UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

# EGGS AND CHICKEN EMBRYOS AS POTENTIAL SENTINELS FOR FLOCK AND HATCHERY HEALTH

RUTE MARINA GARCIA DA NOIVA

Advisors: Doctor Maria da Conceição da Cunha e Vasconcelos Peleteiro
Doctor Hulimangala L. Shivaprasad

Thesis for Attribution of the Philosophy Doctor Degree in Veterinary Sciences
Specialty of Animal Health

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2016

# DEDICATION

*To my, family who raised me up*

*To my friends, who kept me up*

*And to the people who keep me looking up*

"….It is well known that a vital ingredient of success is not knowing that what you're attempting can't be done…"

*Terry Pratchett*

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This project was carried out with in cooperation of a major commercial incubation center and the author would like to thank the company for their availability in providing samples for analysis.

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# RESUMO

A operação eficiente e lucrativa de uma exploração industrial produtora de espécies aviárias (*Gallus gallus*) depende, entre outras coisas, do acesso a pintos saudáveis e com bom potencial produtivo, obtidos por incubação artificial de ovos de um bando reprodutor. É com a incubação destes ovos, levada a cabo em grande escala por máquinas altamente especializadas, que se inicia o desenvolvimento das aves, tendo esta influência directa sobre a viabilidade e futura *performance* do pinto eclodido. Vários factores podem intervir negativamente no sucesso da incubação, estando estes relacionados com o ovo em si, com o seu manuseamento ou com a própria incubadora.

Por essa razão, este trabalho teve por objectivo adquirir, coligir e interpretar informação essencial sobre a resposta embrionária a alterações físicas do ambiente de incubação (temperatura, humidade relativa (RH) e concentração de dióxido de carbono), assim como à infecção com agentes patogénicos com transmissão vertical conhecida (*Salmonella enterica* vars. Enteritidis e Typhimurium). Estes ensaios foram conduzidos em laboratório, em ambiente cuidadosamente controlado. Numa segunda fase, o conhecimento obtido através de experimentação laboratorial foi aplicado na análise de uma amostra de ovos incubados provenientes de um centro de incubação industrial, com o objectivo de verificar a compatibilidade dos dados experimentais com os dados obtidos em situação real.

Com vista a estudar a influência de alterações no ambiente de incubação sobre o desenvolvimento embrionário, procedeu-se à incubação de 2300 ovos *specific-pathogen free*, dividos em dez grupos diferentes. O primeiro grupo foi incubado sob condições controlo (temperatura - 37.8ºC; RH - 50-55% durante os primeiros 18 dias de incubação e 60-65% durante os últimos 3 dias) e os restantes cinco grupos incubados sob: A) temperatura elevada constante (38.9ºC) e RH padrão; B) temperatura inferior constante (36.7ºC) e RH padrão; C) temperatura e RH padrão com um período (3 h) de elevação da temperatura (38.9ºC) aos dias 3 e 18 de incubação; D) temperatura e RH padrão com um período (3 h) de redução da temperatura (36.7ºC) aos dias 3 e 18 de incubação; E) humidade relativa elevada constante (63%) e temperatura padrão; F) humidade relativa inferior constante (43%) e temperatura padrão; G) humidade relativa e temperatura padrão com um período (3 h) de elevação da RH (63%) aos dias 3 e 18 de incubação; H) temperatura padrão com um período (3 h) de redução da RH (43%) aos dias 3 e 18 de incubação; I) ausência

de renovação de ar/concentração de $CO_2$ elevada constante durante incubação sob temperatura e RH padrão.

Para investigar a influência da infecção com microrganismos transmitidos verticalmente, foram utilizados 576 ovos *specific-pathogen free,* divididos por oito grupos, quatro grupos controlo e quatro grupos inoculados. O primeiro grupo tratado foi inoculado com $10^5$ CFU/ml de uma cultura de referência de *S. enterica* var. Enteritidis (CECT-4300) em NaCl (0,9%) estéril. O segundo grupo foi inoculado com $10^2$ CFU/ml (dose de inoculação semelhante à concentração destas bactérias previamente reportada em ovos naturalmente infetados) da mesma cultura de referência. O terceiro grupo foi inoculado com $10^5$ CFU/ml de uma cultura de referência de *S. enterica* var. Typhimurium (CECT-443) em NaCl (0,9%) estéril. Finalmente, o quarto grupo foi inoculado com $10^2$ CFU/ml da mesma cultura de referência. Cada grupo tratado foi incubado juntamente com um grupo controlo inoculado com NaCl (0.9%) estéril. A presença ou ausência de Salmonella em cada grupo foi confirmada através de PCR multiplex convencional.

Em todos os ensaios, foi retirada uma amostra de ovos da incubadora a cada 48 horas de incubação para análise dos efeitos das diferentes manipulações sobre o desenvolvimento embrionário. Uma extensa lista de parâmetros relacionados com o comportamento, metabolismo, crescimento e viabilidade dos embriões foi depois analisada.

Em relação ao estudo de campo, foi efetuada análise morfológica, histológica e microbiológica de uma amostra de 51 ovos não viáveis, obtidos após 21 dias de incubação, num centro de incubação industrial.

Relativamente aos ensaios de manipulação do ambiente físico de incubação, os resultados obtidos permitiram concluir que as alterações de temperatura, em qualquer direção, exerceram efeitos mais marcados sobre o desenvolvimento embrionário do que as alterações na humidade relativa ou na concentração de $CO_2$ no interior da incubadora, enquanto que estas duas últimas variáveis produzem efeitos na sua maior parte sobreponíveis. Dos parâmetros monitorizados, aqueles que mais consistentemente apresentaram alterações encontravam-se relacionados com o comportamento e metabolismo embrionários, nomeadamente com a diminuição do metabolismo embrionário como consequência do stress induzido pela manipulação. O crescimento embrionário, representado pelo peso do embrião e por parâmetros alométricos, foi igualmente afetado, tendo-se verificado que as alterações de temperatura resultaram em embriões mais leves, enquanto as alterações de humidade relativa e $CO_2$ produziram embriões de tamanho semelhante mas peso superior aos dos restantes tratamentos. À análise histológica, os embriões sujeitos a alterações contínuas de temperatura apresentaram ainda atraso na formação do tecido linfoide da bolsa de Fabricius e alterações celulares com distorção da arquitetura do epitélio glandular da moela, estas últimas em embriões sujeitos a aumentos de temperatura). Os embriões sujeitos a modificações da humidade relativa produziram efeitos temporários sobre o desenvolvimento do tecido hepático. As lesões microscópicas encontradas em animais mortos durante a incubação não permitiram o diagnóstico fiável da(s) causa(s) imediata de morte. Nenhum dos tratamentos produziu elevação significativa dos níveis de mortalidade, à exceção da incubação sob temperaturas inferiores ao normal. Dos restantes tratamentos, a elevação da humidade relativa resultou num aumento da mortalidade no final do período de incubação. Ainda relativamente à viabilidade embrionária, verificou-se que apenas as manipulações da temperatura de incubação resultaram num aumento significativo na incidência de malformações espontâneas. Finalmente, é de notar que, para todas as variáveis estudadas, a alteração contínua de uma variável produziu efeitos mais pronunciados e duradouros, enquanto a alteração pontual e temporária de uma variável produziu efeitos mais limitados, tanto em amplitude como em duração.

Ao contrário dos ensaios de manipulação do ambiente de incubação, a inoculação de ovos *specific-pathogen free* não incubados com serovars de *Salmonella enterica* e sua posterior incubação não produziu alterações significativas no comportamento, metabolismo ou desenvolvimento embrionários. A inoculação de *S.* Enteridis, tanto em inóculo alto como em inóculo baixo, resultou num aumento significativo da taxa de mortalidade, sendo que esta se encontrou limitada aos primeiros 7 dias de incubação. A inoculação com qualquer dos inóculos de *S.* Typhimurium produziu, igualmente, níveis de mortalidade superiores comparados com os controlos, limitados aos primeiros dias de incubação, mas inferiores aos produzidos por *S.* Enteritidis. As lesões microscópicas observadas em animais encontrados mortos não permitiram o diagnóstico fiável da(s) causa(s) imediata de morte, sendo que a única diferença significativa encontrada se restringiu a alteração da afinidade tintoral dos tecidos provenientes de embriões infetados, indicativa de decomposição mais acelerada provavelmente devido a maior proliferação bacteriana. Contudo, nenhum dos embriões viáveis inoculados apresentou qualquer alteração macroscópica ou histológica significativa e em nenhum destes animais se observaram agregados bacterianos à análise microscópica, apesar da presença de infeção confirmada por métodos de biologia molecular. Os resultados obtidos sugeriram, ainda, que ambos os serovars de *Salmonella* utilizados apresentam tropismo para o embrião, possuindo a capacidade de não só sobreviver dentro do ovo, mas também de atravessar compartimentos orgânicos em direção ao embrião. Estas observações evidenciam algumas das razões

para a eficiência demonstrada por estes serovars (e particularmente *S.* Enteritidis) na transmissão vertical e estabelecimento de portadores assintomáticos.

Em relação ao estudo de campo, a análise dos ovos não viáveis não evidenciou diminuição de *performance* ou viabilidade embrionária devido a qualquer das variáveis estudadas em laboratório. Contudo, o exame externo, macroscópico dos ovos, assim como a sua análise microbiológica evidenciaram problemas na seleção e calibração dos ovos para incubação, assim como na possível higiene e práticas de recolha dos ovos na exploração de reprodutores, sinalizando potenciais pontos de intervenção futura com vista ao melhoramento da *performance* de incubação que de outra forma não seriam detetados.

Finalmente, este trabalho permitiu a aquisição de informação essencial para a compreensão da resposta embrionária aos desafios da incubação, permitindo ainda confirmar a importância do embriodiagnóstico na monitorização da saúde da incubadora e do bando.

Palavras-Chave: Galinha, Desenvolvimento embrionário, Temperatura, Humidade, Dióxido de carbono, *Salmonella*, Incubação

# ABSTRACT

Successful hatching of strong, healthy chicks with a potential for good productive and reproductive performance depends on the assurance of as normal and ideal as possible embryonic development. The latter, however, can be negatively affected by a series of factors intrinsic to either the egg, its handling, or the incubator.

This project aimed at collecting and interpreting essential and extensive information regarding embryonal response to physical challenges (temperature, relative humidity and carbon dioxide concentration) in the incubation environment, to infectious challenge with vertically transmitted *Salmonella* serovars Enteritidis and Typhimurium and, ultimately, to apply the knowledge obtained to a field survey of a commercial hatchery.

For this purpose, eggs were incubated under different, controlled environments or after infection with a vertically transmitted pathogen, and an extensive list of parameters used to monitor subsequent changes in embryonic behavior, metabolism, growth and viability. The information gathered was then applied to a sample of non-viable eggs incubated at a major hatchery.

Overall, the effects of manipulating temperature were more prominent than those of manipulating humidity and carbon dioxide. The most significantly affected parameters pertained to embryo metabolism, embryo weight, mortality and malformation rates. Changes in the development of the lymphoid follicles in the bursa of Fabricius and of the glandular epithelium of the gizzard were also found.

Vertical infection with *Salmonella* was associated with higher embryonic mortality rates. No changes in performance or growth were observed in infected but viable embryos.

The results obtained during the field survey did not directly correlate to the variables studied. However, the issues detected allowed for the designation of critical control points and opportunities for performance improvement that could have otherwise gone undetected at the hatchery and at the breeder farm, highlighting the importance of embryodiagnosis in the monitoring of hatchery and flock health.

Keywords: Chicken, Embryonic development, Temperature, Humidity, Carbon dioxide, *Salmonella*, Incubation

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# ABBREVIATIONS

| | |
|---|---|
| ACE | Angiotensin I-Converting Enzyme |
| ACTH | Adrenocorticotropic Hormone |
| apo-B | Apoprotein B |
| APP | Acute Phase Protein |
| AR | Albumen Weight To Egg Weight Ratio |
| ATP | Adenosine Triphosphate |
| AVMA | American Veterinary Medicine Association |
| B.C. | Before Christ |
| BHI | Brain Heart Infusion |
| BL | Beak Length |
| BPW | Buffered Peptone Water |
| CECT | Colección Española De Cultivos Tipo |
| CFU | Colony Forming Units |
| CHCO2 | Continuously High Carbon Dioxide |
| CHRH | Continuously High Relative Humidity |
| CHT | Continuously High Temperature |
| CL | Comb Length |
| CLRH | Continuously Low Relative Humidity |
| CLT | Continuously Low Temperature |
| CRB | Calcium Reserve Body |
| DDW | Deionized Distilled Water |
| ED | Eye Diameter |
| EL | Embryo Length |
| EEM | Extra-Embryonic Membranes |
| ER | Embryo Weight To Egg Weight Ratio |
| ESG | Eggshell Gland |
| ESM | Eggshell Membranes |
| FCS | Fetal Calf Serum |
| FL | Feather Length |
| FSH | Follicle-Stimulating Hormone |
| GAG | Glycosaminoglycan |
| GAPDH | Glyceraldehyde 3-Phosphate Dehydrogenase |
| HDL | High Density Lipoprotein |
| HR | Heart Rate |
| IgA | Immunoglobulin A |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IgY | Immunoglobulin Y, Y-Livetin |
| IL | Interleucin |
| IMViC | Indole-Methyl Red-Voges-Proskauer-Citrate |
| INF | Interferon |
| ISM | Inner Shell Membrane |
| LH | Luteinizing Hormone |
| LL | Leg Length |

| | |
|---|---|
| N.Y. | New York |
| NL | Nail Length |
| OC-17 | Ovocleidin-17 |
| OC-116 | Ovocleidin-116 |
| OCX-21 | Ovocalyxin-21 |
| OCX-32 | Ovocalyxin-32 |
| OCX-36 | Ovocalyxin-36 |
| OIH | Ovulation-Inducing Hormone |
| OSM | Outer Shell Membrane |
| OSP | Osteopontin |
| OTrf | Ovotransferrin |
| PBS | Phosphate Buffered Saline |
| PHRH | Pinpoint High Relative Humidity |
| PHT | Pinpoint High Temperature |
| PLRH | Pinpoint Low Relative Humidity |
| PLT | Pinpoint Low Temperature |
| PUFA | Poly-Unsaturated Fatty Acids |
| RBP | Flavoprotein |
| RH | Relative Humidity |
| ROS | Reactive Oxygen Species |
| RVS | Rappaport Vassiliadis Medium |
| SPF | Specific-Pathogen Free |
| SR | Shell Weight To Egg Weight Ratio |
| TE | Tris-EDTA |
| TL | Toe Length |
| TM | Trade Mark |
| TNF | Tumor Necrosis Factor |
| TSB | Tryptic Soy Broth |
| TSI | Triple Sugar Iron Agar |
| U.S.A. | United States Of America |
| VLDL | Very Low Density Lipoprotein |
| VM | Perivitelline Membrane |
| VMM | Voluntary Movements Per Minute |
| VMO | Vitelline Membrane Outer Proteins |
| VTG | Vitellogenin |
| WL | Wing Length |
| XLD | Xylose Lysine Deoxycholate Agar |
| YR | Yolk Weight To Egg Weight Ratio |
| ZP | Zona Pellucida |

# LITERATURE REVIEW

# THE HISTORY OF ARTIFICIAL INCUBATION

The search for the very origins of artificial incubation can take one through a more than 3000-year backward journey of discovery into the sands of time (Petersime, 2011), and past the outspoken fascination of others before us, as demonstrated by the two North American newspapers from the early 20th century displayed in Figure 1.

THE MILWAUKEE JOURNAL

Friday, February 14, 1930

**Incubators Used in Ancient Egypt**

Although the ancient Egyptians lacked artificial light with which modern poulterers perform fake sunrises to make their hens lay overtime, they used incubators to hatch out eggs. The oldtime hatcheries were cone shaped mud huts heated by burning chaff. An attendant always sat within to warn against temperature too hot or too cold. Of a clutch 95 per cent hatched successfully. William D. Mann, United States assistant commercial attache at Cairo, found out about the ancient Egyptian brooders when he was seeking an Egyptian market for the latest type of United States made incubators.

THE READING EAGLE,

TUESDAY, NOVEMBER 19, 1929.

**Ancient Egyptians Had Egg Incubators of Mud**

Washington, Nov. 19.—The ancient Egyptians had artificial egg incubators, according to a report of William D. Mann, assistant commercial attache at Cairo, to the Department of Commerce.

Mr. Mann stated that the incubators the Egyptians employed 6.000 years ago were crude cone-shaped affairs heated by burning chaff and capable of hatching 95 per cent of the eggs.

Similar mud incubators are in use in Egypt today, he said.

The incubators hold about 6.000 eggs on platforms circled by a small trough in which the chaff is burned. Two attendants sleep in the hatchery during the 21-day process and put out the fires after the 14th day.

**Figure 1. North American newspaper articles from the 1920's-30's (Anonymous, 1929, 1930).**

Although its history is deeply scarred by lost knowledge and long periods of apparent lack of activity, an attempt will be made to present an accurate condensation of the most relevant periods and methods recorded for artificial incubation.

## Artificial Incubation in Ancient Egypt

Tradition credits the priests of the temple of Isis as being the inventors of the earliest artificial methods, but when (or even by what nation) the first *eccaleobion* or *mahmal* (so named on account of its life-giving properties) was constructed is not known (Lippincott & Card, 1921; Réaumur, 1750; Sutcliffe, 1909).

The earliest available record of hatching by artificial means is mentioned by Herodotus, about 450 years B.C., in his reference to the egg ovens of Egypt (Halsted, 1884), later followed by Diodorus' (50-30 B.C.) comment in his *Bibliotheca Historica*: *"…and the most astonishing fact is that, by reason of their unusual application to such matters, the men who have charge of poultry and geese, in addition to producing them in the natural* *way known to all mankind, raise them by their own hands, by virtue of a skill peculiar to them, in numbers beyond telling; for they do not use the birds for hatching the eggs, but, in effecting this themselves artificially by their own wit and skill in an astounding manner, they are not surpassed by the operations of nature*" (Diodorus & Mayer, 1933). The Roman emperor Hadrian also makes special mention of it in his description of the uses and customs of Egypt (von Culin & von Culin, 1894).

Yet, the earliest authentic account we have of the early incubation methods of the Egyptians is from the writings of Sir J. Maundville, about the year 1356, in which he describes the heated houses to which women brought the eggs of hens, ducks, and geese to be hatched, returning three weeks or a month later for the newly hatched chicks (Sutcliffe, 1909).

These, according to the early descriptions, were built out of mud, or adobe; in later years they were made of bricks, which were, in fact, sundried mud (Figure 2) (Halsted, 1884; Lippincott & Card, 1921; Réaumur, 1750; Sutcliffe, 1909).

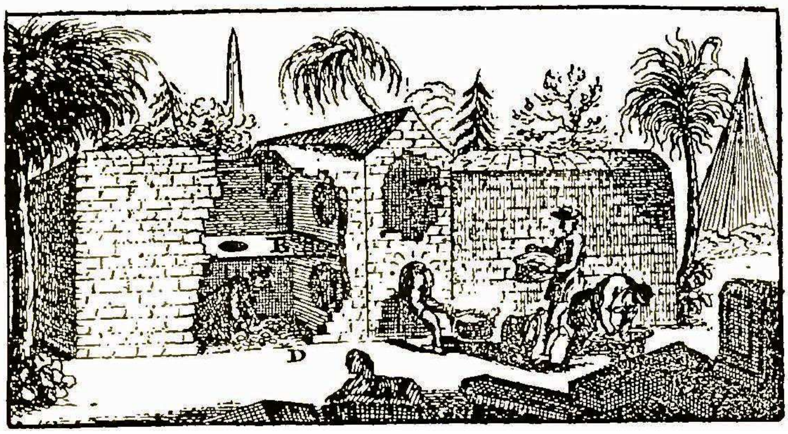


**Figure 2. Ancient Egyptian hatchery (Réaumur, 1750).**

They are described as consisting of two parallel rows of small ovens, and cells for fire, divided by a narrow, vaulted passage. Each oven was about 3 m long, 2.5 m wide, and 1.5 m or 1.8 m high, and had above it a vaulted fire-cell of the same size (Halsted, 1884). Antechambers guarded the incubator-room from being affected by outside temperature (Sutcliffe, 1909). Each oven communicated with the passage by an aperture large enough for a man to enter (Figure 3).

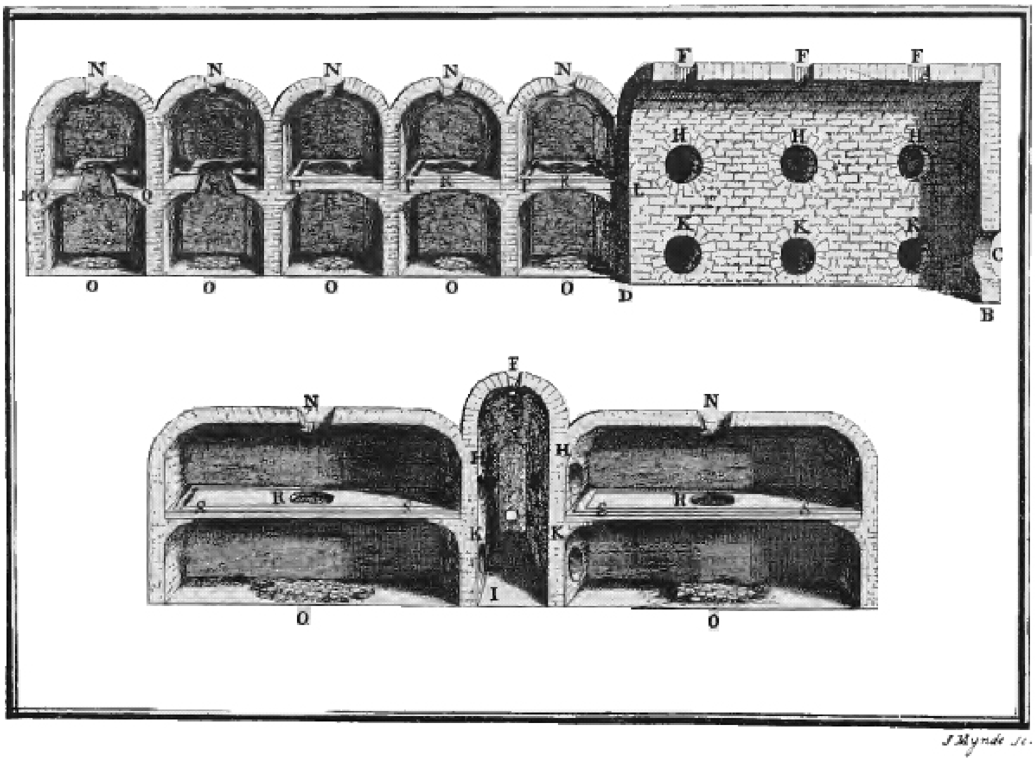


**Figure 3. Lengthwise vertical sections of an Egyptian hatchery (Réaumur, 1750).**
**B – wall between the antechambers and the gallery; C – access door into the gallery; F – roof holes for ventilation and lighting; I – central gallery; K – holes for access to the lower egg chamber; H – holes for access to the upper egg chamber; M – outer wall; N – roof hole; P – separation between the upper and lower chambers; O – heap of eggs being incubated; Q – part of the separation between the upper and lower chambers; R – communication hole between the upper and lower chamber for diffusion of hot air; S – gutter in which the fire is lit.**

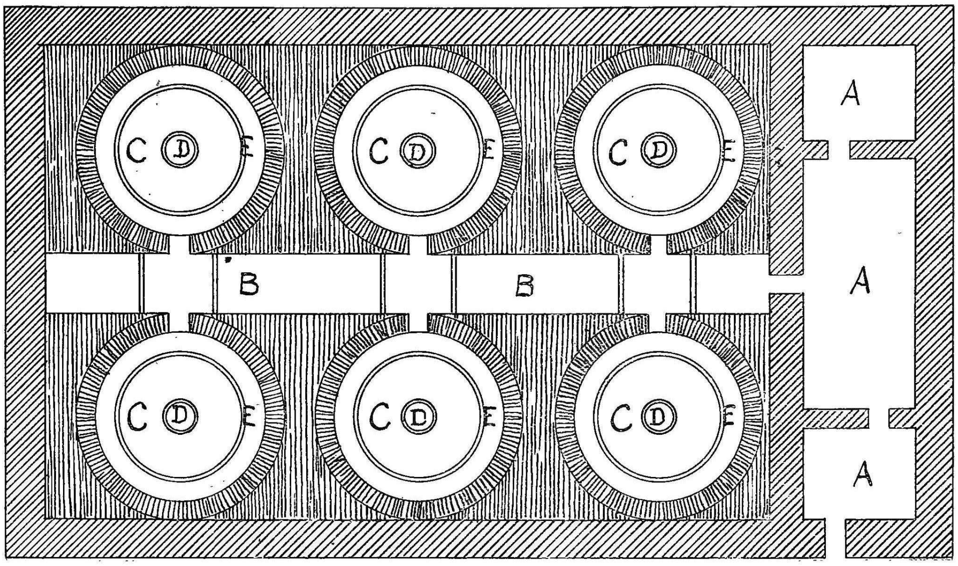


**Figure 4. Bird's-eye view of an Egyptian hatchery (Sutcliffe, 1909).**
**A – antechamber; B – central passage; C – egg chamber; D – central access manhole; E – partition in which fire is lit.**

These were all underground, and connected with the outer air by a long, covered passage, in order to avoid cold drafts. Small circular openings or mouths, which can be seen in Figure 3, about 1 m in diameter led into each oven by a short-arched passage

(Halsted, 1884). Small doors gave access to each tier or egg-chamber. These were kept closed, except when the attendant entered to see the fires, or for the admission of fresh air about three times a day (Sutcliffe, 1909). The ovens were nearly, but not quite circular; the roofs were domed, and contrived with a kind of chamber over them; the apertures leading to the fire-chambers were the same width as the openings to the ovens, and only high enough to admit a boy to pass through (Halsted, 1884). The fires were placed around and close to the wall in each chamber, being partitioned off by a ridge about 15 cm high (Figure 4) (Sutcliffe, 1909). Each fire-chamber also communicated with its respective oven (Halsted, 1884). In the domed roofs of the ovens, and in the roof of the room, there were holes that could be opened or closed at pleasure; these served the threefold purpose of letting out the smoke, letting in air and a dim, hazy light, and, by adjusting their size, also regulating heat (Halsted, 1884; Sutcliffe, 1909). However, regulation of heat was done chiefly by increasing or diminishing the fires in the ovens, which were lighted in one or more places as needed (Sutcliffe, 1909). The material employed for heating, which was called *gelleh*, consisted of dung collected and dried for the purpose, and kept smoldering slowly in the fire-chambers above the eggs. Water was supplied in troughs made of mud bricks, encircling the eggs (Halsted, 1884). No thermometers were used (Sutcliffe, 1909). The men who attended the hatching process were bred into their tasks from childhood, the secrets of their trade handed down from father to son, and lived in the incubators during the time they were in operation, which allowed them a remarkable ability to accurately judge by touch the temperature required, a little higher than that of their own skin (Halsted, 1884; Lippincott & Card, 1921; Réaumur, 1750; Sutcliffe, 1909). The eggs were tested for fertility on the tenth day with the palm of the hand, or by placing them against the upper eyelid (Lippincott & Card, 1921; Réaumur, 1750; Wilkinson, 1841). Those noticeably cold were regarded as infertile (or dead), and discarded, while those warmer than the human skin were considered viable (Lippincott & Card, 1921; Réaumur, 1750; Wilkinson, 1841).

The floors of the ovens were covered with bran to prevent the eggs from rolling, and the attendant changed the position of the eggs twice a day, removing those near the center to the rim (Sutcliffe, 1909).

The whole structure would have been around 21 m long, 18 m wide, and 5 m high, provided with twelve compartments, or incubators, each capable of holding 7500 eggs, making a total capacity of 90,000 eggs undergoing incubation at one time (Lippincott & Card, 1921; Réaumur, 1750).

These enormous incubators functioned so well in hatching such a high percentage of chicks that natural incubation was actually rarely used in Egypt, the Egyptian hen being thought of as having long ago lost its maternal instincts as a sitter (Sutcliffe, 1909).

The Egyptian ovens were public institutions and run on a toll basis, with two chicks being usually expected from three eggs (Lippincott & Card, 1921; Réaumur, 1750). The eggs were supplied by the peasantry, and there were two systems of purchase. Under one system, the hatcher paid down an agreed sum to the peasant for eggs; under the other, the owner of the eggs left them with the hatcher at his own risk, the latter agreeing to return one chicken for every two eggs (Halsted, 1884). The chicks were distributed within a few hours of hatching, in large or small batches, mainly by women, who purchased and reared them, thus resolving the hatchers' obvious inability to rear all the chicks they hatched (Sutcliffe, 1909).

It is essential to notice that the climate of Egypt was (and is) particularly suited for this method, being almost of uniform temperature, and that the incubators functioned for no longer than three months each year, the hatching process being carried out only during the months of April, May and June (Halsted, 1884).

## Artificial Incubation in Ancient China

The Chinese method, still in vogue in the beginning of the 20th century, was equally primitive and probably practiced for as long as (or even longer than) the Egyptian (Halsted, 1884; Lippincott & Card, 1921).

The eggs were purchased out of the produce boats that came down the river, and incubated in a large wooden, barn-shaped building on the river bank (Halsted, 1884). From this point on, descriptions vary and several distinct processes have been reported (Halsted, 1884; Lippincott & Card, 1921).

One of the processes described was singular, using only the natural heat of the egg. Large baskets, each twice the size of an ordinary barrel, and thickly lined with hay to prevent the loss of heat, were filled with the eggs, and then carefully closed with a closely-fitting cover of twisted straw. The eggs were then left for three days, after which they were removed from the basket and replaced in different order – those eggs which were before at the top being then placed on the lowest tier. At the end of three days more the position of the eggs was again altered, and so on for fifteen days, after which time the eggs were taken out of the basket and placed on a shelf in another apartment, and covered with bran. In the course of a day or two, the chicks would burst their shells and make their way out of the bran, being immediately looked after by a constantly watchful attendant. The secret of the process lay in the animal heat of the whole mass of eggs being retained by the basket – made of materials that do not conduct heat – in sufficient amounts to support the animal life of any particular egg, and to foster its development (Halsted, 1884).

Another process described a hatching house for duck eggs, built at the end of a regular cottage, a kind of long shed with mud walls thickly thatched with straw. Along the ends and down one side of the building were a number of round straw baskets, well plastered with mud to prevent them from catching fire (Halsted, 1884). A tile formed the bottom of the basket, a small fireplace being below each basket to act as a source of heat beneath it (Halsted, 1884; Lippincott & Card, 1921). Upon the top of each basket there was a closely fitted straw cover, which was kept shut throughout the whole process. In the center of the shed were a number of large shelves placed one above another, upon which the eggs were laid at a certain stage of the process. No thermometers were used as, not unlike the Egyptians, the Chinese used their experience and senses to appraise temperature (Halsted, 1884).

Four or five days after the eggs had been subject to this temperature they were taken carefully out to a door, in which a number of holes nearly the size of the eggs had been carved; they were then held one by one against these holes, and the Chinese looked through them to tell whether they were viable or not. If viable, they were taken back and replaced in their former quarters; if bad, they were excluded. About fourteen days after the beginning of the process, the eggs were taken from the basket and spread out on the shelves. Here, no fire heat was applied; the eggs were covered over with cotton, and a kind of blanket, under which they remained for about fourteen days more, when the young ducks burst through their shells (Halsted, 1884).

However, it was thought that it would be highly unlikely for any or both these processes to be successful and both were thought to be incompletely described, since it was considered, as Halsted (1884) put it, "*exceedingly improbable that any process so directly antagonistic to the natural system could be successfully carried out. Doubtless there was some unseen means of keeping the eggs warm, as it is impossible that the egg should of itself possess sufficient heat to sustain life, much less develop it*".

## Artificial Incubation in Europe and North America

No attempt seems to have been made in the west of Europe either to erect an incubatory or make an incubator until about the year 1415, when Charles VII of France took some interest in the subject (Sutcliffe, 1909).

Seventy-nine years later, in 1494, both Alphonse II, King of Naples and the Duke of Florence, established Egyptian Incubators. The latter even went as far as to import an

Egyptian architect skilled in the art to construct an incubator after the Egyptian pattern (Halsted, 1884). Although at first it would appear that these efforts would be rewarded with obvious success, neither of them was successful, owing to the differences in climate between the two countries, Italy and Egypt (Halsted, 1884; Sutcliffe, 1909).

As was stressed before, Egypt's climate, with its almost uniform temperature, fulfilled all of the requirements of the Egyptian method of artificial incubation so perfectly and to such an extent that this could not be copied nor used to model an incubator that would function in the European climate (Halsted, 1884; Sutcliffe, 1909). Their gigantic "ovens" could not be adopted in Europe's variable climate, which required a machine capable of automatically adjusting itself to constant fluctuations in ambient temperature and humidity, issues that did not trouble the operators in the Egyptian climate during the three months when the incubatories operated. In fact, all of the European and American incubators later created were developed quite independently of any aid from the ancient methods (Sutcliffe, 1909).

Around 1588, the Italian inventor Jean Baptiste Porta drew on the ancient Egyptian designs as well to build his egg incubator, but was forced to abandon his work by the Inquisition (T. A. Shah, 2011).

Knowledge about egg incubation was later revived and introduced throughout Europe in the 1750's by René-Antoine Ferchault de Réaumur (1683-1757) (Halsted, 1884; T. A. Shah, 2011). The celebrated French naturalist constructed an apparatus for hatching by means of horse manure. His apparatus consisted in wooden casks lined with plaster of Paris or clay and fitted with drawers or movable shelves (on which the eggs were placed), set on end (bottom downward), the whole surrounded with fresh horse manure mixed with straw, making a bed extending to a distance of 0.5 m around their circumference, which was renewed at intervals to keep up the heat (Figure 5). Réaumur's apparatus was quite successful in the hands of the naturalist, but with others it did not do as well, probably owing to lack of attention and knowledge of the requisite care (Halsted, 1884). After Réaumur's death, the incubator was further developed by Abbé Jean-Antoine Nollet (1700-1770) and later by Abbé Copineau, who used alcohol lamps as a source of heat (T. A. Shah, 2011).

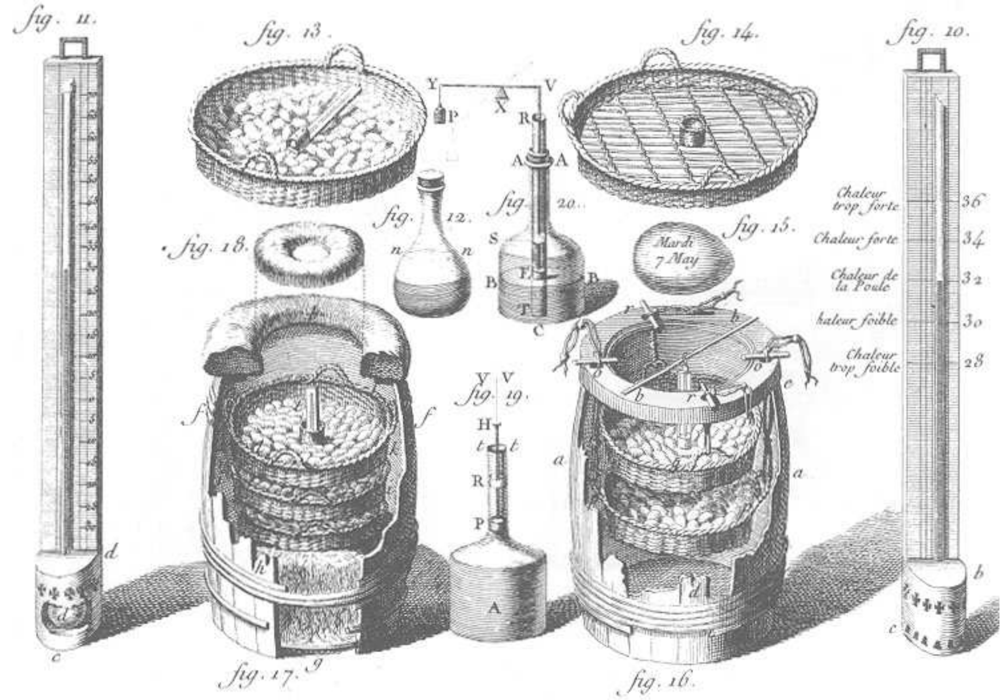


**Figure 5. Réamur’s device for hatching eggs (Réaumur, 1750).**

In 1770, John Champion of Berwick-upon-Tweed, England, erected an incubator house or room, in the center of which the eggs were placed on a table (Lippincott & Card, 1921; Sutcliffe, 1909). Champion hatched eggs by passing flues carrying hot air through this room; the source of heat was a fire that burned in an ante-room, which masked the entrance to the egg-chamber and in which the operator could attend to the flame (Lippincott & Card, 1921; Sutcliffe, 1909). The flues also returned into this anteroom, thus keeping the egg chamber free of smoke (Sutcliffe, 1909).

Later, in 1777, Bonneman, a French physician contemporaneous with Champion, established hatching ovens in Nauterre, in which the ovens were supplied with heat diffused from hot-water pipes and a fire (Halsted, 1884; Lippincott & Card, 1921; Sutcliffe, 1909).

Bonneman used sponges saturated with water placed at the bottom of the egg chamber, to supply moisture to the air. He also constructed a regulating bar, formed of two different metals, (probably iron and brass), which acted upon a damper in the furnace door, thus increasing or decreasing the draft of the fire (Halsted, 1884). The eggs were set on slides, as shown in Figure 6. Although some sources claim this incubator was reputed to be a success, as it supplied the French Court with chickens all year round, others state that it wasn’t, due to the impossibility of keeping the temperature even on the different slides (Halsted, 1884; Sutcliffe, 1909). The French Revolution brought an end to this and similar experiments for a time (Sutcliffe, 1909).

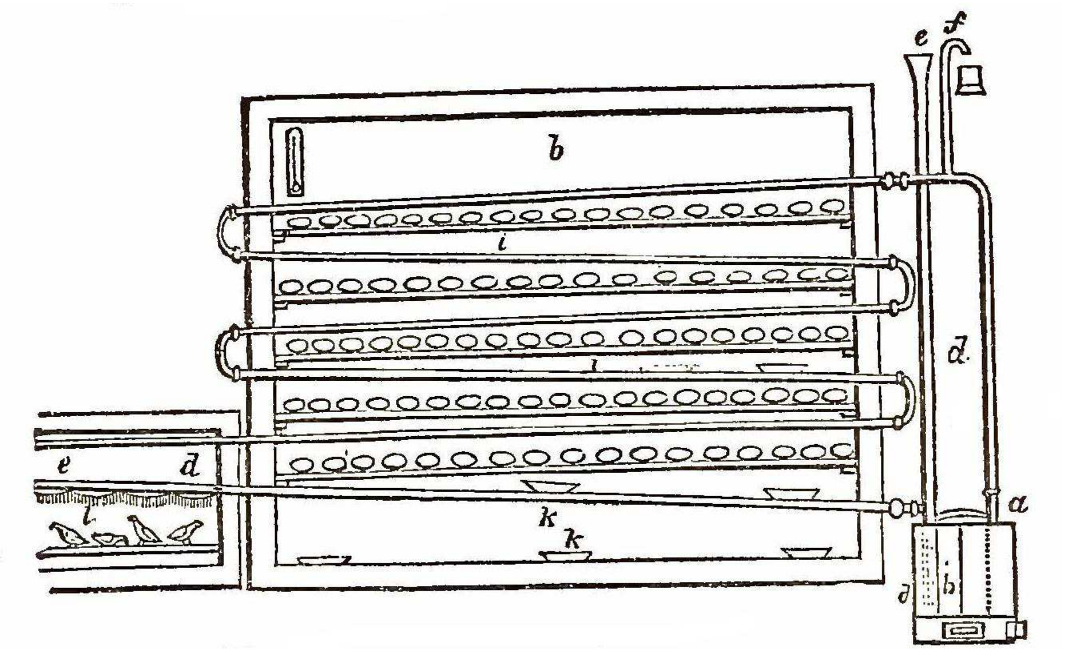


**Figure 6. Bonneman's incubator (Halsted, 1884).**
**A – boiler; b – incubator proper; c – brooder; d – tubes for circulating hot water; e – funnel end of supply tube; f – exhaust pipe; i – slides for placing the eggs; k – water sponges.**

It was not until almost seventy years later that accounts of other incubator designs emerged (Halsted, 1884; Sutcliffe, 1909). Next after Bonneman's was an invention which was shown in London in 1839, and called an Eccaleobion: this was heated by steam pipes, with jugs of water placed in the egg chambers to keep the air moist (Anonymous, 1882; Halsted, 1884).

Three years later, in 1842, a small machine was exhibited in operation in Bristol, England, by Mr. Appleyard and, about the same time, one other was exhibited by Mr. E. Bayer, in New York and Brooklyn, called the Potolokian. However, there are no descriptions available for any of these devices (Halsted, 1884).

The first patent egg incubator for hatching chickens by artificial heat was patented one year later, on March 30th, 1843, by Napoleon E. Guerin of New York City, known for developing and patenting methods of distributing steam heat and purifying air, as well as for creating the very first life preserver made of cork (Kane, Anzovin, & Podell, 1997).

Another American design, Cantelo's Patent Incubator, was invented in 1844 and patented in England. This was also a hot-water-heated machine (the water being heated by a charcoal fire) and proved to be a fairly successful machine (Halsted, 1884; Lippincott & Card, 1921). It was the first attempt to imitate the natural process of applying the heat from above, described as being a very simple apparatus. It consisted of a tank with bottom of India rubber; the drawers of eggs underneath were pressed up so that the eggs came in contact with the rubber cloth. A stove placed on one side heated the water, making it circulate through the machine's piping system (Halsted, 1884).

Contemporary with Cantelo's, Minasi's design was somewhat more elaborate, featuring a boiler heated by a naphtha or spirit lamp, all enclosed in an upright box, which communicated heat to a reservoir or tank of water. The underside of this tank was corrugated, to support a series of small, narrow sand-bags by aid of wires, against which the eggs were pressed by springs under the drawers. This initial device was abandoned and replaced with a series of tubes, through which the hot water circulated. The eggs were placed on these tubes, which were close enough together to prevent the eggs from falling through. Overall, the machine was simply far too elaborate to become popular (Halsted, 1884).

The following year, in 1845, near Paris, a poultryman named M. Vallee designed probably one of the most successful of the many French inventions (Figure 7) (Halsted, 1884; Lippincott & Card, 1921). One of them was in exhibition for a long time at the Museum of Natural History, in Paris, and gave a very fair percentage in hatching rates (Halsted, 1884).

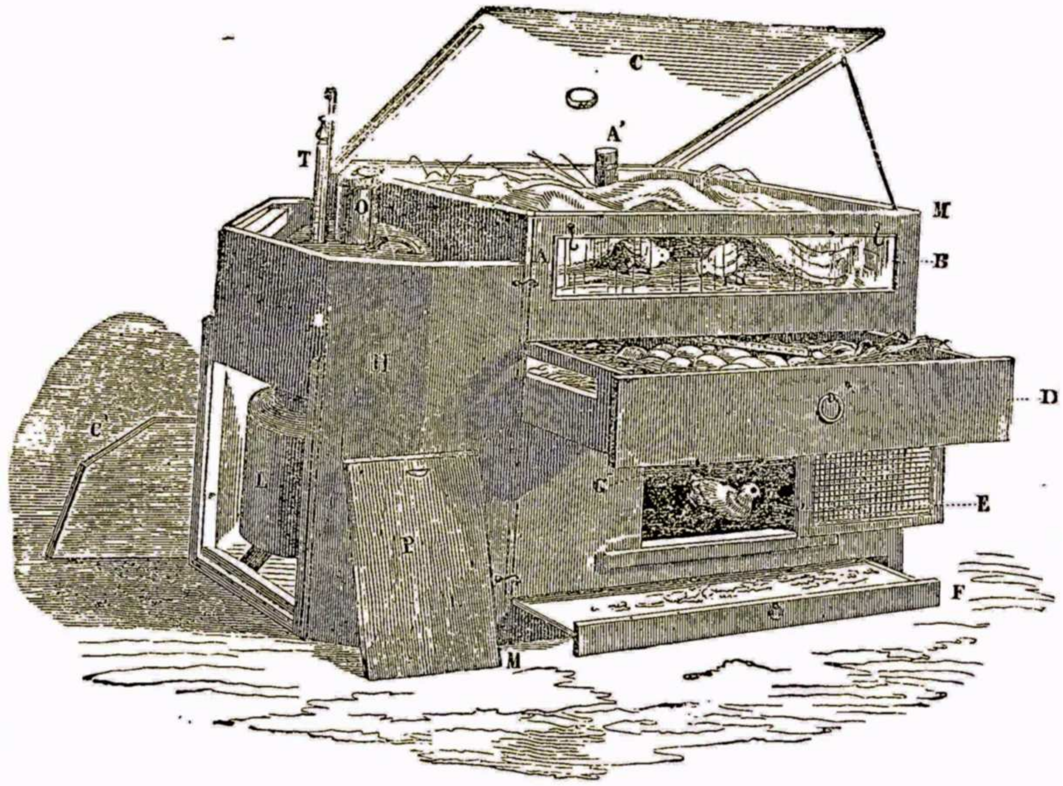


**Figure 7. Vallee's egg hatcher (Halsted, 1884).**
**A – ventilating tube; B – nursery or brooder; C – nursery cover; D – egg-drawer; E – sliding door for extra chick apartment; F – litter tray; G – return pipe; H – boiler cover; L – boiler; O – flue; T – reservoir filling tube.**

The machine, which included a mercurial regulating device with self-acting valves that opened when the temperature of the egg chamber was excessive, was still in use in some parts of France several years later, around 1884 (Halsted, 1884; Lippincott & Card, 1921; Sutcliffe, 1909). Although Vallee's particular valves were soon discarded, and in spite of being still the type of machine which could not be safely left unattended for many hours without danger of overheating, the idea of automatically regulating the temperature was all-important. It was that concept which turned this invention into what may be considered the beginning of the modern incubator (Halsted, 1884; Sutcliffe, 1909).A number of Vallee's machines have actually found their way to America. One Dr. Preterre, of New York City, had one, with which he was very successful. His apparatus was connected to an electrical bell, which signaled an alarm whenever the heat exceeded the proper limit. It was exhibited by the Doctor a number of times before the American Institute, and also at poultry shows in N. Y. City, winning him a gold medal awarded by the N. Y. State Poultry Society in 1870 (Halsted, 1884).

About the year 1846, Carbonnier constructed an incubator in which a hot-water tank, heated by a lamp on one side, was the source of heat (Figure 8). The eggs rested on damp sawdust and no method of automatic regulation was used. Owing to fluctuations in the heat from the lamp, very frequent attention was necessary to increase or diminish the temperature and two thermometers were provided for guidance, one for the tank and one for the egg-chamber. Most tank machines available in the early 1900’s may be said to have been a combination of the principles (with improvements) of Vallee’s automatic heat regulator and Carbonnier’s hot-water tank (Sutcliffe, 1909).

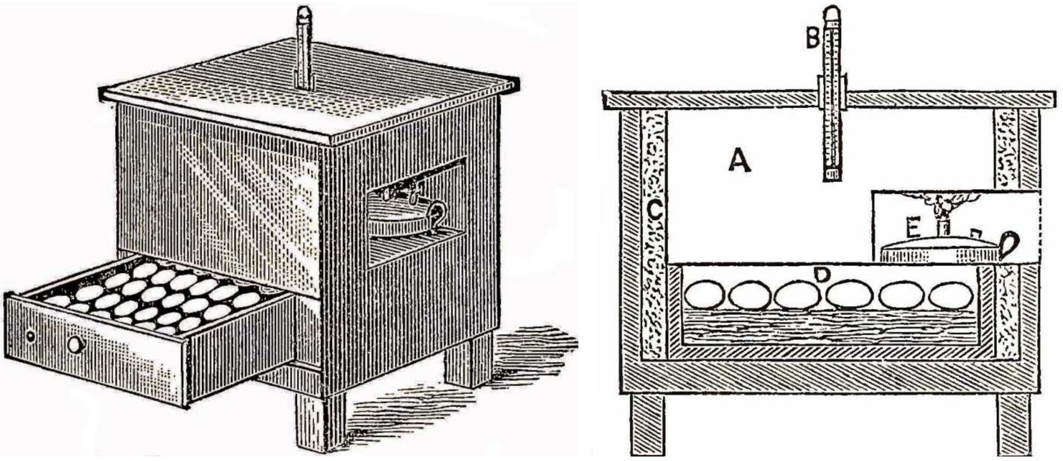


**Figure 8. Carbonnier’s incubator (Halsted, 1884).**
**A – zinc case for water; B – thermometer; C – Non-conducting filling; D – drawer with eggs; E – lamp.**

Similar to Minasi’s first machine was one constructed by Adrien & Trioche, at Van Girard, France, in 1848. This also had the rubber-cloth bottom to the water tank, supported on wooden rods. A sheet-iron cylinder, heated by a charcoal fire, supplied the tank with hot water. The top of the tank was covered with sand, in order to retain the heat. The drawers were in two ranges, placed back to back; the bottoms of the drawers were made of perforated tin or fine wire cloth, covered with bran to keep the eggs level. The machine held fifteen hundred eggs, and was claimed to yield twelve hundred chicks per run. It required attention every four hours (Halsted, 1884).

In spite of all the models emerging, it was not until about 1870 that incubators began to engage serious attention in the United States, when a patent was awarded to Jacob Graves, for an "*incubator and artificial mother*", followed by James Rankin, of southeastern Massachusetts, with a machine that was "*guaranteed to hatch as many chicks as could be done with hens*" (Lippincott & Card, 1921).

From Carbonnier's time to 1877, improvements continued to be made, and new incubators put on the market, each having something to recommend it but still not being practical or profitable enough to come into general use (Sutcliffe, 1909).

In 1877, T. Christy brought out an incubator which was an improvement on all previous ones, in that a fairly steady supply of heat could be maintained, though the method by which this was done was somewhat laborious. A certain quantity of cooled water was withdrawn from the tank twice a day at equal intervals and replaced with hot or boiling water. Thermometers were provided for both tank and egg drawer. As water loses its heat very slowly, the temperature did not fall so far as to seriously affect the hatching between one refilling and the next, the fluctuations between the highest and lowest temperatures being only a few degrees. This incubator was the first to become of actual utility to poultry-keepers (Sutcliffe, 1909).

The attention of inventors was now devoted to finding a practical means of automatically keeping the heat in the egg drawer uniform. In 1881, C. Hearson patented a thermostatic capsule for his own hot-water incubator (Figure 9).

This invention placed the heat of the incubator under perfect control in a simple and effective manner, overcame the chief difficulty of Christy's first machine, and placed Hearson's tank incubator (for at least the full term of patent rights) a long way ahead of others in popularity. The upper part of Hearson's incubator consisted of a hot-water tank, well packed to conserve heat. This tank was kept heated by means of the flue from the lamp at the side, passing through and round the inside of the tank, and having its outlet near the lamp chimney in the hood or lantern. Over the chimney was a valve or damper, operated, through a lever, by a thermostatic capsule placed in the egg chamber. When this valve was down the heat from the lamp was diverted into the tank flues, but when it rose through the expansion of the capsule in the egg-drawer, the hot fumes passed straight out of the lamp chimney and the heat of the water (and correspondingly the heat of the egg-chamber below it) gradually fell (Sutcliffe, 1909).

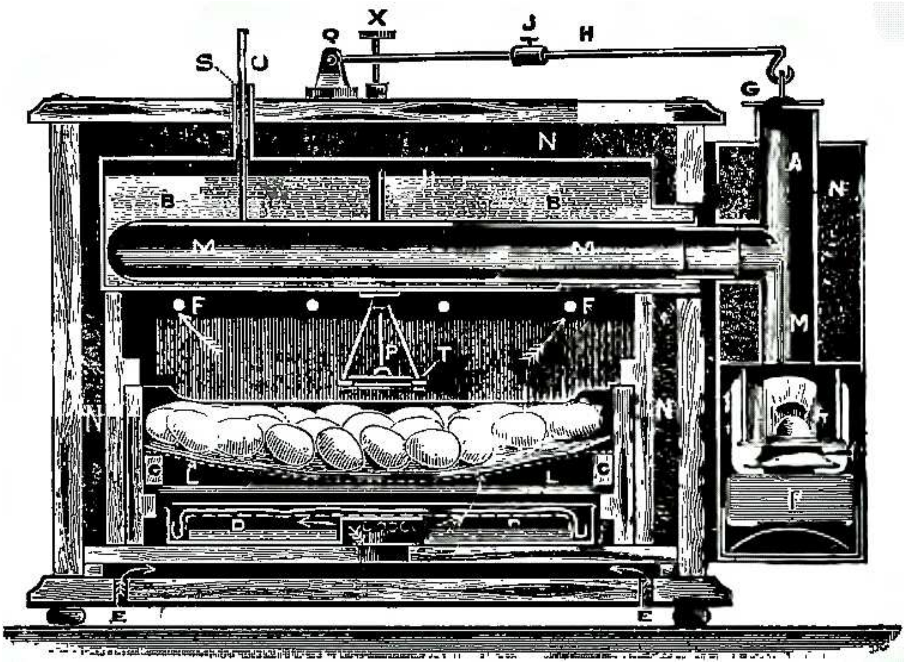


**Figure 9. Sectional view of Hearson's incubator (Sutcliffe, 1909).**
**A – lamp chimney; B – water tank; C – egg tray; D – moisture-distributing tray; E – ventilating holes (intake). F – ventilating holes (exit); G – chimney damper; H – regulation lever; I – lamp reservoir; J – sliding weight adjuster; L – drawer adjusting slips; M – flue for heating tank; N – packing to retain heat; O – tank thermometer; P – capsule regulator rod; Q – lever hinge; R – lamp burner; S – orifice to fill tank; T – capsule regulator and table; X – adjusting screw.**

Fresh air entered the egg-chamber through four small holes in the corners of the double bottom of the case. From these holes, it passed through a central hole in the upper part of the double bottom, passing through perforated zinc covered with canvas kept damp by placing its edges in a water tray. The ingoing air, passing through this damp canvas became charged with moisture, passed between the eggs and exited at the ventilating holes immediately below the tank (Sutcliffe, 1909).

Another incubator which enjoyed a fair degree of popularity, owing largely to its having the next best heat regulator, was Hillier's, in which the heat was at first supplied from the lamp chimney placed below the incubator and afterwards by radiation from a heated iron plate and tubes (Sutcliffe, 1909).

From about 1890 onward, numerous copies or modifications of Hearson's pattern were made. Several attempts at hot-air incubators with ascending ventilation were also made with some level of success, but it was not until the introduction of hot-air incubators, in which the heated air was forced *downwards*, through the eggs, that the supremacy of the hot-water type was finally challenged (Sutcliffe, 1909).

Through at least the first third of the 20th century, hot-air and hot-water incubators pretty much shared the attention of poultry farmers (Lewis, 1913; Lippincott & Card, 1921; Schowengerdt & Colorado Agricultural College. Extension, 1918). In actual efficiency, there appeared to be no difference between the two types. While the hot-water type was less sensitive to outside changes in temperature – with the advantage that when, for any reason, the lamp went out at night, there was less danger of an incubator cooling down to a dangerous degree before it was discovered – once affected it was much slower in regaining the proper temperature (Lewis, 1913; Lippincott & Card, 1921; Schowengerdt & Colorado Agricultural College. Extension, 1918). The hot-water type usually cost more for a given capacity and equal quality and there was always the possibility of the tank rusting out and leaking (Schowengerdt & Colorado Agricultural College. Extension, 1918). A strongly built copper tank, with proper care, would last many years, but a thin copper or tin tank could not be expected to last longer than two or three seasons. Sediment soon formed in the bottom of the tank, especially if hard water was used. The tank was hard to clean, and this sediment accumulated in different parts of the circuit, causing uneven radiation with variation in the temperature of different parts of the machine (Lewis, 1913). Leaky tanks were also difficult to repair (Schowengerdt & Colorado Agricultural College. Extension, 1918).

There was a common belief that hot-water incubators furnished a moister heat than hot-air machines, which appeared to explain much of the special popularity of hot-water types in arid and semi-arid sections (Jackson, 1919; Nisson, 1894). While the idea that moisture in the egg chamber was directly affected by the nature of the heating medium was erroneous, hot-water incubators probably did have less air circulation on the average, than the more adjustable hot-air machines (Jackson, 1919; Nisson, 1894). The practical effect of this was to reduce the evaporation from the egg in hot-water machines, thus giving the impression that more moisture was present in them (Jackson, 1919).

In any case, hot-air incubators seemed to be the most popular, no doubt because they required less attention, were cheaper, less complicated, and less liable to become irreparable for lack of parts (Lewis, 1913; Schowengerdt & Colorado Agricultural College. Extension, 1918). They heated up quicker, were usually easier to regulate and had no water tank to be kept filled with water or to rust out and leak in the middle of a hatch, with water soaking the machine and spoiling the eggs (Lewis, 1913; Lippincott & Card, 1921; Schowengerdt & Colorado Agricultural College. Extension, 1918). On the other hand, they did not retain heat as long as the hot-water type (Lewis, 1913; Schowengerdt & Colorado Agricultural College. Extension, 1918). They were inclined to vary in performance with room temperatures and some claimed they dried the eggs out more rapidly (Schowengerdt & Colorado Agricultural College. Extension, 1918). Still, and although there were a number of excellent hot-water incubators on the market, all things considered the hot-air type was perceived to be the safest and best (Lewis, 1913).

Incubators, according to their size and type, were also classified as individual, or small, and mammoth incubators. The small ones were composed of single compartments for the eggs, with capacities of 50 to 500, each unit being a separate machine heated by its own lamp (Lewis, 1913). Mammoth incubators, so named because of their sheer size and capacity, were first presented to the general public around 1901 (Anonymous, 1901). These machines, later known as sectional-type incubators, had a capacity of 2000 to 15,000 or 20,000 eggs and consisted of a series of sections (ordinary sized incubator units each with its own regulator, nursery, and trays) – each section operating individually but connected with the other to form a single machine, and heated by a central heating plant or boiler which conveyed heat to all the compartments by means of hot-water pipes extending above the egg trays (Figure 10) (Anonymous, 1901; Buckeye Incubator & Newtown Giant Incubator Co, 1926; Hooley, 1919; Lewis, 1913). A series of sections was known as a deck and the machines were so designed that one deck could be placed above the other, making either a two-deck or a three-deck machine. Sectional type incubators were ventilated by natural circulation of air and did not require electric current for incubation. The advantage of this type of incubator was largely its flexibility, in that the capacity of a given machine could be enlarged by simply adding new sections, up to certain limits (Buckeye Incubator & Newtown Giant Incubator Co, 1926). They also made it entirely possible to be bringing off a hatch in one compartment and just starting another one in the next one (Hooley, 1919).

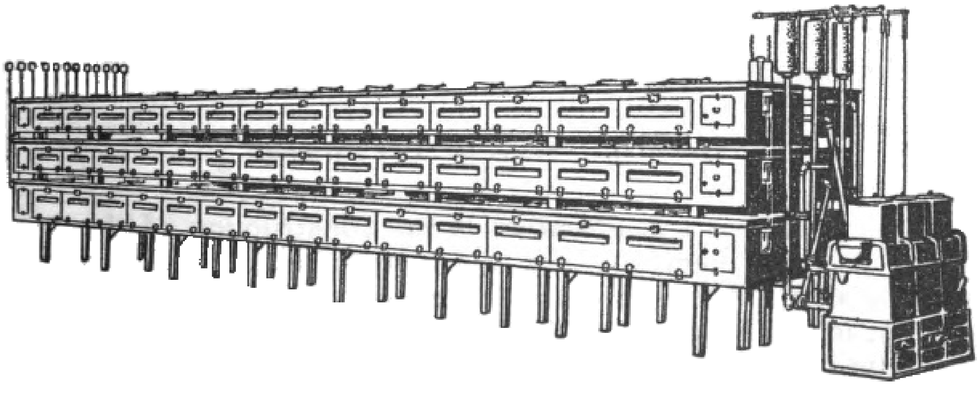


**Figure 10. Three-deck sectional mammoth incubator (Buckeye Incubator & Newtown Giant Incubator Co, 1926).**

Mammoth incubators were best adapted to large poultry plants or community centers, where there was a demand for custom-hatched and day-old chicks. In custom hatching, a compartment or a number of compartments were rented at so much per hatch – the person who hired the compartment supplying the eggs and taking the chicks – with the operator furnishing the heat and doing the work (Lewis, 1913). These community hatching centers were immensely popular in certain areas and the opening of one would merit state honors (Anonymous, 1933b).

Most incubators ranging from 80 to 400-egg capacity or more, were lamp-heated, the latter value apparently being the practical limit for machines of this type (Jackson, 1919). For larger capacities (500 to 2,500 eggs) there were incubators heated with oil (kerosene) or gas stoves; while mammoth incubators were heated with hot water, supplied from a central coal (anthracite), oil- or gas-burning boiler or heater, and furnished in any desired capacity up to many thousands of eggs (Hooley, 1919; Jackson, 1919).

Interestingly enough, in spite of all this evolution, natural incubation was still considered to be the best means of incubating eggs and producing strong and healthy chicks – those resulting from hatching via artificial means being considered frail and sickly – and so-called inventors would spend a tremendous amount of time and effort trying to imbue incubators with an animal or "henish" quality (Anonymous, 1903, 1932). Around 1903, for instance, one Mr. Boyes claimed to have "*secured a quantity of the perspiration of a large number of hens feverish with the maternal instinct...*" and found *"…that it consisted of a fatty matter, moisture, and an ethereal substance of acetic odor, with a little dust or dirt".* Boyes introduced this newly found substance in the form of "hen oil", and claimed that *"by putting a little of this curious concoction into the moisture tray under the egg drawer in the incubator, it will be automatically evaporated and diffused by the internal heat, and will do all the instinctive work of a sitting hen."* Mr. Boyes also said to have invented a tablet which would *"provide the lifeless incubator with 'animal magnetism.'"* and aided *"in bringing forth a larger percentage of healthy chicks than is at present obtainable" (Anonymous, 1903).* Although apparently miraculous, no further reports were found on the matter and knowledge of Mr. Boyes' invention was buried in the archives.

The first three decades of the 20th century also saw the first steps in the business of building/selling industrial mammoth incubators of several famous companies, such as Buckeye (the first commercial incubator company founded in Ohio, in 1882), Cyphers (founded in Buffalo, U.S.A., in 1884, first mammoths released in 1907), Jamesway (founded in Canada in 1890, first incubator released in 1915), Petersime (founded in the United States in 1912, first incubators released shortly after), Pas Reform (founded in 1919, first incubators released shortly after), and Multiplo (founded in Australia in 1933, first incubators released shortly after) (Anonymous, 2010a, 2010b, 2012; Chapple, 2012; Cyphers Incubator, 1912; Martin, 2001; Petersime, 2011).

The first forced-draught incubator was invented in 1911, by a famous American inventor called Milo Hastings (Boerjan, 2012). In this new kind of machine, hot air was forced to circulate within the incubator by means of fans instead of relying on the natural diffusion of hot air from areas of higher temperature (and hence higher pressure) to areas of lower temperature (lower pressure) used in the so-called still-air machines (Anonymous, 1923).

In 1922, Ira Petersime, an American engineer, launched the first all-electric incubator (Figure 11) (Petersime, 2011).

**Figure 11. Petersime's first all-electric incubator (Petersime, 2011).**

His company, fueled by Ira's and his brother Ray's patent designs, introduced the concept of incubator design based on scientific enquiry and statistics instead of trial-and-error, a laboratory-based approach to technological evolution that soon became popular amongst incubator-building companies (Marques, 2012; I. M. Petersime, 1925; I. M. Petersime & Petersime, 1939; R. M. Petersime, 1932, 1941).

Forced-draught electric incubators soon evolved into a new kind of machine: the cabinet-type mammoth incubator (Figure 12). As the name indicates, this type of incubator resembled a cabinet in appearance. The outstanding advantages of such machines were larger capacity in a smaller amount of space, and greater simplicity in operation. One thermometer and one regulator cared for the temperature of the entire machine. Additionally, the mechanical means for handling the eggs reduced the labor to a minimum. Electric current was required for the operation of the fans that were used to circulate the air within the incubator (Buckeye Incubator & Newtown Giant Incubator Co, 1926).

The first incubators featuring automatic, "programmable" egg-turning mechanisms also hit the major markets in the 1920's. These relied on clockwork arrangements connected to a revolving motor to initiate turning at the desired hours (Anonymous, 1925a).

These first mammoth incubators were multi-stage incubators: by repositioning the eggs during incubation, hatchery managers balanced the load in the incubators, taking into account the heat production of the eggs (Petersime, 2011).

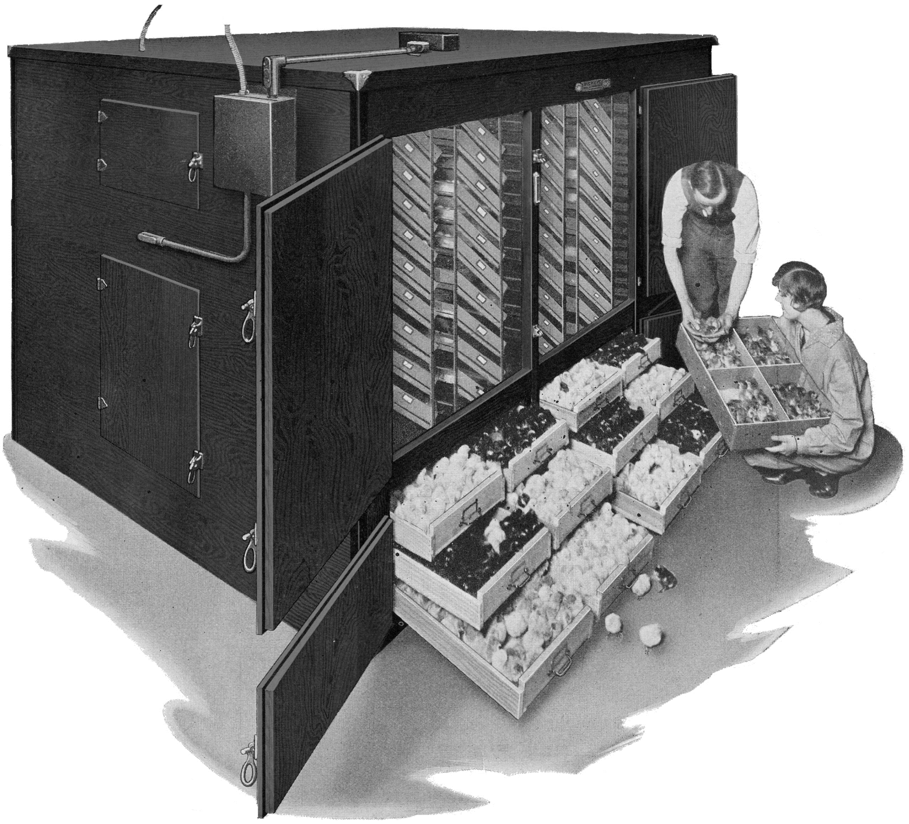


**Figure 12. Cabinet type Buckeye Electric Mammoth Incubator, Model No.46 (courtesy of Chick Masters).**

This enabled even a farmer with a relatively small number of hens to use these machines, as some required only 60 eggs daily to operate them to full capacity. One did not need to wait three weeks for every setting, as with other incubators, which entailed either the setting of stale eggs or the loss of part of the production from selected breeders. They saved their owners floor space, labor and operating expenses. The advantages of getting a convenient lot of chickens every three days against a large number in three weeks was obvious, especially when the day-old chick had to hatch at times convenient to transport trains. They were claimed to be so simple to operate that no previous experience was required and maintenance expenses were considered negligible. Some machines even offered automatic ventilation and moisture (Anonymous, 1925b).

In spite of all these advances, it was not until the 1930’s that artificial incubation became widely accepted as being far superior to natural incubation, although still with a number of handicaps, as was pointed out in The Central Queensland Herald, published in 1932: “*…There is a lower percentage of fertile eggs hatched, and a tendency for* *artificially brooded chicks to suffer a greater mortality. To put this another way, one may say that artificially brooded chicks require more care through the critical first few weeks. There is a danger, especially during the latter part of incubation, that the incubator will become overheated, due partly to the increased animal heat from the developed chicks. Lastly, the cost of equipment for artificial methods may be prohibitive in the case of smaller flocks…" (Anonymous, 1932)*

Regardless of these minor drawbacks, the artificial incubator was finally regarded as having defeated the old-fashioned hen in all matters short of laying eggs, as was stated in an article published by an unknown author in the Advocate (1936) "…*the fretful hen is at a great disadvantage. It is her pea-sized brain against the brain of a man. It is her long list of bodily ills against insensible wood and metal. It is her patient breast, probed by the feeding of a thousand vermin, against a machine which has no fear of such pests. Both practically and theoretically the modern incubator can easily outdistance the hen* (…) *In a properly constructed incubator the temperature is uniform throughout the egg chamber; there is no such thing as eggs getting out from under the hen and getting chilled. An incubator does not jump up and down on the nest and break half the eggs. The incubator does not leave its eggs, go out and get wet, and come back to finish the work of chilling the eggs. An incubator does not eat eggs, an incubator does not trample a large percentage of the chickens to death, nor start them out in life covered with vermin; an incubator does not change its mind before the twenty-one days are over, nor does it die on the nest" (Anonymous, 1936a).*

By that time, successful hatching with an incubator was already considered to be a simple matter, provided a little elementary knowledge of the principles of incubation was gained. Machines were now user-friendly and said to be "thoroughly reliable in every way" (Anonymous, 1933a). By 1934, all-electric mammoth incubators had already become commonplace in poultry farms and, in 1937, the first electric incubators appeared in which eggs were incubated in upright position and tilted instead of turned, as is common practice nowadays (Anonymous, 1937; Petersime, 2011).

The recent popularity of electrical components and machines and the spread of this resource to the various corners of developed and developing countries at the time led to the appearance of rather innovative and, for lack of a better word, quaint electrical inventions. One such invention, reported in 1936, consisted in an electric ray apparatus which was claimed to "…*show whether an egg is fertile and what the sex would be if hatched*". The machine was said to sex approximately 1000 eggs per hour; and had a clock face which would indicate infertile, male or female. It was also said to be "…*small, compact, and scientifically perfect*…", and did not require any special or expert attention (Anonymous, 1936b). No other records regarding the success or failure of this invention were found.

Quaint inventions aside, incubators evolved dramatically, although individual evolutionary steps in technology became increasingly harder to trace, due to the limited information available and corporate secrecy frequently associated with markets ruled by great companies (Petersime, 2011).

The wood that was used as the main building material for incubators was replaced with metal (Figures 13 and 14) (Marques, 2012).

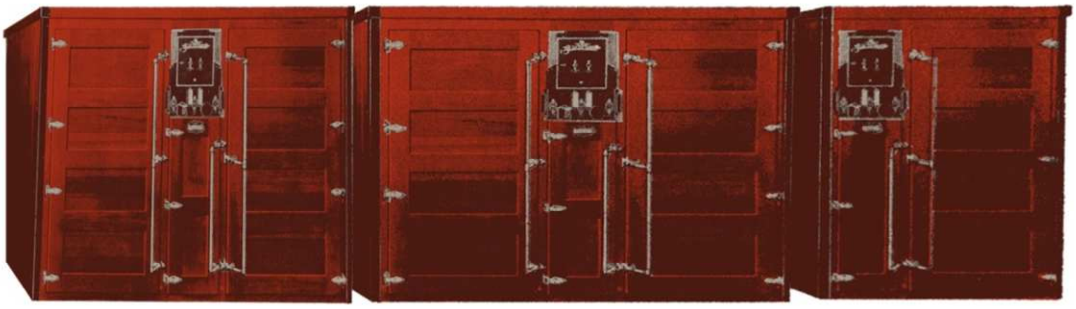


**Figure 13. Old Buckeye circa 1940 built out of wood. Middle and left units are setters while the rightmost unit is a hatcher (courtesy of Chick Masters).**

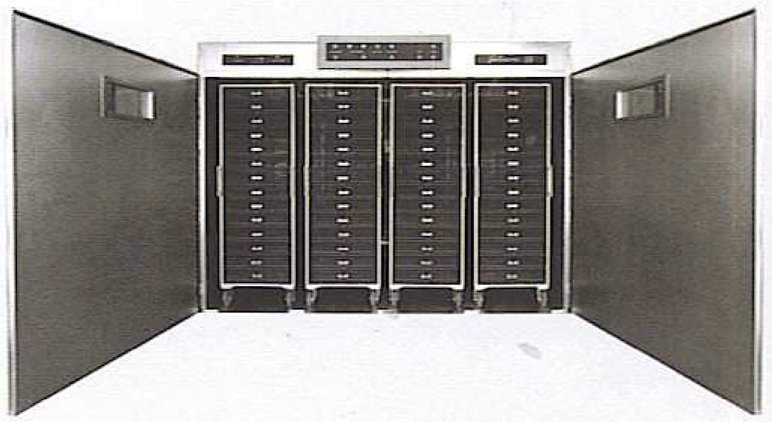


**Figure 14. Petersime incubator circa 1968, built out of metal (Petersime, 2011).**

Hatching was also separated from the setting process, allowing for differential control of temperature, ventilation and humidity. Gradually, large walk-in or drive-in forced draught incubators were introduced. These allowed for intensification and mechanization of the hatching process and thus became the cornerstones for mass production of day-old chicks (Petersime, 2011). The late 1960's saw the introduction of single-stage incubators on an industrial scale, first in Europe and later in the rest of the world (Petersime, 2011; van der Sluis, 2004a).

Single stage incubators were filled with eggs only once, so that these eggs were all virtually at the same point in their incubation process, even if some of the eggs might be chronologically several days or even a week older than others. The primary advantage of this was that all of the embryos were in need of the same environmental conditions. This differed significantly from multi-stage machines, which attempted to maintain the setter at the average temperature and humidity called for by the overall needs of newly set eggs ranging up to eggs reaching the point of transfer to the hatcher (ChickMaster, 2006).

Throughout the 1970's and 80's, an incubator was still often seen as a simple box with a fan, heater and humidifier. The trick was to put in as many eggs as possible in the hope that nearly all would hatch. During the nineteen-eighties, discussions began about whether single- or multi-stage incubation would dictate the future. Dr. Charles Deeming expressed in a 1992 issue of World Poultry that single-stage would be the future, but many in the incubation business questioned that statement (van der Sluis, 2004a). To this day, both multi- and single-stage machines are still in use, the choice between one or the other falling on the shoulders of the farmer (Petersime, 2011).

Despite the higher initial investment cost and the initially slightly lower hatchability compared to multi-stage, single-stage incubation has become the preferred method, since those disadvantages are more than compensated by better hygiene, reduced labor and avoidance of weekend work (van der Sluis, 2004a).

In multi-stage incubators, eggs at different stages of incubation share the same space within a single incubator (ChickMaster, 2006). Unincubated eggs containing heat-seeking embryos are placed alternately with eggs containing older, heat-producing embryos. Hot eggs heat the cold eggs and the energy absorbed by the cold eggs helps to keep the hot eggs from overheating. The humidity given off by the hotter eggs keeps the environment moist, so the demands on the humidification system are lower. The proximity of different ages of eggs to each other means that the air system does not have to move huge volumes of air to ensure a good mix of warm and cool air (ChickMaster, 2006). This also means that in multi-stage machines, the temperature, humidity and ventilation are set at a fixed point and the climate is "controlled" by the eggs (Boerjan, 2004, 2012). New eggs are placed regularly, once or twice a week (Boerjan, 2012). The advantage of multi-stage incubation is its simplicity, both with respect to the control system of the incubator and in the management of incubation. The main disadvantage, however, is that the multi-stage incubation environment cannot, by its nature, create optimum conditions for every egg set. Consequently, in multi-stage incubation, it is impossible to optimize both hatchability and chick quality, especially when dealing with variable egg quality (Boerjan, 2004). The need for humans to work for fairly long periods in a warm and humid environment and the difficulty of cleaning the entire incubator at one time constitute further disadvantages (ChickMaster, 2006). "Older" eggs are not only a source of heat. They are also a source of microorganisms, like bacteria or fungi, which can contaminate the “younger” eggs. Add to this the risk of exploding or gaseous eggs, and that contamination early in life may have lasting implications, leading to contaminated broilers with decreased performance and higher mortality (and, ultimately, contaminated meat products). Thus, from a hygiene and food safety point of view, the multi-stage incubator becomes a source of contamination, which may lead to economic losses at hatchery level due to lower hatching rates and chick mortality (Boerjan, 2012).

In single-stage incubators (Figure 15) all of the embryos are in need of the same environmental conditions (Boerjan, 2012).

**Figure 15. Modern single-stage incubators (ChickMaster, 2012; PasReform, 2012). Right-side – Chick Master Avida S series model; Left-side – Pas Reform SmartSet$^{TM}$ model.**

Single-stage incubation is based on climate-control technology, geared specifically to meeting the demands of the growing embryo. The incubator climate controller provides the embryo with heat and cooling as required. Set points of temperature, relative humidity and ventilation are adjusted, according to embryonic age. The fact that all eggs are taken into and out of the incubator at the same time also allows for the implementation of all-in-all-out policies. This means that each incubator can be emptied, sanitized and disinfected every 18 days and that each new batch of eggs is placed in empty, disinfected incubators (Boerjan, 2012).

During the last decade, constant efforts have been invested in optimizing single-stage incubation of modern high-yield breeds, targeting the improvement of hatchability, chick quality and uniformity and, consequently, post-hatch performance (growth and feed conversion). As a result of this, major companies have focused their attention and studies in the idea of adapting incubator environment to the ever-changing needs of developing embryos. An example of this is patent in Petersime’s so called “bio-response incubation”. Bio-response incubation$^{TM}$ consists of a method of incubating each flock by measuring the embryo’s physiologic parameters and constantly and interactively optimizing the incubation environment by controlling ventilation, humidity and temperature according to the embryo's particular needs (Barnwell, 2007; Degraeve, 2009). In other words, by developing better sensors to take measures closer to the embryo and reacting appropriately, the embryo's needs can be fulfilled in an interactive and immediate way (Degraeve, 2009).

Another example of such efforts is Pas Reform's concept of "circadian incubation". Circadian incubation™ is a single-stage protocol that includes periodic stimulation, by increasing temperature during certain sensitive periods of embryonic development. This is based on the idea that during natural incubation, the embryo is exposed to a daily rhythm, with the hen routinely shifting incubation conditions with its coming and going to and out of the nest, and that these daily changes adapt the embryos to certain stress factors (high or low temperatures, for example), improving robustness and delivering better performance later in life. In contrast to nature, embryos hatched in a conventional incubator are not exposed to a daily rhythm. The Circadian Incubation™ technique proposes to change this (PasReform, 2010).

Regardless of which technologies and incubation protocols are supported and advertised by whom, nowadays, artificial incubation is focused on the embryo and what is known about the embryo's metabolic needs and responses to changes in the incubation environment is currently being used to optimize and manipulate embryonic growth. The main challenge now lies in optimizing embryonic development for each egg at every stage of its development. This means providing every one of a very large number of eggs in a closely packed environment with optimal conditions for their development in an adaptive and "interactive" manner (PasReform, 2010).

As the art of incubation gives way to the science in incubation, large companies secure the collaboration of major universities, ensuring a constant flow and production of knowledge that can be used to better understand the physiology of embryonic development and optimize machines accordingly. Embryologists, poultry-integration experts, hatchery-management specialists, electro-mechanical engineers and industrial designers now form multidisciplinary teams to decipher the future of artificial incubation, trying to find the key to this mystery deep within the egg, flowing through the veins of the embryo (van der Sluis, 2004b).

## Artificial Incubation in Modern Egypt

Curiously enough, the ancient Egyptian artificial incubation techniques that captivated so many people throughout history is still kept alive to this day, providing a cheap source of animal protein in rural areas of Egypt (Abd-Elhakim, Thieme, Schwabenbauer, & S. Ahmed, 2009).

Most traditional hatcheries are dome-shaped structures. The foundations are made of red brick, while the rest of the building is made of sun-dried mud bricks, which assist with isolation and temperature regulation. The hatchery is divided longitudinally into two sections of egg houses (or ovens) with a midpassage (called the *qasaba*) between them. The *qasaba*'s floor is concrete, to facilitate cleaning and disinfection, and is covered with a layer of wood shavings where the hatched birds are left until dispatch to dry and fluff out. The shavings minimize leg deformities resulting from the birds slipping on the smooth floor (Abd-Elhakim et al., 2009).

On each side of the *qasaba*, there are 3 to 13 egg houses, each approximately 3.5 m wide and long, and 3 m high. Each egg house (*bait* or oven) is divided into two levels (upper and lower units) by a wooden platform (Figure 16) (Abd-Elhakim et al., 2009).

**Figure 16. Insights into the upper unit of egg house featuring a central manhole to the lower unit (Images graciously provided by Prof. Hafez, Mohammed).**

Each unit has a separate entrance (called the *moadem*) and a trap door (the *friz*) between the upper and lower units that allows workers to slip from one unit to the other to set and turn the eggs. The floor of each unit is covered with a layer of wood shavings and either straw or a plastic carpet (called the *lasa*). These protect the eggs from damage during the turning process and prevent the hatched birds from slipping (Abd-Elhakim et al., 2009).

The roof of each egg house is humped in shape with a central hole (called the *razona*) for ventilation and heat regulation (Figure 17) (Abd-Elhakim et al., 2009).

Two or three rooms connected to the hatchery provide workers with rooms for resting and cleaning, as well as for storage. Most hatchery workers have obtained none or only a low level of education (Abd-Elhakim et al., 2009).

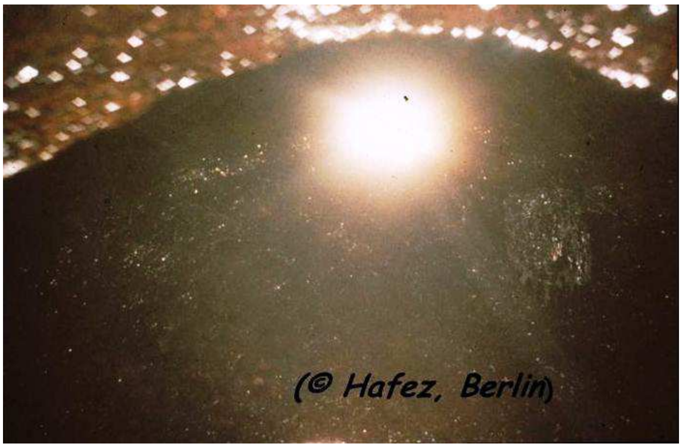


**Figure 17. Ventilating hole in egg oven. (Image graciously provided by Prof. Hafez, Mohammed)**

A large hatchery of at least eight egg houses needs two workers, and a smaller hatchery needs a chief worker and an assistant (Abd-Elhakim et al., 2009).

Recently-built traditional hatcheries are made of red brick, with walls lined on both sides with gypsum, which acts as a heat insulator; the ceilings are flat and made of wood. All other features and dimensions are the same as for older traditional hatcheries (Abd-Elhakim et al., 2009). Some hatchery owners have converted the traditional hatcheries' rest and storage rooms into egg setting and hatching units similar to those in modern hatcheries. Eggs are set on metal trolleys, each carrying about 5,000 chicken eggs. Depending on the size of the room, it may contain four or more trolleys. All the eggs on one trolley are turned manually at the same time, using a metal bar. Heat is generated by flaming petrol gas and temperatures are controlled by a thermostat, which activates the gas in suboptimal temperatures or operates a suction fan when the temperature becomes too high; relative humidity is measured by a hygrometer. The hatching stage takes place in a separate room, to which eggs are transferred in plastic or metal baskets three days before hatching (Abd-Elhakim et al., 2009).

Traditional hatcheries usually operate throughout most of the year, with a break of one to three months for rest, thorough cleaning and disinfection, usually starting in September or December (Abd-Elhakim et al., 2009).

Hatching of the eggs starts in the upper unit. Later, the eggs are either moved to the lower unit or divided equally between both units. The units are warmed for 12 days. Warming is stopped when the embryos' organs are complete and the embryos are producing enough of their own internal heat to continue the incubation process (Abd-Elhakim et al., 2009).

No instruments are used to measure either temperature or humidity. The hatchery worker judges when the egg has reached the proper temperature by placing it in his/her eye socket (Figure 18). Excessive egg temperature is reduced by spraying the eggs with warmed water from a perforated plastic bottle or the mouth (Abd-Elhakim et al., 2009).

**Figure 18. Workmen measuring egg temperature and turning the eggs (Images graciously provided by Prof. Hafez, Mohammed).**

The eggs are turned by hand two to four times a day, until two to three days before hatching (Figure 18). The process of candling is used to identify infertile and/or non-viable eggs at five to seven days of incubation, using a simple wooden box – the candler – with an electric lamp inside and a small hole in one side to emit light. Working in the dark, the hatchery worker holds each of the eggs up to the light of the candler to observe its contents. Hatched birds are transferred to the *Qasaba* to dry and await dispatch (Abd-Elhakim et al., 2009).

Although traditional hatcheries succeed in incubating hatching eggs and providing a low cost source of animal protein in rural areas, neither their structures nor their practices provide either the bio-exclusion or biocontainment that can be relatively easily applied to modern hatcheries as we know them (Abd-Elhakim et al., 2009).

# INDUSTRIAL INCUBATION AND HATCHING PROCESS

In order to understand the different risks to which egg, embryo and chick are exposed through their journey into and through the hatchery, it is necessary to have a basic understanding of hatchery operations and layout. Hatcheries are designed so that work and egg flow patterns contribute to biosecurity – dividing space into clean and dirty areas which must be separated to avoid cross-contamination – and built so that hatching eggs enter one end of the building and chicks leave from the other end, without ever crossing paths (Iowa State, 2013). Figure 19 shows an example of hatchery design (layout will vary according to size and specifications of the hatchery owner), presented here to facilitate comprehension of the egg's journey through the various stages described below.

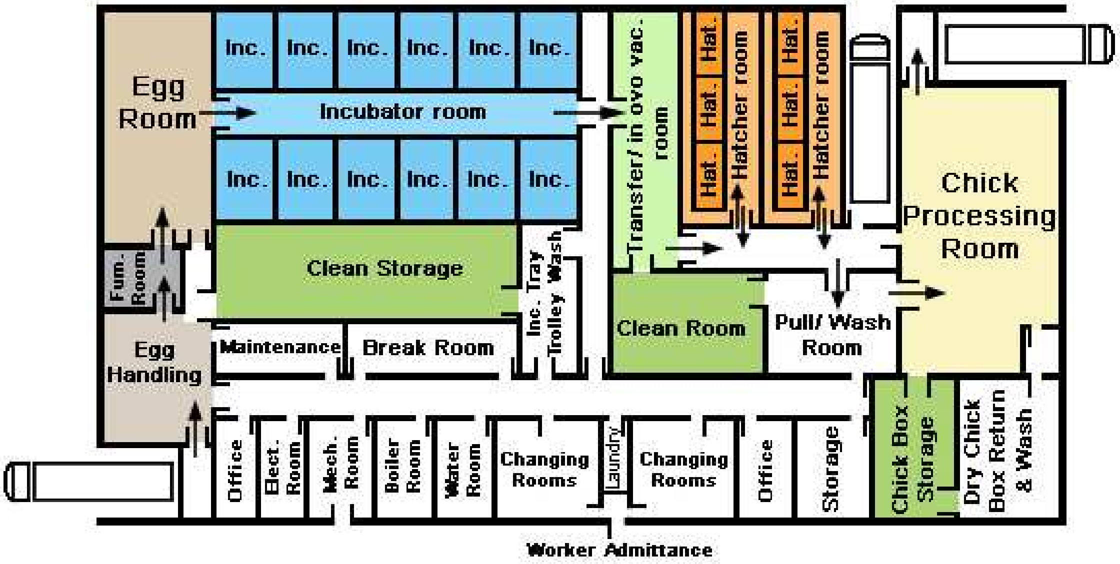


**Figure 19. Example of a simple hatchery layout [drawn from (Mahfudz, 2015)].**
**The arrows depict the movement of eggs and chicks through the building. Note how all processes flow in a single direction, reducing cross-contamination between newly arrived eggs and newly hatched chicks.**

## Breeder Farm

Embryonic development begins while the egg is still in the oviduct, stopping (or rather, pausing) as the egg cools, at the time of oviposition. At this point, the number of embryonic cells will not increase, but only decrease with each challenge or aggression the diapause embryo faces before incubation takes place. For that reason, the preservation of hatchability should be seen as starting at the breeder farm, with the proper collection and handling of eggs (Iowa State, 2013).

Frequent collection of eggs reduces the probability of their contamination with bacteria from feces or nest materials, and prevents overheating or chilling of the eggs, in summer and winter, respectively (Iowa State, 2013; ISA, 2009). Clean nest eggs should be collected first and submitted to the hatchery for incubation, while dirty nest eggs, cracked eggs, and floor eggs should be collected and packaged separately, since they are not suitable for incubation. Eggs are then moved into an air-conditioned room for storage until they are picked up and taken to the hatchery (Iowa State, 2013).

## Reception and Processing at the Hatchery

After arrival at the hatchery, the eggs are unpacked and processed before being set into trays (in vertical position) (Iowa State, 2013). Figure 20 shows an example of this selection. Processing may (and should) involve a second selection (or first if no selection is carried out at the breeder) so as to eliminate eggs that failed to be discarded at the breeder farm (Hill, 1998). This eliminates eggs that are less likely to hatch and produce quality chicks, as well as eggs contaminated with yolk, dried blood, or feces, which pose a biological threat against their cohorts (Tullett, 2008). Candling may also be used in order to more easily discard eggs fractured during transport. Since many hatcheries feature automated machinery (and since size and shape are relevant for hatchability), performing egg calibration during selection is also desirable (ISA, 2009). In addition, a sample of eggs can be taken from a batch in order to estimate its fertility rate (Tullett, 2008).

**Figure 20. Selection of eggs suitable for incubation and eggs set vertically in incubation trays after selection (Images graciously provided by DVM Menezes, António).**

## Disinfection

As soon as the eggs are set in the trays in which they will be incubated, sanitation should take place. Disinfection aims to kill bacteria, molds and some viruses on the eggshell to prevent these microorganisms from penetrating the eggshell (D. C. Deeming, 1998). This is best done in a special designed room between the receiving/processing room and the egg storage room, so that only sanitized eggs may enter the clean area of the hatchery (ISA, 2009). Fumigation with formalin is commonly used to disinfect eggs (D. C. Deeming, 1998; Iowa State, 2013; ISA, 2009).

## Clean Storage

After processing and sanitization, the egg trays are moved (in wheeled trolleys) into a clean, environmentally controlled storage area (Figure 21), where they will be held and concentrated (if they are not in sufficient number to fill an incubator cabinet or if a large order of chicks has been placed), awaiting incubation (Iowa State, 2013; Mayes & Takeballi, 1984).

**Figure 21. Eggs stored in trays and trolleys prior to incubation (Images graciously provided by DVM Menezes, António).**

## Pre-Warming

There is advantage in pre-warming eggs before incubation, as storage rooms tend to be under cool temperatures, in order to better preserve stored eggs (Shaver, 1980). Pre-warming eggs prior to setting is advisable to avoid temperature shock to the embryo and prevent condensation of moisture on the external surface of the egg’s shell (which facilitates microbial contamination) (R. Jones, 1970). Thus, a separate, dedicated pre-warming room may be included in the hatchery layout (as there is no interest in raising the temperature in the whole egg storage room, where eggs not yet to be incubated would suffer detrimental oscillations in temperature) (Iowa State, 2013).

## Incubator

After pre-warming, trolleys are moved to a designated incubator unit (or setter), where they will remain for the first 18 days of embryonic development (Figure 22) (ISA, 2009).

**Figure 22. Incubator room and egg trolleys for machine loading (Images graciously provided by DVM Menezes, António).**

In single-stage incubators, each incubator unit will be completely filled with a single load of eggs only once per incubation cycle and completely vacated by day 18. In multi-stage incubators, each unit may be loaded multiple times, with smaller amounts of eggs, during its operation period, so that eggs at different stages of the incubation cycle co-exist at every moment in operation. This also means that, in multi-stage units, the incubator is progressively unloaded as each batch of eggs reaches day 18 of embryonic development and that *all-in-all-out* operations are impossible to carry out (Boerjan, 2012).

Either way, all eggs are moved to the hatcher before the end of incubation, and hatching does not occur in these units (Iowa State, 2013; ISA, 2009).

## Transfer

At 18 days of incubation, the egg trolleys are taken out of the incubator unit and moved to a transfer room, where the eggs are removed from their trays and set (horizontally) in hatching baskets (Iowa State, 2013). In some settings, the process is almost fully automated and machines may be installed to candle eggs, remove infertile or early dead eggs and perform *in ovo* vaccination, before transferring the eggs into the hatcher baskets (Figure 23) (Iowa State, 2013; Tullett, 2008). Removal of these non-viable eggs has the advantage of reducing exposure of live chicks to potential sources of pathogenic bacteria after hatching (Tullett, 2008). *In ovo* injection systems are routinely used for administration of vaccines to protect broiler chickens against Marek’s disease, infectious bursal disease, and fowl pox (Giamborne, 1997; Iowa State, 2013; Sharma, Zhang, Jensen, Silke, & Yeh, 2002).

**Figure 23. Automated egg transfer machine (Images graciously provided by DVM Menezes, António).**

## Hatcher

After transfer procedures are completed, the egg baskets are moved into the hatcher unit. It is in the hatcher that the eggs will spend the remaining three days of the incubation cycle, until the chicks break the shell and emerge from the eggs (Iowa State, 2013). Hatchers are kept in a separate room in order to isolate the down, egg debris, and microorganisms generated during hatching from the rest of the hatchery rooms (Iowa State, 2013; R. Jones, 1970).

## Chick Processing

As soon as most of the chicks have hatched, the hatching baskets are removed from the hatcher and transferred to the chick processing room. Here, chicks are separated from broken shells and hatching debris (either by hand or automated machinery), graded, sexed and culled, if necessary. After being separated from hatching tray debris, chicks are graded into first quality or culls. Chicks that possess anatomical deformities, partially closed navels, crooked toes, excessively wet down, or twisted legs are culled. The gender of day old chicks can be determined by feather sexing or vent sexing (Iowa State, 2013). Chicks are also counted, weighed and vaccinated (Figure 24) (Giamborne, 1997; Tullett, 2008). Spray vaccination of day-old chicks in the hatchery is commonly used for mass administration of vaccines against Newcastle disease and infectious bronchitis (Giamborne, 1997; Iowa State, 2013).

After vaccination, the chicks are placed in boxes for dispatching (Iowa State, 2013). This is illustrated in Figure 24.

**Figure 24. Automated chick counting and chick packaging prior to dispatch (Images graciously provided by DVM Menezes, António).**

## Holding and Dispatch

After being placed into chick boxes, the chicks are moved into the chick holding room where the flock to be delivered is assembled and then transferred to the delivery vehicles (Hoad, 1983; Iowa State, 2013; Meijerhof, 1997).

# NORMAL STRUCTURES OF THE EGG AND THEIR INTERACTIONS

## Vitellum (yolk)

The part of the egg commonly known as the yolk is actually a single cell, the female sex cell (Patten, 1971). The ovary is the site of gathering of the primordial germ cells in the embryo, which are later transformed into oocytes and, after being covered with the granulosa and theca layers, in follicles (Figure 25) (Mine & Kovacs-Nolan, 2004).

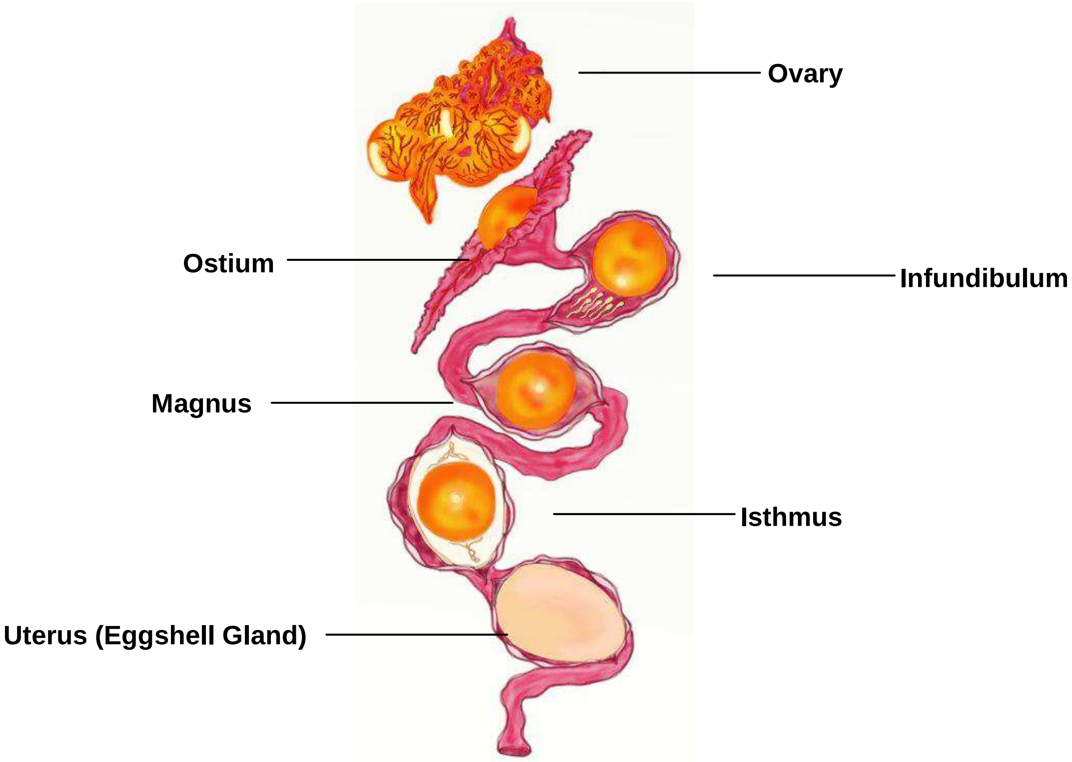


**Figure 25. Schematic representation of the hen's oviduct during egg formation [drawn from (Bellairs & Osmond, 2005)].**

The ovary of the laying hen contains several million follicles of varying size (depending on if and how much yolk they possess), of which several thousand are macroscopically visible (Bellairs & Osmond, 2005; Gilbert, Perry, Waddington, & Hardie, 1983; Patten, 1971). In a hen that has gone "off-lay", no large oocytes are visible, though small ones measuring up to about 5 mm in diameter are still plentiful (Bellairs & Osmond, 2005). In a full laying life of about 8 years, a laying hen may produce up to about 1500 eggs. In common with other vertebrates, therefore, the hen produces more oocytes than are required and eliminates the excess through atresia. Atresia usually occurs only in the smaller follicles, long before they reach ovulable size. Consequently, the hierarchy, which determines the number of follicles which can be ovulated in any period, is the product of the number of follicles produced for growth (those about 1 mm) and the rate of atresia during the increase in size up to about 8 mm. (Gilbert et al., 1983)

In the chicken, the growth of individual oocytes (vitellogenesis) can be divided into three phases: first, the growth of young oocytes (60-80µm) to ~3 mm in diameter is characterized by the absence of typical (yellow) yolk; second, several of these latter oocytes begin a slow growth phase, then involute and become atretic; third, one of the second-phase oocytes (diameter, 6-8 mm) undergoes an extremely rapid final growth phase, to a size of ~35 mm, followed by ovulation (Gilbert et al., 1983; Shen, Steyrer, Retzek, Sanders, & Schneider, 1993).

When the oocyte is about 2.5 mm in diameter, the inner layer of the vitelline membrane is laid down, but the outer layer is not formed until after ovulation. By the time the oocyte is about 25 mm in diameter, the inner layer has reached its maximum thickness of about 2.5 mm. In the final stage of oocyte growth, large vacuoles appear beneath its cell membrane (oolemma) and the number of villi at the surface decreases (Bellairs & Osmond, 2005).

The process is under complex hormonal control from gonadotropins produced by the anterior pituitary gland. An ovulation-inducing hormone (OIH), which is thought to correspond in many ways to the luteinizing hormone of mammals, is released by the pituitary. If there is a sufficiently mature follicle available, it will respond to the OIH by ovulating within about 48 hours. Progesterone also plays a role, probably acting through the hypothalamus and thus controlling the release of the OIH by the pituitary (Bellairs & Osmond, 2005).

While the egg cell is still in the ovary, the materials which will be later used as food by the embryo are brought in through the blood vessels of the theca, absorbed by the follicular cells, transferred by them to the oocyte and deposited in its cytoplasm. These are then elaborated into deutoplasm, a viscid fluid in which granules and globules of various sizes are suspended. As the deutoplasm increases in amount, it forces the nucleus and the cytoplasm toward the surface so that eventually the deutoplasm comes to occupy nearly the entire cell. This abundance of deutoplasm accumulated in the ovum provides a readily assimilable food supply, making possible the extremely rapid development of the chicken embryo (Patten, 1971).

The region of the ovum containing the nucleus and the bulk of the active cytoplasm is known as the animal pole because it subsequently becomes the site of greatest protoplasmic activity. The region opposite to the animal pole is called the vegetative pole because, while material for growth is drawn from this region, it remains itself relatively inactive. Enclosing the ovum is a thin non-cellular membrane, the vitelline membrane, which is a secretory product of the cytoplasm of the ovum (Patten, 1971).

The bulk of the ovum itself is made up of the yolk, which is deposited in concentric spheres around the periphery of the oocyte (Lipar, Ketterson, Nolan, & Casto, 1999; Patten, 1971). Most yolk deposition occurs rapidly over a period of 7 to 9 days just prior to ovulation (Lipar et al., 1999; McIndoe, 1959) .

Despite its apparently homogeneous appearance to the naked eye, the egg yolk possesses a well-defined structure, consisting of concentrically arranged bands of yellow and white yolk (Bellairs, 1961; Bellairs & Osmond, 2005). The banding is thought to correspond to a diurnal rhythm in the formation of the yolk, the wide yellow bands being laid down during the day, the narrower white bands during the night (Bellairs, 1961; Bellairs & Osmond, 2005). Its center is marked by a ball of white yolk, the latebra, and from this a column of white yolk, the neck of the latebra extends upwards to join with a disc of white yolk, the nucleus of Pander, which lies beneath the blastoderm (Bellairs & Osmond, 2005; Halsted, 1884; Sutcliffe, 1909). The latebra is surrounded predominantly by yellow yolk. When the blastoderm is dissected from the yolk, the nucleus of Pander is left behind and can be mistaken by the inexperienced worker for the blastoderm itself (Bellairs & Osmond, 2005).

Yellow pigments are present in the yellow yolk but not in the white (Bellairs, 1961). The yolk, both yellow and white, is an emulsion, an aqueous fluid in which float yolk spheres (also known as globules or yolk droplets consisting, at least in part, of fat) and lipid droplets (known also as yolk granules), forming the dispersed phase (Bellairs, 1961; Bellairs & Osmond, 2005). There are two main types of yolk sphere, the yellow yolk sphere (found in yellow yolk) and the white yolk sphere (found in white yolk). Both contain structures called subdroplets, but those of the white yolk spheres are highly refractive, larger and less abundant, whereas those of the yellow yolk are smaller, more numerous and less refractive (Bellairs, 1961).

Yolk consists of approximately 48% water, 33% lipid, 17% protein and 1% carbohydrates (Bellairs, 1961; K. Mann & Mann, 2008; Milinsk, Murakami, Gomes, Matsushita, & de Souza, 2003). The raw materials from which yolk is manufactured are formed in the liver of the laying hen, under the control of estrogen, transported in the blood plasma and transferred into the ovary via receptor-mediated endocytosis of low-density lipoprotein (LDL) apoproteins (apo) (Bellairs & Osmond, 2005; K. Mann & Mann, 2008; Nielsen, 1998; Vieira et al., 1995). It is well known that a hen’s diet influences yolk composition and that, through dietary manipulation, certain phytochemicals with important health benefits can be enriched in egg yolk (Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2011).

## Proteins and lipids

The growth of oocytes in oviparous species requires the rapid, efficient, and specific transport of large amounts of exogenously synthesized macromolecules across the plasma membrane. In the chicken, in the last phase of oocyte development, this single cell achieves an uptake of up to 2 g of protein per day, with a concomitant increase in volume by a factor of ~3500 (Shen et al., 1993).

Large quantities of lipids and proteins that closely resemble (and are often identical to), similar substances in the yolk are present in the blood of the laying hen but absent in the blood plasma of male or juvenile birds nor of hens that have gone "off-lay" (Bellairs & Osmond, 2005). Because there is no real synthesis of yolk in the oocyte but merely a rearrangement of materials, the substances that pass through the capsule of follicle cells into the oocyte are the definitive proteins and lipids from which the yolk is formed (Bellairs & Osmond, 2005).

The major yolk components of the chicken ovum are vitellogenin (VTG) derived proteins, and very low density lipoprotein (VLDL), which mainly functions in the transport of triacylglycerols, phospholipids, and cholesterol (Shen et al., 1993; Vieira et al., 1995). The plasma levels of VLDL and VTG increase dramatically and the apolipoprotein composition of VLDL changes at the onset of egg laying in hens, in response to estrogen (Vieira et al., 1995).

Vitellogenins I, II and III, which are stable phosphoglycolipoproteins, are constituents of blood high density lipoprotein (HDL) particles and are cleaved on entering the oocyte to yield mature proteins (Bellairs & Osmond, 2005; K. Mann & Mann, 2008; Vieira et al., 1995). The function(s) of the HDL in the oocyte's yolk is unknown. Considering the inability of the avian ovary to synthesize lipids, yolk HDL might be a source, in particular, for phospholipids in the very demanding biosynthesis of surface membrane in the growing oocyte or in the very early stages of embryo development. Its localization in the compartment of yolk that appears to be phagocytized by early embryonic cells might point to a source of lipid for utilization by these cells (Vieira et al., 1995).

Cleavage of VTG-I and II in yolk produces the lipovitellins I and II, and phosvitins. These proteins are established components of the water-insoluble yolk granular lipoprotein (K. Mann & Mann, 2008). The expression of VTG-II in the liver is stimulated by estrogen at the onset of laying and can also be induced in roosters by a single injection of estradiol (Speake, Murray, & Noble, 1998).

Lipovitellins I and II (or α and β) are lipoproteins which constitute the main non-aqueous component of the yolk and are therefore an important component of the yolk granules (Bellairs & Osmond, 2005; Speake et al., 1998). Phosvitin is the second most plentiful constituent of the yolk granules (Bellairs & Osmond, 2005). It is a highly phosphorylated protein, containing 6.5% carbohydrates, that binds iron, an ability for which it is considered a storage source of iron in the yolk, presenting it in the ferric form to the developing embryo (Bellairs & Osmond, 2005; Jiang & Mine, 2001; Speake et al., 1998).

Yolk components, such as polyunsaturated lipids, are very stable in spite of the presence of oxygen in yolk. This has been attributed to metal-binding proteins, such as phosvitin and ovotransferrin. These metal chelators remove free metal ions which could catalyze the production of reactive oxygen species (ROS) and oxidize yolk lipids (K. Mann & Mann, 2008).

The source of yolk LDL is blood VLDL. Yolk LDL apoproteins are also synthesized in the liver and transported in blood to the ovary as constituents of very low density lipoprotein particles (K. Mann & Mann, 2008). The main proteins in this class are the apovitellenins, AI and AII (Bellairs & Osmond, 2005; K. Mann & Mann, 2008).

A second major apoprotein of yolk LDL is apoprotein B. During or after the transfer from blood to yolk, the major apolipoprotein of VLDL, apoprotein B (apo B), is cleaved into several fragments, the apovitellenins III–VI (K. Mann & Mann, 2008). In contrast to these modifications to the protein components, the lipids of the VLDL and vitellogenin are deposited in the yolk essentially unaltered (Speake et al., 1998).

The major egg yolk proteins and lipoproteins are thus distributed among two yolk compartments: the plasma contains the water-soluble fraction, which mainly consists of α-livetin (serum albumin), β-livetin (α2-glycoprotein), and γ-livetin (IgY) (Bellairs & Osmond, 2005; K. Mann & Mann, 2008); the granular (or globular) fraction contains lipovitellins as components of high-density lipoprotein (HDL), phosvitin, and low-density lipoprotein apoproteins (K. Mann & Mann, 2008).

In addition to major proteins, yolk was reported to contain inorganic ions and many enzymes, which were often characterized by their activity only. Important exceptions were cathepsin D, which was suggested to be a key enzyme in yolk protein precursor cleavage and protein maturation, and aminopeptidase Ey (Bellairs & Osmond, 2005; K. Mann & Mann, 2008).

### Hormones

Avian eggs contain a variety of hormones. In female birds, the ovary is the main source of sexual steroids, and the three cell theory suggests that the granulosa cells are the predominant source of gestagens, which are used by the theca interna to produce androgens that are subsequently metabolized to estrogens by the theca externa. During the course of follicular maturation the steroidogenic capacity of the cells changes, which results in the production of different amounts of hormones during egg development. At later stages, aromatase activity decreases and estrogens are no longer produced. In the large preovulatory follicle cells, the enzymatic activity shuts down completely, resulting in an accumulation of progesterone and the preovulatory progesterone peak in the plasma. Besides entering the bloodstream, from where they signal the current reproductive status to the rest of the body, a share of the ovarian hormones also ends up in the yolk, by either passive diffusion or a still unknown mechanism (Rettenbacher, Mostl, & Groothuis, 2009).

The distribution and proportion of steroid hormones found in the yolk of freshly laid, non-incubated eggs strongly reflect the metabolic activity of the ovarian cells. If the yolk is analyzed in concentric layers, gestagens are found mostly in the outer layers, androgens in the middle layers and estrogens in the center of the yolk (Lipar et al., 1999; Rettenbacher et al., 2009). Overall concentrations in the yolk are highest for gestagens and lowest for estrogens, whereas androgens are found in intermediate concentrations (Rettenbacher et al., 2009).

### Antibodies

Birds transmit maternal antibodies to their offspring by depositing them in the egg (Hamal, Burgess, Pevzner, & Erf, 2006; X. Li et al., 1998). There are 3 classes of antibodies in chickens: IgY (IgG), IgA, and IgM (Hamal et al., 2006; X. Li et al., 1998). Chicken IgA and IgM are similar to their mammalian homonyms in terms of molecular weight, structure, and immunoelectrophoretic mobility. Although structural differences exist between IgY and mammalian IgG, IgY is considered the avian equivalent to mammalian IgG. In eggs, IgY is present predominantly in the egg yolk (and actually present in greater concentration here than in the hen’s serum), whereas IgA and IgM are present in the albumen as a result of mucosal secretion in the oviduct (Hamal et al., 2006). In chickens, the transfer of IgY from the hen to her offspring takes place in a 2-step process. In the first step, IgY is taken from the hen’s blood into the egg yolk by the IgY receptors on the ovarian follicle (Hamal et al., 2006). In the second step, IgY is transferred from the egg yolk to the offspring via the embryonic circulation (Hamal et al., 2006). IgY is continuously absorbed by the embryo during embryogenesis until the second day after hatching (X. Li et al., 1998).

Although IgA and IgM are predominantly egg white Ig there is transfer of IgA and IgM antibodies from the egg white into the non-incubated egg yolk in very low concentrations (Hamal et al., 2006).

## Vitamins and antioxidants

Avian embryo development is associated with a progressive accumulation in certain tissues of lipids which are rich in polyunsaturated fatty acids (PUFA), making such tissues vulnerable to free-radical attack and lipid peroxidation. Antioxidant systems are expressed in the embryonic tissues and are responsible for maintaining antioxidant protection during different stages of embryonic development, in particular during hatching, when oxidative stress is highly pronounced (Surai, Speake, & Sparks, 2001).

The antioxidant system of the developing embryo includes vitamins A, E and C, carotenoids and antioxidant enzymes superoxide dismutase, glutathione peroxidase and catalase. These antioxidants probably act synergistically with each other, performing their protective effects against lipid peroxidation and preserving cell membrane integrity (Surai et al., 1998). Of the three vitamins mentioned above, vitamin E is the most important fat-soluble, chain-breaking antioxidant in cell membranes (Blount, Houston, & Møller, 2000; Surai et al., 1998).

In general the main bulk of vitamin A transfer to the embryonic liver from the egg yolk takes place at an earlier stage of incubation than that of vitamin E. Taking into account the vitamin A concentration in egg yolk it is possible to calculate that the vitamin A accumulated in the hen’s liver is sufficient to supply more than 100 eggs. Conversely, with each egg, a hen releases an amount of vitamin E which is more than 2 times greater than its hepatic reserve of the vitamin (Surai et al., 1998).

The yolk is also considered to be an important source of vitamin D, since in addition to cholecalciferol it contains significant amounts of vitamin D’s 5 times more active hydroxylated metabolite, 25-hydroxycholecalciferol (Mattila, Lehikoinen, Kiiskinen, & Piironen, 1999).

Carotenoids are biologically active pigments responsible for the typical yellow-orange colors of the egg yolk. Since they can only be synthesized *de novo* by plants, certain bacteria and fungi, laying birds must obtain the large quantities they need to transfer into egg yolk either directly from the diet or, in conditions of low dietary availability, from endogenous reserves (Blount et al., 2000; Karadas, Pappas, Surai, & Speake, 2005). Carotenoids are efficient, natural lipid-soluble quenchers of singlet oxygen and scavengers of free radicals (Karadas et al., 2005). They reduce lipid peroxidation in the vulnerable, lipid-rich tissues of developing embryos and chicks, thus protecting the cell membranes and/or cell functions of important organs, and the passively acquired antibodies, against damage (Blount et al., 2000; Karadas et al., 2005).

The developing avian embryo relies on the oxidation of fatty acids from the lipid-rich yolk for almost all of its energy requirements. Yolk-derived fatty acids also serve specific functions in developing tissues; for example, to ensure optimal development and function of the brain and retina (Blount et al., 2000). This means that, although embryonic tissues depend on a rich substrate of oxidizable, unsaturated fatty acids, their abundance makes the tissues highly susceptible to peroxidation caused by reactive oxidative metabolites and by free radicals, which are produced as normal by-products of metabolism, that damage cell membranes and cell function, inducing DNA breakage and, thus, mutation (Blount et al., 2000; Karadas et al., 2005).

The risk of lipid peroxidation will probably increase as embryonic growth proceeds because of the accelerating rate of oxygen diffusion through the shell to fuel metabolism, and, ultimately, because of the rapid exposure to high concentrations of atmospheric oxygen (and the onset of pulmonary respiration) at hatching (Blount et al., 2000; Karadas et al., 2005; Surai, Speake, Wood et al., 2001). Large amounts of antioxidants are transferred from the yolk to the embryo during the last few days of embryonic development. Most of these antioxidants are stored in the embryonic liver just before hatching, from where they are readily mobilized into the circulation during the neonatal period, thus preventing lipid peroxidation (Blount et al., 2000). For example, carotenoids repair the vitamin E radical that is produced when this antioxidant quenches an oxyradical, attenuating the depletion of vitamin E during embryonic development (Blount et al., 2000; Surai, Speake, Wood et al., 2001). It is also apparent that antioxidants protect passively-acquired IgY against catabolism. (Blount et al., 2000).

### Other molecules

In addition, yolk is known to contain various vitamin-binding proteins (like retinol-binding protein, biotin-binding proteins, riboflavin-binding protein, vitamin D-binding protein, and two presumed cobalamin-binding proteins), a2-macroglobulin, the complement component C3, and the C-type lectin tetranectin (Bellairs & Osmond, 2005; K. Mann & Mann, 2008).

Several egg white proteins have also been detected in yolk. These are ovalbumin which is among the most abundant proteins of yolk plasma, ovomucoid, ovotransferrin, and ovoinhibitor. A possible route of entry of egg white proteins into the yolk may be receptor-mediated endocytosis from the blood, which is the established mechanism of yolk protein accumulation (K. Mann & Mann, 2008).

In fertilized eggs, the extra-embryonic sources of calcium come from the yolk and the eggshell (Chien, Hincke, & McKee, 2009). The embryo begins to mobilize calcium between days 7 and 8 of incubation, corresponding to the start of mineralization of skeletal tissues (Chien et al., 2009; Karlsson & Lilja, 2008). However, until day 10, the egg yolk is the sole source of calcium, providing around 20% of the hatchling’s total somatic calcium (Chien et al., 2009; Karlsson & Lilja, 2008). While the majority of the shell calcium is used by the embryo to mineralize its skeleton, a significant amount is also transferred into phosvitin-rich platelets residing in the yolk sac. The storage of this latter calcium in the form of high-density, calcium-phosphate lipoprotein platelets is particularly important for newborn hatchlings to continue their skeletal development and mineralization uninterruptedly after loss of chorioallantoic membrane attachment to the shell causing aborted access to shell calcium near the time of hatching (Chien et al., 2009).

## Perivitelline layer

Avian oocytes in ovarian follicles are surrounded by a layer of proteinaceous extracellular matrix termed perivitelline layer (K. Mann, 2008; Takeuchi et al., 1999). During ovulation a split occurs between the perivitelline layer and the granulosa cells; the granulosa cells and basal lamina remain in the follicle while the ovulated ovum, enveloped by the perivitelline layer, is engulfed by the infundibulum of the oviduct, where fertilization is thought to occur (Takeuchi et al., 1999).

The perivitelline layer (VM), a transparent casing that encloses the yolk of the hen's egg and separates it from the albumen, preventing the mixing of the two compartments and forming the last barrier to microbial infection, consists of three layers: the inner layer, which is laid down in the ovary and faces the oocyte, the outer layer, which is secreted in the oviduct and faces the albumen, and a thin continuous layer sandwiched between the previous two (Bellairs & Osmond, 2005; K. Mann, 2008).

The components of the inner layer, which is the avian equivalent of the mammalian zona pellucida (ZP), are secreted by the granulosa cells surrounding the oocyte in the follicle or in the liver from where they are transported to the follicle via blood circulation (K. Mann, 2008). The major proteins of the inner layer are the glycoproteins GPI, GPII and GPIII, which may correspond to major ZP proteins (Bellairs & Osmond, 2005; K. Mann, 2008). The inner VM was shown to contain ZP proteins ZPC/ZP3, ZP1 (ZPB1), and ZPD (K. Mann, 2008). GPII may be concerned with the structural integrity of the inner layer (Bellairs & Osmond, 2005).

The outer layer varies in thickness and is composed of a variable number of sublayers lying one above the other. Strands of the outer fibers of this layer are extended out into the chalazae, where they are spirally arranged and coated with thick albumen. The material between the sublayers of the outer layer of vitelline membrane is probably albumen, and it is likely that there is a penetration of the outer layer by albumen, or that the secretion of the albumen starts to take place before the entire thickness of the outer layer has been laid down (Bellairs & Osmond, 2005). The outer layer possibly contains ovomucin, lysozyme C, and the vitelline membrane outer proteins (VMOs) VMO-I, and VMO-II (Bellairs & Osmond, 2005; K. Mann, 2008).

Ovomucin makes up the fibrous framework on which the outer layer is built, whilst lysozyme forms an electrostatic complex with the ovomucin that provides bulk and strength (Bellairs & Osmond, 2005). The VMOs are major components and among the most abundant proteins in the VM, second only to ovalbumin and lysozyme C. The complete sequence of VMO-I was determined and this protein was also identified in eggshell matrix, egg white, yolk, and cerebrospinal fluid. VMO-II is a small protein of unknown function, which may present some antimicrobial activity (K. Mann, 2008).

In addition, several enzymatic activities, such as $Na^+/K^+$-transporting ATPase, phosphatase, nucleotidase, pyruvate kinase, and GAPDH, were determined in VM preparations.

Although a predominantly proteinaceous structure, the VM also contains some lipid which may be derived from adhering oocyte plasma membrane patches and lipoproteins, and a carbohydrate-containing fraction which includes lectins (K. Mann, 2008).

During the first week of incubation, the albumen loses water (more than could be attributed to evaporation), while, at the same time, the yolk acquires water (Bellairs & Osmond, 2005). It is therefore assumed that water is passed through the vitelline membrane into the yolk and it has been calculated that about 16 g of fluid are drawn through the vitelline membrane during the first week of incubation and collects as sub-blastodermal fluid (Babiker & Baggott, 1995; Bellairs & Osmond, 2005). During the first two days of incubation, albumen penetrates through the vitelline membrane and accumulates distal to the outer area of the blastoderm. The possibility that the vitelline membrane itself selectively controls the passage of materials through it has frequently been investigated, but no clear evidence has been found (Bellairs & Osmond, 2005).

## Germinal disc

Almost coincidently with ovulation, the nucleus of the ovum undergoes its first maturation division. Maturation is a process occurring before fertilization, in which there is an equal mitotic division of the nucleus of the ovum but a markedly unequal division of the cytoplasm and its contents, resulting in the formation of one very large cell containing the entire dower of deutoplasm and one very small cell containing practically no deutoplasm (the polar body). After another such division, the nucleus of the ovum, now ready for fertilization, is called the female pronucleus (Patten, 1971).

When liberated from the ovary, the ovum soon begins to undergo changes which can be characterized as aging or deterioration. These changes progress rapidly to a point where the ovum, although technically still alive, can no longer be fertilized. If, however, the ovum is fertilized reasonably promptly after its liberation, these deteriorative changes are checked and the protoplasm increases its activity in a way that is often described as “being rejuvenated”. These changes symbolize, of course, the beginning of the period of tremendously rapid growth which is destined to end in the production of a new individual. Interestingly enough, fertilization in the usual manner by the male sex cell is not the only way an ovum becomes rejuvenated; a variety of other stimuli may replace that of spermatozoa (Patten, 1971).

## Albumen

The remaining structures of the avian egg, like the albumen, shell membranes, and eggshell are secreted around the ovum during its subsequent passage along the oviduct toward the cloaca (Patten, 1971). Albumen plays the role of a shock-absorber, keeps the yolk in place, constitutes a major antimicrobial barrier before and after the formation of the shell, and provides water, protein, and other nutrients to the developing embryo (Giansanti, Leboffe, Pitari, Ippoliti, & Antonini, 2012; K. Mann, 2007).

Approximately half the albumen of a freshly laid egg consists of a gel (thick albumen) interposed between two liquid fractions, the outer thin and inner thin albumen (Bellairs & Osmond, 2005; Kemps et al., 2010; Patten, 1971).

The albumen-secreting regions of the oviduct, respectively called infundibulum and magnum, constitute about one-half of its entire length (Bellairs & Osmond, 2005; K. Mann, 2007; Patten, 1971; Sugimoto et al., 1999). In the infundibulum, the part of the oviduct adjacent to the ovary, a mass of stringy albumen is produced, in order to block further sperm contact with the ovum after fertilization, should it occur (Bellairs & Osmond, 2005; Patten, 1971; Pines, 2007; Pines, Knopov, & Bar, 1995; Soledad Fernandez, Moya, Lopez, & Arias, 2001). This mass contains fibers apparently identical (and adhering closely) to the fibers in the outer layer of the vitelline membrane, projecting beyond it in two masses extending in either direction along the oviduct (Bellairs & Osmond, 2005; Patten, 1971; Sutcliffe, 1909). Because of the spirally arranged folds in the walls of the oviduct, the egg is rotated as it moves toward the cloaca, causing the adherent albumen to twist into spiral strands, spring-like structures known as the chalazae (Figure 26) (Leeson & Caston, 1997; Sutcliffe, 1909).

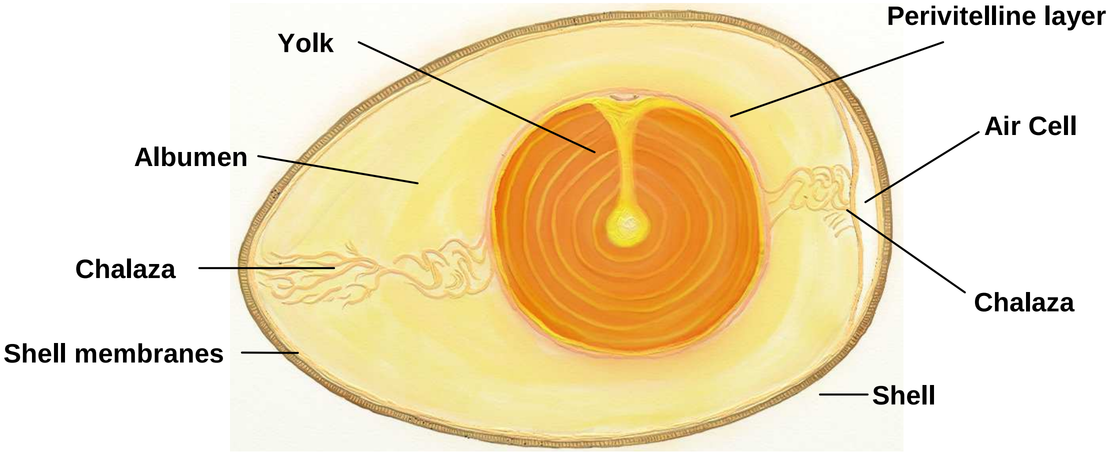


**Figure 26. Schematic representation of a hen's egg [drawn from (Wright, 1902)].**

These project from the equatorial region of the vitelline membrane into the albumen and are considered to act as balancers, maintaining the yolk in a steady position in the laid egg (Bellairs & Osmond, 2005; Patten, 1971; Sutcliffe, 1909). Typically, they are illustrated as passing to the blunt and pointed ends of the egg. If an egg is placed in an upright position, however, the yolk rotates so that the embryo remains at the uppermost side, and the chalazae shift their position accordingly (Bellairs & Osmond, 2005). During the formation of the chalazae, liquid is squeezed from the albumen to form the inner thin albumen (Leeson & Caston, 1997).

As the ovum travels along the oviduct, thick albumen which has been secreted abundantly in advance of the ovum's arrival by the glandular lining of the infundibulum is caught in the chalazae and wrapped about it in concentric layers (Patten, 1971).

The outer thin albumen appears to be added in the magnum but does not reach its final volume until the egg enters the shell gland, and 15 to 16 g of fluid pass into the albumen, expanding it to its full size (Bellairs & Osmond, 2005; Leeson & Caston, 1997; Soledad Fernandez et al., 2001). This event is often referred to as "plumping", and the magnitude of its effects seems to be positively correlated to the length of time the egg spends in this region of the oviduct (Bellairs & Osmond, 2005; Soledad Fernandez et al., 2001).

The albumen consists of approximately 88.5% water, 10.5% protein, and 1% carbohydrate and inorganic ions (K. Mann, 2007). Newly laid eggs are saturated with $CO_2$ (approximately 0.35%, wt/vol) most of which is present as bicarbonate (Banerjee, Keener, & Lukito, 2011; Lapao, Gama, & Soares, 1999). After oviposition, carbon dioxide is quickly released from the egg, resulting in an increase in albumen pH from about 7.6 to 9.5 within a short period of time, whereas the yolk remains slightly acidic, at a pH around 6.5 (Banerjee et al., 2011; Lapao et al., 1999). This pH gradient means that the blastoderm faces a very alkaline environment on the dorsal side of the epiblast, whereas its basal surface faces a slightly acidic environment. A specific pH gradient over the blastoderm, attained in association with an albumen pH of approximately 8.2, is optimal for early embryo development and subsequent hatching (Lapao et al., 1999).

The rise in albumen pH with storage time and hen age is associated with a decrease in albumen height and viscosity (Lapao et al., 1999). Albumen liquefaction probably facilitates the movement of nutrients from the albumen to the blastoderm and may reduce resistance to gaseous diffusion (Benton, Walsh, & Brake, 2001; Lapao et al., 1999). The alkaline pH of egg white also inhibits the growth of microorganisms (Giansanti et al., 2012).

## Major Albumen Proteins

The principal proteins of albumen are ovalbumin (54% of total protein), ovotransferrin (12%), ovomucoid (11%) and lysozyme (3,4%) (Banerjee et al., 2011; Bellairs & Osmond, 2005; Guerin-Dubiard et al., 2006; Sugimoto et al., 1999).

Synthesized in the hen oviduct, ovalbumin, the major hen egg white protein, is consumed as a nutrient supplemental to the yolk in developing avian embryos (Mine & Kovacs-Nolan, 2004; Sugimoto et al., 1999; Tian, Gautron, Monget, & Pascal, 2010). Its two related proteins, ovalbumin X and ovalbumin Y, are also concentrated in the egg white (Tian et al., 2010). Although functionally ovalbumin is important for the gelling, foaming, and emulsifying properties of egg albumen, its biological role in the egg remains unknown. Ovalbumin may possess some immunomodulatory activity, as it was found to induce the release of tumor necrosis factor (TNF) alpha *in vitro*. A vasorelaxing peptide, ovokinin, was isolated by the peptic digestion of ovalbumin. Ovokinin, a peptide produced by chymotrypsin digestion, was also found to possess vasorelaxing activity. Two angiotensin I converting enzyme (ACE)-inhibitory peptides were also identified in ovalbumin by peptic and tryptic digestions. Finally, the phagocytic activity of macrophages was increased by the addition of peptides, derived by peptic and chymotryptic digestions of ovalbumin, respectively (Mine & Kovacs-Nolan, 2004).

Ovotransferrin (OTrf) is a monomeric iron ($Fe^{3+}$) scavenging and binding glycoprotein with the capacity to reversibly bind two iron ions per molecule (Giansanti et al., 2012; Lapao et al., 1999; Mine & Kovacs-Nolan, 2004). It seems to be the only member of its protein family, the transferrins, present in avian albumen and exhibits several antimicrobial properties (Giansanti et al., 2012; Guerin-Dubiard et al., 2006). It exerts a bacteriostatic effect by preventing iron use by microorganisms and may also exert antibacterial activity by permeating bacterial outer membranes, reaching the inner membrane and causing the selective permeation of ions and dissipation of electrical potential (Giansanti et al., 2012; Mine & Kovacs-Nolan, 2004). Ovotransferrin has demonstrated antibacterial activity against a wide spectrum of bacteria, including *Pseudomonas* spp., *Escherichia coli*, *Streptococcus mutans*, *Staphylococcus aureus*, *Bacillus cereus* and *Salmonella* Enteritidis (Mine & Kovacs-Nolan, 2004).

Findings also suggest that ovotransferrin may play a crucial role in protecting against infection with Marek's disease virus (MDV), playing an important part of the innate immune response in birds (Giansanti et al., 2012).

In relation to the antifungal activity of OTfr, a direct interaction of iron-loaded protein with *Candida* cells has been reported (Giansanti et al., 2012; Mine & Kovacs-Nolan, 2004). In birds, ovotransferrin increases in concentration during inflammation and is therefore a positive acute phase protein (APP). Ovotransferrin levels in blood remain elevated as long as the inflammation persists, acting as immunomodulator, preventing microbial growth, and possibly acting as an antioxidant (Giansanti et al., 2012). Finally, OTrf has also been shown to be associated with angiogenesis processes in chickens, particularly during endochondral bone formation (Giansanti et al., 2012).

Ovomucoid is a prominent albumen glycoprotein that appears in blood and continues to be detectable after hatching (Moran, 2007). It is also one of four egg white proteinase inhibitors, namely inhibiting trypsin (Feeney, Rhodes, & Anderson, 1960; Mine & Kovacs-Nolan, 2004; Moran, 2007). Synthetic ovomucoid peptides have also demonstrated immunomodulating activity, inducing T-cell secretion of cytokines interleukin (IL)-4, IL-10, IL-13, interferon-(IFN) gamma, and IL-6 (Mine & Kovacs-Nolan, 2004).

Lysozyme is a ubiquitous enzyme found in humans, other mammals, and poultry (Banerjee et al., 2011; Guerin-Dubiard et al., 2006; Mine & Kovacs-Nolan, 2004). Lysozyme is a natural antimicrobial that hydrolyzes bonds found in the peptidoglycan layer of the bacterial cell wall, causing cell lysis (Banerjee et al., 2011; Gautron et al., 2007; Giansanti et al., 2012; M. T. Hincke et al., 2000; Maxwell T. Hincke, Nys, & Gautron, 2010; Mine & Kovacs-Nolan, 2004). The bactericidal effect of lysozyme is primarily limited to gram-positive bacteria, including pathogens such as *Listeria monocytogenes, Bacillus stearothermophilus*, *Bacillus* spp., *Enterococcus faecalis*, *Weissella viridescens*, *Brochothrix thermosphacta*, *Lactobaccilus sakei*, *Leuconostoc mesenteroides*, *Carnobacterium* sp., and certain *Clostridium* species, as well as some spoilage organisms, including thermophilic spore-forming bacteria and certain yeasts (Banerjee et al., 2011; Gautron et al., 2007; M. T. Hincke et al., 2000; Maxwell T. Hincke et al., 2010). The gram-negative bacteria are more resistant to lysozyme action since the envelope of lipoproteins and lipopolysaccharides on the cell wall of gram-negative bacteria makes the peptidoglycan layer inaccessible to lysozyme (Banerjee et al., 2011). However, lysozyme is also known to bind and detoxify LPS (Meade, Higgs, Lloyd, Giles, & O'Farrelly, 2009). Lysozyme has also been shown to exert antiviral activity, reportedly associated with its charge, rather than its lytic ability. Finally, lysozyme has also been shown to act as an immunemodulating and stimulating agent, enhancing immunoglobulin production in immunedepressed human patients (Mine & Kovacs-Nolan, 2004).

### Other albumen proteins

Ovomucin is a macromolecular and heavily glycosylated glycoprotein mostly present in the thick albumen and bound in a complex with lysozyme, in an insoluble form (the thin albumen contains only soluble ovomucin) (Guerin-Dubiard et al., 2006; Hammershoj & Qvist, 2001). Ovomucin serves physical functions within the egg, such as maintaining the structure and viscosity of the egg white albumen, thus preventing the spread of microorganisms and stabilizing the foam of both thick and thin albumen against liquid drainage (Feeney et al., 1960; Hammershoj & Qvist, 2001; Mine & Kovacs-Nolan, 2004). The content and nature of ovomucin appear to be primarily responsible for determining albumen height (Silversides & Budgell, 2004). It has shown antiviral activity against Newcastle disease virus, bovine rotavirus, and human influenza virus *in vitro* (Banerjee et al., 2011; Mine & Kovacs-Nolan, 2004).

Moreover, ovomucin possesses anti-angiogenic activity, inhibits cholesterol uptake and may also act as immunomodulator, showing macrophage stimulating activity *in vitro* (Mine & Kovacs-Nolan, 2004).

Ovoinhibitor represents about 0.1-1.5% of total protein in the albumen and, like ovomucoid, inhibits enzymes such as trypsin, chymotrypsin, and elastase, as well as various bacterial and fungal proteinases (Guerin-Dubiard et al., 2006; Mine & Kovacs-Nolan, 2004). It has also been found to inhibit the formation of ROS by human polymorphonuclear leukocytes (Mine & Kovacs-Nolan, 2004).

Tenp is a recently identified antimicrobial protein that could participate in the antibacterial activity of hen egg albumen against Gram-negative bacteria (Guerin-Dubiard et al., 2006; K. Mann, 2007). Avidin, another protein with antimicrobial activity, has been found to inhibit the growth of biotin-requiring bacteria and yeasts (Banerjee et al., 2011; Giansanti et al., 2012; Mine & Kovacs-Nolan, 2004). Avidin’s antimicrobial activity has also been attributed to its ability to bind to various gram-negative and gram-positive bacteria, including *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermis* (Mine & Kovacs-Nolan, 2004). Gallin is another newly discovered protein belonging to a new class of antimicrobial peptides found in albumen, the ovodefensins, which are related to the betadefensins. Gallin may be, like avidin, another egg white protein involved in innate immunity (Gong et al., 2010). Chicken cystatin, which has been implicated in the antimicrobial properties of hen egg albumen, has been shown to possess antibacterial activity, preventing the growth of group A streptococcus, *Salmonella* Typhimurium, and the periodontis-causing *Porphyromonas gingivalis* (Mine & Kovacs-Nolan, 2004).

Ovomacroglobulin, also referred to as ovostatin, has demonstrated broad-spectrum inhibitory activity against various types of proteases, including serine proteases, cysteine proteases, thiol proteases, and metalloproteases (Mine & Kovacs-Nolan, 2004). The antimicrobial effects of ovomacroglobulin against *Serratia marcescens* and *Pseudomonas aeruginosa*, due to its proteinase inhibitory action, have been demonstrated both *in vitro*, and *in vivo* (Giansanti et al., 2012; Mine & Kovacs-Nolan, 2004).

RBP, also referred to as flavoprotein or ovoflavoprotein, is a member of the folate receptor family. This protein binds riboflavin (vitamin B2), an essential vitamin for embryo development (Guerin-Dubiard et al., 2006).

Finally, a non-proteic compound, thiamin (vitamin B1), must also be mentioned as a normal constituent of the chicken egg, participating in a number of biochemical reactions vital to embryonic development and survival. Deprivation of this vitamin results in the accumulation of lactate and pyruvate in embryonic tissues (Muniyappa & Adiga, 1979).

## Eggshell

The avian eggshell is a unique, sophisticated, mineralized dynamic structure produced by birds in a circadian fashion to isolate and protect the developing embryo from the exterior environment (Chien et al., 2009; Hernandez-Hernandez et al., 2008; Jonchere et al., 2010; Lavelin, Meiri, Genina, Alexiev, & Pines, 2001). This hard, calcareous shell protects the growing embryo from physical trauma, invasion by microorganisms and dehydration, all while allowing gaseous exchange and buffering against temperature fluctuations to maintain an appropriate growing temperature for the embryo (Chien et al., 2009; Jonchere et al., 2010). In addition, the eggshell provides, via its physiologic dissolution, a primary source of calcium for the developing embryonic skeleton of the chick and may prevent respiratory acidosis in the embryo (Chien et al., 2009).

Undoubtedly, the main function of the eggshell is to shelter the embryo from external aggression, a function that must be compatible with easy breakage from inside to allow hatching. These requirements are fulfilled by the eggshell, because it is a ceramic material displaying a texture gradient. In the outer zone of the shell, there is a tough structure made of large crystals where the external impacts are absorbed by thin inter-crystalline organic layers that make intracrystalline crack propagation difficult. However, the inner region of the eggshell is composed of microcrystals of calcite arranged in a texture that facilitates the propagation of cracks during piping, when the embryo breaks out of the eggshell with its beak. Moreover, this facilitates the mobilization of calcium to nourish the embryo by dissolution of highly reactive calcite microcrystals (Nys, Gautron, Garcia-Ruiz, & Hincke, 2004).

The eggshell is classically defined as a six layer structure consisting, from the inside out, of: two eggshell membranes, a mammillary layer, a palisade layer, a vertical crystal layer and, finally, an organic cuticle. While this is a classical description the eggshell is, in fact, a single structure from the viewpoint of its mechanism of formation (Nys et al., 2004).

### Eggshell membranes

As the yolk and albumen complex next travel through the proximal (white) isthmus, about 2-3 hours after ovulation, the eggshell membranes (ESM) are acquired in a 1 to 2-hour period (Maxwell T. Hincke et al., 2010; Lavelin, Meiri, & Pines, 2000; Pines, 2007; Pines et al., 1995). Two shell membranes are present – a thick outer membrane attached to the shell (the outer shell membrane (OSM)) and a thin inner membrane surrounding the albumen (the inner shell membrane (ISM)) – and they adhere tightly to each other except at the blunt end of the shell where they are separated by an air space (the air cell) (Bellairs & Boyde, 1969; Hunton, 2005; Kodali et al., 2011; Tsai et al., 2006).

Avian eggshell membranes play a key role in the formation and structure of the eggshell and carry out several important functions (Bellairs & Boyde, 1969; X. Wang, Ford, Praul, & Leach, 2002). They provide a substrate on which the shell is laid down during its formation (Hunton, 2005; Kodali et al., 2011; Nakano, Ikawa, & Ozimek, 2003); they act as a bag enclosing the albumen and preventing it from clogging the pores of the shell (Bellairs & Boyde, 1969; Nakano et al., 2003); they separate the chorioallantoic membrane from the shell during incubation, while allowing the passage of carbon dioxide out of the egg and of oxygen into it (Bellairs & Boyde, 1969); and, finally, acting as a barrier to the passage of bacteria into the egg (Bellairs & Boyde, 1969; Nakano et al., 2003).

The shell membranes, formed in the isthmus region of the avian oviduct, are two dense nets of fibrils composed of a core surrounded by a fuzzy material referred to as a mantle (Fernandez, Araya, & Arias, 1997). This meshwork of interwoven fibers, known to emerge from tubular gland cells in the isthmus region of the oviduct, is considered to be the innermost component of the eggshell (Ha, Son, Yun, & Kim, 2007; Maxwell T. Hincke et al., 2010; Kodali et al., 2011). The fibers are deposited sequentially in inner and outer layers, both of which are similar in general morphology and amino acid composition, although the inner shell membrane, contacting the albumen, has thinner fibrils than the outer one (Fernandez et al., 1997; Tsai et al., 2006). Each membrane consists of an intricate lattice network of stable and water-insoluble fibers that are arranged so as to form a semi-permeable membrane (Bellairs & Boyde, 1969; Tsai et al., 2006).

Each fiber consists of a core surrounded by a region of different density and it has been suggested that they are mainly made of keratan covered with mucopolysaccharide (Bellairs & Boyde, 1969; Kodali et al., 2011). Individual fibers do not weave in and out from one part of the membrane to another, but they remain at a constant level. The fact that they also extend for considerable distances and appear to be randomly orientated suggests that the egg may execute a random roll or spin during its journey in the isthmus, or that any "peristaltic" movements of the oviduct wall in contact with the egg are localized and random in direction (Bellairs & Boyde, 1969).

The membranes are composed of 10% collagens (types I, V and X) and 70-75% of other proteins and glycoproteins containing lysine derived cross-links, with small amounts of carbohydrates and lipids (M. T. Hincke, Chien, Gerstenfeld, & McKee, 2008; Maxwell T. Hincke et al., 2010; Kodali et al., 2011; Nakano et al., 2003; Nys et al., 2004; Pines, 2007; X. Wang et al., 2002).

Type X collagen is specifically located in the core of every shell membrane fiber, while keratan sulfate, a major glycosaminoglycan (GAG) in eggshell membranes, is located especially in the core of the outer shell membrane fibers (Ha et al., 2007; Nys et al., 2004; Soledad Fernandez et al., 2001). Keratan sulfate content of shell membranes has an effect on eggshell strength (Ha et al., 2007). Together with sialic acid, other glycosaminoglycans, such as uronic acid and sulfated GAGs are positively correlated with breaking strength in shell membranes (Ha et al., 2007; Nakano et al., 2003).

Osteopontin (OSP), a phosphorylated glycoprotein present at high concentration in bone, kidney and various body secretions is also present in the eggshell, localized in the core of the eggshell membrane fibers. It partially inhibits hydroxyapatite formation and is, therefore, likely to be a potent and phosphorylation-dependent inhibitor of calcium carbonate precipitation (Gautron et al., 2001; M. T. Hincke et al., 2008; Jonchere et al., 2010; Nys et al., 2004; Panheleux et al., 1999). The cortex of each fiber is thought to consist of mucopolysaccharides. Its nature is of particular interest since it is highly impenetrable both to bacteria and to the chorioallantoic membrane (Bellairs & Boyde, 1969).

Molecules such as ovotransferrin, ovocalyxin-36 and lysozyme are some of substances responsible for protecting the egg contents (and hence the embryo) from microbes (Gautron et al., 2007). Ovotransferrin is located particularly in the eggshell membranes, where it functions as a bacteriostatic filter to reinforce inhibition of *Salmonella* growth in egg white and is found in the lumen of the distal oviduct, becoming incorporated into the eggshell membranes and the eggshell. Ovocalyxin-36 (OCX-36) may be involved in the innate defense of the egg to keep the egg free of pathogens and safe for the developing embryo. Lysozyme is abundant in the limiting membrane that circumscribes the egg white and forms the innermost layer of the shell membranes (Gautron et al., 2007).

While the inner membrane remains uncalcified, providing a barrier to prevent inward mineralization, the fibers of the outer shell membrane penetrate the mammillary cones of the calcified shell (Maxwell T. Hincke et al., 2010; Nys et al., 2004). The outer shell membrane is so firmly attached to the so-called mammillary knobs on the inside of the shell in the unincubated egg that it is impossible to separate them without damage to one or the other (Bellairs & Boyde, 1969).

The mechanisms that prevent calcification towards the inner membranes and albumen are not well understood; one proposal is that collagen type X prevents a generalized calcification of the shell membrane (Maxwell T. Hincke et al., 2010). Intact eggshell membranes are, however, a prerequisite for shell calcification in laying hens and any modification of the eggshell membranes alters eggshell formation and its mechanical properties (Maxwell T. Hincke et al., 2010; Nys et al., 2004).

## Eggshell proper

Three different stages can be differentiated during eggshell formation: (a) initial, (b) active-fast growth, and (c) terminal (Hernandez-Hernandez et al., 2008). Eggshell mineralization is initiated, about 5.25 to 5.5 hours post ovulation, in the distal (red) isthmus (tubular shell gland), where organic aggregates are deposited on the surface of the outer eggshell membrane at quasi-periodic, but seemingly randomly located, sites to form the mammillae (Figure 27) (Fernandez et al., 1997; Fernandez, Escobar, Lavelin, Pines, & Arias, 2003; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010).

**Figure 27. Schematic representation of a mammilum attached to the outer eggshell layer [drawn from (Tan, Chen, Chan, & Ng, 1992)].**

These are the sites where the initiation of calcium carbonate crystal nucleation takes place, in the form of polycrystalline aggregation (stage I), and contain mammillan, a highly sulfated keratan sulfate proteoglycan, as the main component (Fernandez et al., 1997; Fernandez et al., 2003; Ha et al., 2007; Hernandez-Hernandez et al., 2008; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010; Nys et al., 2004; Panheleux et al., 1999; Soledad Fernandez et al., 2001).

Other proteoglycans of lesser calcium affinity and other proteins with calcium affinity (ovotransferrin, ovalbumin) are also found in mammillae (Panheleux et al., 1999). Osteopontin is another component of mammillae; it is immediately secreted out of the epithelial cells of the eggshell gland (ESG) and becomes localized in the core of the unmineralized shell membrane fibers in the bases of the mammillae but not on their surface (Arazi et al., 2009; Fernandez et al., 2003; Maxwell T. Hincke et al., 2010; Soledad Fernandez et al., 2001). The base of the mammillae does not mineralize, suggesting that osteopontin could contribute to the compartmentalization of the calcium reserve bodies (Fernandez et al., 2003; Soledad Fernandez et al., 2001).

The incomplete egg, with its membranes and initial mineral deposits, now enters the uterus (shell gland pouch), where fluid is pumped into the albumen, causing it to swell to its final size and shape (M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010; Nys et al., 2004). Calcium carbonate deposition continues outward to give rise to the inner mammillary (cone) layer and the outer palisade (calcitic prism) layer during the 16 to 17-hour period of shell formation (Figure 28) (Hernandez-Hernandez et al., 2008; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010).

Mineralization occurs while the egg is bathed in uterine fluid, an acellular milieu containing high levels of ionized calcium, bicarbonate, and the native and soluble organic precursors of the shell matrix (Gautron et al., 2001; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010; Nys et al., 2004).

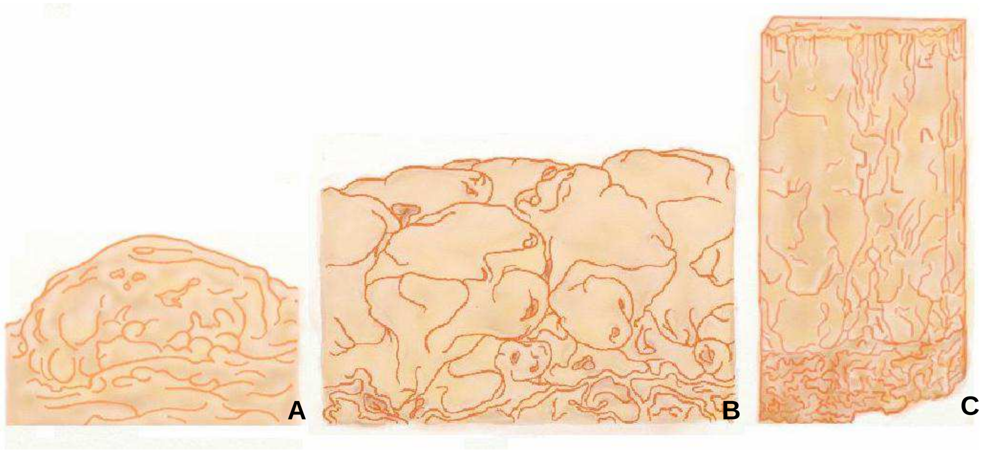


**Figure 28. Schematic representation of the process of shell formation [drawn from (Tan et al., 1992)]**
**A – mammillary body formation; B – growth of the mammillary layer; C – palisade layer formation**

The ionic and organic constituents of the uterine fluid change progressively during eggshell formation and can be subdivided into the successive stages of initiation (five to ten hours post-ovulation in hen), growth (10 to 20 h post-ovulation) and termination (1.5 h pre-oviposition) of eggshell mineralization (Gautron et al., 2001; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010; Nys et al., 2004; Pines, 2007). The egg rotates during the linear deposition of calcium carbonate as the mammillary and palisade layers are sequentially formed (Nys et al., 2004).

Eggshell formation constitutes one of the most rapidly mineralizing biological processes known, in which ≈6 g of shell are deposited in 20 h, in a highly ordered ultrastructure, consisting of a mineral phase (95%) of calcitic calcium carbonate (98% of the mineral phase), magnesium carbonate and calcium phosphate, and an organic proteinaceous matrix (3.5% with the remainder as water), of which approximately one half can be readily solubilized by decalcification of the shell (Gautron et al., 2001; Hernandez-Hernandez et al., 2008; M. T. Hincke et al., 2008; Lavelin et al., 2000; Pines, 2007; Tsai et al., 2006; Wu et al., 1995).

$Ca^{2+}$ transport across the ESG mucosa is dependent on at least three different mechanisms: *1*) secretion of $Ca^{2+}$-rich granules, *2*) a $Ca^{2+}$ pump localized in the ESG epithelium, and *3*) an ATP-dependent $Na^{+}$/ $Ca^{2+}$ exchanger, which increases the $Ca^{2+}$ efflux indirectly by creating an $Na^{+}$ gradient across the ESG epithelium membranes via $Na^{+}$-$K^{+}$-ATPase, an enzyme important in preserving the volume, pH, and electrical resting potential of cells (Arazi et al., 2009; Lavelin et al., 2001). The deposition of calcium carbonate is accompanied by large changes in the flux of $K^{+}$, $Mg^{2+}$, and glucose into and $Na^{+}$ and $Cl^{-}$ out of the ESG fluid, while they recirculate within the ESG mucosa via an active transport system (Lavelin et al., 2001).

To recapitulate, the calcified eggshell consists primarily of calcite, the most stable polymorph of calcium carbonate ($CaCO_3$) and is progressively composed of the inner mammillary cone layer, central palisades and the outer vertical crystal layers (Maxwell T. Hincke et al., 2010; Nys et al., 2004).

The mammillary layer is a regular array of hemispherical nucleation centers, termed cones or knobs, with highly organic cores, within which microcrystals of calcite are arranged with spherulitic texture which facilitates the propagation of cracks during piping as well as the mobilization of calcium to nourish the embryo by dissolution of highly reactive calcite microcrystals (Maxwell T. Hincke et al., 2010; Nys et al., 2004). The mammillary layer is, in fact, the main calcium reserve for the chicken embryo; by the time of hatching, over 80% of the embryo's calcium requirements will have been supplied by the mammillary layer, a great part of which will have been used by the embryo to mineralize its skeleton, while a significant amount will also have been transferred into high-density calcium-phosphate lipoprotein platelets residing in the yolk sac to act as calcium reserves for newborn hatchlings to continue their skeletal development and mineralization (Chien et al., 2009; Fernandez et al., 1997; Karlsson & Lilja, 2008). The mammillary layer has been described as consisting of basic structural components referred to as calcium reserve assemblies. These are distinctively formed by a baseplate partially integrated into the outer shell membrane, a calcium reserve body (CRB) attached to the baseplate, and a cover or crown, which is the base of each column of the palisade (Fernandez et al., 1997). The CRB sac is a unique compartmental unit with unknown function which is physiologically depleted of calcium and ultimately lost between days 11 and 16 of incubation; this leaves behind empty CRB sacs as centrally located hollows within the mammillae, with an exposed rich proteinaceous network that might then serve as a conduit increasing internal surface area and acid diffusion to facilitate resorption of the mammillary tips (Chien et al., 2009; Chien, Hincke, Vali, & McKee, 2008; Karlsson & Lilja, 2008).

As $CaCO_3$ is deposited on mammillary knobs, in a radial arrangement displaying concentric growth banding, but, due to competition for space between adjacent sites of growth, only crystals growing perpendicular to the egg surface have space to grow, the probability of surviving at a given thickness depending on how disoriented a crystal is with respect to this axis (Maxwell T. Hincke et al., 2010; Nys et al., 2004). Thus, during the fast growth stage (stage II), adjacent spheruliths fuse together until apparently single-crystal columnar calcite units arise to form the palisade layer, the thickest layer of the shell, made of groups of calcite columns that are perpendicular to the eggshell surface, and thus to the supporting eggshell membranes (Hernandez-Hernandez et al., 2008; Nys et al., 2004; Soledad Fernandez et al., 2001). This type of process in a restricted space is denominated competitive crystal growth as crystals compete for the limited available space (Gautron et al., 2001; Hernandez-Hernandez et al., 2008). Although competitive growth surely explains significant features of the eggshell pattern, the refined, repetitive and organized structure of eggshell can only come about from additional influences, such as those provided by an organic matrix closely associated with the crystalline phase of the columns (Fernandez et al., 1997; Soledad Fernandez et al., 2001; Tsai et al., 2006). This matrix is organized into vesicular structures with electron-lucent cores dispersed along and between flocculent sheets of organic material that align generally parallel, or slightly angled, to the eggshell surface (Gautron et al., 2001; M. T. Hincke et al., 2008). These lamellae, found in the palisades region, demonstrate not only an abundant matrix that intermingles with the calcitic mineral phase, but also a periodic structure most obviously apparent after full decalcification of the eggshell. While some of this arrangement is likely artifactual, arising from partial collapse of the matrix when the mineral phase is removed by decalcification there is a strong indication that much of this organization is real (Chien et al., 2008).

The native and soluble precursors of the eggshell matrix are present in the uterine fluid, from which they become incorporated into the calcifying shell, comprising proteins (of which approximately 500 have already been identified by proteomics analysis), glycoproteins, and proteoglycans (Gautron et al., 2001; Gautron et al., 2007; Hernandez-Hernandez et al., 2008; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010; Odabas, Miles, Balaban, & Portier, 2007; Pines, 2007).

It is widely accepted that organic matrix components play a role in the control of calcite crystal shape, size, and orientation, and therefore in the mechanical properties of this material, as well as in the antimicrobial defenses of the eggshell (Gautron et al., 2001; Maxwell T. Hincke et al., 2010; Lavelin et al., 2000; Lavelin, Yarden, Ben-Bassat, Bar, & Pines, 1998; Pines, 2007). The nature of the interactions between the mineral phase and the eggshell matrix proteins has been intensely investigated (Maxwell T. Hincke et al., 2010). Such studies led to the concept that eggshell matrix protein components form three characteristic groups:

#### “Egg white” proteins

“Egg white” proteins present in the eggshell include ovalbumin, lysozyme, and ovotransferrin (Gautron et al., 2001; Gautron et al., 2007; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010; Wellman-Labadie, Lakshminarayanan, & Hincke, 2008). Ovalbumin is mainly secreted by the magnum and not by the shell-secreting region of the oviduct and probably becomes entrapped within the mineral layer (in the mammillary knobs) during the process of eggshell formation (Gautron et al., 2001;

Panheleux et al., 1999). Ovalbumin does not influence crystal morphology but it appears to slightly accelerate the nucleation of calcium carbonate by driving calcium ions on to calcium-affinity molecules and so influence the initiation of eggshell formation (Panheleux et al., 1999). Lysozyme is abundant in the innermost layer of the shell membranes (Gautron et al., 2007). Although eggshell lysozyme is present at highest levels in the non-calcifying collagenous membranes, particularly the inner limiting membrane, and to a lesser extent within the shell matrix, weak intramineral expression is evident at the level of the mammillary knobs, which extends in a graded fashion towards the palisade layer. Some lysozyme expression is also evident on the surface of the shell. Lysozyme is a potent antibacterial agent which can also modify calcite crystallization (M. T. Hincke et al., 2000). Ovotransferrin is located in the calcified mammillae and particularly in the eggshell membranes, where it functions as a bacteriostatic filter to reinforce inhibition of *Salmonella* growth in egg white (Arazi et al., 2009).

#### Ubiquitous proteins

The eggshell contains ubiquitous proteins - *i.e.* proteins that are expressed in a variety of tissues – such as osteopontin, calbindin and clusterin (Gautron et al., 2001; Gautron et al., 2007; Jonchere et al., 2010; Lavelin et al., 2000).

Osteopontin is a prominent phosphoprotein of the eggshell matrix, synthesized and secreted by the nonciliated granular epithelial cells of the shell gland mucosa (Chien et al., 2008; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010). The oviduct expression of osteopontin is entirely uterine-specific and is temporally associated with eggshell calcification through coupling of physical distension of the uterus by the egg with osteopontin gene expression (Gautron et al., 2001; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010; Lavelin et al., 2000; Lavelin et al., 1998; Pines, 2007). Studies show that OSP is localized in the core of the unmineralized shell membrane fibers and in the bases of the mammillae but its greatest concentration is found in the highly mineralized palisades layer of the eggshell (M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010).

OSP is intimately involved in the regulation of both physiological and pathological mineralization and is thought to regulate crystal growth (*e.g.*, form, size and orientation) (Arazi et al., 2009; Chien et al., 2008). It binds tightly to hydroxyapatite, partially inhibiting calcium carbonate precipitation, and may regulate eggshell formation by inhibiting calcite growth at specific crystallographic faces and compartmental boundaries, thereby creating a biomineralized architecture whose structure provides for the properties and functions of the eggshell (Arazi et al., 2009).

Whereas the primary function of osteopontin may be to regulate crystal growth patterns and speed by binding to mineral in the eggshell, its incorporation into calcite as an occluded crystal protein might serve the secondary function of also providing some resistance to fractures (M. T. Hincke et al., 2008). Reduced or absent OSP expression in specific regions of the uterine luminal epithelium is correlated with eggshell defects such as corrugations, pimples and cracks (Arazi et al., 2009; Maxwell T. Hincke et al., 2010; Jonchere et al., 2010).

Calbindin is a calcium-binding protein, which also fluctuates in a circadian fashion during the daily egg cycle, in close temporal association with eggshell calcification (Arazi et al., 2009; Lavelin et al., 2001; Lavelin et al., 2000; Lavelin et al., 1998; Pines et al., 1995). Calbindin gene expression in the ESG marks the onset of calcification and is predominantly regulated by the calcium flux (Arazi et al., 2009; Lavelin et al., 2000). Calbindin is a known facilitator of calcium diffusion, closely related to eggshell quality, expressed around 2 h after osteopontin gene expression begins (approximately 3 hours after the egg reaches the eggshell gland) (Arazi et al., 2009; Lavelin et al., 1998).

Clusterin is a widely expressed secretory glycoprotein which probably functions as a chaperone for one or more members of the protein matrix, acting to prevent their premature aggregation and precipitation before and/or during assembly of the eggshell organic matrix. Thus, clusterin may regulate the assembly of eggshell matrix components into the protein scaffold associated with ordered mineralization. Moreover, since clusterin also becomes incorporated into the calcified shell, it may play a structural role itself (Karlheinz Mann et al., 2003).

#### Eggshell-specific matrix proteins

Several matrix proteins are unique to the shell calcification process and only secreted in regions of the oviduct where eggshell calcification occurs (Gautron et al., 2007; Jonchere et al., 2010). These matrix components are termed Ovocleidins or Ovocalyxins, with distinction based on apparent molecular weight when initially characterized (Maxwell T. Hincke et al., 2010).

Ovocleidin-17 (OC-17) was the first eggshell-specific matrix protein to be isolated and characterized (Gautron et al., 2007; Maxwell T. Hincke et al., 2010; Jonchere et al., 2010; Wellman-Labadie et al., 2008). OC-17 is an abundant soluble eggshell-matrix specific protein which is secreted by the tubular gland cells in the shell gland and is distributed throughout the shell matrix, but concentrated in the mammillary bodies (Maxwell T. Hincke et al., 2010; Panheleux et al., 1999). It is not detected in other tissues but its role in eggshell calcification is still to be established (Maxwell T. Hincke et al., 2010; Panheleux et al., 1999). OC-17 is potently bactericidal against Gram-positive bacteria such as *B. subtilis* and *S. aureus*, binding to peptidoglycan and LPS; it exhibits enhanced activity in the presence of calcium (Wellman-Labadie et al., 2008). It has also been suggested that OC-17 could modify the crystalline habit of calcium carbonate and the pattern of calcite nucleation and crystal growth via direct interaction with carbonate anion (Maxwell T. Hincke et al., 2010; Wellman-Labadie et al., 2008).

Ovocleidin-116 (OC-116) is the most abundant eggshell matrix protein. It is relatively eggshell-specific but can also be found in young chick cortical bone, laying hen medullary bone and growth plate hypertrophic chondrocytes (Maxwell T. Hincke et al., 2010). OC-116 is incorporated into, and widely distributed throughout, the palisade region of the calcified eggshell, being predominately associated with the periphery of the vesicular structures that probably correspond to the walls of microvesicular holes (voids) in the calcitic eggshell (M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010).

Ovocalyxin-21 (OCX-21) is another eggshell specific protein, which may play a role as molecular chaperone. Chaperone proteins in uterine fluid could play an important role in proper folding of the eggshell matrix, which is the crucial template for eggshell calcification (Jonchere et al., 2010).

Ovocalyxin-32 (OCX-32) is abundant in the terminal phase of shell formation and is concentrated in the outer palisade layer, the vertical crystal layer and the cuticle of the calcified shell (Gautron et al., 2001; Gautron et al., 2007; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010). The timing of OCX-32 secretion into the uterine fluid has been interpreted to suggest that it plays a role in the processes that lead to termination of eggshell calcification and which morphologically manifest themselves as a change in the direction of calcite crystal growth (Maxwell T. Hincke et al., 2010).

Ovocalyxin- 36 (OCX-36) is a prominent present in the uterine fluid collected during the active calcification stage of shell mineralization and only detected in the regions of the oviduct where eggshell formation takes place (Maxwell T. Hincke et al., 2010; Jonchere et al., 2010; Tian et al., 2010). Its sequence displays significant identity with mammalian proteins that are associated with the innate immune response and act as the first line of host defense against bacteria, such as lipopolysaccharide-binding proteins and bactericidal permeability-increasing proteins (M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010; Tian et al., 2010). OCX-36 may therefore participate in natural defense mechanisms that keep the oviduct and the egg free of pathogens and safe for the developing embryo (Gautron et al., 2007; Maxwell T. Hincke et al., 2010; Tian et al., 2010).

The organic matrix also contains several glycosaminoglycans and proteoglycan molecules, such as keratan sulfate (mammillan), dermatan sulfate (ovoglycan) the distribution of which varies spatially in the mineralized parts of the shell and temporally with the sequence of shell deposition (Panheleux et al., 1999). Eggshell strength depends on its GAGs content as they are assumed to be involved in the binding of calcium ions and in regulation of the mineralization processes controlling eggshell mineralization (Ha et al., 2007; Nakano, Ikawa, & Ozimek, 2001).

The avian eggshell contains a large number of pores, up to several tens of thousands. These pores form a shell-traversing channel network that allows water and gas exchange necessary for the growing embryo, while at the same time not significantly diminishing the mechanical properties of eggshell required to protect the embryo, and resisting microorganism invasion (Chien et al., 2008; Maxwell T. Hincke et al., 2010; Tsai et al., 2006). Pore formation begins at the level of the mammillary layer with the grouping of 4-5 mammillary bodies. As their associated columns grow laterally and vertically, their orientation is such that a central space is left which in functional exchange sites persists through the entire depth of the shell (Solomon, 2010). OSP has been proposed to be involved in the formation of eggshell pores as a prominent component of a distinct, delimiting protein layer at the margins of the calcitic columns, defining the pore space in such a way that calcification is not only inhibited and/or terminated at the calcite column boundary, but is done so in a definitive way that preserves the patency of the pore channel (Chien et al., 2008).

The palisade layer ends in a thin vertical single crystal layer where the crystallites are aligned perpendicular to the shell surface (Hernandez-Hernandez et al., 2008; Nys et al., 2004). The crystalline structure of the vertical crystal layer has a higher density than that of the palisade layer, providing a tough structure made of large crystals where the external impacts are absorbed by thin inter-crystalline organic layers that make intracrystalline crack propagation difficult (Maxwell T. Hincke et al., 2010).

Shell calcification halts prior to egg expulsion in a milieu that remains supersaturated in calcium and bicarbonate (Gautron et al., 2001; Nys et al., 2004). The mechanism by which calcite deposition from this milieu is inhibited has not yet been elucidated but it is likely that shell mineralization is inhibited by a specific process or component of the uterine fluid at this stage (Gautron et al., 2001; Nys et al., 2004).

## Cuticle

A thin, noncalcified outer layer, called the cuticle, completes the shell (Maxwell T. Hincke et al., 2010; Hunton, 2005). This layer, which covers the entire mineral surface of the shell, is of variable thickness and may even be missing (Maxwell T. Hincke et al., 2010; Solomon, 2010). It is composed of glycoproteins (*e.g.*, osteopontin, lysozyme), polysaccharides (*e.g.*, keratan sulfate), lipids, the majority of the pigments in colored eggs (around 2/3, the remainder of which is present in the underlying shell), and hydroxyapatite crystals (Chien et al., 2008; Fernandez et al., 1997; Fernandez et al., 2003; M. T. Hincke et al., 2008; Maxwell T. Hincke et al., 2010; Mikšík, Holáň, & Deyl, 1996; Soledad Fernandez et al., 2001).

The cuticle is a two layered structure, the inner layer of which presents with a vesicular appearance (Solomon, 2010). This layer has a basic task in maintaining the integrity of the shell's gaseous diffusion pathway against obstruction by nest debris, flooding with water and in reducing the probability of bacterial penetration of the shell post-oviposition (Mikšík et al., 1996; Solomon, 2010; Tsai et al., 2006).

Pigments are applied into the cuticle layer of the shell as an egg passes through the shell gland of the oviduct (Shafey, Al-Batshan, Ghannam, & Al-Ayed, 2005). This pigmentation seems to have cryptic reasons; changes in eggshell coloration may reflect physiological condition like egg laying or nesting, but they may possibly result from exogenous (environmental) factors as well (Mikšík et al., 1996). Hens’ eggs show enormous variation in the intensity of eggshell pigment, even between single eggs of the same clutch (Mikšík et al., 1996; Shafey et al., 2005). As the hen ages, the egg weight and size increases with no proportionate increase in shell or pigment deposition (Odabas et al., 2007). This means that the eggshell pigments are deposited on a larger surface area, resulting in older hens laying paler eggshells (Odabas et al., 2007).

## Air cell

As previously stated, two delicate porous membranes line the shell, enclosing the albumen (Halsted, 1884; Sutcliffe, 1909). These two membranes remain in contact, except at the blunt pole of the egg, where they separate and form the air cell, so small in a newly-laid egg as to be almost invisible, but which gradually increases in size as the contents of the egg condense, whether incubated or not, by the evaporation of the water (Halsted, 1884; Sutcliffe, 1909). This is the basis for the utilization of air chamber height as one of the non-destructive methods to determine the degree of freshness of eggs for human consumption (Comission, 2008; Sutcliffe, 1909).

Thus, while in a fresh, unincubated egg the air cell should not exceed 6 mm, in one ready for hatching, the chamber can occupy from one-sixth to one-fifth the interior space (Comission, 2008; Sutcliffe, 1909). It is from the air cell that the embryo will extract its first inhalations of atmospheric air (upon internal pipping) as it transitions into pulmonary respiration, in preparation for hatching (Maatjens et al., 2014).

# NORMAL *IN OVO* DEVELOPMENT

Knowledge of the normal process of embryonic development is essential in the study of incubation. Placing the time of embryonic death at a specific day or period of development, for instance, requires elementary comprehension of the various different and complex changes taking place at any given time in incubation. Unlike most works of avian embryology, which focus on a more extensive, in-depth description of events taking place in each system and organ separately, the main goal of this chapter lies in facilitating diagnosis of the time of embryonic death to be used later, in the experimental work chapters. Therefore, an attempt has been made to describe the most important changes taking place in the embryo in chronological order. However, it should be taken into consideration that biological diversity does mean that not all embryos will behave in the exact same manner, and certain changes are not as yet fully studied, regarding their timing of occurrence. A schematic summary of the evolution taking pace in the embryo is provided in Annex 1.

## 0-2 days of incubation

Fertilization occurs (and must occur) immediately after ovulation, in the brief period (≈15 min) before the outer layer of the vitelline membrane is laid down, blocking sperm entry (Bellairs & Osmond, 2005). As the egg yolk, enclosed in its vitelline membrane, passes down the oviduct and becomes covered first by the albumen, then by the shell membranes and finally by the shell, the embryo undergoes cleavage, this process differing greatly in birds from that found in mammals (Bellairs & Osmond, 2005; Wittler & Kessel, 2004).

Cleavage in bird’s eggs begins as it does in the eggs of Amphibia, but the mass of the inert yolk material is much greater than in the latter, so great in fact that the yolk is not divided. The process of segmentation is limited to the small disk of protoplasm lying on the surface of the yolk at the animal pole, and is for this reason referred to as discoidal cleavage (Arendt & Nubler-Jung, 1999). The cells formed in the process of cleavage are known as blastomeres (Arendt & Nubler-Jung, 1999; Patten, 1971).

In the bird’s egg about to undergo cleavage, the disk of active protoplasm at the animal pole (blastodisc) is a whitish, circular area about 3 mm in diameter (Patten, 1971).

The central portion of the blastodisc is surrounded by a somewhat darker appearing marginal area known as the periblast. The protoplasm of the blastodisc, especially in the periblast region, blends into the underlying white yolk so that is it difficult to make out any line of demarcation between the two. It is in the central area of the blastodisc that cleavage furrows first appear. Neither the nuclei resulting from the early cleavages nor the cleavage furrows invade the marginal periblast until very late in the process of segmentation (Patten, 1971). In each of the two blastomeres resulting from the first cleavage division, mitotic spindles initiating the second cleavage arise at right angles to the position which was occupied by the first cleavage spindle (Arendt & Nubler-Jung, 1999; Patten, 1971).

Cleavage furrows are visible in fertilized eggs taken from the oviduct although the first 16 cleavage cells are not completely enclosed in their own cell membranes but are open ventrally to the yolk mass; they are known as ‘open cells’ (Bellairs & Osmond, 2005; Lemaire & Kessel, 1997). With each mitotic division the cell membrane becomes completed around one of the daughter ‘cells’, while the other migrates into the adjacent yolky cytoplasm, carrying some cytoplasm with it, so that a new open cell is formed. By the 64-cell stage the most centrally situated cells have each become completely surrounded by a cell membrane, but there is still a region of mixed yolk and cytoplasm (ooplasm) beneath and around the embryo known as the subgerminal and marginal periblast, respectively. During this time, fluid is drawn from the albumen through the vitelline membrane and accumulates beneath the embryo, forming the fluid in the subgerminal cavity (Bellairs & Osmond, 2005).

The most important event for the cleaving embryo is the establishment of the axes of polarity (Bellairs & Osmond, 2005). First, the dorsal and ventral sides become apparent (the dorso-ventral axis) and then the antero-posterior (the cranio-caudal axis) (Bachvarova, 1999). With the interaction of these two axes, the embryo acquires a left and a right side (Bellairs & Osmond, 2005).

The establishment of the antero-posterior axis results from a shifting of the ooplasmic components by gravity (Bachvarova, 1999; Page, Maini, Monk, & Stern, 2001). This takes place during the 20 or so hours spent in the shell gland (uterus), when the entire egg rotates at about 10-12 turns per hour as the shell is laid down and, in so doing, tips the blastoderm from its original position on top of the yolk. The future anterior end (head) forms in the region that has been tipped lowest, whereas the future posterior end (tail) forms from the highest region. This tilting is accompanied by a shifting of the yolky ooplasm beneath the oocyte so that the more lightweight of its components come to lie beneath one side of the blastoderm (Bellairs & Osmond, 2005).

At the end of segmentation, the chick embryo consists of a disc-shaped mass of cells several strata in thickness (the blastoderm) lying closely applied to the yolk (Patten, 1971). Its lower surface rests on the yolk and becomes the ventral side of the embryo, while the cells at the upper side lie immediately beneath the vitelline membrane and become the dorsal side. There is a potential difference of 25 mV across the blastoderm between the acidic ventral side and the basic dorsal side, and the differing environments at the two sides of the blastoderm may play a role in bringing about the dorso-ventral polarity. In the center of the blastoderm the cells are smaller and completely defined; at the periphery the cells are flattened, larger in surface extent, and are not walled off from the yolk beneath (Bellairs & Osmond, 2005).

By the time of laying, the unincubated blastoderm already consists of several regions and the embryo possesses several thousand cells (Bellairs & Osmond, 2005; Patten, 1971). Estimates range from 32,000-42,000 cells to as many as 60,000 (Lemaire & Kessel, 1997; Patten, 1971).

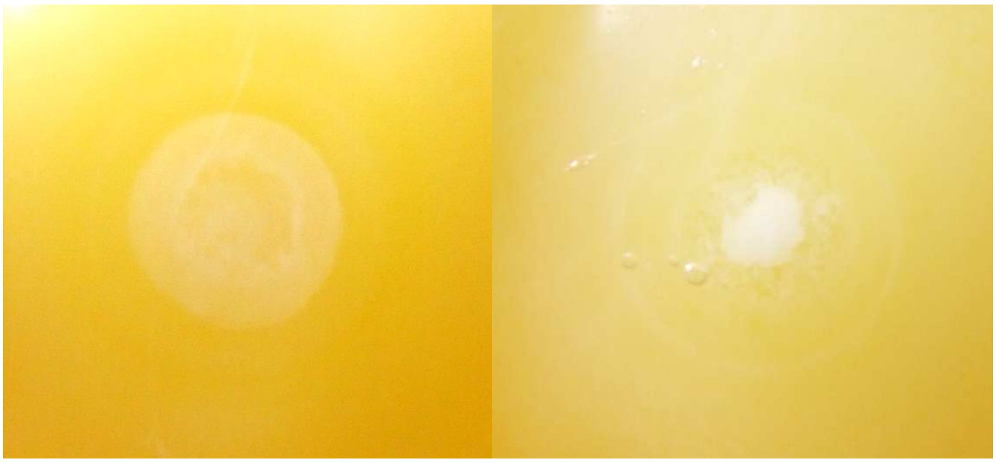


**Figure 29. Appearance of a fertile unincubated egg (left) in contrast with an infertile egg (right).**

The most striking feature of the first 24 hours of embryonic development is the division into the *area pellucida*, the almost transparent central region, and the *area opaca*, the more opaque peripheral ring (Figure 29) (Wittler & Kessel, 2004). The opacity of the *area opaca* is due to the presence of large numbers of intracellular yolk droplets in the lower layer. Intracellular yolk droplets are present in the *area pellucida* also, but they are much smaller and fewer than those in the *area opaca* so that the tissue is relatively transparent (Bellairs & Osmond, 2005; Wittler & Kessel, 2004). The *area pellucida* gives rise mainly to embryonic tissues, whereas the lateral borders of the *area pellucida* together with the entire *area opaca* form extra-embryonic tissues only (Bellairs & Osmond, 2005). Between the two regions is a ring of cells called the marginal zone (Page et al., 2001; Wittler & Kessel, 2004).

At this point, the embryo is a bilaminar disc composed of a single-cell thick epiblast underlain by a looser layer, the hypoblast (Page et al., 2001; Pera, Stein, & Kessel, 1999). The epiblast consists of the *area pellucida* or central disc, surrounded by the *area opaca* (Page et al., 2001). Although morphologically the epiblast appears largely as a homogeneous, single layer, it is already patterned along an axis of bilateral symmetry, which became established in the uterus under the combined influence of gravitational and mechanical forces (Pera et al., 1999). Under normal conditions, antero-posterior polarity is specified fairly early on by the action of gravity, but the axis is not irreversibly determined (i.e. external cues may alter its orientation) until the appearance of the primitive streak (Page et al., 2001).

The primitive streak is the first axial structure to appear during embryonic development in amniotes (Bellairs & Osmond, 2005; Page et al., 2001). It forms the scaffold on which the main body axis will be built. Under normal circumstances, the blastodisc develops a single primitive streak, which marks the position of the future backbone of the embryo. The streak normally forms adjacent to the posterior marginal zone and there is evidence that this structure causes streak induction. All (or at least most) of the marginal zone has the ability to initiate streak formation, but most of the marginal circumference appears to do so only in the absence of a more potent inducer (Page et al., 2001).

The primitive streak starts to be visible in the area pellucida at about 10 hours of incubation, when cells begin to migrate from lateral posterior parts of the *area pellucida* towards the central midline, accompanied by a thickening of cells in the posterior region of the upper layer (Bellairs & Osmond, 2005; Wittler & Kessel, 2004). This thickening forms the beginnings of the primitive streak, appearing as a dark triangular region in the upper layer, its apex lying in the *area pellucida* and its base along the border with the *area opaca* (Bellairs & Osmond, 2005; Wittler & Kessel, 2004). The streak then elongates anteriorly (becoming thinner) towards the center of the blastodisc (Bellairs & Osmond, 2005).

Meanwhile, by about 12 hours of incubation the periphery of the *area opaca* has become attached to the inner surface of the taut vitelline membrane, using it as substrate for the migrations of cells from peripheral cells of the *area opaca* - the so-called ‘margin of overgrowth’ - so that the entire blastoderm gradually expands outward (Bellairs & Osmond, 2005; Sanders & Wride, 1997). The blastoderm itself is under tension at this point. If that tension is released, abnormal development follows (Bellairs & Osmond, 2005).

The primitive streak keeps extending anteriorly until about 13 hours of incubation, when it reaches the center of the *area pellucida* (Bellairs & Osmond, 2005). The primitive streak also extends posteriorly, so that the posterior end eventually moves into the area opaca, causing the area pellucida as a whole to become pear-shaped (Hamburger & Hamilton, 1992).

By about 18 h of incubation it has reached its full length, extending about two-thirds of the way across the *area pellucida* (Bellairs & Osmond, 2005; Page et al., 2001). When the primitive streak is fully formed, the first stages of gastrulation are complete and the tissues that will develop into the body are arranged around the primitive streak's anterior end, especially around the extreme end, a swollen tip known as Hensen's node (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992). All these presumptive areas then ingress through the primitive streak, leaving the epiblast and becoming part of the primitive streak before migrating away from it to give rise to a number of tissues: (1) the definitive endoderm, which will form the lining of the gut and the respiratory system; (2) the head process and notochord; (3) the paraxial mesoderm (somites), the intermediate mesoderm (which forms the urino-genital system) and the lateral plate mesoderm (which lines the coelom and contributes to the extra-embryonic membranes) (Bellairs & Osmond, 2005; Pera et al., 1999; Stern, 2005).

The presumptive notochord cells in Hensen's node and the anterior primitive streak give rise to the notochord proper (Bellairs & Osmond, 2005; Catala, Teillet, De Robertis, & Le Douarin, 1996). The most anterior part of the notochord is the head process, formed by the anteriorward migration of cells from Hensen's node, whereas the trunk notochord is formed by the caudalward movement of cells during the process of regression (Bellairs & Osmond, 2005). The first appearance of the head process is as a small dark triangle anterior to Hensen's node, which marks the completion of the formation of the primitive streak (Bellairs & Osmond, 2005; Lemaire & Kessel, 1997). The onset of regression initiates the disappearance of the primitive streak (Bellairs & Osmond, 2005; Catala et al., 1996).

Hensen's node regresses posteriorly, leaving notochord structures in its wake (Page et al., 2001; Patten, 1971). Two elementary mechanisms may account for notochordal elongation: addition of cells coming from Hensen's node and cellular proliferation (and rearrangement) in the caudal part of the already formed notochord (Catala et al., 1996). Differentiation of the tissues takes place gradually from anterior to posterior as regression occurs, so that the development of the anterior tissues is always in advance of the posterior ones. The cephalic end of an embryo will always be found precocious in differentiation as compared with the more posterior portions of the embryo. The head is actually older in development, since the structures posterior to the head are laid down by cells which proliferated from the caudally located areas of rapid growth subsequently to the establishment of the head itself. Differentiation occurs more quickly in the head, preventing other regions from passing it in developmental progress (Patten, 1971).

The first indication of the body proper is at about 24 hours of incubation (Figure 30), when the head fold develops (Hamburger & Hamilton, 1992; Lemaire & Kessel, 1997). Anterior to the tip of the head process a crescent-shaped groove appears in the entire thickness of the blastoderm. The result is that the region of the future head, together with the developing foregut, becomes raised above the flat blastoderm (Bellairs & Osmond, 2005).

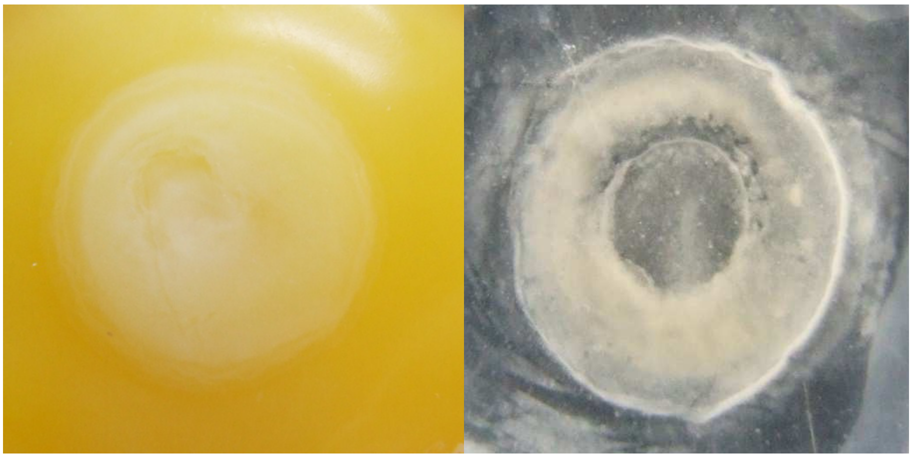


**Figure 30. Appearance of a 24-hour embryo in the egg and after removal from the yolk.**

The first pair of somites appears at around the same time and succeeding pairs are laid down sequentially further and further posteriorly (Hamburger & Hamilton, 1992). The cells that will become somatic mesoderm leave the primitive streak and enter a segmental plate, a strip of condensed mesoderm lying on one of the sides (left or right) of the neural tube. Groups of cells at the anterior end of the two segmental plates then become separated off simultaneously to form the left and the right of a pair of somites (Bellairs & Veini, 1984). Each newly formed somite is a ball of columnar epithelium, the walls consisting of a single layer of cells surrounding a cavity, the somitocoele, which contains mesenchyme cells. While a steady stream of mesenchyme cells is added to the posterior end of the segmental plate, a new pair of somites forms from the anterior end of each segmental plate at about every 90-100 min. Thus, the segmental plate remains visible, even though there is a continuous turnover of the individual cells which constitute its population (Bellairs & Osmond, 2005).

At this point, the basic embryonic axis consists of ectoderm, including neural plate and neural crest, notochord, somites, lateral plate and intermediate mesoderm, and endoderm (Bellairs & Osmond, 2005; Catala et al., 1996; Hamburger & Hamilton, 1992). The neural plate initially forms as a pear-shaped area. As soon as it is formed, however, it starts regressing, narrowing and extending posteriorly at the trunk region and widening at the brain region (Bellairs & Osmond, 2005). The notochord is attached to the floor of this neural plate (known as the floor plate) and acts as a hinge around which the neural plate folds and bends to form a tube (Monsoro-Burq, Stieber, Bontoux, Le Douarin, & Gonatas, 1998). This tube, known as the neural tube, begins to form at around 26 hours of incubation, at the level of the midbrain and is rapidly followed by fusion of the neural folds throughout the entire brain and the anterior regions of the trunk (Bellairs & Osmond, 2005; Grapin-Botton & Melton, 2000).

Along the dorsal side of the early neural tube, a strip of cells lies forming a ‘crest’ down the tube’s entire length: the neural crest (Wahl & Noden, 2001). As the neural tube closes, the neural crest becomes lodged between it and the overlying ectoderm, later settling down onto the dorsal surface of the neural tube (Bellairs & Osmond, 2005). Neural crest cells will eventually give rise to cartilage and bone of much of the head and face skeleton, connective tissue and Schwann cells, cranial sensory ganglia, the entire enteric (gut) nervous system, pigment cells of the dermis, the dorsal roots of ganglia, sympathetic ganglia, and adrenomedullary cells (Schneider, 1999; Wahl & Noden, 2001).

About the same time, the foregut starts to appear. The first part of it to form lies anteriorly, ending blindly at the stomatodaeal (oral) plate, where it is fused to the ectoderm. The open, posterior end of the foregut diverticulum, the anterior intestinal portal, is not a fixed point but merely an opening into the foregut at this stage (Bellairs & Osmond, 2005).

As the foregut forms, the precardiac cells are moved into the midline and begin to condense into bilateral tubular structures (Aleksandrova et al., 2012; Bellairs & Osmond, 2005). As the developing foregut loses contact with the yolk sac, its sides joining medially to form the pharynx, the two endocardial tubes of the heart fuse in the midline to form a simple tube, anterior and ventral to the anterior intestinal portal (Aleksandrova et al., 2012). Blood islands become visible in the proximal region of the yolk sac, the area vasculosa (Dieterlen-Lievre & Le Douarin, 2004). These develop from isolated clumps of cells in the splanchnic mesoderm, which will form the first blood vessels, extra-embryonic in nature. The peripheral cells of each blood island become the endothelium of the vessels, while the remaining cells become the hematopoietic (primitive) blood cells (Dieterlen-Lievre, 1975). Paired dorsal aortae soon become visible beneath the somites, as well. The anterior cardinal veins and the median head vein, which will give rise to the main venous vessels of the head and neck, also begin to form at this stage (Garriock & Mikawa, 2011).

The heart proper begins to develop after hour 29 of incubation, after the endocardial tubes have fused into a single tube, which then loops into an S-shape (Aleksandrova et al., 2012; R. E. Poelmann, Gittenberger-de Groot, & Hierck, 2008).

Even as it forms, the basic regions become apparent, first the truncus and ventricle, then the atrium and lastly the sinus venosus (Bellairs & Osmond, 2005; Mortola, 2009). Action potentials in the cardiac mesoderm can already be recorded at this stage, first appearing in the left caudal region of the ventricle, before either the atrium or the sinus venosus have formed (Bellairs & Osmond, 2005).

The first pair of aortic arches forms at around the same time (Bellairs & Osmond, 2005). The aortic arches are blood vessels formed mainly from neural crest cells, which supply the pharyngeal arches, serving as a communication between the ventral and dorsal aortae (Garriock & Mikawa, 2011; Wahl & Noden, 2001). The ventral aorta is the main artery into which the truncus arteriosus leads. It bifurcates into left and right vessels which extend forward as the paired external carotids, while the paired dorsal aortae extend forward as the internal carotids. The aortic arches do not all develop at once and some are of a transitory nature, disappearing before others have even begun to form. They are paired, serving the left and right pharyngeal regions. Intersomitic arteries sprout from the dorsal aorta at this time as well (Garriock & Mikawa, 2011).

Meanwhile, the basic pattern of the yolk sac vessels (which will carry blood from the embryo to the yolk sac and back again, to transport partly digested yolk to the embryo) begins to form, with the blood islands in the splanchnic mesoderm of the proximal part of the *area opaca* joining those in the distal part of the *area pellucida* to form a meshwork of capillaries (Figure 31) (Bellairs & Osmond, 2005; Moonen & Villamor, 2011).

The eyes have also begun to form, with the appearance of the rudiments of the primary optic vesicles, two bulgings at the lateral sides (one on each side) of the prosencephalon (Goodall et al., 2009).

At about 30 hours of incubation, the nephric duct (which will serve the pronephric and, later, the mesonephric kidney) has also begun to develop (Bellairs & Osmond, 2005).

The first heart beats begin  around hours 33 and 36 of incubation (Hamburger & Hamilton, 1992). Looping of the cardiac tube begins during the same time period, when the right ventricular wall bulges outwards and becomes convex at the same time as the left wall becomes concave (R. E. Poelmann et al., 2008; Voronov, Alford, Xu, & Taber, 2004). The cardiac tube is capable of looping as soon as it has formed, normally looping to the right. Many factors have been associated with this but no definitive reasons have yet been found to this asymmetry in looping. (Bellairs & Osmond, 2005; R. E. Poelmann et al., 2008; Schlueter & Brand, 2009; Voronov et al., 2004)

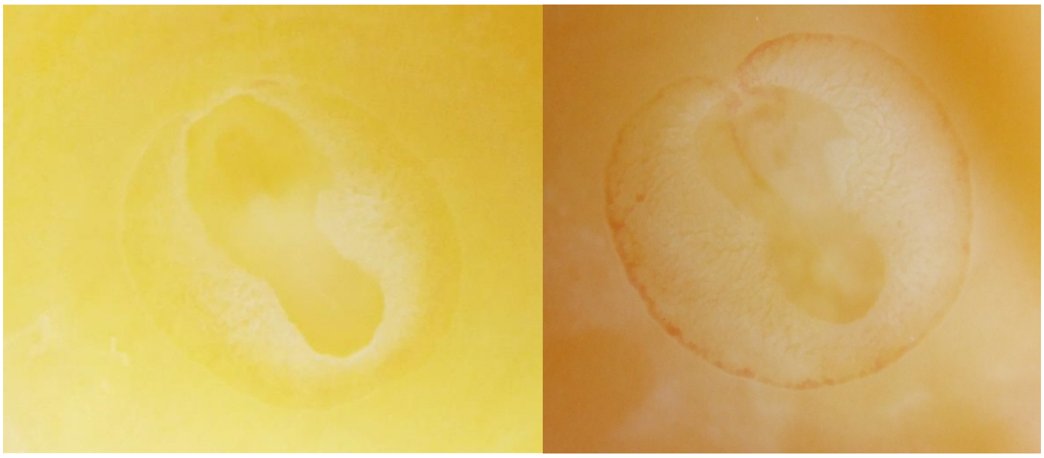


**Figure 31. Examples of 48-hour embryos.**

At about 36 hours of incubation, the pronephros, the most cranial region of the embryonic region (and the earliest in development of three distinctive regions) begins to form (Bellairs & Osmond, 2005; James & Schultheiss, 2003). The mesonephros and then the metanephros will later follow (James & Schultheiss, 2003).

By this stage, the brain has become divided into three primary regions, the prosencephalon (forebrain), the mesencephalon (midbrain) and the rhombencephalon (hindbrain) (Bellairs & Osmond, 2005; Rodriguez-Gallardo, Sanchez-Arrones, Fernandez-Garre, & Puelles, 2005). The ciliary ganglia (from which nerve fibers will emerge to innervate the oculomotor muscles) also appear at this time. Each is situated two-thirds of the distance from the proximal to the distal end of the oculomotor nerve (Bellairs & Osmond, 2005). The left and right otic (ear) placodes, precursors of the auditory nerves, are visible in embryos of this age as thickened regions on either side of the head just anterior to the somites (Bellairs & Osmond, 2005; Pera et al., 1999; Shamim & Mason, 1998).

By around 40 to 45 hours of incubation, the remnants of Hensen’s node and the primitive streak lie at the posterior end of the area pellucida and have become aggregated into a mass of cells, the tail bud (Catala et al., 1996). As the tail fold (posterior body fold) forms, it undercuts the node, which, therefore, becomes wrapped dorsally, ventrally and posteriorly in ectoderm (Bellairs & Osmond, 2005).

At this point, the rhombencephalon has become subdivided into the metencephalon (cerebellum and *pons*) and the myelencephalon (medulla oblongata), and the facial nerve is now recognizable (Bellairs & Osmond, 2005; Hsu & Jeng, 1995).

Meanwhile, the segmental (intersegmental) arteries, branches of the dorsal aorta that contribute to the formation of the vertebral and subclavian arteries, have formed between all existing somites (Garriock & Mikawa, 2011).

At around 45 hours of incubation, the embryo’s head bends ventrally toward the yolk sac, creating the cranial flexure (Hamburger & Hamilton, 1992). At this point, the head has also begun to rotate, so that its left side comes to lie against the yolk sac and its right side lies uppermost. However, at this stage the trunk region has not turned yet, so, while the head already lies on its side, the rest of the body still lies with its ventral side against the yolk sac (with the dorsal side lying uppermost). The trunk itself soon becomes curled around, so that the tail bud comes to lie close to the head (Bellairs & Osmond, 2005).

By this time, the anterior neuropore becomes obliterated, marking the ending of the fusion of the neural folds that make up the brain. The walls of the telencephalic vesicles have now begun to thicken in order to later form the cerebral hemispheres. Their lumina will give rise to the lateral ventricles (Xu et al., 2011).

The connection between the optic vesicles and the brain has meanwhile thinned into a narrow stalk (Bellairs & Osmond, 2005; Goodall et al., 2009). The distal part of each optic vesicle (the future sensory layer), invaginates and presses against the proximal part (the future pigment layer of the retina, iris and ciliary body), forming the optic cup, eliminating the original lumen of the optic vesicle and forming a new lumen, the future vitreous chamber (Bellairs & Osmond, 2005; Goodall et al., 2009). The wall of the cup is absent on the ventral side and the invagination is able to continue along the ventral side of the optic stalk, to form a groove along which the optic nerves and blood vessels subsequently pass (Bellairs & Osmond, 2005). The gap in the ventral wall of the optic cup is called the choroid fissure (Bellairs & Osmond, 2005; Goodall et al., 2009). Concurrently, the lens placode, a thickening of the ectoderm which will give rise to the lens proper, has just become visible and the cells of the neural crest that will become the melanophores of the choroid have also reached the eye (Bellairs & Osmond, 2005; Wahl & Noden, 2001).

The left and right otic placodes have, in the meantime, invaginated to form the otic (auditory) pits (Robertson & Mason, 1995).

Meanwhile, in the circulatory system, the common cardinal veins, which form the initial H-shaped structure of the venous system, have already begun to form and fused with the omphalo-mesenteric (vitelline) veins, which have meanwhile formed a meshwork of capillaries and channels over the yolk sac (van den Berg & Moorman, 2011). Waves of excitation have also started to sweep through the heart from the caudal pacemaker, leading to the rhythmical contraction of the muscles and initiating blood circulation through the blood vessels (Bellairs & Osmond, 2005; de Jong, Geerts, Lamers, Los, & Moorman, 1987).

Three to four pairs of pharyngeal arches are present by the end of day 2 of incubation, as well as the stomatodaeum, an ectodermal depression that appears beneath the forebrain and will later form the buccal cavity (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992). The liver primordium is also visible at the end of day 2 (Bellairs & Osmond, 2005).

## 3-4 days of incubation

At the beginning of the third day of incubation, the neural tube is progressively closing down the trunk. The process will become almost complete by 48 to 52 hours into the incubation process, with a single opening remaining at its posterior end, the posterior neuropore (Bellairs & Osmond, 2005). As the neural folds fuse, the cells of the neural crest come to lie at the dorsal side of the neural tube (Wahl & Noden, 2001).

Differentiation of the prosencephalon into telencephalon and diencephalon (thalamus) has also begun to take place (Bellairs & Osmond, 2005). Paired swellings, have begun to appear just anterior to the optic vesicles, to form the telencephalic vesicles, each of which communicates with the median telocoele (the central lumen of the brain in that region) by the foramen of Monro (Bellairs & Osmond, 2005; Mey & Thanos, 2000). At about the same time, the cranial flexure has begun to appear as a bend at the anterior end of the mesencephalon (Hamburger & Hamilton, 1992). Along the spine, the ventral roots of the spinal nerves have emerged in the cervical region (Bellairs & Osmond, 2005).

Meanwhile, communication between the optic vesicles and the anterior end of the diencephalon becomes reduced to a stalk (Goodall et al., 2009; Mey & Thanos, 2000). The various branches of the trigeminal nerve appear as well, together with the large, neural crest-derived trigeminal ganglion, situated at the widest part of the mesencephalon (Bellairs & Osmond, 2005; Wahl & Noden, 2001).

A branch from the facial ganglion (cranial nerve VII) has reached the upper end of the hyoid arch by this stage, and a large ganglionic complex begins to form at the root of the IX and X cranial nerves, the most rostral portion of it being the superior ganglion of the glossopharyngeal nerve, and the more caudal part the jugular ganglion of the vagus.

The pro-epicardial serosa, which will give rise to the epicardium, develops from the pericardial wall between hours 48 and 52 of incubation (Bellairs & Osmond, 2005; Schlueter & Brand, 2009). As it does so, cells from the pro-epicardial serosa are transferred across the pericardial cavity to the surface of the developing ventricles, either as free-floating aggregates or by the formation of bridges across the lumen

(Bellairs & Osmond, 2005; Duband, Volberg, Sabanay, Thiery, & Geiger, 1988). The primordial epicardium then spreads as a continuous epithelial sheet over the myocardium (Bellairs & Osmond, 2005; Schlueter & Brand, 2009).

In the urinary apparatus, the pronephros is now starting to degenerate (Bellairs & Osmond, 2005).

The hindgut begins to appear at around the same time. By 50 to 53 hours of incubation, the hindgut is present as a simple tube, its anterior border being the posterior intestinal portal. A small section of the hindgut, lying at its most posterior end, extends into the tail region at this time, becoming an integral part of it. This section is known as the tailgut and will later become obliterated, largely as a result of cell death. The cloacal (proctodaeal) membrane, which will later form the cloaca, is visible at about this stage as well (Bellairs & Osmond, 2005).

Meanwhile, the head is beginning to bend in the neck region, so that it lies at a right angle with the trunk, its ventral side lying against the ventral side of the pharynx, thus forming the cervical flexure (Hamburger & Hamilton, 1992). The first cellular precursors of the ribs are also starting to proliferate (Bellairs & Osmond, 2005; Nowicki, Takimoto, & Burke, 2003).

In the brain, the neural or posterior lobe of the hypophysis, named infundibulum, starts to appear. Development of the ears is also well under way, with the opening of the auditory pits now becoming greatly restricted to form the otic vesicles or otocysts (Bellairs & Osmond, 2005).

In the heart, the interatrial septum begins to form as a small ridge at the cranio-dorsal end of the, so far, single atrium (Bellairs & Osmond, 2005). Brought into proximity by the enlargement of the coelom and the formation of the dorsal mesentery, the right and left dorsal aortae start to fuse together (Garriock & Mikawa, 2011; Y. Wang et al., 2009). Fusion remains incomplete in the extensions of the aortae anterior to the heart, which subsequently become the internal carotids (Garriock & Mikawa, 2011; Y. Wang et al., 2009). At its most posterior end, the dorsal aorta extends into the tail as a small vessel, the caudal artery (Garriock & Mikawa, 2011; Y. Wang et al., 2009).

The potential limb regions become visible from about 50 to 55 hours into the incubation process, lying at the level of somites 15-20 (wing buds) and 26-32 (leg buds). As the lateral body folds form, the limbs come to lie along the sides of the body wall (Bellairs & Osmond, 2005).

**Figure 32. Appearance of a 3-day old embryo.**

At 3 days of incubation (Figure 32), each limb bud is about 1 mm in length by about 1 mm in width (Hamburger & Hamilton, 1992; J. B. Miller & Stockdale, 1986). Meanwhile, the allantois begins to arise as an outgrowth from the hindgut (Hamburger & Hamilton, 1992).

On the head, the nasal (olfactory) placodes (the first structures to appear in the formation of the nose) now become visible as shallow pits on either side of the fronto-nasal process (Bellairs & Osmond, 2005; Drapkin & Silverman, 1999; Pera et al., 1999). Soon, these pits give rise to two thickened regions anterior to the eyes, which, like the optic and otic placodes, will later invaginate to form the nasal pits (Bellairs & Osmond, 2005).

Between hours 51 and 56 of incubation, blood circulation has become well established in the embryonic body. The vascular area of the yolk sac has meanwhile become surrounded by the sinus terminalis, a vein that sharply delineates the area vasculosa from the non-vascular area vitellina, which lies more peripherally. The capillaries that form near the body are relatively large and gradually become transformed into a major pair of vessels, the left and right omphalo-mesenteric (vitelline) arteries. These soon fuse together, to form a single vessel. Blood moves from the dorsal aorta into the right and left omphalo-mesenteric arteries and then into the area vasculosa, where it enters a series of smaller and smaller branching arteries, eventually moving through a meshwork of fine capillaries that extends over the proximal part of the area vasculosa (the developing yolk sac). It is then collected up into the anterior or posterior omphalo-mesenteric (vitelline) veins, either directly or by way of the sinus terminalis, before returning to the heart (Bellairs & Osmond, 2005).

The heart itself is beginning to divide into a left and right channel by the growth of a series of septa, so that after hatching, the two sides of the heart are completely separate. Two apposed ridges, the atrioventricular cushions, project into the lumen of the atrio-ventricular canal from its dorsal and ventral walls. The cushions are initially formed from acellular cardiac jelly which then becomes invaded by cells from the endocardium to form the cardiac septa (Robert E. Poelmann et al., 2014; Qayyum et al., 2001). The future left ventricle bulges from the, so far, single ventricle at this point, possibly due to the force of the blood streams (Bellairs & Osmond, 2005).

At a region just posterior to the omphalo-mesenteric artery, primordial germ cells are collecting, brought here via the blood stream, which they now leave to enter the splanchnopleure at the site of the future gonad (Nakamura et al., 2007). The nephrogenous mesenchyme has enlarged and given rise to a pair of ridges that hang down from the body wall into the peritoneal cavity on either side of the gut. As the genital components develop, each ridge becomes known as an uro-genital, nephrogenic or genital ridge. In the spine, spinal nerves can now be identified (Bellairs & Osmond, 2005).

The amnion and chorion are also developing, stretching as a fold from the anterior to the posterior ends of the embryo, with additional lateral folds that rise up and meet to fuse in the midline (Hamburger & Hamilton, 1992). The inner part of this fold will become the amnion, while the outer will form the chorion, the inner and outer layers becoming separated from each other as the lateral folds fuse together (Bellairs & Osmond, 2005). By 51 to 56 hours of incubation, the amniotic fold extends as far as somites 10-18. In the next eight to twelve hours, a similar fold will begin to form immediately posterior to the tail bud and later converge with the anterior one to form the amniotic vesicle (Hamburger & Hamilton, 1992).

A temporary layer of squamous epithelial cells, the periderm, forms over the outer layers of the skin at about this time, to act as a barrier between the internal tissues and the amniotic fluid (Bellairs & Osmond, 2005).

By 52 to 64 hours into incubation, the posterior cardinal veins are fully formed. From them arise the segmental veins which pass into the intersomitic spaces, along with the segmental arteries, as well as other branches, which anastomose to form the left and right subcardinal veins, which lie close to the left and right posterior cardinal veins respectively, on the medial side of the mesonephroi and lateral to the aortae. Branches from the subcardinal veins collect blood from the hind limbs and tail as well as from the intestines, and pass it via the mesonephric veins on to the posterior cardinal veins. Further veins, the left and right vena capitis lateralis (lateral head vein), have also formed by this time to ensure drainage of the forebrain (Bellairs & Osmond, 2005).

In the head, the olfactory nerve is beginning to form from cells that extend from the nasal placode to the olfactory lobes. Fibers of this nerve will become visible in the next eight to 24 hours (Drapkin & Silverman, 1999). The first axons of the hypoglossal nerve (Cranial Nerve XII), which supplies the muscles at the base of the tongue, are also starting to appear (Bellairs & Osmond, 2005).

Meanwhile, Rathke's pouch, which is destined to become the anterior lobe of the hypophysis and the pars tuberalis, has arisen to fuse, at its anterior end, with the infundibulum (Hamburger & Hamilton, 1992; Reijntjes, Blentic, Gale, & Maden, 2005).

The thyroid forms at about this time, from a median invagination from the floor of the pharynx at the level of the second pair of pharyngeal pouches (Hopkins, 1935; McNabb, 2006). During day 3, it becomes a sac open to the lumen of the pharynx (McNabb, 2006).

In the embryonic kidney, the first glomeruli of the pronephric region have become visible. However, the nephric tubules in this region are not yet fully patent, nor are the blood vessels in the glomeruli continuous with the aorta. The same cannot be said about the nephric ducts, which have lengthened since their appearance at 30 hours of incubation, and are now fused with the cloaca (Bellairs & Osmond, 2005).

The dorsal pancreas, one of three rudiments from which the pancreas of birds develops, forms at around this time as well, as an outgrowth from the dorsal wall of the gut (Bellairs & Osmond, 2005). The α-cells of the organ already secrete glucagon at this stage (Maňáková & Titlbach, 2007).

The pyloric sphincter appears to be induced at the caudal end of the developing gizzard at this time (Bennett & Cobb, 1969). By day 3 of incubation, the embryonic gut consists of three sections: foregut, midgut and hindgut. The midgut is similar to the foregut and hindgut but differs from them in that, although possessing lateral body walls, it lacks a ventral wall and is in communication with the yolk sac. The allantoic diverticulum starts to arise from the hindgut in embryos at about 65 to 69 hours of incubation, though there is considerable individual variation. Shortly after its appearance, the allantois forms a ramifying network of capillaries that receive blood via the allantoic (umbilical) arteries and return it to the body via the allantoic (umbilical) veins. It also fuses with the chorion, a structure consisting of ectoderm underlain by somatic mesoderm, continuous at its lateral edges with the splanchnopleure covering the yolk sac, but associated elsewhere with the allantois, to form the chorioallantoic membrane (Bellairs & Osmond, 2005).

The vitelline membrane breaks down over the embryo during day 3 and its value as a substratum is lost (Bellairs & Osmond, 2005). Meanwhile, the anterior, lateral and posterior regions of the amniotic folds have united above the region of the hind limbs, forming initially a small oval opening, the amniotic umbilicus (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992). Although this soon closes, the inner and outer layers remain attached to one another at this single point (Bellairs & Osmond, 2005).

In the cranial pole of the embryonic body, fibers from the retina are beginning to enter the optic stalk (Goodall et al., 2009; Mey & Thanos, 2000). Cells are also leaving the auditory epithelium to form the acoustic ganglion. The nodose ganglion, an integral part in the formation of the vagus nerve, begins to appear as well, as a group of cells lateral to the dorsal aorta, where it is joined by the anterior cardinal vein. It subsequently shifts caudally (Bellairs & Osmond, 2005).

A few hours later (between 68 and 72 hours into incubation), the pineal gland begins to form as an evagination from the median part of the roof of the diencephalon (Bellairs & Osmond, 2005). Distinct areas in Rathke's pouch precursor to the pars distalis and pars tuberalis can already be recognized histologically at around this time (Bellairs & Osmond, 2005; Reijntjes et al., 2005).

By 70 to 72 hours of embryonic development, the notochord extends from the level of Rathke's pouch to the tip of the tail bud and is one of the few medial structures in the body (Bellairs & Osmond, 2005; Catala et al., 1996; Hamburger & Hamilton, 1992).

At this stage, the trochlear nerve (Cranial Nerve IV), which innervates the superior oblique muscle of its contralateral eye, is just beginning to arise, followed by the abducens nerve (Cranial Nerve VI), another oculomotor nerve. Meanwhile, in the circulatory system, the sinus terminalis is beginning to regress and the posterior vena cava starting to form, with contributions from both the posterior cardinal veins and the subcardinal veins (Bellairs & Osmond, 2005).

The first morphological sign of the development of the respiratory tract is the laryngo-tracheal groove, which becomes visible in the midline of the floor of the pharynx during this period of incubation, arising posterior to the fourth pharyngeal pouches. As it extends caudally, it becomes closed off from the pharynx along most of its length, forming a tube, the trachea. It remains open to the pharynx at its most anterior end, however, this opening constituting the glottis. Cartilaginous rings begin to form in the mesoderm surrounding the trachea and bronchi around this time (J. N. Maina, 2003b).

By 3.5 days in incubation, about 50 pairs of somites have been laid down. The five pairs of pharyngeal pouches, the fifth of which is smaller, are fully formed as well, and coincide with a series of grooves, the ectodermal grooves, that indent the ectoderm (Hamburger & Hamilton, 1992).

The mesencephalon is now covered with fibers and has grown so large that it projects over the metencephalon (Bellairs & Osmond, 2005). The mesencephalon is associated with vision, among other things, and its great dimension foreshadows the enormous size of the optic lobes in the adult, correlating with the importance of vision in birds (Goodall et al., 2009; M. P. Jones, Pierce, & Ward, 2007; Mey & Thanos, 2000).

As the third day of incubation reaches its end, the stomatodaeal (oral) plate breaks down connecting the anterior (ectodermal) and posterior (endodermal) parts of the oral cavity and bringing the lumen of the foregut into communication with the amniotic cavity. Further development of the mouth will include formation of the jaws from the first pair of pharyngeal arches. The region of the foregut which will form the stomach has begun to swell and shift toward the left of the midline (Bellairs & Osmond, 2005). Shortly afterwards the division into proventriculus and gizzard begins (Bennett & Cobb, 1969). Mucosubstances are already present in the gizzard at this time, even though the secretory glands will not begin to form until day 12 of incubation (Bellairs & Osmond, 2005).

In the spine, the first signs of the primary sympathetic chain are visible as two rows of cells lying one on either side of the neural tube, ventral to the somites and immediately lateral to the aorta (S. Bhargava, 2007; Kulesa, Lefcort, & Kasemeier-Kulesa, 2009).

By the fourth day of incubation, the cervical flexure has become fully formed and the embryo’s head now lies at a right angle with the trunk (Hamburger & Hamilton, 1992). The notochord has reached its maximum diameter, of 115-160 μm, due to the development of vacuoles which subsequently secrete material that supplements the notochordal sheath (peri-notochordal tube) (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992).

The ciliary nerve (Cranial Nerve III), which runs from its homonymous ganglion to the iris, is now visible and the lens has begun to acquire its characteristic, lentoid, shape (Bellairs & Osmond, 2005; Mey & Thanos, 2000). The choroid fissure usually begins to close in the region near the lens about this time, while a ridge of mesoderm migrates along the choroid fissure into the posterior chamber of the eye, carrying with it a blood vessel which will give rise to the pecten (Bellairs & Osmond, 2005; Goodall et al., 2009; Thanos & Mey, 2001).

As day 4 of incubation progresses, the interatrial septum, which began to form at about 50 to 52 hours of incubation, begins to fuse with the atrioventricular cushions to divide the atrium into left and right compartments (Bellairs & Osmond, 2005; R. E. Poelmann et al., 2008). The right ventricle bulges from the still single ventricle at this time, leaving a part of the original wall of the single ventricle between it and the left bulge. This remainder of the original wall will later be the source of the interventricular septum (Robert E. Poelmann et al., 2014; Qayyum et al., 2001). All the main chambers have thus been formed, and the heart is now situated at the level of the wing buds. The ‘descent’ of the heart is largely the result of an increase in the length of the neck and brain, which shift forward in relation to the heart (Figure 33). All the arterial arches have now formed as well (Bellairs & Osmond, 2005).

**Figure 33. Appearance of a 4-day old embryo.**

The caeca begin to form during day 4 as bilateral swellings approximately midway between the umbilical wall and the base of the allantois (Bellairs & Osmond, 2005). The large intestine, which lies posterior to the caeca begins to form soon after, as does the bursa of Fabricius, which forms as a diverticulum on the dorsal side of the cloaca (Bellairs & Osmond, 2005; Dieterlen-Lievre & Le Douarin, 2004). Haemopoietic cells reach the spleen at about this time (Bellairs & Osmond, 2005).

The left and right ventral pancreatic rudiments (which join the already formed dorsal rudiment to form the pancreas) are now clearly visible as evaginations from the base of the hepatic diverticulum, and insulin is starting to be secreted by its b-cells (Bellairs & Osmond, 2005; Maňáková & Titlbach, 2007).

Other hormones being produced at this time are estrogens and testosterone, secreted by the yet indifferent gonad. Estrogenic hormones are secreted by the interstitial cells of the medulla, whereas testosterone is produced by the cord cells (Nomura, Nakabayashi, Nishimori, Yasue, & Mizuno, 1999).

Meanwhile, the thyroid becomes bilobed, as the enlarging esophagus, pressing the trachea against the thyroid, reduces the glands connection to the pharynx to a single, solid stalk, the thyroglossal duct (McNabb, 2006).

The trachea itself has its lumen temporarily closed at this point, whereas the lungs are now small, smooth-surfaced, sacs lying on either side of the esophagus (Makanya, Hlushchuk, & Djonov, 2011).

Solid nodular structures lying between the mesonephros and the dorsal aorta announce the formation of the adrenal glands. The metanephric diverticulum, which will later form metanephric (ureteric) duct – in itself a precursor of the medullary (collecting) tubules and ureter - now begins to arise, from the posterior end of the nephric duct, just short of its junction with the cloaca. In the area which will later become the pelvic girdle, a mesenchymal condensation begins to separate into its three components, the dorsally situated ilium, the laterally situated ischium and the latero-ventrally situated pubis (Bellairs & Osmond, 2005).

In the nervous system, the two halves of the cerebellum now begin to form (Bellairs & Osmond, 2005). The retinal fibers of the optic nerve have already crossed through the optic chiasm, coming from the optic stalk while, along the spine, the ganglia of the primary and secondary sympathetic chains are beginning to form (Bellairs & Osmond, 2005; Goodall et al., 2009; Thanos & Mey, 2001).

## 5-12 days of incubation

At the beginning of the 5th day of incubation, the wing and leg buds have already begun to acquire their individual characteristics (Hamburger & Hamilton, 1992). Both the scapula and the coracoid have become established in membranous bone by this time, and the pre-cartilaginous precursors of the femur (already undergoing chondrification), tibia and fibula have been already laid down (Newman & Frisch, 1979). The first indication of muscle development in a limb bud is the formation of two pre-muscle masses which form close to the girdle (whether pectoral or pelvic) (Caplan & Koutroupas, 1973; Shellswell & Wolpert, 1977). They become visible in the wing bud at this stage, lying dorsal and ventral to the condensation of cartilage that will form the wing skeleton. Lobulations in the ventral mass indicate some of the future muscles (Caplan & Koutroupas, 1973; Shellswell & Wolpert, 1977).

The vertebrae are cartilaginous and the cranial flexure (which bends the head ventrally toward the yolk sac) is well defined, the cerebral hemispheres and eyes now being prominent structures in the head. The nasal pits – fated to become the external nares - are visible open to the surface of the head, while the oral cavity, which lies beneath the forebrain, is bordered above by the fronto-nasal process (which separates the two nasal pits), and laterally by the maxillary processes, which have meanwhile fused together and with the sides of the fronto-nasal process, to form the palate and the upper jaw (beak). Similarly, the two mandibular processes also fuse in the midline and become extended forward to form the lower jaw. A slight depression present on the medial wall of each nasal pit is thought to be the organ of Jacobson (vomeronasal organ). Ventrally, the cavities of the nasal pits are still open into the oral cavity. Eventually, the palate that now begins to form will reduce this communication to the caudally located internal nares. The middle nasal concha (turbinal) appears at this time, preceding the superior and inferior conchae in development (Bellairs & Osmond, 2005).

On day 5, the anterior end of the notochord lies just posterior to the developing hypophysis and the parachordal cartilages lie on either side of it, beneath the midbrain and hindbrain, continuous laterally with the chondrified wall of the otic vesicle, the pars cochlearis (Bellairs & Osmond, 2005; Robertson & Mason, 1995; Wahl & Noden, 2001). The remainder of the otic vesicle has, meanwhile, become loosely divided into a superior chamber, which will form the semicircular canals and utricle, and an inferior chamber, which will give rise to the saccule and cochlea (Bellairs & Osmond, 2005; Robertson & Mason, 1995). The semicircular canals start to develop as three grooves in the wall of the superior chamber, each of which invaginates and closes over to form a tube. The lumen of each tube is continuous with that of the superior chamber of the otic vesicle. The anterior semicircular canal (which lies in the sagittal plane) forms first, followed by the external semicircular canal (which lies in the horizontal plane) and finally by the posterior canal (which lies in the frontal plane). The acoustic ganglion (Cranial Nerve VIII) is now a large bilobed structure, closely applied to the auditory epithelium (Bellairs & Osmond, 2005).

The retinal fibers of the optic nerve have, in the meantime, reached the surface of the optic lobes, and the pecten has become fully formed (Bellairs & Osmond, 2005; Gerhardt, Liebner, & Wolburg, 1996). This is a structure characteristic of birds, thought to act not only in bringing oxygen and nutritive materials to the eye but also playing a role in vision (Gerhardt et al., 1996).

In the hypophysis, the pars distalis has already appeared and production of the luteinizing (LH) and follicle-stimulating (FSH) hormones commenced (Bellairs & Osmond, 2005; Veerle Bruggeman, Van As, & Decuypere, 2002).

The adrenal medulla is being formed from the penetration of cells originating in the neural crest and individually migrated from the sympathetic chain into the cortical nodules (Bellairs & Osmond, 2005; Wahl & Noden, 2001). The primary sympathetic chain itself is beginning to disappear, while the secondary sympathetic chains sprouts from it and become connected with one another to form the single secondary chain, which runs close to the spinal ganglia (Bellairs & Osmond, 2005). Rami communicantes pass from the secondary sympathetic ganglia of the chain to the roots of the spinal nerves as the secondary chain becomes a continuous nerve cord (the paravertebral), running from the superior cervical ganglion to the coccyx and connecting with the plexuses in the aortic plexus (which becomes modified further into the celiac plexus around the celiac artery, as well as the pelvic and hypogastric plexuses), the medullary plexus of the adrenal gland, the splanchnic plexus and Remak's ganglion in the rectal region (N. M. Le Douarin, Teillet, Ziller, & Smith, 1978).

In the gastrointestinal system, the esophagus is being innervated by the invasion of fibers from the vagus nerve (Bellairs & Osmond, 2005; Kuratani, Tanaka, Ishikawa, & Zukeran, 1988). Its lumen has meanwhile become reduced and will ultimately become temporarily obliterated apparently due to the rapid growth of the epithelial lining. The crop begins to form around this time, as a swelling at the posterior end of the esophagus at about the level of the seventh vertebra (Allenspach, 1966). The foregut and hindgut have, meanwhile extended greatly, while the midgut has been reduced to a narrow region known as the yolk sac stalk (Bellairs & Osmond, 2005).

The pharyngeal arches and pouches have also begun to undergo changes (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992). Each of the first three pouches is subdivided into a dorsal and a ventral diverticulum, which give rise to different tissues. The thymus forms principally from the dorsal wall of the third pharyngeal pouch and lengthens along the jugular vein (Graham, 2001). The dorsal wall of the third pouch and of the fourth pouch, together with mesenchyme of the pharyngeal wall, contribute to it so that it becomes a series of glandular patches running down either side of the neck (Graham, 2001; Grevellec & Tucker, 2010).

In the respiratory system, pulmonary veins and arteries have become visible, while the distal end of the mesobronchus has swollen to become the abdominal air sac (Anderson-Berry et al., 2005; J. N. Maina, 2003a; John N. Maina, 2006; Makanya et al., 2011).

At this time, the mesonephros, which contributes with tissue to form both kidneys, overlaps the pronephric region, forming sequentially, from anterior to posterior (Friebová-Zemanová, 1981; James & Schultheiss, 2003; Moritz & Wintour, 1999). It is already functioning and, although originally continuous with the nephrogenic tissue of the metanephros - the most caudal section (and the youngest in development) of the embryonic kidney -, has already separated from the latter so that two sets of kidneys now exist independently (Friebová-Zemanová, 1981; James & Schultheiss, 2003; Moritz & Wintour, 1999). The cloacal wall, on its end, is expanding to engulf the posterior end of the nephric duct so that its junction with the metanephric duct becomes absorbed, the result being that the metanephric duct acquires a separate opening into the cloaca from the nephric duct, which serves the pro- and mesonephros (Friebová-Zemanová, 1981; James & Schultheiss, 2003; Moritz & Wintour, 1999). The metanephric duct will become the ureter (Bellairs & Osmond, 2005).

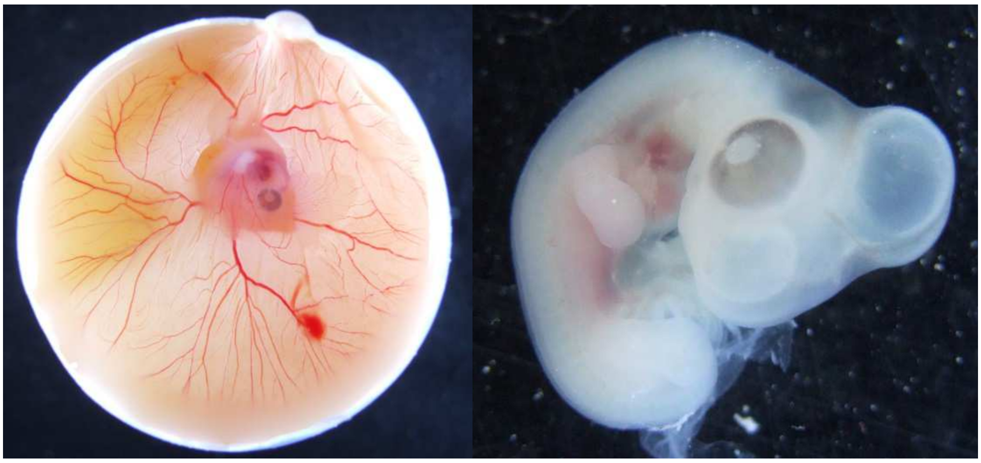


**Figure 34. Appearance of a 5-day old embryo.**

Toward the end of day 5 (Figure 34), the dorsal and ventral atrioventricular cushions have become fully fused in the heart, leaving a channel, the atrio-ventricular canal, on either side (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992; Robert E. Poelmann et al., 2014; Qayyum et al., 2001). However, the division into right and left heart will not become complete until the time of hatching (Bellairs & Osmond, 2005; Robert E. Poelmann et al., 2014; Qayyum et al., 2001). Various interatrial foramina break through the septum and allow communication between the left and right atria, balancing blood flow in the two sides of the heart and enabling their equivalent growth (Bellairs & Osmond, 2005; Robert E. Poelmann et al., 2014; Qayyum et al., 2001). Strands of tissue overlap the foramina so that they open only when the right atrium becomes expanded during diastole (Bellairs & Osmond, 2005; Qayyum et al., 2001). Blood then passes from the right to the left atrium, so that, when the foramina close as the atrial wall contracts during systole, equal pressure is already re-established in the two atria (Bellairs & Osmond, 2005; Qayyum et al., 2001).

Still during this day of incubation, the proximal part of the truncus arteriosus becomes incorporated into the right atrium, while the distal part becomes divided into the aorta and the pulmonary arteries by the growth of the aortico-pulmonary septum (Laane, 1979). The valvulae venosae and the sinu-atrial valves develop from the wall of the right atrium at its junction with the sinus venosus (Qayyum et al., 2001). The atrio-ventricular valves are formed partially from the endocardial cushions and partially from the atrial and ventricular muscles, while the atrio-ventricular node and the upper part of the atrio-ventricular bundle (conducting tissue) develop in the interatrial septum (Argüello, Alanís, Pantoja, & Valenzuela, 1986; Qayyum et al., 2001). The ventricular walls also become greatly thickened and muscular, with extensive trabeculae carnae. By day 5 the hepatic portal vein has formed from the omphalomesenteric vein (Bellairs & Osmond, 2005).

Stem cells are, meanwhile, arising in the embryonic organs to generate the secondary lineage of erythrocytes (Bellairs & Osmond, 2005; Jaffredo, Gautier, Brajeul, & Dieterlen-Lievre, 2000).

The first feather rudiments appear at about 5 days as a single row in the lumbar region, separated by spaces where no feathers develop (Hamburger & Hamilton, 1992). As additional rows are laid down, the new rudiments will always appear opposite the spaces between the rudiments in the preceding row (Parakkal & Matoltsy, 1968; Wessells, 1965). The two sides grow unequally so that the apex moves posteriorly and the entire structure lies almost flat on the surface of the skin (Parakkal & Matoltsy, 1968; Wessells, 1965). The dermis will be present as a distinctive tissue from this point onward (Parakkal & Matoltsy, 1968).

Meanwhile, in females, the migration of primordial germ cells into the gonads has shifted from its initially balanced state to favor the left ovary, in detriment of the right gonad (Nakamura et al., 2007). The same soon takes place in males as well, with the left testicle being favored as well (Nakamura et al., 2007). The primary sex cords appear toward the end of day 5, later differentiating into the seminiferous tubules in the male and the medullary cords in the female (Bellairs & Osmond, 2005). They form by proliferation of the epithelial cells that invade the mesenchymal stroma and carry the primordial germ cells with them into the substance of the gonad (Bellairs & Osmond, 2005; Nakamura et al., 2007).

The Müllerian ducts, which begin as placode-like thickenings in close contact with the Wolffian ducts (derived from the nephric ducts), are now becoming canalized, a process that will be almost completed late in the fifth day of incubation (Bellairs & Osmond, 2005).

On the sixth day of incubation (Figure 35), genital folds in both sexes arise around the cloaca. In the meantime, paired ‘genital eminences’ have formed in the cloacal membrane at the base of the tail, fused together and grown to form the genital tubercle (Perriton, Powles, Chiang, Maconochie, & Cohn, 2002). The cloacal wall itself expands to engulf the posterior end of the nephric duct so that its junction with the metanephric duct is absorbed and separate openings are created for the metanephric and nephric ducts, respectively (Bellairs & Osmond, 2005).

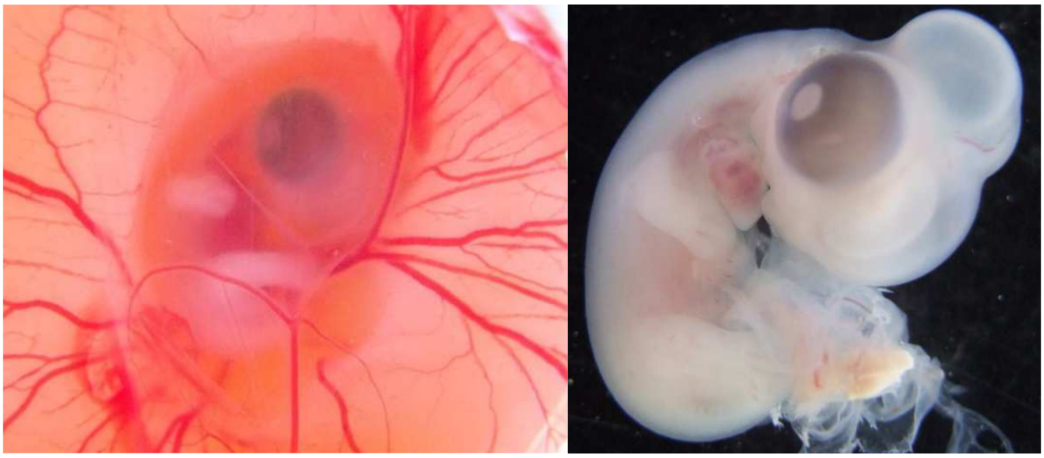


**Figure 35. Appearance of a 6-day old embryo.**

The lumen of the large intestine, which will later open into the cloaca, occludes at this time, not becoming recanalized until 6 days later. Upstream, in the small intestine, the future divisions that will be present in the day-old chick, are now recognizable and the intestinal loop has just begun to rotate 90 degrees, the anterior section moving to the right and the posterior section to the left (Bellairs & Osmond, 2005). The thick gizzard is now also apparent in gross sections, eventually becoming more marked as its circular muscles form (Bennett & Cobb, 1969). Further cranially, the esophagus begins to reopen, after its original occlusion at day 5 (Bellairs & Osmond, 2005).

The three rudiments of the pancreas start to fuse together during day 6 of incubation, when cell differentiation of the exocrine pancreas is also beginning to take place. A pancreatic duct is usually maintained per rudiment, all three opening near the end of the duodenum, close to the two bile ducts (Kim, Hebrok, & Melton, 1997).

In the circulatory system, more specifically in the heart, the interventricular septum, which initially lies at the apex of the ventricles, extends towards the atrio-ventricular cushions and fuses with them, leaving only a small gap, the interventricular canal (Robert E. Poelmann et al., 2014; Qayyum et al., 2001). Extensive apoptosis meanwhile occurs in the myocardium of the outflow tract, which, in association to extensive local remodeling, is thought to enable the great vessels to align properly over their respective ventricles (Robert E. Poelmann et al., 2014; Qayyum et al., 2001).

The communication of the fourth arterial arch with the dorsal aorta is now lost on the left side (retained on the right to form the arcus aortae). The cranial extensions of the dorsal aortae become the internal carotid arteries in all young amniote embryos, while the cranial extensions of the ventral aortae become the external carotid arteries (Y. Wang et al., 2009). In birds, the internal carotid arteries in the head anastomose with the external carotid arteries at this point, and the remnants of the internal carotid arteries in the neck region disappear. Much of the posterior cardinal (venous) system has disappeared and the subclavian veins have formed connections with the anterior cardinal veins. The jugular veins on each side join the vertebral and subclavian veins near the heart to form the anterior vena cava (Baldwin, 1996).

With the fusion of the allantois with the chorion, the pattern of vascularity in the chorioallantois now begins to differ from that in the inner layer of the allantois, with the capillaries of the outer layer of the allantois proper penetrating into the chorion and passing through it to lie between the shell and the shell membranes (DeFouw, Rizzo, Steinfeld, & Feinberg, 1989). This will make them primely located for gas exchange with the outside air moving in through the shell pores (Mortola, 2009).

The respiratory tract is far from being functional at this point, with the pulmonary veins and arteries just becoming visible and the trachea having just lengthened enough and expanded at its anterior end to form the larynx (Bellairs & Osmond, 2005; J. N. Maina, 2003b). The nasal pits have also closed with the appearance of a plug formed from the proliferation of epithelial cells, and will not reopen until the plug degenerates, at hatching (Bellairs & Osmond, 2005). In the closed off nasal cavity, the second of three nasal conchae (the superior concha) has meanwhile formed (Bellairs & Osmond, 2005; Sneddon, Hadden, & Hepper, 1998).

In the meantime, the ectoderm of the beak region has started to thicken along the upper and lower jaws (Bellairs & Osmond, 2005). A ridge along the head, no more than a vascularized dermal thickening beneath the ectoderm, now announces the formation of the comb (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992). Small papillae will develop along the posterior two-thirds of the region, the forerunners of the adult serrated comb (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992). During the following days, this region will become highly vascularized (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992).

Still in the head, the cochleae and cochlear ducts start to form at this point by growing out from the ventral region of the inferior chamber (Bellairs & Osmond, 2005; Robertson & Mason, 1995; Wahl & Noden, 2001). The cartilaginous predecessor of the avian single auditory ossicle, the columella, is now present, lying between the tympanic membrane and the cochlea (Bellairs & Osmond, 2005; Robertson & Mason, 1995; Wahl & Noden, 2001).

In the ocular region, scleral papillae are beginning to appear in the sclera, around the margin of the cornea (Coulombre, Coulombre, & Mehta, 1962; Franz-Odendaal, 2008). These will induce formation of the scleral ossicles, a ring of 14 overlapping bony plates present in this region, in the days to come (Coulombre et al., 1962; Franz-Odendaal, 2008).

The embryonic hypophysis now secretes prolactin and thyroptropin, while the thyroid, which consists entirely of an epithelium of endodermal origin that is just becoming arranged into cords at this point, becomes invaded by mesenchyme that will give rise to sinusoids (Bellairs & Osmond, 2005; Hopkins, 1935). The thyroglossal duct meanwhile degenerates, each thyroid lobe becoming further lobulated and extensively vascularized by vessels originating in the subclavian arteries (derived from the third aortic arches) (Bellairs & Osmond, 2005; Garriock & Mikawa, 2011).

In the cervical region, the thymus rudiment is receiving the first wave of lymphocyte precursors, in a process that will take about 36 hours until the organ is fully colonized (M. A. S. Moore & Owen, 1967). The ultimo-branchial bodies, the embryonic precursors of the ultimo-branchial glands responsible for producing calcitonin, develop from the fifth pharyngeal pouches after migration and invasion by the ultimo-branchial gland progenitor cells migrate originating in the neural crest (N. Le Douarin, Fontaine, & Le Liévre, 1974; Merida-Velasco, Espin-Ferra, Valdecasas, & Garcia-Garcia, 1988; Scanes, 2015).

In the skeleton, the dorsal spines of the vertebrae become cartilaginous, while the ribs begin to appear in membrane. Although processes comparable to ribs form from the lumbar and sacral vertebrae they do not separate from the vertebrae. Chondrification of the scapula, humerus, radius, ulna and coracoid, as well as of the tibia, fibula and the bones in the pelvic girdle begins at about the end of day 6. The carpal and metacarpal bones as well as the precartilaginous precursors of the tibiotarsus and metatarsus also begin to appear around this period. In the femur, ossification has already started (Bellairs & Osmond, 2005).

Development of the leg muscles now starts to take place and voluntary movement can be seen if the egg is candled (Tong et al., 2013). The dorsal and ventral pre-muscle masses are well defined, and each then undergoes a series of subdivisions comparable to those of the pre-muscle masses of the wing bud (Caplan & Koutroupas, 1973; Shellswell & Wolpert, 1977). The tendons begin to form in the lower leg at the end of day 6 or at the start of day 7 (Bellairs & Osmond, 2005; Kieny & Chevallier, 1979).

By day 7 (Figure 36), ossification has begun in the humerus, radius and ulna. The clavicle (furculum, wishbone) is just beginning to appear as a condensation of mesenchyme, while the ribs are beginning to become cartilaginous. In the skull, two regions of cartilage have formed, the interorbital and the ethmoidal regions, the whole structure extending from the hypophysis fossa to the tip of the head. The ethmoidal region itself is divided into the pre-nasal cartilage from which the beak will form, and the ali-nasal cartilage. The egg tooth has begun to develop as a projection from the tip of the upper jaw (Bellairs & Osmond, 2005).

**Figure 36. Appearance of a 7-day old embryo.**

The palate has meanwhile formed and separated the upper nasal cavity from the oral cavity, forming the choanae (internal nares), located at the posterior end of the buccal cavity. The final of the nasal conchae, the inferior (or vestibular) concha has also formed (R. M. Shah & Crawford, 1980).

In the eye, the lens capsule is starting to form, while the melanophores of the choroid are beginning to develop pigment. The eyelids also start to form from a circular fold of skin surrounding the eye which will later become modified to form the upper and lower eyelids. A semicircular fold within this circular fold becomes the nictitating membrane (Bellairs & Osmond, 2005). Many fibers of the optic nerve have, in the meantime, spread over the surface of the optic lobes (Bellairs & Osmond, 2005; Goodall et al., 2009; Wahl & Noden, 2001).

The caudal end of the hypophysis now breaks away from the roof of the mouth. Adrenocorticotropic hormone (ACTH)-producing cells are already present at this time (Bellairs & Osmond, 2005). In the thyroid the first colloid droplets are just appearing (Hilfer, 1979; Hopkins, 1935).

In the medulla of the adrenal glands, extensive cellular proliferation has meanwhile occurred and the chromaffin cells have since become arranged in cords between the cortical cells (N. M. Le Douarin et al., 1978; Wahl & Noden, 2001).

In the cloaca, the enlarged genital tubercle has acquired a bi-lobed appearance. The gonads of males now become recognizable as testes, as the sex cords proliferate abundantly while, in the females, the ovary has also become a recognizable structure by the expansion of the primary sex cords (Bellairs & Osmond, 2005). The primordial germ cells lie in the secondary sex cords of the left ovary and differentiate into oogonia

(Nakamura et al., 2007). The right gonad fails to form a cortex and remains rudimentary unless the left ovary is removed (Nakamura et al., 2007).

The distal nephron, unlike the proximal, is capable of active transport at this stage, and excretory products from the blood are filtered through the glomerulus and pass into the nephric duct and, subsequently, the cloaca (James & Schultheiss, 2003; Moritz & Wintour, 1999).

In the gastrointestinal system, the crop now begins to bend to the right and has since acquired a lumen about 0.25 mm wide, as opposed to that of the esophagus proper, which is about 0.16 mm. The crop continues to grow in size as a diverticulum at the right side of the esophagus, its walls and plicae being thinner than those of the main part of the esophagus (Bellairs & Osmond, 2005). The stomach, on the contrary, shifts leftwards and, by day 7, the gizzard has swollen into a thick-walled sac that points posteriorly (Bennett & Cobb, 1969). Meanwhile, the glands of the proventriculus have begun to form. Downstream, the caeca have already grown to 2-4 mm in length (Bellairs & Osmond, 2005).

By the end of day 7, the heart is completely enclosed in the thoracic cavity (Tong et al., 2013). The aortico-pulmonary septum has extended back into the interventricular canal and become continuous with the interventricular septum, so that the right ventricle now opens directly into the pulmonary artery while the left ventricle opens into the aorta (Robert E. Poelmann et al., 2014; Qayyum et al., 2001). The carotid arteries have also branched to supply all the arteries of the head (Garriock & Mikawa, 2011).

As the next day of incubation progresses, fibers of the Auerbach's plexus begin to invade the esophagus, adding to its innervation (Aarestrup et al., 2000; N. M. Le Douarin & M.-A. e. Teillet, 1973). There are now 38 pairs of spinal nerves and the secondary sympathetic chains have fused into a single chain, a continuous nerve cord (the paravertebral) running from the superior cervical ganglion to the coccyx. It bifurcates around each rib but remains as a single structure between the ribs. Almost every part of the vertebrae has become cartilaginous and the sternum is beginning to form, while, in the pectoral girdle the clavicle is starting to ossify (Bellairs & Osmond, 2005).

By day 8 of incubation, the metacarpals have become a set of four cartilages, and metacarpals 3 and 4 become fused distally (Hamburger & Hamilton, 1992). In the femurs, the bone marrow cavity begins to form while, distally, the membrane mold that will give rise to the patella forms and the leg muscles continue to develop (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992).

Cartilage is likewise beginning to form in the sclera of both eyes (Coulombre et al., 1962; Franz-Odendaal, 2008). In the septal wall of the nasal cavity, a solid mass starts to form. This will later give rise to the lateral nasal glands. The lumen of the larynx meanwhile becomes temporarily obliterated, only to be recanalized a three days later in incubation (Bellairs & Osmond, 2005).

In the urinary system, the pronephric region of the nephric kidney has now completely disappeared (Hiruma & Nakamura, 2003). At this time, each individual, regardless of gender, possesses paired male (Wolffian, nephric) and paired female (Müllerian or paramesonephric) ducts. However, both of the Müllerian ducts of the male and the right Müllerian duct of the female will now begin to degenerate (Gasc & Stumpf, 1981). In the cloaca, the genital swellings have fused with each other and have since extended like a collar around the genital tubercle (Bellairs & Osmond, 2005).

By day 8 (Figure 37), the size of each adrenal mass, as seen in transverse sections, is approximately twice that of the dorsal aorta (Bellairs & Osmond, 2005). In the adrenal medulla, a meshwork of nerve fibers has formed (N. M. Le Douarin et al., 1978; Wahl & Noden, 2001). The hypophysis now secretes mesotropin and vasotocin (Bellairs & Osmond, 2005).

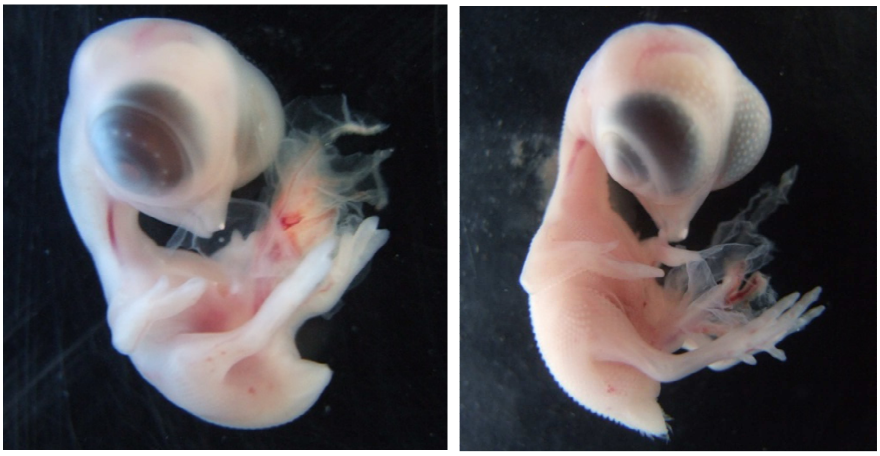


**Figure 37. Appearance of an 8-day old (left) and a 9-day old (right) embryo.**

By day 9 of incubation (Figure 37), the two halves of the cerebellum have fused together in the midline and the myelencephalon has acquired the characteristic shape of the medulla oblongata (Bellairs & Osmond, 2005; Hallonet, Teillet, & Le Douarin, 1990).

In the eye, 14 papillae are now visible on the conjunctival sclera, each composed of a solid cord of epithelium (Coulombre et al., 1962; Franz-Odendaal, 2008).

The first rudiments of sternum, which formed during day 8 in the dorso-lateral wall of the thorax immediately posterior to the pericardial cavity, have now migrated ventrally and fused together, forming not only the main body of the sternum but also the keel. They have also become chondrified. However, ossification of the sternum and keel will not begin until about the time of hatching (Bellairs & Osmond, 2005).

Tendons are also now well-defined in the legs (apart from those of the toes), having grown forward from each muscle mass as it formed from its respective pre-muscle mass and stretching toward their future places of insertion. Each tendon grows along a digital ray, which is essentially a band of collagen around which the tendon condenses (Kardon, 1998).

In the organs annex to the digestive system, the three rudiments of the pancreas have now become fully fused (Kim et al., 1997).

In the respiratory tract, muscles are forming in the region of the syrinx, closely accompanied by the syringeal cartilages (Bellairs & Osmond, 2005). The abdominal air sacs, formed from a swelling of the mesobronchus at around day of incubation, now start to migrate into the abdomen, penetrating the pleuroperitoneal septum (J. N. Maina, 2003b; John N. Maina, 2006).

In the skin, the melanocytes that lodge in the feather buds have proliferated and are now secreting melanin. The parathyroid glands have already formed, their anterior lobes looking semicircular in shape and containing epithelial cords separated by mesenchyme in which capillaries run. The adrenal glands are now mostly formed and their development from this point on mainly consists of further growth (Bellairs & Osmond, 2005).

By 10 days of incubation (Figure 38), another gland is just beginning to form, the uropygial gland. Birds possess no integumentary glands apart from the uropygial (preen) gland, which forms on the dorsal side of the body at the base of the tail. It starts as a bilobed structure that arises from a pair of ectodermal invaginations, one on either side of the vertebrae. These become paired ducts and each buds off further sacs (Farner, King, & Parkes, 2013).

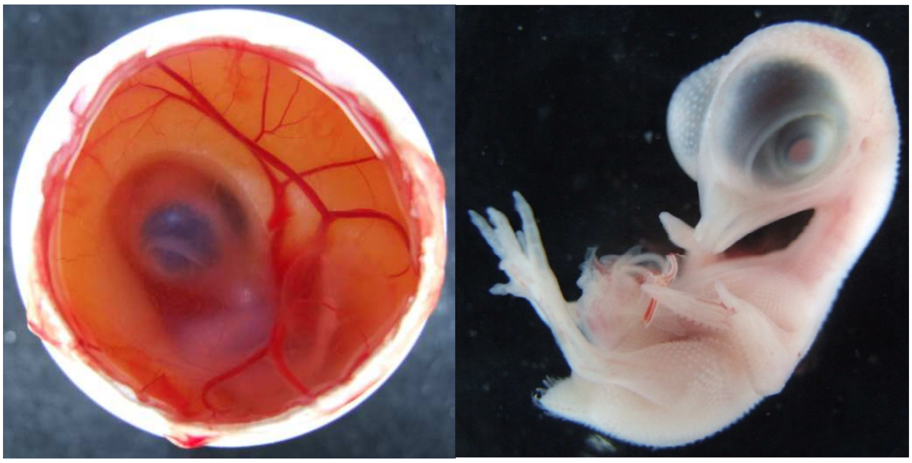


**Figure 38. Appearance of a 10-day old embryo.**

At this time, the first adenohypophysis-thyroid interactions have just become established and, with the near disappearance of the cranial flexure and the reduction of the cervical flexure, the embryo’s head no longer lies tucked against its thorax (Hamburger & Hamilton, 1992; Thommes, 1987). Its beak is also starting to become cornified (Bellairs & Osmond, 2005).

At about day 10 of incubation, the diameter of the notochord starts to be reduced. The ribs start to ossify and a transitory thumb-like claw is visible on the first digit of the wing. The patella is just starting to chondrify (Bellairs & Osmond, 2005). In the eye, the scleral ossicles are now fully visible (Coulombre et al., 1962; Franz-Odendaal, 2008).

Hematopoiesis begins in the spleen at about 10 days (Dieterlen-Lievre, 1975). In the male and female gonads, the primordial germ cells are undergoing proliferation (Nakamura et al., 2007). However, just as before, when migration favored accumulation of these cells in the left gonad, the greater degree of cell death in the right gonad again favors greater accumulation of germ cells in the left (Bellairs & Osmond, 2005).

In the skin of the legs, the broad, overlapping scales on the anterior and posterior surfaces of the tarsometatarsus begin to arise as a series of plates (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992). Claws, which are modified scales, are visible on the toes (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992).

The first rudiments of the scutellate scales, which appear on the dorsal side of the foot, have formed plates by day 11 (Figure 39) (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992). By this day in incubation, all leg tendons have completed their insertions, with the formation of the final skeletal cartilages (Kardon, 1998).

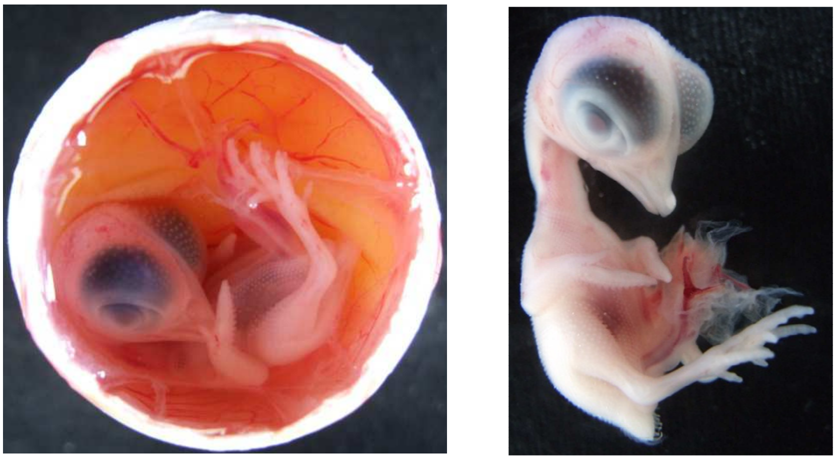


**Figure 39. Appearance of an 11-day old embryo.**

In the head, the wattles start to appear at this time, developing in a similar way to that previously observed for the comb. The cerebral hemispheres have now expanded so much that they overlap the diencephalon. The syrinx of the male is larger than that of the female by now (Bellairs & Osmond, 2005).

In the embryonic urinary system, the entire metanephric kidney has formed secreting tubules, and has begun to excrete, while the mesonephros has reached the end of its functional period (James & Schultheiss, 2003; Moritz & Wintour, 1999). The glomeruli are supplied by segmental branches of the dorsal aorta (James & Schultheiss, 2003).

In the female, the Müllerian ducts fuse with the cloaca at about this day. The left duct will then undergo modification into ostium, glandular regions and the shell gland, but it will not open into the cloaca until after hatching. The Müllerian ducts have usually disappeared in the male by about day 12 (Bellairs & Osmond, 2005).

During the previous 10 days of incubation, the embryo had obtained its calcium from the yolk sac. However, the onset of bone ossification, such as that of the vertebrae, now pushes its requirements regarding this mineral beyond the yolk's content in calcium, forcing the embryo to turn to the shell as its main calcium supplier and to the chorioallantoic membrane for transport of this ion (Tuan & Ono, 1986).

By day 12 of incubation (Figure 40), the ribs are ossifying, as are the ilium, the scapula and coracoid. However, the junctional region of the latter two remains permanently cartilaginous. The left and right clavicles meet and fuse at about this time (Bellairs & Osmond, 2005). Ossification of the scleral ossicles has likewise begun, and the conjunctival papillae which first gave rise to them have now disappeared (Coulombre et al., 1962; Franz-Odendaal, 2008).

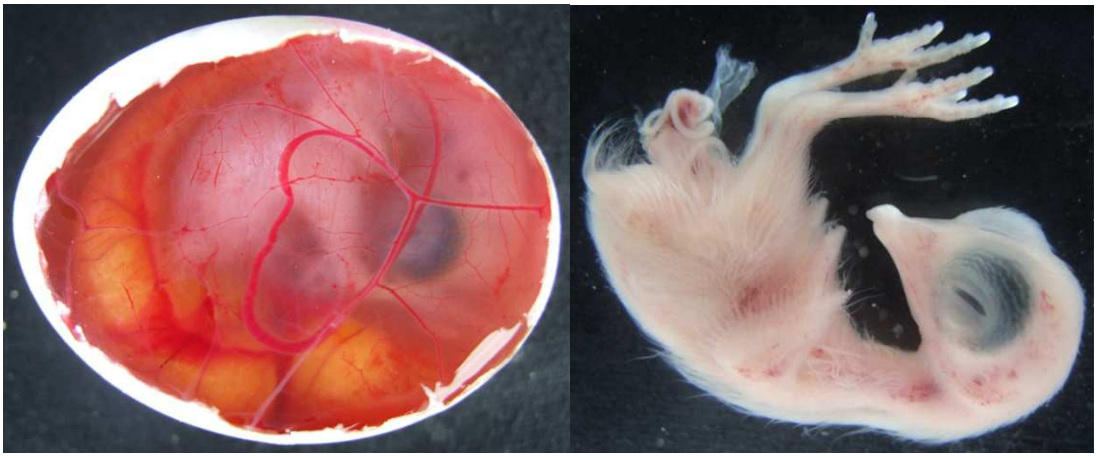


**Figure 40. Appearance of a 12-day old embryo.**

Meanwhile, in the gizzard, the secretory glands are beginning to form (Bennett & Cobb, 1969). The large intestine, occluded at day 6 of incubation, has now recanalized (Bellairs & Osmond, 2005).

At this point in incubation, the hypophysis is beginning to secrete growth hormones and large amounts of insulin are being secreted by the β-cells of the pancreas (Kim et al., 1997).

## 13-18 days of incubation

By day 13 of incubation (Figure 41), innervation of the esophagus is completed, with the formation of the Meissner's plexus (N. M. Le Douarin & M. A. Teillet, 1973). By this time, lymphoid tissue has begun to develop in the bursa of Fabricius and the synthesis of immunoglobulins will soon start taking place (Bellairs & Osmond, 2005; Dieterlen-Lievre & Le Douarin, 2004).

In the testis, the primordial germ cells begin to divide and differentiate into spermatogonia (Nakamura et al., 2007). The hypophysis-gonadal axis is now established and levels of LH in the bloodstream are becoming sufficient to stimulate steroidogenesis in the gonads (Veerle Bruggeman et al., 2002; Woods, Mennella, & Thommes, 1981).

In the wing, what were originally thirteen separate carpal bones have fused either with each other or with the metacarpals, forming the carpo-metacarpus, while ossification of the rest of the skeleton progresses, at this time affecting the pubis (Hamburger & Hamilton, 1992).

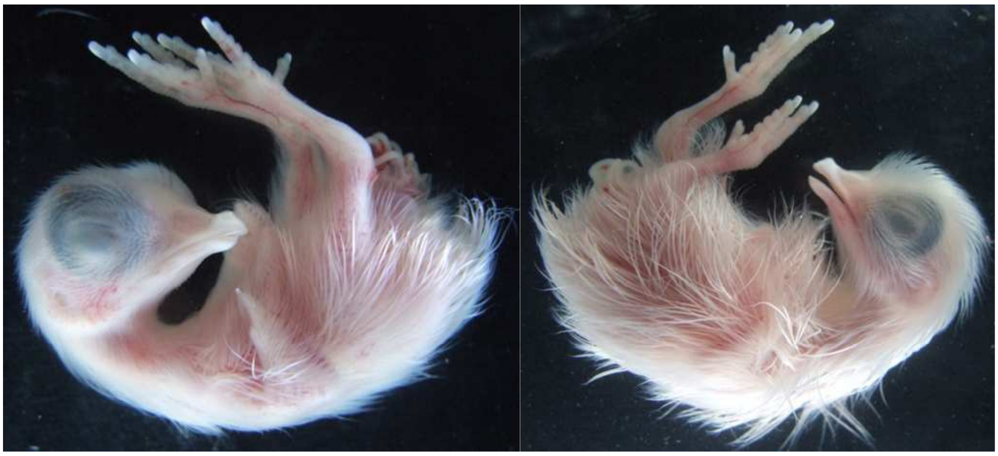


**Figure 41. Appearance of a13-day old (left) and a 14-day old (right) embryo.**

A day later, the ischium begins to ossify as well. This bone will then fuse with the pubis and with the ventral part of the ilium, while the ilium itself fuses with the synsacrum (Bellairs & Osmond, 2005).

By day 14 of incubation (Figure 41), the beak has hardened and the eggtooth, essential in aiding the hatching chick in breaking through the shell, has reached its maximum size. The claws have started to become cornified (Bellairs & Osmond, 2005).

The two, paired rudiments of the uropygial gland have now fused to form a single, multilobular gland that will not begin secreting until the bird has hatched (Cater & Lawrie, 1951).

Meanwhile, in the kidney, three types of nephrons begin to differentiate: the mammalian type I (juxtamedullary) with long loops of Henle, the mammalian type II with short loops of Henle, and the reptilian type (Bellairs & Osmond, 2005; Braun & Scanes, 2015).

By day 15 of incubation (Figure 42), the metanephric kidneys are fully functional and the mesonephros is no longer active. The latter structure will soon start to degenerate (James & Schultheiss, 2003; Moritz & Wintour, 1999).

**Figure 42. Appearance of a 15-day old (left) and a 16 day-old (right) embryo.**

By 16 days of incubation (Figure 42), the chorioallantoic membrane has grown to cover most of the yolk sac. It is also closely pressed against the shell membranes, which enables it to act as a gas-exchange organ receiving oxygen and eliminating carbon dioxide through the pores in the shell (Mortola, 2009).

In the brain, the cerebellum almost abuts the cerebral hemispheres, while, in the tongue, taste buds begin to develop. Their numbers at hatching will be approximately half that of the adult bird (Bellairs & Osmond, 2005). However, the moment at which they become functional is not yet known (Sneddon et al., 1998). In the esophagus, solid epithelial buds are growing into the tunica propria, giving rise to the mucous glands (Ventura et al., 2013). Radial folds have also arisen around the periphery of the cloaca, its aperture having become transversely oriented with the long axis of the body, its smaller, dorsal lip overhanging the ventral one (Bellairs & Osmond, 2005).

By day 17 (Figure 43), the gut starts to be gradually drawn back into the abdomen (Hamburger & Hamilton, 1992). The parathyroids are now completely vascularized by branches of the carotid body artery and have already become fully differentiated (Bellairs & Osmond, 2005; Hamburger & Hamilton, 1992). Still, they are probably not yet necessary for calcium uptake, as they are later on (Bellairs & Osmond, 2005).

The cerebral hemispheres have expanded so much by day 18 of incubation (Figure 43), that they overlap the mesencephalon (Bellairs & Osmond, 2005). Provided the egg has been exposed to light and darkness rhythms, the embryo will already exhibit a circadian clock to its melatonin production in the pineal gland at this time (Okabayashi et al., 2003).

**Figure 43. Appearance of a 17-day old (left) and an 18-day old (right) embryo.**

The embryo’s skin is just losing the periderm, the protective, outer layer which sits over the epidermis. The feathers now form their characteristic barbs and associated barbules, acquiring a hollow region at the base, the calamus, where the barbed ridges remain fused (Bellairs & Osmond, 2005). The remaining portion of the albumen and the amniotic fluid are now being ingested (Mortola, 2009).

At this point, the embryo, its neck thus far bent so that the head sits between its thighs, turns its body to the correct hatching position: a folded neck and head under the air space membrane (Tullett, 2008). Vocalization soon begins (Mortola, 2009).

## 19-20 days of incubation

By day 19 of incubation (Figure 44), the olfactory epithelium is mature, though it is probably precocious in its development when compared to the central processing of olfactory signals (Sneddon et al., 1998). By this time, a series of breathing movements can already be observed in the embryo. These are: (1) a series of rapid deflections produced by bouts of beak clapping just before the onset of breathing; (2) bouts of irregularly intermittent breathing movements; (3) more regular, medium-amplitude movements; and (4) more rapid breathing movements of greater amplitude (Chiba et al., 2002).

The gut has now been completely internalized into the abdomen and most of the yolk has been absorbed (Hamburger & Hamilton, 1992). The remnants of the yolk sac and its contents start to be drawn up into the body as well (by contraction of the abdominal muscles) and will serve to nourish the hatchling during its first day (Bellairs & Osmond, 2005).

**Figure 44. Appearance of a 19-day old (left) and a 20-day old (right) embryo.**

About day 20 (Figure 44), absorption of allantoic fluid is complete (Hamburger & Hamilton, 1992). In the skin, the base of the feather germ sinks into the feather follicle and what is left of the periderm remains only as a thin sheath of epidermal cells around the feather germ until after hatching but splits as the chick dries and so releases the barbs and barbules (Bellairs & Osmond, 2005).

Ossification in the synsacral, as well as in the caudal vertebrae, is not completed until about this time. Ossification of the sternum has just begun to take place (Bellairs & Osmond, 2005).

In the neck, the hatching (or pipping) muscle (muscularis complexus), the primary muscle responsible for propelling the embryo’s head forward in its attempts to pierce the shell, matures and hepatic glycogen stores start being mobilized (Pulikanti et al., 2010). Postural reflexes are fully developed and the sense of hearing becomes similar to that of a fully hatched chick. Lymphocytic homing in the spleen has now begun (Bellairs & Osmond, 2005).

The embryo is now in hatching position, its spine running parallel to the long axis of the egg, beak positioned underneath the right wing. The tip of the beak is directed towards the air cell in the blunt pole of the egg (Tullett, 2008).

## 21 days of incubation – Hatching day

About 8 to 9 hours before effectively breaking through the shell, the embryo uses the egg tooth to pierce the portion of inner shell membrane that separates it from the air cell (internal pipping) (Figure 45) and begins to draw air into its lungs from the air cell. The onset of pulmonary respiration marks its transition into a chick. Gas exchange still takes place via both the chorioallantoic membrane and the lungs, however (Mortola, 2009). As the circulation in the chorioallantoic membrane gradually ceases and the allantois begins to dry up (Tong et al., 2013), the exchange becomes restricted to the lungs. A period of relative inactivity follows, punctuated by occasional vigorous wholebody and head movements (Mortola, 2009; Tong et al., 2013).

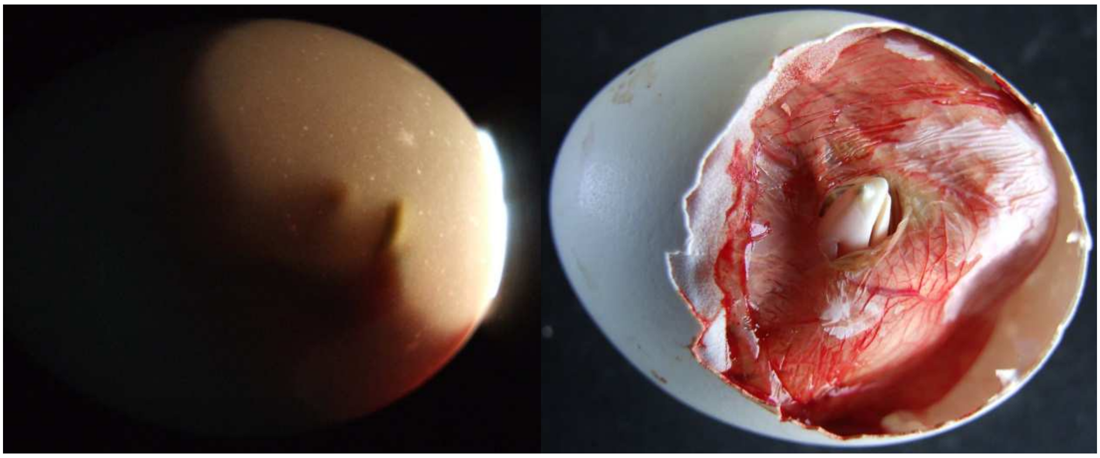


**Figure 45. Appearance of an internal pip-stage embryo on candling (left) and on break-out (right).**

The yolk sac should be fully withdrawn into the abdomen at this time, if the chick is to survive post hatching (Hamburger & Hamilton, 1992). The omphalo-mesenteric vessels will continue to absorb yolk for the first day, after which they begin to degenerate (Bellairs & Osmond, 2005).

At this time, the large intestine is still only about 20 mm long, while the caeca are around 30 mm in length (Bellairs & Osmond, 2005). The cloacal membrane perforates just before hatching and the ectodermally derived part becomes incorporated as the proctodaeum into the cloaca, as opposed to the endodermally derived components of the cloaca, which will form the two remaining sections of the cloaca, known as the coprodaeum and the urodaeum, respectively (S. A. Miller, Clark, Cooney, Crary, & Payzant, 1998).

At the time of hatching important changes take place in the circulation that result in an equalization of pressures in the two sides of the heart and which in turn results in the closure of the interatrial foramina, as well as in that of the ductus arteriosi (the vascular bypasses between the aorta and pulmonary arteries), increasing blood flow to the recently ventilated lungs (Bellairs & Osmond, 2005; Greyner & Dzialowski, 2008). The external naris has once again opened, with the degeneration of the epithelial plug that once occluded it (Sneddon et al., 1998).

In the females, the right ovary has undergone considerable atrophy. In the skin, the base of the feather germ sinks into the feather follicle and what is left of the periderm remains only as a thin sheath of epidermal cells around the feather germ (Bellairs & Osmond, 2005).

As hatching progresses, the chick continues to push its head outward using the sharp egg tooth and the hatching muscle on the back of the neck to open a spot in the eggshell (external pipping) (Mortola, 2009; Tong et al., 2013). This is followed by another quiescent period until a series of back thrusts with the beak and egg tooth against the shell ensues, enlarging the hole made by external pipping, which, together with repeated partial body rotations achieved by stepping movements of the feet against the shell, lead to the top of the shell being loosened sufficiently to be pushed off (Mortola, 2009; Tong et al., 2013).

The chick finally emerges from the shell and is completely freed from it, leaving the chorioallantoic membrane attached to the inside of the discarded shell. As the chick dries, the periderm remnants around the follicles dry as well, detaching and releasing the barbs and barbules of the feathers of the hatching plumage, which consists of the down feathers that give the chick its characteristic fluffy appearance (Bellairs & Osmond, 2005).

Once dried, the chick is ready to be taken out of the hatcher and prepared for its future journey to the farm.

# OBJECTIVES

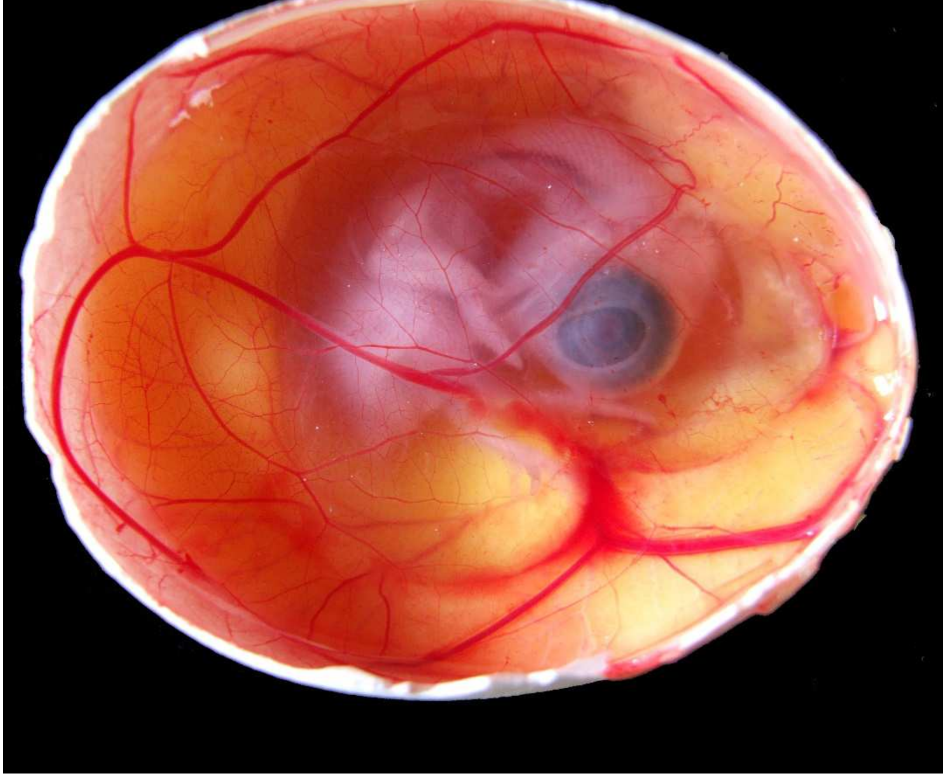
Successful hatching of strong, healthy chicks with a potential for good productive and reproductive performance depends on the assurance of as normal and ideal as possible embryonic development. The latter, however, can be negatively affected by a series of factors intrinsic to either the egg, its handling, or the incubator. Understanding how the embryo is affected by such factors, such as changes in the incubation environment or infection with vertically transmitted pathogens, should enable an easier and earlier detection of ongoing issues in the hatchery and/or upstream production. Using histopathological and microbiological analysis of egg candling break-out eggs and/or embryos may thus prove to be an economically interesting form of using non-viable eggs as flock health and hatchery sentinels.

For that reason, this project aimed at collecting and interpreting essential and extensive information regarding embryonal response to physical challenges in the incubation environment by characterizing physiological and pathological gross and microscopic changes in chicken embryos after manipulation of incubator temperature, relative humidity and carbon dioxide concentration.

It further aimed at observing the effects of vertical infection with *Salmonella enterica* serovars Enteritidis and Typhimurium on embryonic development.

Finally, this study aimed at applying the information gathered during experimental manipulation to the real-life scenario of a field screening of samples collected from an industrial hatchery, in order to determine the viability of their application in the industry.

# EXPERIMENTAL WORK



# PART ONE - PHYSICAL INCUBATION ENVIRONMENT MANIPULATION UNDER LABORATORY CONDITIONS

## INTRODUCTION

After oviposition, embryonic development is mainly dependent upon the energy provided to the avian embryo in the form of heat (Barri, 2008; Feast, Noble, Speake, & Ferguson, 1998). Similarly to most species, the chicken embryo cannot regulate its own temperature; its thermoregulatory system begins as an open system without feedback control, meaning that it cannot compensate the input of too much or too little heat by, respectively, dispersing or generating the necessary heat to maintain its body temperature stable (Mortola, 2006; Tzschentke, 2008). The development and activation of feedback-regulated mechanisms of thermoregulation, essential for the transition into homeothermy, begins to occur late in incubation, becoming fully competent only after hatching (Black & Burggren, 2004b; Tzschentke, 2008). Thus, if temperature is ideal during incubation, developmental processes should proceed with maximum efficiency if no other obstacles are presented (Lourens, 2008). If, however, temperature drifts above or below optimum values, the embryo is bound to respond and adapt (by mechanisms other than thermoregulation) in order to survive and keep growing (Black & Burggren, 2004a; Leksrisompong, Romero-Sanchez, Plumstead, Brannan, & Brake, 2007; von Blumroder & Tonhardt, 2002). In regard to chickens, this interval of optimum incubation temperatures is generally accepted to be 37.8ºC±0.3ºC and large deviations from this, in either direction, have been known to interfere with development, hatchability and post hatch survival and performance (Lourens, 2008; Yalcin & Siegel, 2003).

In nature, how much or how little heat is available to the developing embryo is dependent on the hen’s body heat, the egg’s proximity to the hen, the size and condition of the nest, the size of the clutch, environmental temperature around the nest, etc. (Conway & Martin, 2000; D.C. Deeming, 2008; Mueller, Burggren, Tazawa, & Scanes, 2015). In the carefully controlled conditions of the industrial environment, the amount of heat (and therefore) energy that an egg is exposed to is initially almost exclusively dependent on the temperature inside the incubator (French, 1997; Lourens, 2008). However, from mid-incubation onwards, metabolic heat production by the embryo increases embryonic temperature above that of the incubator (French, 1997; Hulet, Gladys, Hill, Meijerhof, & El-Shiekh, 2007). At this point, embryonic temperature is not only determined by incubator temperature but also by the embryo’s own heat production and by the efficiency with which it transfers heat into the incubator environment – itself determined by airflow direction and speed around the eggs, number and size of shell pores and shell thickness and egg size (larger eggs have greater difficulty losing metabolic heat) (Barri, 2008; Hulet et al., 2007; Lourens, Meijerhof, Kemp, & van den Brand, 2011).

Thus, as incubation progresses and the embryo grows, so does the risk of overheating and so, although raising temperature initially accelerates embryonic growth and utilization of nutrients and energy from yolk and albumen, it also leads to smaller, lighter and weaker chicks at hatching (Molenaar et al., 2011; Molenaar et al., 2013; Piestun, Halevy, & Yahav, 2009). Chick quality problems often found associated with high incubation temperatures include pale skin and feathering, excessive blood inside the eggshell, blood on the down and feathers, short feathers, increased incidence of malformation (e.g., red hocks, unhealed navels, externalized yolk sac remnants, cross beaks, ectopic viscera), weakness, unsteady gait, apathy and a general abnormal and unthrifty appearance (Leksrisompong et al., 2007; Lourens, van den Brand, Meijerhof, & Kemp, 2005; Romanoff & Romanoff, 1972). Chicks incubated at high temperatures have also been found to have smaller hearts, bursa of Fabricius, intestines, proventriculi and gizzards, but heavier livers (Barri, 2008; Leksrisompong et al., 2007; Maatjens et al., 2014; Oznurlu, Celik, Telatar, & Sur, 2010; Sozcu & Ipek, 2015).

Another observation, which may partially explain the decreased survival and development rates in the peri- and posthatch period that embryos exposed to high temperatures present, has to do with glucose metabolism (Maatjens et al., 2014; Molenaar et al., 2010; Molenaar et al., 2013). During the first week of egg incubation, glucose is the predominant energy source because the chorioallantois is not sufficiently developed to provide the required $O_2$ for complete fatty acid oxidation. During the second half of incubation, fatty acids are the predominant substrate for adenosine triphosphate (ATP) production and proteins are presumably deposited. At the end of incubation, around day 19 of incubation, the embryo begins the energy-demanding hatching process, in which glucose acts as the main energy source (Molenaar et al., 2010). Because glucose is hardly available in the egg at the start of incubation (0.1%), gluconeogenesis is indispensable with amino acids, glycerol, or lactate being used as potential precursors to build up glycogen stores during the incubation period in the heart, liver, muscle, and yolk sac membrane (K. Mann & Mann, 2008; Milinsk et al., 2003; Molenaar et al., 2013). Embryos incubated at high temperatures oxidize more glucose than embryos incubated at normal temperatures, depleting their hepatic glycogen stores and thus reducing the amount of all-important glucose available to the nervous system and muscles at hatch. These embryos have also been shown to be less efficient in using their protein stores for growth, which could help explain their reduced body weight at hatch (Molenaar et al., 2013).

Exposure to low temperatures early in incubation not only appears to affect embryonic heat production (by influencing embryonic metabolic rate), but is also reported to influence post-hatch development (Feast et al., 1998; Janke, Tzschentke, Hochel, & Nichelmann, 2002; Noiva, Menezes, & Peleteiro, 2014). Embryonic development can be sustained to some extent at temperatures as low as 27°C (the physiological zero) - below this threshold, growth and development are suspended but may resume when normal incubation temperatures are reinstated. Such reductions in temperature are accompanied by proportionate decreases in growth rate and consequential increases in the length of the developmental period. It is generally agreed that 30±40% of the embryo's energy is spent maintaining development, while 32% of the energy originally stored in the egg fuels embryo development. As growth is delayed, more energy is shifted to fuel maintenance needs, reducing the stores that should have been used in growth and partially explaining the lower body weight found in chicks incubated under low temperatures (Feast et al., 1998).

Although far less important than temperature, relative humidity is another key factor in development (Leksrisompong et al., 2007; M. V. Nechaeva, 2011). As incubation (and therefore embryo development) progresses and nutrients (*e.g.*, lipids) are incorporated into the embryo and metabolized, the egg loses water through evaporation (Burnham et al., 2001; van der Pol, van Roovert-Reijrink, Maatjens, van den Brand, & Molenaar, 2013). This is essential to create an air cell sufficient to allow embryonic lung ventilation after internal pipping and a successful hatch (Burnham et al., 2001). As previously stated, lipid oxidation constitutes a primary mechanism of energy production in the embryos (Feast et al., 1998; Molenaar et al., 2013). This results in the production of large amounts of water, much of which evaporates into the surrounding atmosphere (Burnham et al., 2001). By day 18 of incubation, the total diffusive water loss should amount to 10-14% of the fresh egg weight for the best chances of successful hatching (Burnham et al., 2001; van der Pol et al., 2013). Embryonic mortality increases when water loss is lower than 9.1% or higher than 18.5%. Water evaporation from the egg can be manipulated by changing the relative humidity (RH) inside the incubator, as water vapor deficit determines the direction of $H_2O$ exchange (van der Pol et al., 2013).

The optimal RH range established is quite wide, between 40 and 70% RH (Peebles et al., 2001). However, studies on the effects of incubation RH on the posthatch broiler chick have shown that overall, optimal performance was achieved when eggs were incubated at 53% RH (Bruzual, Peak, Brake, & Peebles, 2000a, 2000b; Peebles et al., 2001). Lower RH values increase water loss, decreasing hatchling body weight by lowering the water content of the chick’s body (van der Pol et al., 2013). Conversely, higher RH values result in higher chick body weight and in lower embryonic metabolism, by decreasing the need for yolk-derived water and subsequently inhibiting lipid oxidation (without causing an apparent effect on embryonic growth) (Burnham et al., 2001). Late embryonic mortality is also higher in embryos incubated at high (63%) RH values (Bruzual et al., 2000a).

It can also be speculated that RH in the incubator influences embryo temperatures since the energy required to evaporate water from the eggshell is subtracted from the egg. This means that heat loss could be increased in eggs incubated at a low RH when compared with a high RH (van der Pol et al., 2013). Moreover, an increased RH could lower oxygen availability, because of additional water vapor in the air, increasing the risk of hypoxia at the end of incubation (Bruzual et al., 2000a). Thus, lower hatching rates and poorer early chick performance can generally be expected whenever relative humidity values are less than ideal.

Carbon dioxide concentration, the third variable in this study, has been reported to generate contradicting information. The composition of the ambient gaseous environment in the incubator plays an important role during embryonic development (De Smit et al., 2008). As the embryo develops, its metabolism increases, while the permeability of the eggshell remains constant, leading to increasingly limited gas exchange and progressively greater hypoxia and hypercapnia (von Blumroder & Tonhardt, 2002). Embryos can survive mild hypercapnia (4% $CO_2$) across the last half of incubation and severe hypercapnia (10% $CO_2$) for 1 day (Andrewartha, Tazawa, & Burggren, 2011). However, sudden increases to levels above 1% have been shown to be lethal during the first 4 days of incubation, indicating a higher sensitivity of the embryo to higher $CO_2$ levels at this period, possibly related to lack of buffering capacity of the very young embryo to cope with high $CO_2$ (V. Bruggeman et al., 2007). This early buffering is provided by the albumen, since the chorioallantoic membrane is not yet developed at this stage of incubation (V. Bruggeman et al., 2007; Reijrink, Meijerhof, Kemp, & van den Brand, 2010). Higher $CO_2$ levels lower the pH of the initially alkaline albumen by reacting with the abundant water in the albumen to form protons and bicarbonate ions (V. Bruggeman et al., 2007). A number of possibly detrimental effects may arise from this: reduction or inhibition of mitosis during early embryonic development; interference with the establishment of the dorsoventral polarity of the single-cell thick epiblast; lower number of somites; smaller, defective area vasculosa of the yolk sac; malformations; blood-island developmental inhibition; and lower embryonic length and weight (Han, Li, Yu, & Lian, 2011; Reijrink et al., 2010). An increased percentage of malpositioned embryos has also been reported, as well as a higher heart and lung weight (Reijrink et al., 2010).

However, other studies have reported a positive effect of exposure to modest hypercapnia during the first half of incubation (Banerjee et al., 2011; Mortola, 2009). This is usually related to accelerated embryonic growth and faster hatching, presumably via activation of steroid and thyroid hormones (Mortola, 2009). The levels and time of initiating the $CO_2$ rise should play a determining role in the positive or negative outcome of exposing embryos to elevated concentrations of carbon dioxide during incubation (V. Bruggeman et al., 2007).

A thorough search through the available literature has yielded a relatively large number of works published in the field of incubation, many of them having to do with the three variables described here. Many such studies have even been conducted in industrial settings or with access to industrial-grade incubators, using thousands of eggs as samples. However, most if not all of these studies have focused on specific periods of incubation or even exclusively on the chicks hatched from eggs subjected to trial conditions. Moreover, most if not all of these studies focus on no more than two or three parameters of embryonic response to challenge conditions, such as hatchability, hatchling weight, organ weight/size, carcass weight at slaughter, etc. Information obtained from these multiple experiments in differing conditions is thus fragmentary and not always easy to piece together. Additionally, the parameters studied, undoubtedly crucial for industrial yield, tend to be related only to either mortality or hatchling performance, providing very little information as to what is happening during incubation and how the embryo is adapting to the challenges it faces. Histological analysis is also rarely used, for which it is not possible to know at what physical cost challenged embryos take their development to term, or to understand why performance is not ideal in the resulting hatchlings. For this reason, and to make the most efficient use possible of a considerably high number of subjects and samples, this study has taken into account a large number of parameters in embryonic response to changes in the incubation environment. These include physiological markers connected to embryonic distress, nutrient usage and growth, as well as physical changes, evaluated grossly and microscopically.

# MATERIALS AND METHODS

## Establishing control conditions (Normal Pattern of Embryonic Growth under Laboratory Conditions)

Four-hundred seventy first-grade specific-pathogen free (SPF) hatching eggs (Lohmann Selected Leghorn-White Leghorn), were obtained, in two separate batches, from a commercial supplier of Vaccine Eggs (VALO BioMedia, Spain). The eggs weighed between 49.6 g and 64.1 g (average weight 54.8 g ± 3.4 g).

The eggs were stored for less than 7 days, at 14-16ºC, 75-80% relative humidity (RH), and pre-warmed at 22-24ºC for 12 h before incubation. An OVA-EASY® Advance 380 (Brinsea) (Figure 46) cabinet incubator with a maximum capacity of 384 eggs was used to incubate the eggs. The incubator was calibrated (±0.1ºC) previously to the trials.

Eggs were randomly divided and set in vertical position (blunt end up) across 6 incubator trays before incubation.

Figure 46. OVA-EASY® Advance 380, countertop incubator used in the study.

Incubation conditions were as follows: from day 0 to day 18, eggs were incubated at 37.8ºC, RH 50-55% (turning – 1x/hour); from day 18 onwards the eggs were set on the hatcher trays (in horizontal position) and incubated at 37.8ºC, RH 60-65% (no turning).

A random sample of 5% of the initial egg number was removed from the incubator at each day of incubation (from day 0 to day 20) and submitted to the procedures described in Annex 2 Table 1.

A PowerLux® Egg Candler (Lyon Technologies, Inc.) (Figure 47) was used for candling. Heart rate was assessed by visual inspection upon candling from day 3 to day 6 of development and via an electronic digital cardiac egg monitor (Buddy Mk2®, Avitronics) from day 7 onwards (Figure 47). Voluntary movements were assessed by visual inspection upon candling.

**Figure 47. PowerLux® Egg Candler and Buddy Mk2® digital cardiac egg monitor.**

Euthanasia was performed according to the European Parliament (Communities, 1986; Parliament, 2010) and the American Veterinary Medical Association (AVMA) guidelines (AVMA, 2007). Briefly, embryos between days 0 and 14 of incubation were placed in a refrigerated environment (<4ºC) for 4 hours prior to break-out, while embryos between days 16 and 20 were placed in a chamber with 100% environmental $CO_2$ concentration for 20 minutes prior to break-out. Death was confirmed through exsanguination without maceration, in order to preserve embryos for future histopathological study. On opening the eggs, albumen and yolk were separately collected and weighed. After visual detection, the embryos were measured, weighed and photographed, and subsequently collected for fixation in 10% neutral buffered formalin, for routine histopathological analysis and observation of histological embryonic structure (Table 1).

**Table 1. Average number of paraffin blocks per embryo/egg**

| Incubation Day | Number of histology slides per specimen |
|---|---|
| **1-3** | 1 |
| **5** | 1-2 |
| **7** | 2 |
| **9** | 3 |
| **10** | 4 |
| **12** | 6 |
| **14** | 12 |
| **16** | 15 |
| **18** | 17 |
| **20** | 19 |

## Trial 1 – Challenge with Continuously High Temperatures (CHT)

Two-hundred seventy first-grade SPF hatching eggs were obtained, from a commercial supplier of Vaccine Eggs (VALO BioMedia, Spain). The eggs weighed between 58.6 g and 66.3 g (average weight 62.2 g ± 1.7 g).

The eggs were stored for 12 h before incubation and then randomly divided and set in vertical position across 6 incubator trays before incubation, as described for control eggs.

Incubation conditions were as follows: from day 0 to day 18 eggs were incubated at 38.9ºC, RH 50-55%; from day 18 onwards the eggs were set on the hatcher trays (in horizontal position) and incubated at 38.9ºC, RH 60-65%.

A random sample of 5% of all eggs was removed from the incubator every 48 hours of incubation (from day 0 to day 20) and treated as described previously for control eggs.

## Trial 2 – Challenge with Continuously Low Temperatures (CLT)

Two-hundred seventy first-grade SPF hatching eggs were obtained, from a commercial supplier of Vaccine Eggs (VALO BioMedia, Spain). The eggs weighed between 50.7 g and 66.2 g (average weight 58.8 g ± 2.9 g).

The eggs were stored for 12 h before incubation and then randomly divided and set in vertical position across 6 incubator trays before incubation, as described for control eggs.

Incubation conditions were as follows: from day 0 to day 18 eggs were incubated at 36.7ºC, RH 50-55%; from day 18 onwards the eggs were set on the hatcher trays (in horizontal position) and incubated at 36.7ºC, RH 60-65%.

A random sample of 5% of all eggs was removed from the incubator every 48 hours of incubation (from day 0 to day 20) and treated as described for control eggs.

### Trial 3 – Challenge with Sudden Temperature Rises (PHT)

Two-hundred seventy first-grade SPF hatching eggs were obtained, from a commercial supplier of Vaccine Eggs (VALO BioMedia, Spain). The eggs weighed between 47.1 g and 64.3 g (average weight 57.2 g ± 2.3 g).

The eggs were stored for 12 h before incubation and then randomly divided and set in vertical position across 6 incubator trays before incubation, as described for control eggs.

Incubation conditions were as described for control eggs. The eggs were challenged with a pinpoint (3 hour-long) rise in temperature (38.9ºC) during days 3 and 18 of standard incubation. Because embryo mortality has been observed to peak at days 3-4 and day 18 of incubation, time periods which correspond to important moments in embryonic development, when the circulatory system (namely the heart) is developing (day 3) and when the embryo begins to turn into the right position for lung inflation with air from the air chamber (day 18), eventually followed by internal pipping, these days were considered critical challenge points for pinpoint manipulation (Bellairs & Osmond, 2005) (Tullett, 2008).

A random sample of 5% of all eggs was removed from the incubator every 48 hours of incubation (from day 0 to day 20) and treated as described for control eggs.

### Trial 4 – Challenge with Sudden Temperature Drops (PLT)

Two-hundred seventy first-grade SPF hatching eggs were obtained, from a commercial supplier of Vaccine Eggs (VALO BioMedia, Spain). The eggs weighed between 57.9 g and 79.8 g (average weight 65.5 g ± 4.00 g).

The eggs were stored for 12 h before incubation and then randomly divided and set in vertical position across 6 incubator trays before incubation, as described for control eggs.

Incubation conditions were as described for control eggs. The eggs were challenged with a pinpoint (3 hour-long) drop in temperature (36.7ºC) during days 3 and 18 of standard incubation.

A random sample of 5% of all eggs was removed from the incubator every 48 hours of incubation (from day 0 to day 20) and treated as described for control eggs.

### Trial 5 – Challenge with Continuously High Relative Humidity (CHRH)

One-hundred fifty first-grade SPF hatching eggs were obtained, from a commercial supplier of Vaccine Eggs (VALO BioMedia, Spain). The eggs weighed between 63.8 g and 78.3 g (average weight 68.1 g ± 2.8 g).

The eggs were stored for 12 h before incubation and then randomly divided and set in vertical position across 6 incubator trays before incubation, as described for control eggs.

Incubation conditions were as follows: from day 0 to day 18 eggs were incubated at 37.8ºC, RH 60-65%; from day 18 onwards the eggs were set on the hatcher trays (in horizontal position) and incubated at 37.8ºC, RH 60-65%.

A random sample of 5% of all eggs was removed from the incubator every 48 hours of incubation (from day 0 to day 20) and treated as described for control eggs.

### Trial 6 – Challenge with Continuously Low Relative Humidity (CLRH)

One-hundred fifty first-grade SPF hatching eggs were obtained, from a commercial supplier of Vaccine Eggs (VALO BioMedia, Spain). The eggs weighed between 50.7 g and 62.3 g (average weight 57.9 g ± 2.4 g).

The eggs were stored for 12 h before incubation and then randomly divided and set in vertical position across 6 incubator trays before incubation, as described for control eggs.

Incubation conditions were as follows: from day 0 to day 18 eggs were incubated at 37.8ºC, RH 40-45%; from day 18 onwards the eggs were set on the hatcher trays (in horizontal position) and incubated at 37.8ºC, RH 40-45%.

A random sample of 5% of all eggs was removed from the incubator every 48 hours of incubation (from day 0 to day 20) and treated as described for control eggs.

### Trial 7 – Challenge with Sudden Relative Humidity Rises (PHRH)

One-hundred fifty first-grade SPF hatching eggs were obtained, from a commercial supplier of Vaccine Eggs (VALO BioMedia, Spain). The eggs weighed between 55.6 g and 65.4 g (average weight 61.6 g ± 2.0 g).

The eggs were stored for 12 h before incubation and then randomly divided and set in vertical position across 6 incubator trays before incubation, as described for control eggs.

Incubation conditions were as described for control eggs. The eggs were challenged with a pinpoint (3 hour-long) rise in relative humidity (60-65%) during days 3 and 18 of standard incubation.

A random sample of 5% of all eggs was removed from the incubator every 48 hours of incubation (from day 0 to day 20) and treated as described for control eggs.

### Trial 8 – Challenge with Sudden Relative Humidity Drops (PLRH)

One-hundred fifty first-grade SPF hatching eggs were obtained, from a commercial supplier of Vaccine Eggs (VALO BioMedia, Spain). The eggs weighed between 55.3 g and 68.5 g (average weight 60.3 g ± 2.6 g).

The eggs were stored for 12 h before incubation and then randomly divided and set in vertical position across 6 incubator trays before incubation, as described for control eggs.

Incubation conditions were as described for control eggs. The eggs were challenged with a pinpoint (3 hour-long) drop in relative humidity (40-45%) during days 3 and 18 of standard incubation.

A random sample of 5% of all eggs was removed from the incubator every 48 hours of incubation (from day 0 to day 20 and treated as described for control eggs.

### Trial 9 – Challenge with Continuously High Carbon Dioxide Concentration (CHCO2)

One-hundred fifty first-grade SPF hatching eggs were obtained, from a commercial supplier of Vaccine Eggs (VALO BioMedia, Spain). The eggs weighed between 60.5 g and 76.8 g (average weight 66.2 g ± 2.6 g).

The eggs were stored for 12 h before incubation and then randomly divided and set in vertical position across 6 incubator trays before incubation, as described for control eggs.

Eggs were incubated at 37.8ºC, RH 50-55% from day 0 to day 18; from day 18 onwards the eggs were incubated at 37.8ºC, RH 60-65%. All air circulation was impeded and the $CO_2$ levels allowed to rise naturally within the incubator (Annex 3), reaching up to 1200 ppm (twice the normal values). $CO_2$ levels were monitored using an appropriate data logger (desktop $CO_2$ data logger, model ZG106A-M, ZyAura™, Radiant™, Taiwan). A random sample of 5% of all eggs was removed from the incubator every 48 hours of incubation and treated as described for control eggs.

A summary of all trial conditions is presented in Table 2 for easier reading.

**Table 2. Summary of the trials and conditions applied.**

| | Temperature | Relative Humidity | | Carbon Dioxide |
|---|---|---|---|---|
| | | **Day 0-18** | **Day 18-20** | |
| Control | 37.8ºC | 50-55% | 60-65% | Normal air input<br>(max. 665 ppm) |
| CHT | 38.9ºC | 50-55% | 60-65% | Normal air input |
| CLT | 36.7ºC | 50-55% | 60-65% | Normal air input |
| PHT | 37.8ºC<br>2x 3h, 38.9ºC (day 3&18) | 50-55% | 60-65% | Normal air input |
| PLT | 37.8ºC<br>2x 3h, 36.7ºC (day 3&18) | 50-55% | 60-65% | Normal air input |
| CHRH | 37.8ºC | 60-65% | 60-65% | Normal air input |
| CLRH | 37.8ºC | 40-45% | 40-45% | Normal air input |
| PHRH | 37.8ºC | 50-55%<br>2x 3h at 60-65% (day 3&18) | 60-65% | Normal air input |
| PLRH | 37.8ºC | 50-55%<br>2x 3h at 40-45% (day 3&18) | 60-65% | Normal air input |
| CHCO2 | 37.8ºC | 50-55% | 60-65% | No air renewal<br>(max. 1200ppm) |

## Data Analysis

Data was subjected to one-way ANOVA and to the all-pairs Tukey-Kramer-HSD test, and to Chi-square test where appropriate, by means of the SPSS Statistics 19 software (IBM. 2013).

# RESULTS

## *PRACTICAL CONSIDERATIONS IN RESULT ORGANIZATION AND PRESENTATION*

**Treatments**

In an effort to facilitate result presentation and interpretation, treatments are designated by a simple identifier code. Physical environment treatments are thus designated:

$$\frac{\text{C (Continuous change)}}{\text{P (Pinpoint manipulation)}} + \frac{H\ (Higher\ than\ ideal)}{L(Lower\ than\ ideal)} + Manipulated\ Variable$$

*Variable codes: T – Temperature*

*RH – Relative Humidity*

*CO2 – Carbon Dioxide*

*Example: Continuously High Temperature - CHT*

**Parameters**

All information recorded is organized here as a set of monitored parameters falling under two major types: ante mortem observations and post mortem observations.

Ante mortem observations mostly pertain to behavioral indicators of embryonic stress, namely heart rate (HR) and voluntary movements per minute (VMM). These parameters were deemed as indicators of embryonic stress due to ease of monitoring without direct manipulation, as well as due to their linking to embryonic homeostasis and liveliness.

Post mortem observations were further divided into three categories: indicators of nutrient reserve mobilization, indicators of embryonic growth and indicators of embryonic viability. Nutrient mobilization indicators used were the albumen weight to whole-egg weight ratio (AR), the yolk weight to whole-egg ratio (YR) and the eggshell weight to whole egg weight ratio (SR). The indicators of embryonic growth used pertained to embryo weight gain (here expressed as embryo weight to whole-egg weight ratio) (ER), morphometric analysis (Embryo Length (EL), Wing Length (WL), Leg Length (LL), Beak Length (BL), Comb Length (CL), Eye Diameter (ED), Feather Length (FL), Toe Length (TL), Nail Length (NL). Histological analysis was also performed to monitor the microscopic development of the different organs in their original anatomic positions. Malformation rates and types, and mortality rates constituted the selected indicators of embryonic viability.

### Ratios

Because absolute standardization in egg size (and therefore weight) could not be ensured due to logistical limitations and natural in-flock variation, all weight-based parameters are presented here as ratios and not as absolute values, in order to normalize results as much as possible. Consequently, albumen, yolk, shell and embryo weights are always presented as a ratio of the absolute value recorded for the parameter and the weight of the egg from which the data was collected prior to manipulation.

### Malformations

All malformations were characterized and classified in accordance with both human and veterinary works of reference in embryology and teratology (Drews, 1995; Romanoff & Romanoff, 1972; Sadler, 2011). Classification of malformations varies between authors and thus care has been taken in consistently classifying malformations. However, alternative designations are also presented here.

Cases of spontaneous malformation did occur in the embryos incubated under ideal conditions (≈1.3%). These affected primarily the cranium, which failed to develop, leaving the brain exposed (exencephaly) (Figure 48H). The spine was also affected, namely by posterior twinning (cephalopagus/lambdoid embryo) (Figures 48B), as well as the eyes (duplication of the left eye) (Figures 48C) and beak (maxillary hypoplasia) (Figure 49F). In some cases, the abdominal wall failed to close, causing protrusion of the abdominal organs (gastroschisis/celosomia), with or without protrusion of the heart (ectopia cordis) (Figure 49D). Cases of dwarfism (Figure 49G), twinning (Figure 48E) and cystic embryos (presence of a fluid-filled vesicle surrounding embryonic tissues without development of an actual embryo) (Figure 48A) were also identified.

Compound malformations (co-existing malformation in more than one embryonic structure at a time, as opposed to simple malformations) were also found (Figure 49B).

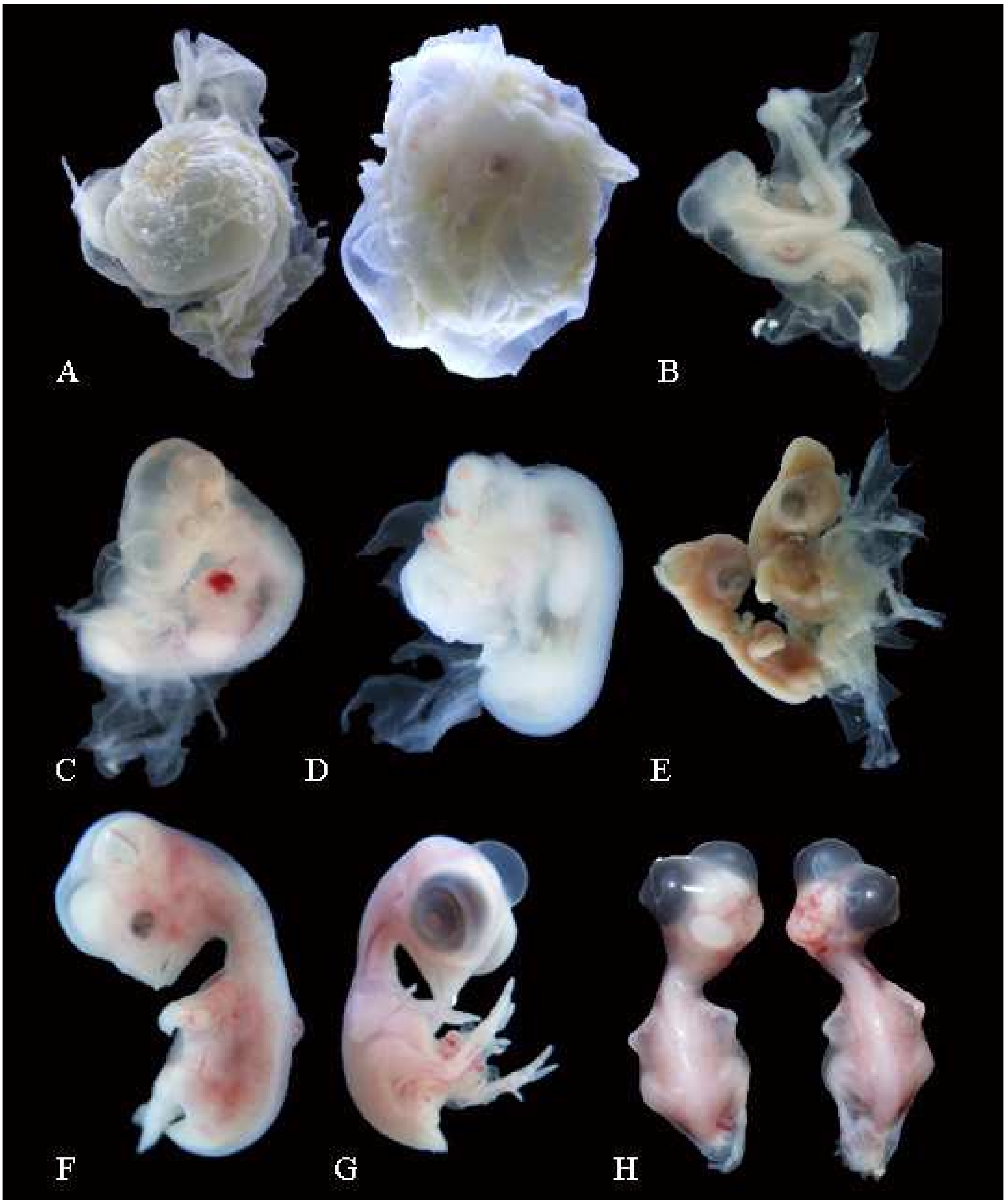


**Figure 48. Examples of malformations.**
**Legend: A) Day 2-3 - Cystic embryos; B) Day 4 – Cephalopagus; C) Day 4 - Duplication of the left eye; D) Day 5 – Anencephaly; E) Day 6 – Twinning; F) Day 7 – Microphthalmia; G) Day 9 - Cutaneous vesicle; H) Day 9 – Exencephaly.**

**Figure 49. More examples of malformations.**
**Legend: A) Day 11 - Cephalopagus with ectopia cordis (not shown in the image); B) Day 12 - Compound malformation affecting the skull (exencephaly), both eyes (bilateral anophthalmia) and the abdominal wall (gastroschisis and ectopia cordis); C) Day 14 - Compound malformation affecting both eyes (bilateral anophthalmia) and the upper beak (maxillary hypoplasia); D) Day 15 - Gastroschisis with ectopia cordis; E) Day 15 – Plantigrady; F) Day 17 - Compound malformation affecting the skull (exencephaly), the left eye (duplication) and the upper beak (maxillary hypoplasia); G) Day 18 – Dwarfism; H) Day 20 – Exencephaly; I) Day 20 - Unhealed navel.**

### Dead-in-shell embryos

Analysis of dead-in-shell embryos yielded no significant changes in lesion patterns between challenged and control embryos. In the embryos examined during this study, no gross lesions were apparent and microscopic observation has only yielded images of: 1) apparent arrest in embryonic development (Figure 50A); 2) failure in embryo formation in spite of extra-embryonic membranes being present (Figure 50B); and 3) tissue instability with possible dysfunction and blood loss (Figure 50C).

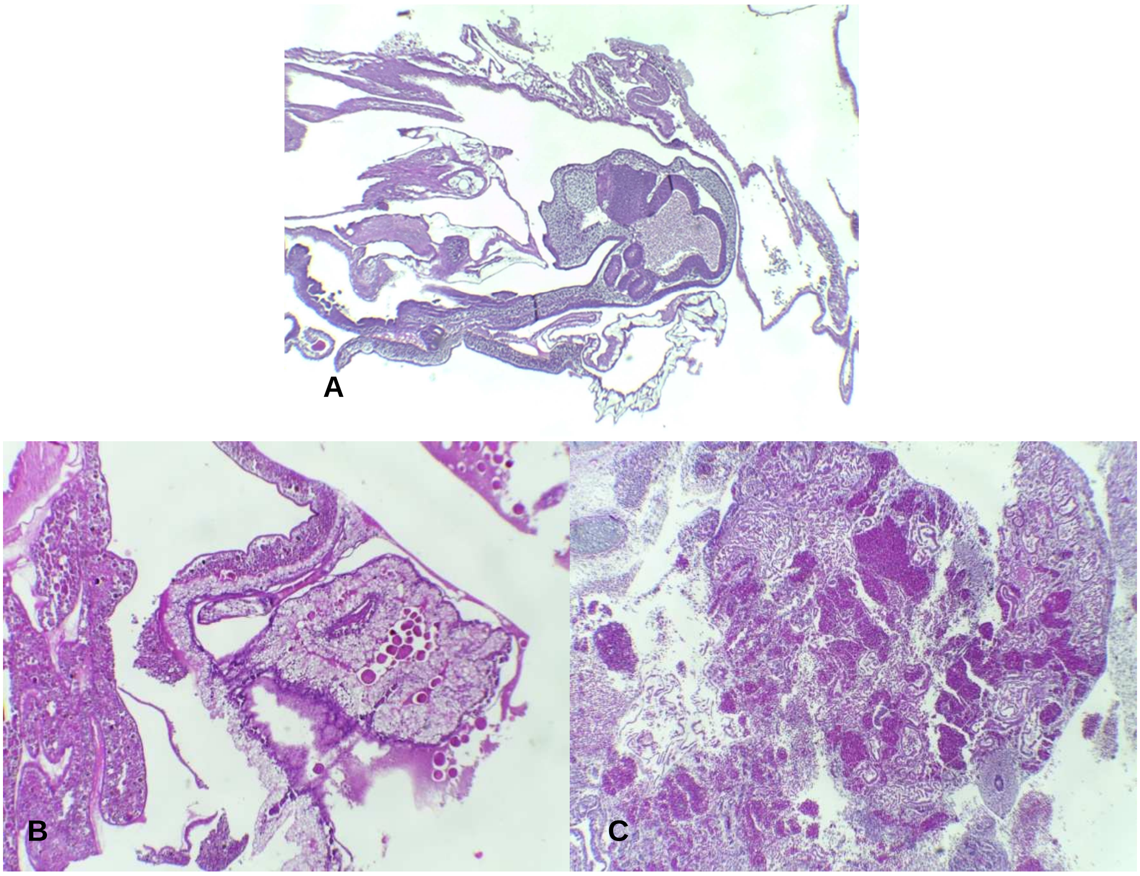


**Figure 50. Microscopy images of dead-in-shell embryos.**
**A) Embryo dead at day 3 of incubation. No apparent cause of death was found (H&E, 40x); B) Embryo dead at day 2-3 of incubation. Only extra-embryonic membranes can be seen without formation of the embryo proper (H&E, 40X); C) Embryo dead at day 6-7 of incubation. The tissues corresponding to the kidney and liver appear loosely arranged, with extensive areas of hemorrhage (H&E, 40x)**

## TEMPERATURE-RELATED TRIALS

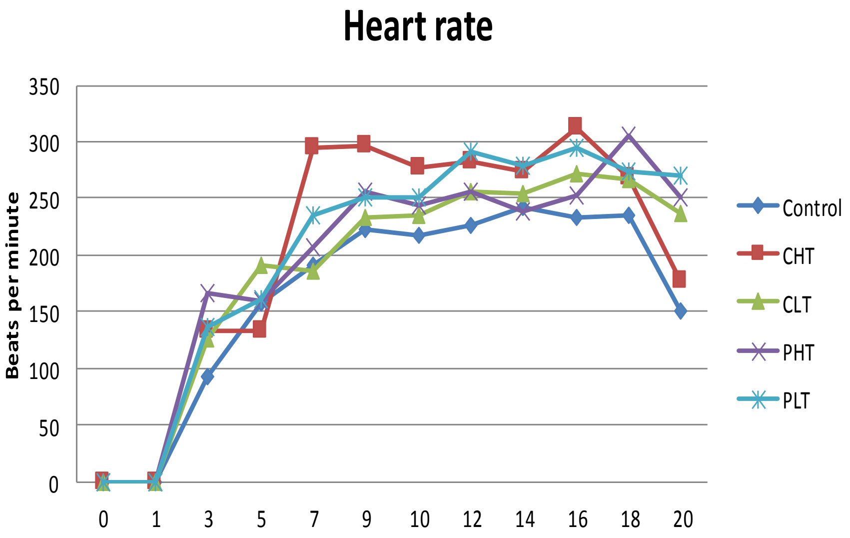
A total of 1109 trial embryos were analyzed, described and catalogued, and later compared against 442 control embryos. During the four separate trials, over 24,000 images were taken, over 8000 histology slides produced, and compared against 10,000 images and 2600 control-embryo histology slides. For effects of simplifying reading, all statistical data has been compiled and is present in Annex 4.

### Ante-mortem observations

*Behavior*

***Heart rate***

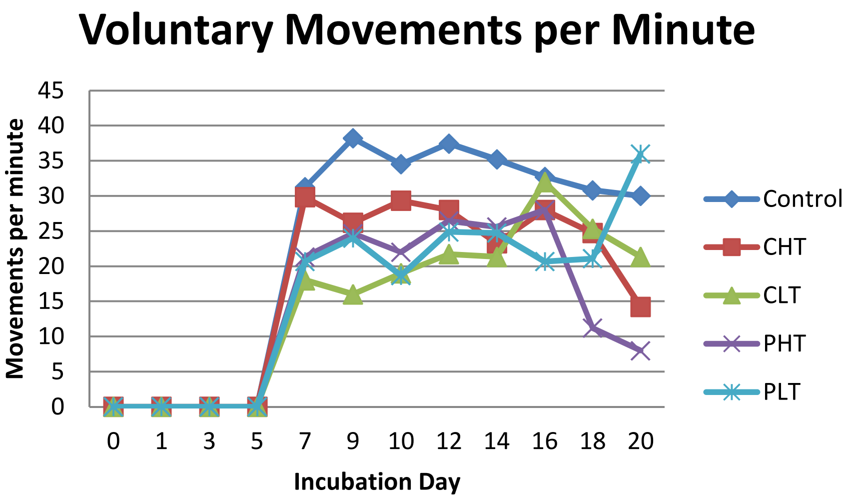
Heart rate (Graph 1) was consistently higher than control values for all challenged groups, particularly from day 5 onward.



**Graph 1. Heart rate (beats/minute) throughout incubation for control and temperature-challenged eggs.**

***Voluntary movements per minute***

In contrast to heart rate, VMM were consistently lower than control-group VMM for all challenged groups (Graph 2), prominently from day 7 onward. However, Treatment CLT embryos exhibited a sudden rise in VMM to values similar to those of the control group, between days 14 and 16, dropping back below control VMM values from day 16 onward.



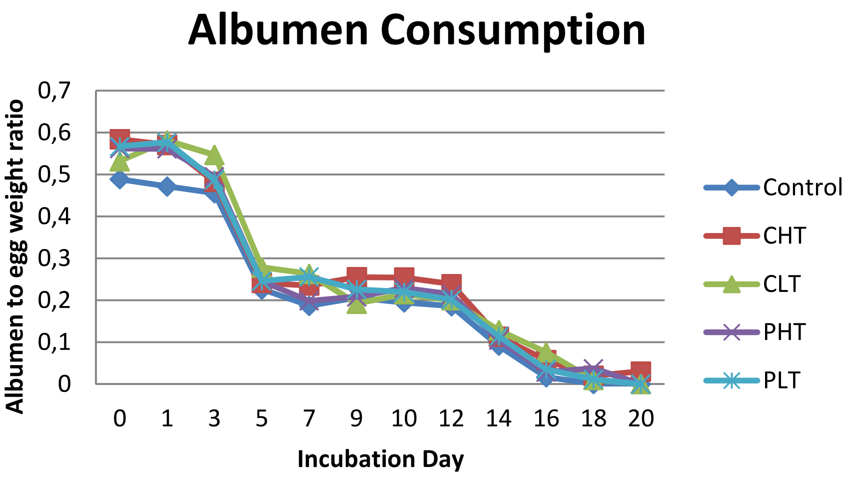
**Graph 2. Voluntary movement (movements/minute) throughout incubation for control and temperature-challenged eggs.**

**Post-mortem observations**

*Nutrient reserves usage*

***Albumen consumption (AR)***

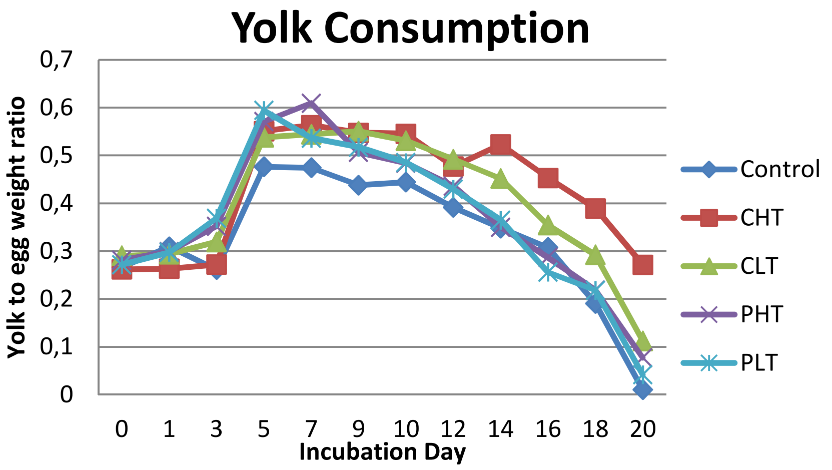
In Graph 3 it is possible to observe that the eggs in treatment CHT exhibited higher initial AR then controls, that dropped suddenly to values similar to the control group by day 3 of incubation and followed the same tendency of control eggs until day 5, when the AR for Treatment CHT eggs stabilized at values higher than those for control eggs, remaining higher than the latter until day 20 (in spite of showing a similar trend). Treatment CLT eggs had AR values that were higher than those for control eggs between days 0 and 3 of incubation and did not follow the tendency to drop shown by the latter. The values dropped between days 3 and 5, although remaining slightly above AR values for the control group and stabilizing between days 5 and 7 instead of following the control group’s tendency to drop. The above mentioned drop in AR values took place between days 7 and 9, from which point on AR values for Treatment CLT eggs followed the values for the control group closely, despite exhibiting a less accentuated drop between days 12 and 20. Results for Treatment PHT eggs were similar to Treatment CLT eggs, albeit exhibiting a sharper drop between days 6 and 9. Treatment PLT eggs showed a similar trend to Treatment CLT eggs.



**Graph 3. Albumen consumption (albumen weight to egg weight ratio) throughout incubation for control and temperature-challenged eggs.**

### *Yolk consumption (YR)*

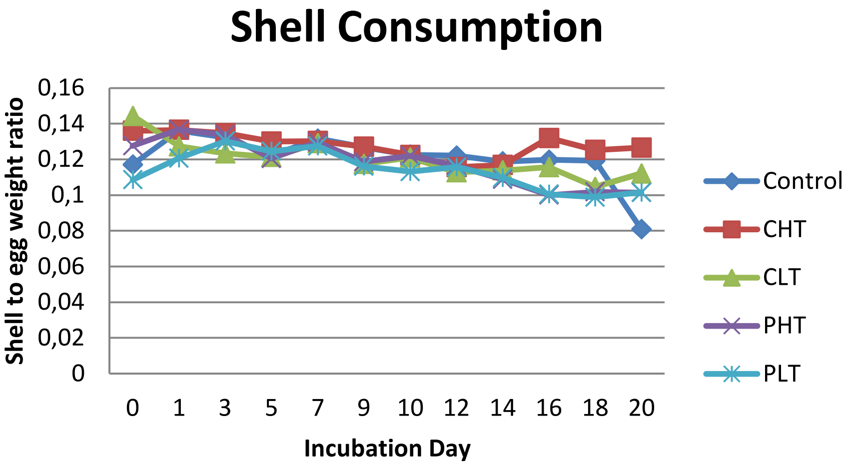
Regarding yolk to egg-weight ratio (Graph 4), no significant differences were evident between Treatment CHT and control eggs before day 3 of incubation when the YR rose (until day 5) and then dropped as incubation progressed with a tendency similar to control eggs, albeit always maintaining higher values than the latter. Treatment CLT eggs showed a similar trend to Treatment CHT eggs, though with a smaller discrepancy between YR values for challenged and control eggs. Both Treatment PHT and PLT eggs showed a similar trend, parallel to the control eggs, with values that remained consistently higher than the latter until day 13 of incubation, from which point on YR values dropped and then closely followed those obtained for the control eggs.



**Graph 4. Yolk consumption (yolk weight to egg weight ratio) throughout incubation for control and temperature-challenged eggs.**

***Shell consumption (SR)***

Shell to egg-weight ratios very closely followed those observed for control eggs for the whole of the incubation process, without statistically significant deviance from the control SR (Graph 5).

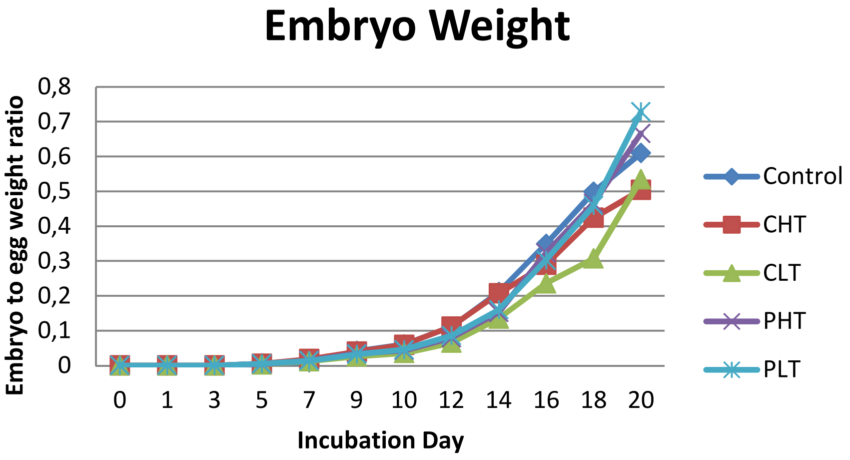


**Graph 5. Shell consumption (shell weight to egg weight ratio) throughout incubation for control and temperature-challenged eggs.**

*Embryonic development*

***Embryo weight gain (ER)***

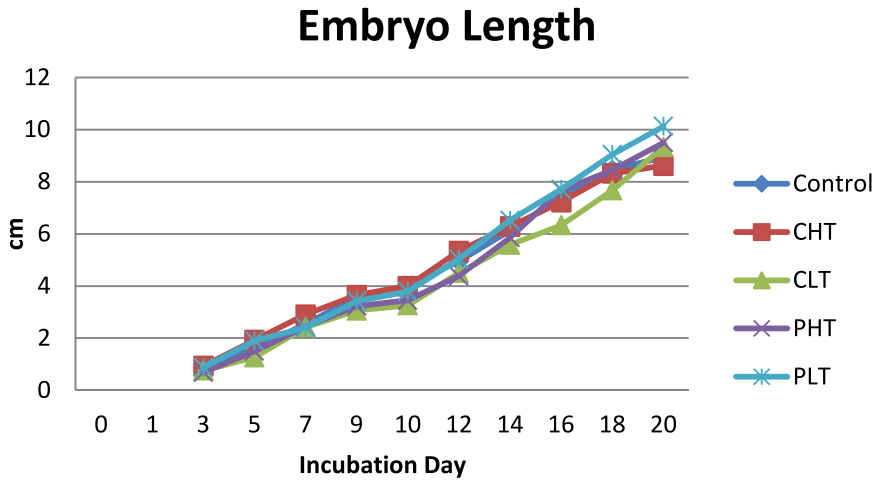
Embryo to egg-weight ratios for Treatment CHT eggs showed very similar patterns and values to control up to day 14, at which point ER values for challenged eggs began to exhibit slower growth rates than the eggs in the control group (Graph 6). Treatment CLT eggs exhibited a similar tendency for lower ER growth rates which started on day 7 of incubation. Treatments PHT and PLT produced limited, non-significant effects on ER.



**Graph 6. Embryo growth (embryo weight to egg weight ratio) throughout incubation for control and temperature-challenged eggs.**

***Morphological indicators of embryo growth***

Most of the morphometric indicators measured showed no statistically significant changes when compared to controls. These were namely Embryo length (Graph 7), Wing length (Graph 8), Leg length (Graph 9), Comb length (Graph 11) and Eye diameter (Graph 12).



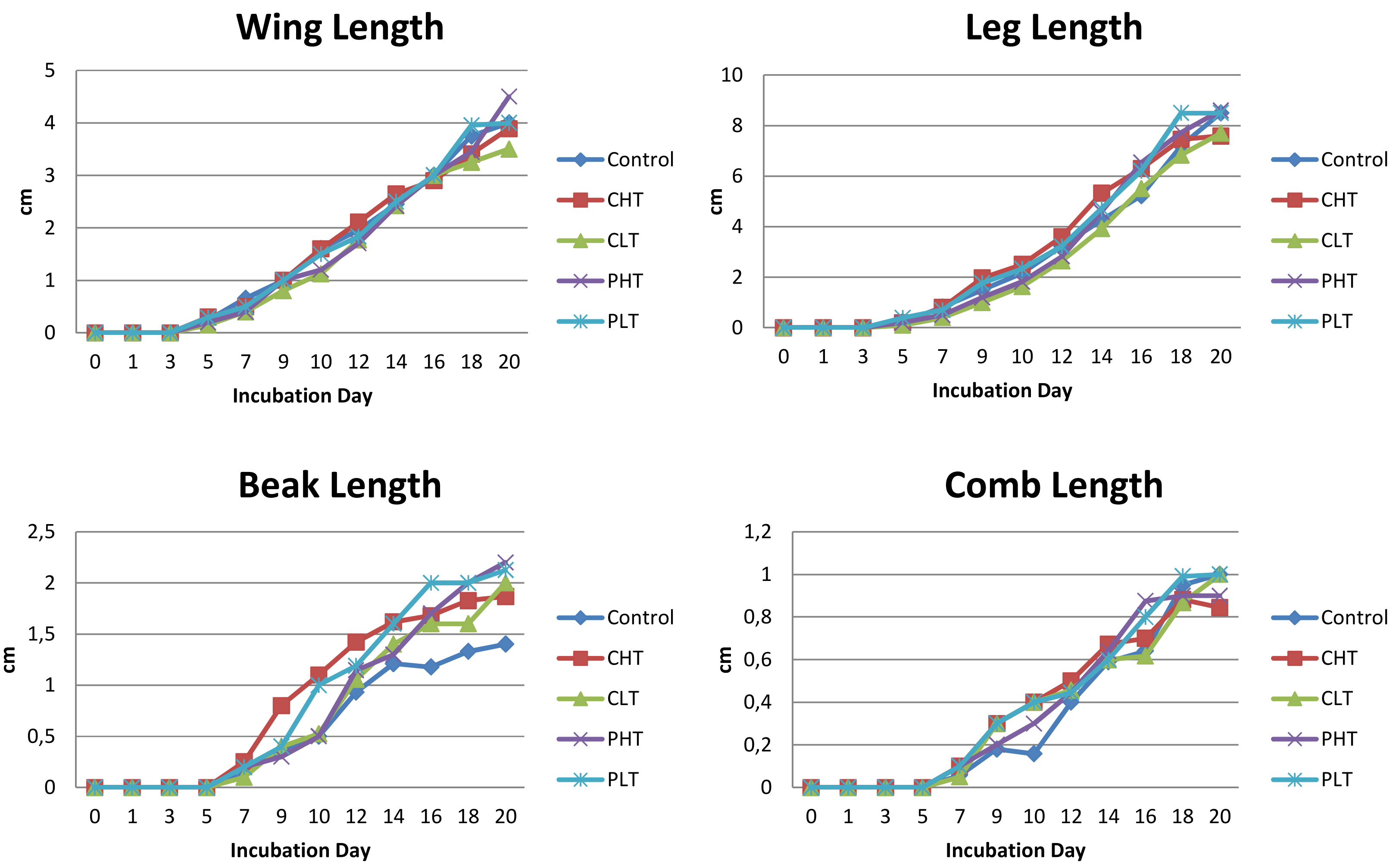
**Graph 7. Embryo growth (embryo length) throughout incubation for control and temperature-challenged eggs.**

Beak length (BL) presented measurements consistently higher than controls for all treatments, with final values rather similar between treatments, with departure from control values occurring at day 9 for CHT embryos, day 10 for PLT embryos and at day 12 for CLT and PTH embryos (Graph 10).

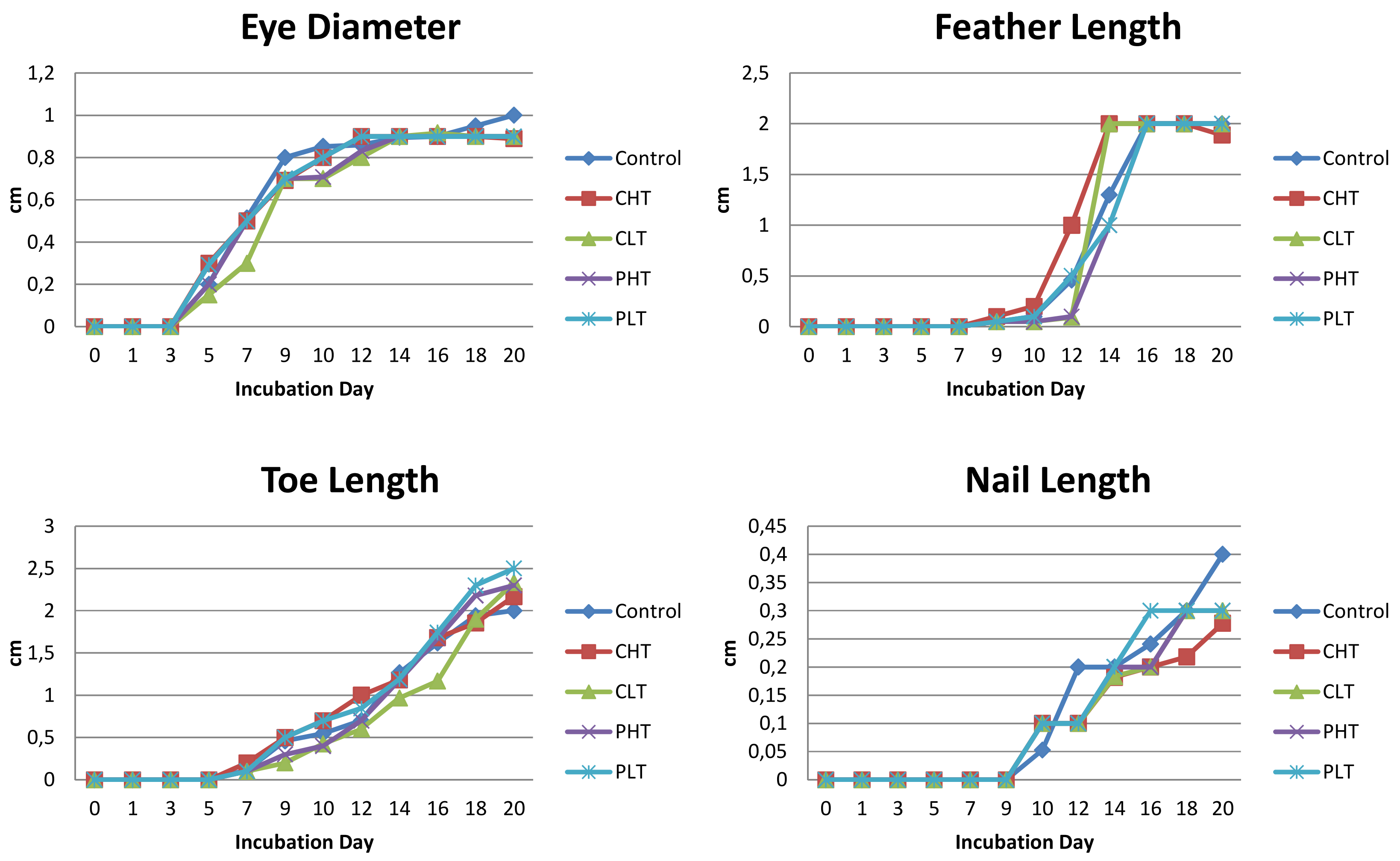
As shown by Graph 13, speed of feather formation and growth, as measured by Feather length (FL), varied between challenged and control groups, and among treatments, even though the final feather length was similar for all groups. Differences between treatments mostly pertained to different speeds of feather growth, with earlier or delayed feather growth. CHT embryos had consistently faster feathering, with the first feathers developing at day 9 and reaching their final length at day 14 of incubation, two days before control embryos. Similarly, CLT embryos reached final FL at day 14 after an initial appearance at day 9. However, CLT embryos presented with feathers shorter than 0.5 cm until day 12, after which they abruptly grew to final FL, between days 12 and 14. PHT embryos reached final FL at day 16, in synchrony with control embryos, but did not start to grow significantly before day 12. PLT embryos showed no significant difference in feathering from control embryos.

Toe length was mostly unaffected, except for a period between days 12 and 18 of incubation, when CLT embryos registered shorter toe lengths than the remaining challenged and control groups (Graph 14). This difference, disappeared by day 18 of incubation and was completely absent by day 20.

Nail growth presented variable growth patterns between challenged and control groups (Graph 15). Although all groups exhibited nail formation by day 10, final NL was significantly lower for all challenged groups when compared to control embryos, while being similar between challenged groups. Nail growth was fastest for PLT embryos, which reached final NL by day 16, and shortest for CHT embryos, which did not reach final NL until day 20.



**Graphs 8 - 11. Embryo growth throughout incubation for control and temperature-challenged eggs.**
**Upper Left – Wing Length; Upper Right – Leg Length; Lower Left – Beak Length; Lower Right – Comb Length**



**Graphs 12 - 15. Embryo growth throughout incubation for control and temperature-challenged eggs.**
**Upper Left – Eye Diameter; Upper Right – Feather Length; Lower Left – Toe Length; Lower Right – Nail Length**

### *Histological analysis*

Microscopic morphological changes found were limited to the gizzard and the Bursa of Fabricius, with no other organ showing any evident changes in morphology or growth patterns.

### Gizzard

Embryos exposed to the CHT Treatment exhibited vacuolization of the glandular epithelium of the gizzard mucosa. The cytoplasm of these cells exhibited large vacuoles which displaced the nucleus against the cell membrane, giving the cells a signet-ring appearance (Figure 51). In some cells, these vacuoles were filled with mildly basophilic content. Cellular overdistension and rupture were frequent findings, with formation of irregularly-shaped cavities in the mucosa. These cavities varied in number and size, and occasionally contained the same mildly basophilic, amorphous substance seen in smaller vacuoles. The cavities were consistently present in nearly all embryos (>85%) analyzed between days 12 and 16, in most cases affecting between 15% and 50% of the extension of the mucosal layer, which appeared vacuolated at lower magnification.

By day 18, the cavities were not as evident, instead appearing as areas of dystrophy and apparent detachment of the more superficial layers of the mucosa with formation of vesicles. In some areas, these layers of the mucosa also appeared thickened, with cells containing large vacuoles in their cytoplasm. Additionally, the glandular mucosa in these embryos appeared disorganized, irregular and less cellular, when compared to the well-formed, column-like architecture found in control embryos.

Control embryos only occasionally exhibited similar findings, always in smaller in size, affecting smaller areas (<5% of mucosa affected) and present only between days 14 and 16 of incubation. Two out of thirteen day 14 embryos belonging to the PLT Treatment also exhibited a small number of these lesions, in a close parallel to control embryos.

### Bursa of Fabricius

A greater number of lymphoid follicles was observed in the bursa of Fabricius of CHT embryos between days 12 and 14 of incubation, when compared to control embryos of the same age (Figure 52). These follicles were also bigger in size than controls. At day 16, however, the bursal follicles of CHT embryos appeared slightly less cellular (but not less abundant) when compared to controls. This became more evident by day 18, when the cellular scaffolding for bursal follicles was found outlining follicles in similar numbers and of similar dimensions to control-embryo bursas but containing scarcer numbers of lymphoid cells. In some follicles, no evidently lymphoid cells whatsoever could be found (Figure 53).

In embryos challenged with CLT, the bursa of Fabricius consistently appeared to have a lower number of follicles (which were also smaller in size) and a diminished number of lymphoid cells when compared to controls. These differences became evident at day 12 and were found to persist throughout incubation. In both PHT and PLT, a lower number of lymphoid cells in the bursal follicles was only observed between days 12 and 14, after which lymphoid populations mirrored those of control embryos.

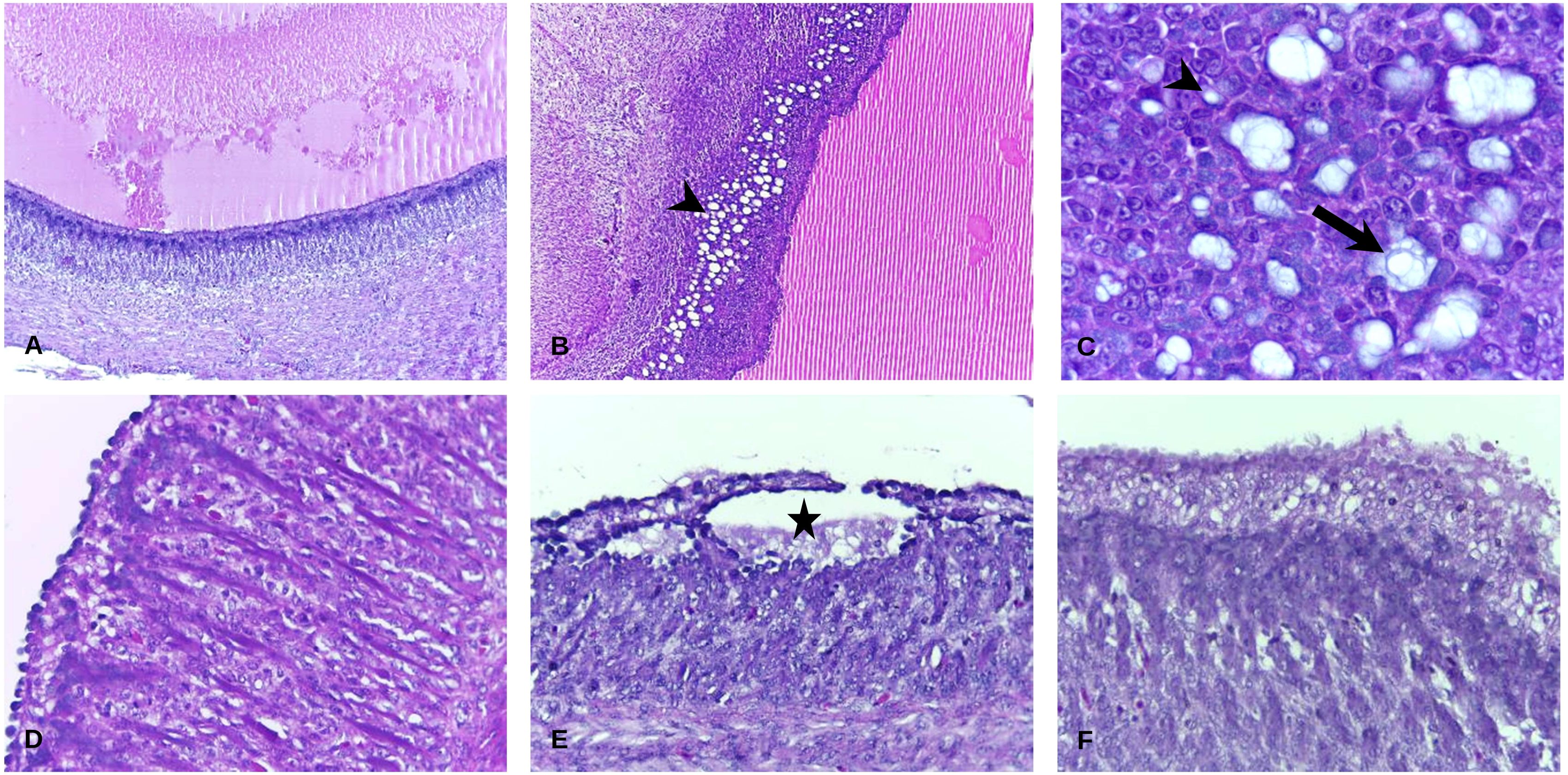


Figure 51. Gizzard.

A) Control embryo at day 16 (H&E, 40x); B) CHT embryo at day 16. Note the vacuolar aspect of the mucosa (arrowhead) (H&E, 40x); C) CHT embryo at day 16. At a higher magnification, it becomes apparent that the vacuolar aspect of the mucosa is caused by the presence of large vacuoles that distend the cytoplasm of glandular cells, giving them a signet-ring appearance (arrowhead) and leading to cell membrane rupture with cavity formation (arrow) (H&E, 1000x); D) Control embryo at day 18. Note the regular columns formed by the mucosal glands (H&E, 100x); E) CHT embryo at day 18 with detachment of the upper layers of the glandular epithelium and vesicle formation (star) (H&E, 100x); F) CHT embryo at day 18. Note the irregularly shaped glands and foamy aspect of the superficial epithelial layers. (H&E, 100x)

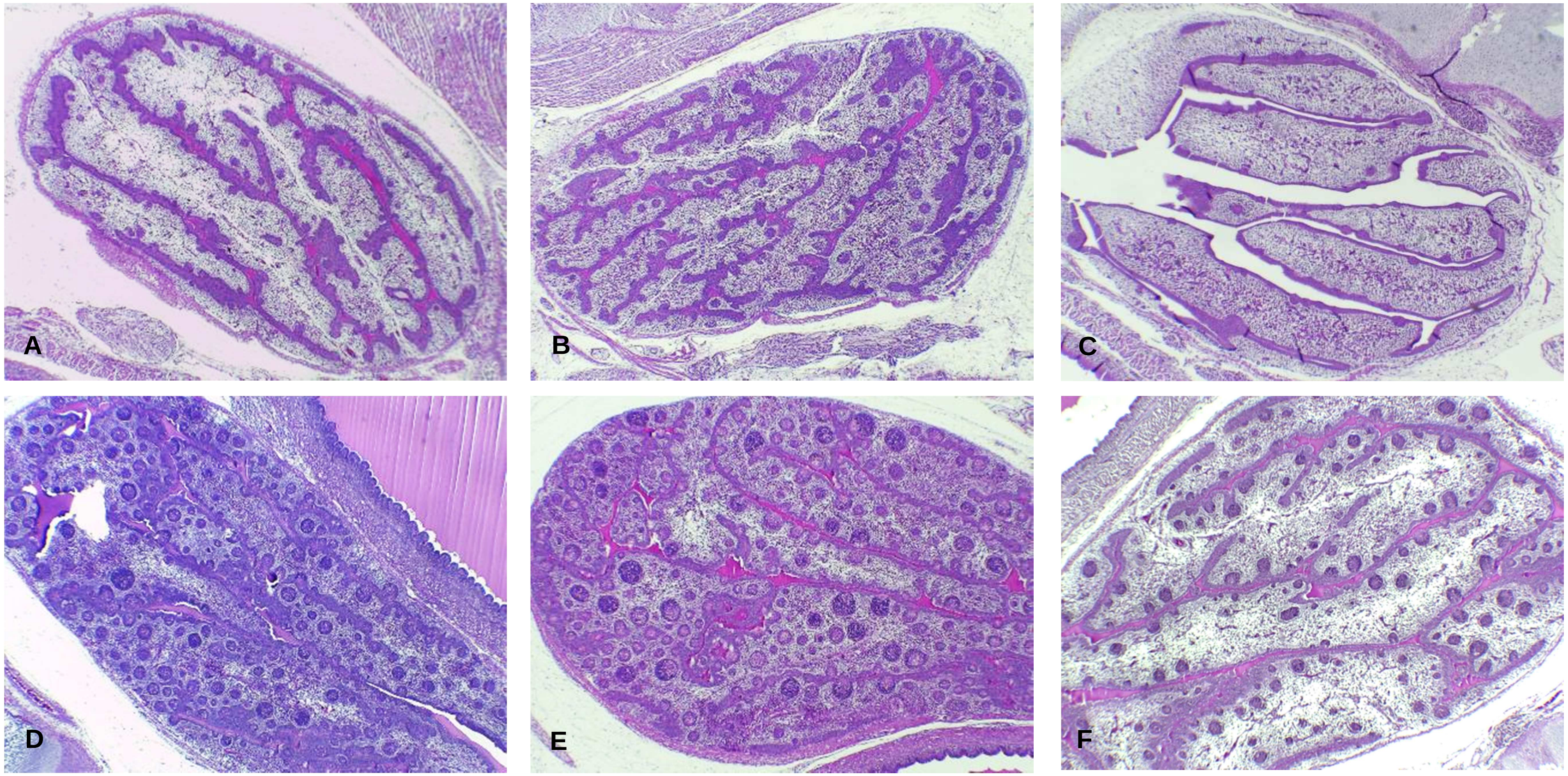


**Figure 52. Bursa of Fabricius.**
**A) Control embryo at day 14 (H&E, 40x); B) CHT embryo at day 14 (H&E, 40x); C) CLT embryo at day 14 (H&E, 40x); D) Control embryo at day 16 (H&E, 40x); E) CHT embryo at day 16 (H&E, 40x); F) CLT embryo at day 16 (H&E, 40x)**

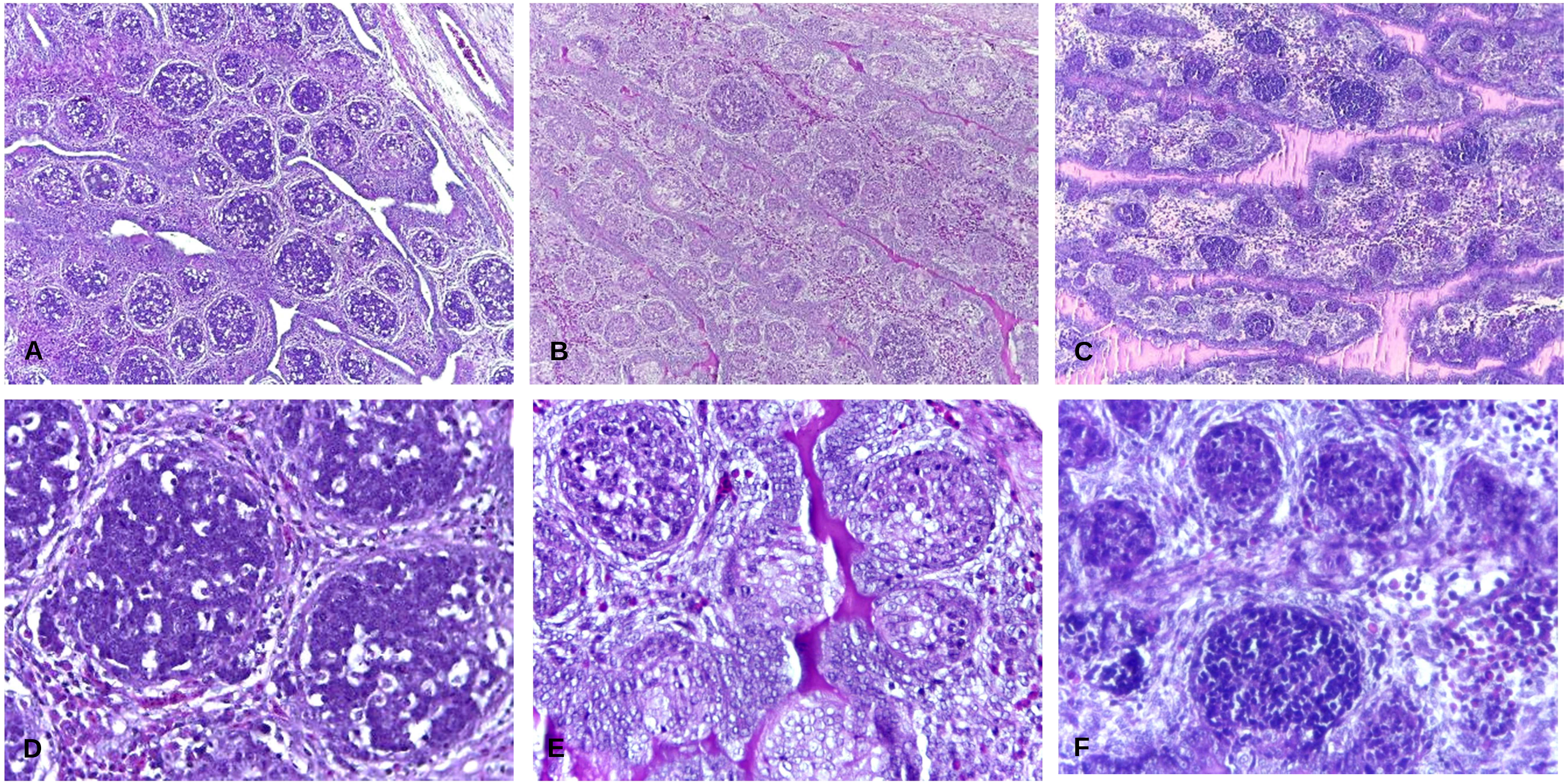


**Figure 53. Bursa of Fabricius at day 18 of incubation.**
**A) Control embryo (H&E, 100x); B) CHT embryo (H&E, 100x); C) CLT embryo (H&E, 100x); D) Higher magnification of a Control embryo. Note the consistent, lymphocyte-rich appearance of follicles (H&E, 400x); E) Higher magnification of a CHT embryo. Note the differences the scarce cellular population of the follicles (black stars) (H&E, 400x); F) CLT embryo, higher magnification. Note the small size of the follicles, which are nevertheless more cellular than in CHT embryos (H&E, 400x).**

*Embryonic viability*

### ***Malformation rates and types***

Malformation rates (Graph 16) were highest for embryos submitted to high incubation temperatures (2.6%). Among the malformations catalogued for this group were cases of gastroschisis (associated with ectopia cordis), plantigrady (Figure 49E), dwarfism, cutaneous vesicle formation (Figure 48G), exencephaly and unhealed navels (failure in the closure of the umbilical ring) (Figure 49I).

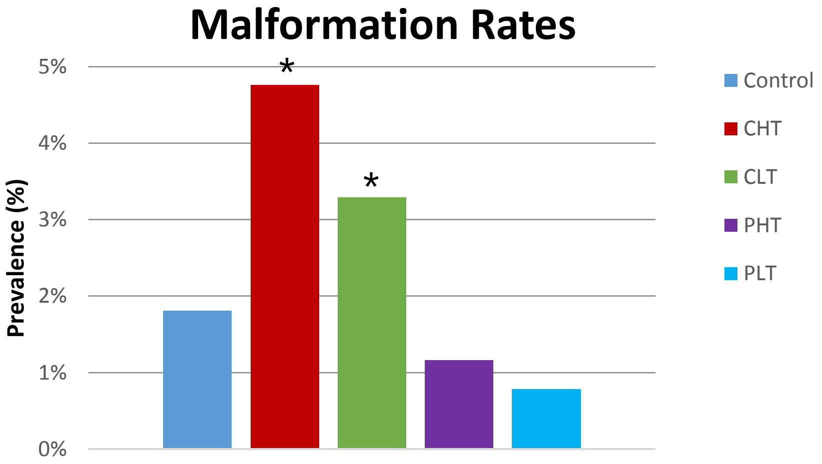
Malformations identified in embryos challenged with treatment CLT (≈1.8%) consisted of cystic embryos, gastroschisis and ectopia cordis, anencephaly (absence of a cranial vault associated with degeneration and necrosis of brain tissue) (Figure 48D), anophthalmia (absence of one or both eyes) (Figure 49C), maxillary hypoplasia and polymelia (presence of extra limbs) (Graph 17).

Prevalence of malformations was only significantly higher for treatments CHT and CLT ($p$<0.05).

The pinpoint rises in temperature in treatment PHT were associated with cases of exencephaly, gastroschisis, anophthalmia, microphthalmia (Figure 48F) and duplication of the left eye, affecting approximately 1.2% of all embryos.

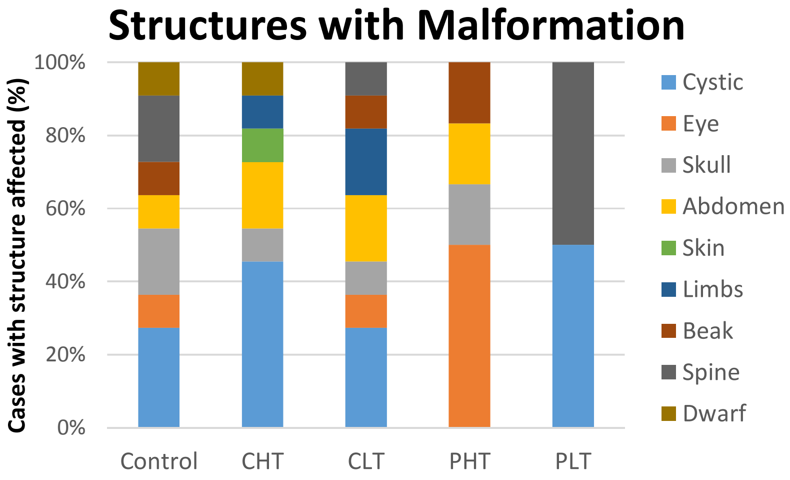
Treatment PLT was linked to the occurrence of cystic embryos and a single other example of malformation (cephalopagus) (≈0.4%).

Compound malformations were found in treatments CLT and PHT, where they represented, respectively 25% and 33% of all malformations found in each treatment.



***($p$<0.05)**

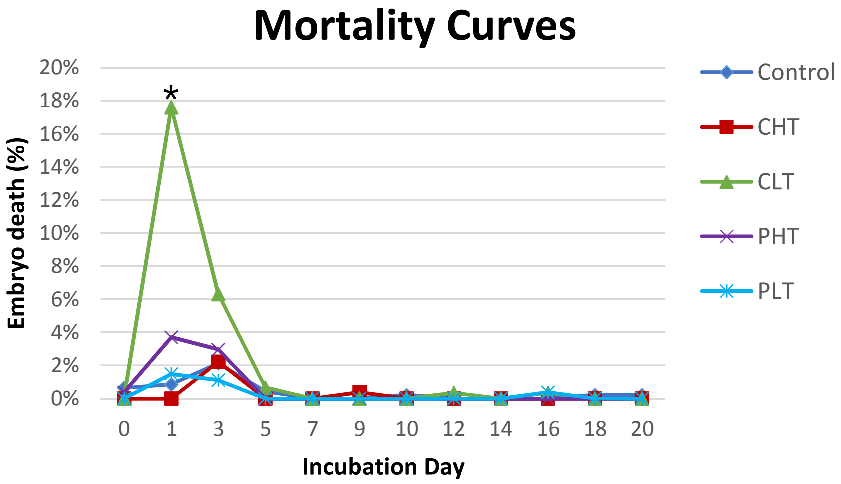
**Graph 16. Prevalence of malformed embryos (percentage) for control and temperature-challenged eggs.**



**Graph 17. Organs affected by the malformations found in control and temperature-challenged embryos.**

### *Mortality rates*

Incubation under continuously low temperatures caused the highest mortality rates of all temperature-related treatments (p<0.05), with embryonic death occurring between days 1 and 3 of incubation and at day 5. CHT embryos mortality rates (Graph 18) peaked at day 2 of incubation, between days 3 and 4 and between incubation days 13 and 17 (not statistically significant). Mortality rates for embryos challenged with sudden rises in temperature were highest during the first four days of incubation, while embryos challenged with sudden drops in temperature displayed a mortality curve very similar to that of the control eggs (not statistically significant).



***(*p*<0.05)**

**Graph 18. Distribution of mortality incidence (percentage) during the incubation period for control and temperature-challenged eggs.**

## HUMIDITY-RELATED TRIALS

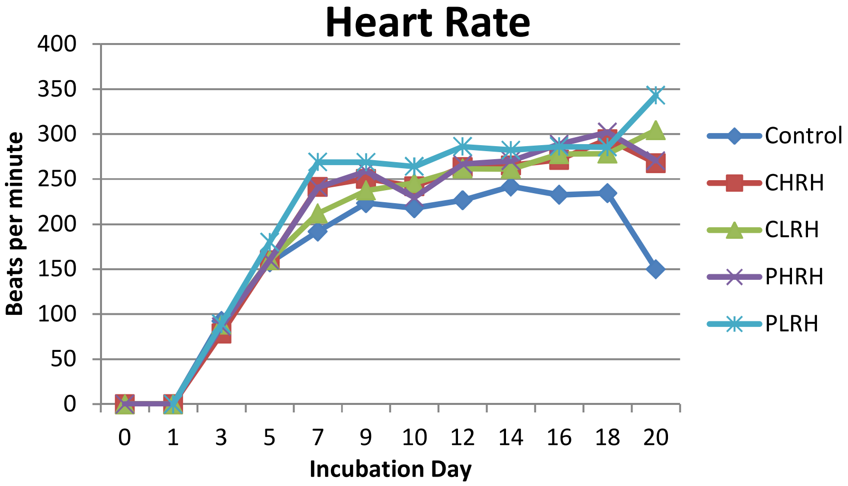
A total of 570 trial embryos were analyzed, described and catalogued, and later compared against 442 control embryos. During the four separate trials, nearly 11,000 images were taken, over 4200 histology slides produced, and compared against 10,000 images and 2600 control-embryo histology slides. For effects of simplifying reading, all statistical data has been compiled and is present in Annex 4.

#### Ante-mortem observations

*Behavior*

***Heart rate***

Heart rate was consistently higher than control values for CHRH and CLRH groups, particularly from day 5 onward (Graph 19). PHRH embryos showed HR values that were consistently higher than control embryos and similar to CHRH embryos. PLRH embryos exhibited the highest HR values of all challenged groups, consistently above control embryo HR from day 5 until the end of incubation.

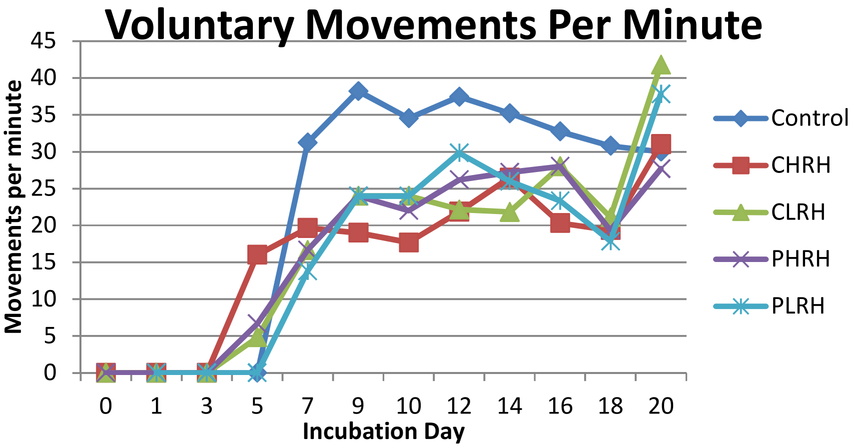


**Graph 19. Heart rate (beats/minute) throughout incubation for control and humidity-challenged eggs.**

***Voluntary movements per minute (VMM)***

Treatments CHRH, CLRH and PHRH embryos exhibited early onset of voluntary movement (on day 5 instead of the usual day 6-7), albeit consistently presenting VMM values lower than the embryos in the Control group, until a sudden rise was registered between days 18 and 20. PLRH embryos initiated voluntary movement by day 6-7. Similarly to the other challenged groups, VMM values for these embryos remained below

Control group VMMs, except for the last two days of incubation. The evolution in VMM throughout incubation is evident in Graph 20.



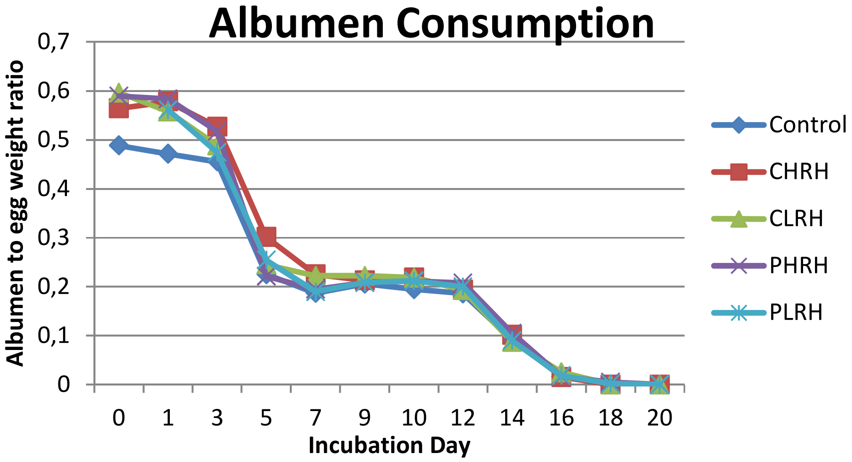
**Graph 20. Voluntary movement (movements/minute) throughout incubation for control and humidity-challenged eggs.**

**Post-mortem observations**

*Nutrient reserves usage*

***Albumen consumption (AR)***

Albumen to egg-weight ratio values for Treatment CHRH eggs remained above those of the control eggs until day 14, as shown in Graph 21. AR values for Treatment CLRH, PHRH and PLRH eggs were initially higher than control AR values but then plunged at day 3 until reaching values similar to the latter, which they followed closely as incubation progressed.

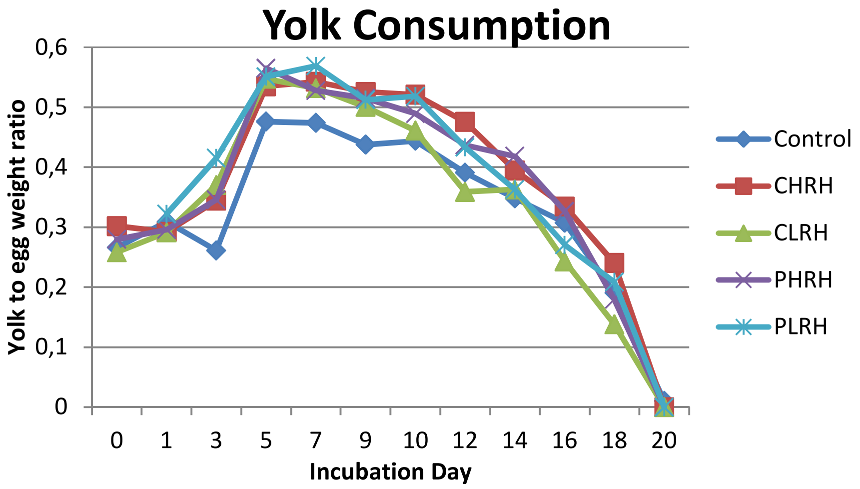


**Graph 21. Albumen consumption (albumen weight to egg weight ratio) throughout incubation for control and humidity-challenged eggs.**

### *Yolk consumption*

Yolk to egg-weight ratio values for Treatment CHRH remained above Control YR values throughout incubation. Treatment CLRH eggs exhibited YR values that were consistently higher than Control YR values, except for the last days of incubation (days 18-20), when YR values were significantly lower than those registered for Control eggs.

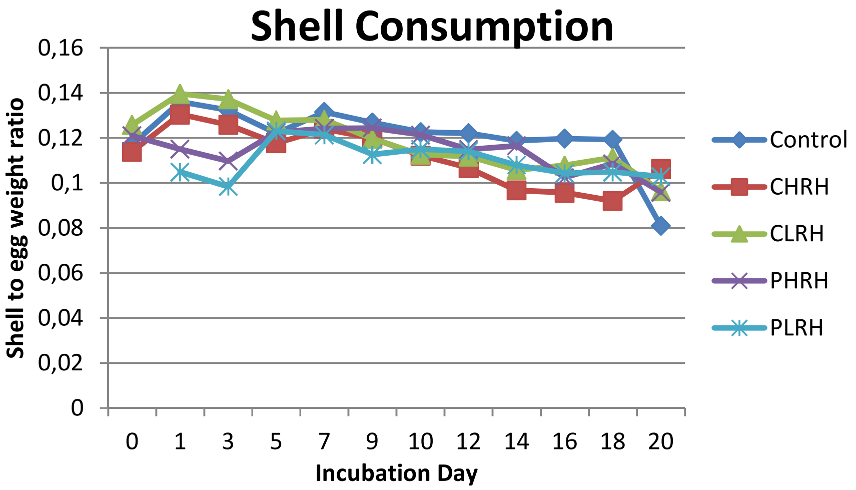
Graph 22 shows that YR values for Treatment CHRH eggs were consistently higher than those for control eggs between days 5 and 16. Treatment CLRH eggs followed this same trend until day 10. PHRH eggs followed a trend very similar to CHRH eggs. Regarding PLRH eggs, YR closely followed that of CHRH eggs until day 10 of incubation, at which point YR values dropped more sharply, reaching values slightly below control egg YR at day 16. From day 18 onward, PLRH eggs followed a similar trend and presented similar values to control eggs.



**Graph 22. Yolk consumption (yolk weight to egg weight ratio) throughout incubation for control and humidity-challenged eggs.**

### *Shell consumption*

Shell to egg-weight ratios very closely followed those observed for control eggs throughout the whole of the incubation process, without statistically significant deviance from the control SR (Graph 23).

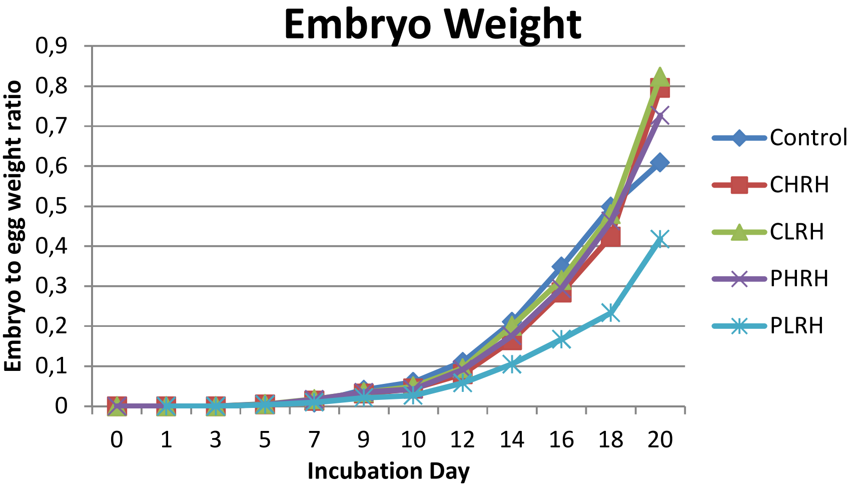


**Graph 23. Shell consumption (shell weight to egg weight ratio) throughout incubation for control and humidity-challenged eggs.**

*Embryonic development*

***Embryo weight gain (ER)***

Graph 24 shows that humidity-related treatments had limited effects on ER until late in incubation (day 20), when embryos suddenly exhibited higher ER values than control eggs. PLRH embryos were an exception, maintaining consistently lower ER values from day 14 until the end of incubation.



**Graph 24. Embryo growth (embryo weight to egg weight ratio) throughout incubation for control and humidity-challenged eggs.**

***Morphological indicators of embryo growth***

Most of the morphometric indicators measured showed no statistically significant changes when compared to controls. These were namely Embryo Length (Graph 25), Wing Length

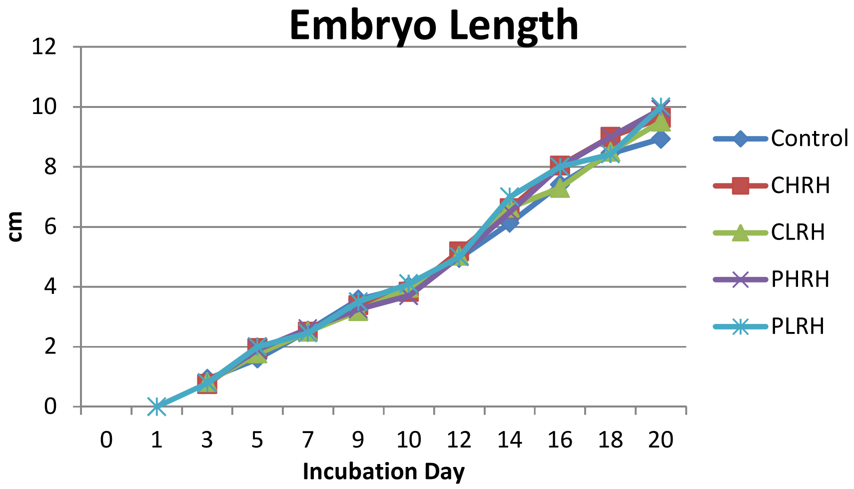
(Graph 26), Toe Length (Graph 32), Feather Length (Graph 31) and Eye Diameter (Graph 30).

Leg Length (Graph 27) mostly followed that of control embryos for all challenged groups except for a single time point at day 16, where embryos subject to treatments CHRH, CLRH and PHRH had higher LL values than control embryos.

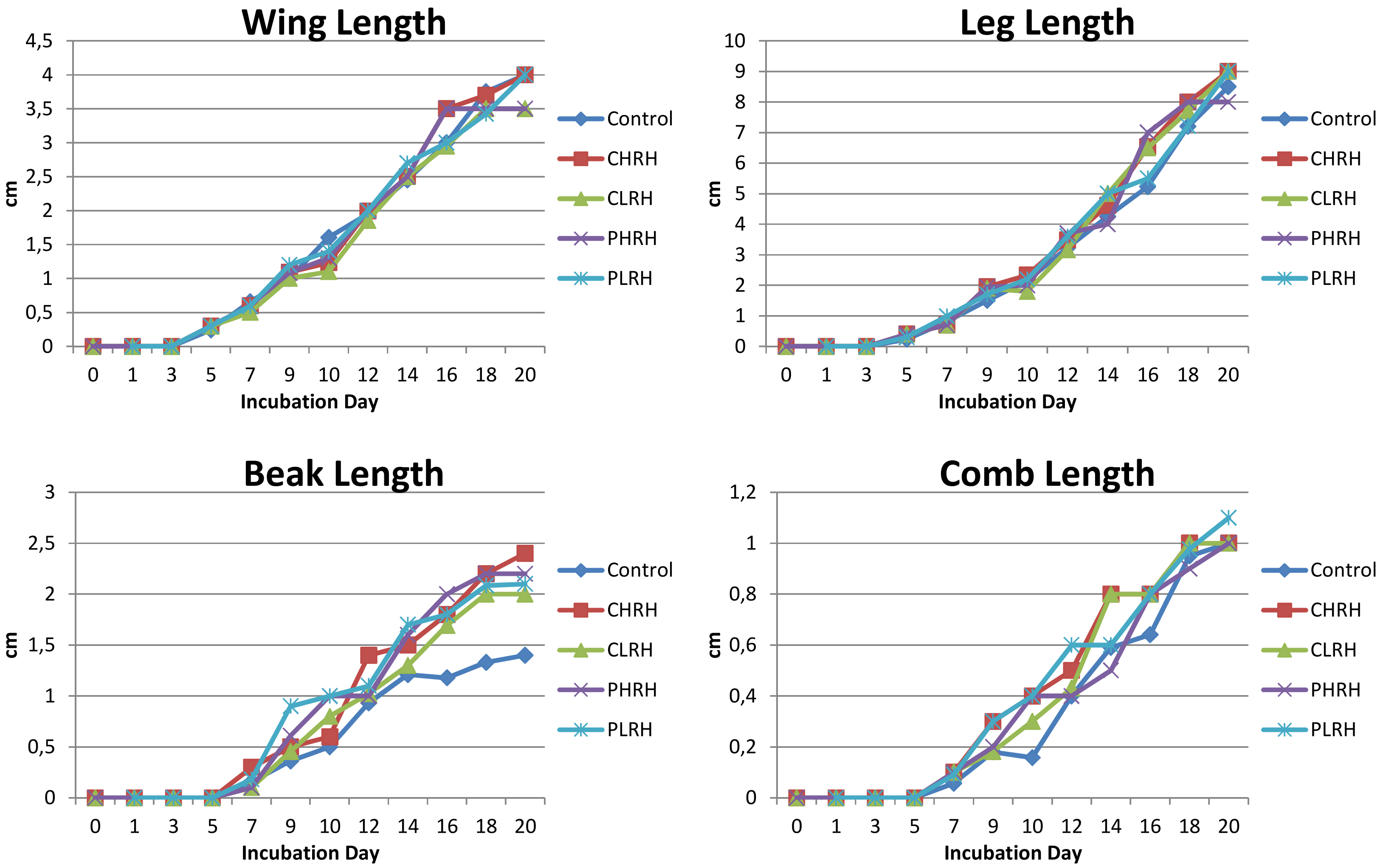
Beak Length presented with measurements consistently higher than controls for all treatments, with final values rather similar between treatments by the end of incubation, with departure from control values occurring at day 9 for CHRH embryos, at day 12 for PHRH and PLRH embryos and at day 16 for CLRH embryos, as seen on Graph 28.

Comb Length did not vary significantly between treatment and control embryos, except for one time point at day 10 (all treatments) and one at day 14 (CHRH and CLRH embryos), where CL values were significantly higher than those registered for control (Graph 29).

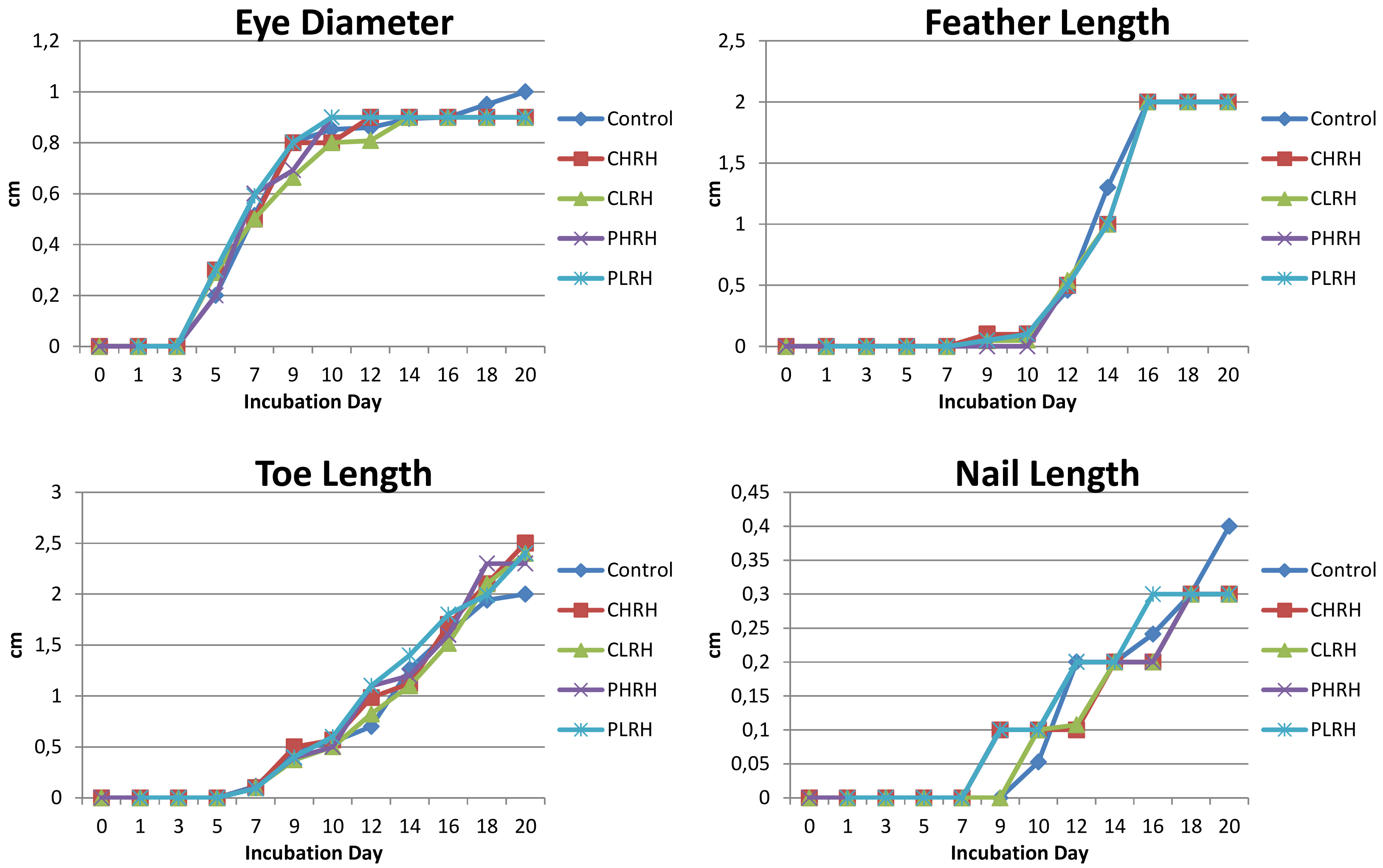
Nail growth presented with variable growth patterns between challenged and control groups. CHRH, PHRH and PLRH embryos exhibited nail formation by day 9, one day before control groups (Graph 33). Onset of nail growth/differentiation for CLRH embryos was similar to controls. However, nail growth stagnated for both CHRH and CLRH embryos until day 12, with values below control embryo NL. Nail growth was fastest for PLRH embryos, which reached final NL by day 16. All the challenged groups reached a final NL lower than control embryos.



**Graph 25. Embryo growth (embryo length) throughout incubation for control and humidity-challenged eggs.**



**Graphs 26 - 29. Embryo growth throughout incubation for control and humidity-challenged eggs.**
**Upper Left – Wing Length; Upper Right – Leg Length; Lower Left – Beak Length; Lower Right – Comb Length**



**Graphs 30 - 33. Embryo growth throughout incubation for control and humidity-challenged eggs.**
**Upper Left – Eye Diameter; Upper Right – Feather Length; Lower Left – Toe Length; Lower Right – Nail Length**

### *Histological analysis*

Histological analysis revealed limited effects of humidity-related challenges on embryonic development. These were only found in the liver of CHRH embryos between day 7 and day 10, and consisted of a lower density in hepatic plates, with wider sinusoids and lower hepatocyte numbers (Figure 54). After this period, CHRH embryos showed no significant morphological differences from control embryo livers.

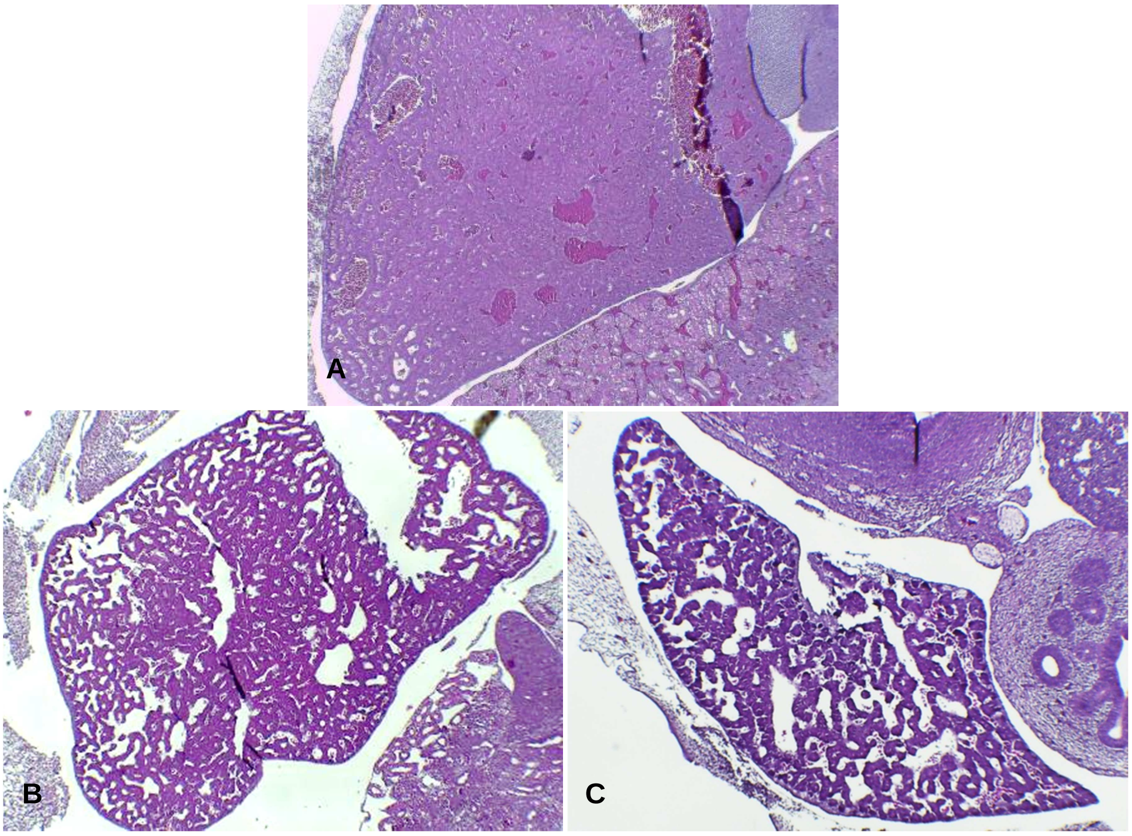


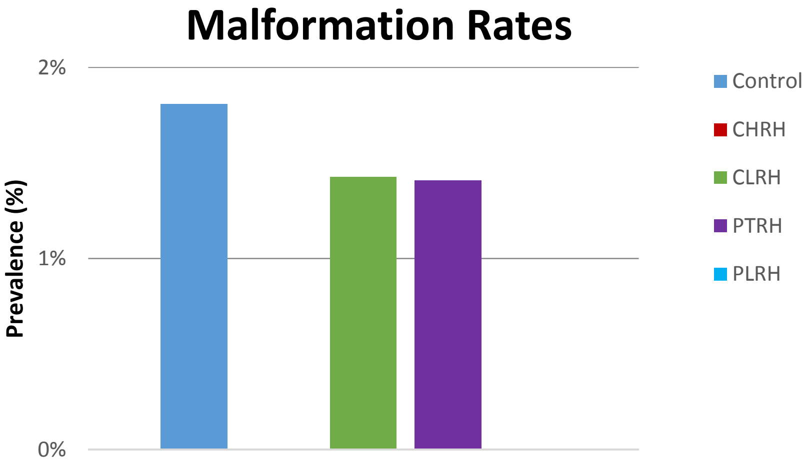
**Figure 54. Microscopy images of liver at day 7.**
**A) Control embryo (H&E, 40x); B) CHRH embryo (H&E, 40x); C) CLRH embryo (H&E, 40x). Note the comparatively less cellular appearance of the livers in B and C, causing a false image of sinusoid distension.**

*Embryonic viability*

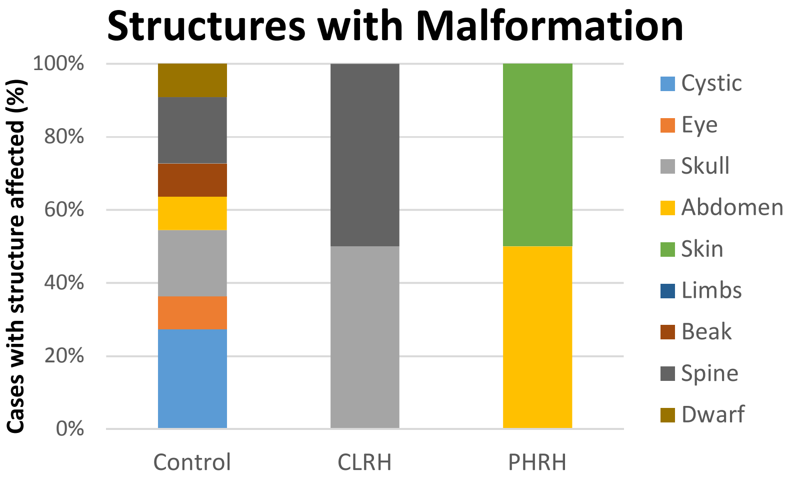
***Malformation rates and types***

No malformations were observed in embryos challenged with Treatments CHRH or PLRH. Prevalence of malformations was similar for treatments CLRH and PHRH (≈1.4%) and not statistically different from Controls (Graph 34).

Malformations present in embryos subjected to Treatment CLRH included head and skull malformations (exencephaly) and posterior twinning (cephalopagus). Malformations found in Treatment PHRH embryos were found to involve the skin and subcutaneous tissue (anasarca) and the abdominal wall (gastroschisis) (Graph 35).



**Graph 34. Prevalence of malformed embryos (percentage) for control and relative humidity-challenged eggs.**

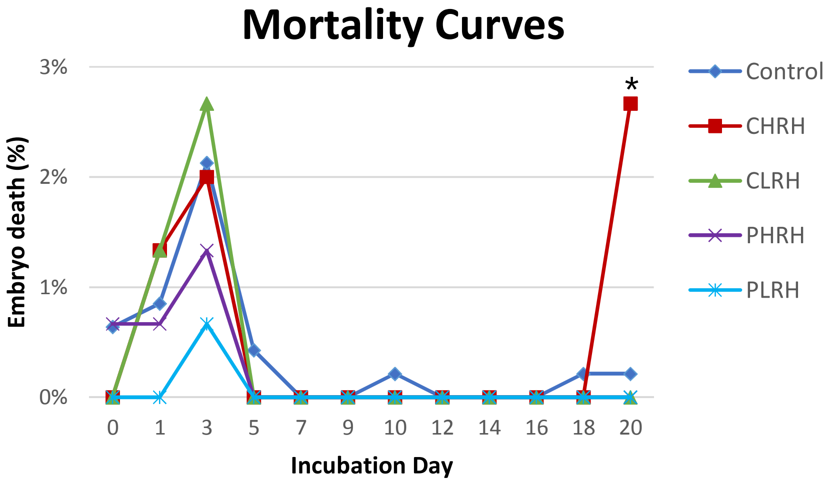


**Graph 35. Organs affected by the malformations found in control and humidity-challenged embryos.**

***Mortality rates***

Mortality rates were higher during the first 4 days (not statistically significant) and on the last day of incubation ($p<0.05$) for CHRH embryos (Graph 36). Embryos incubated under Treatment CLRH exhibited greater mortality rates during the first 4 days of incubation (not statistically significant).

Mortality for embryos subject to pinpoint rises in relative humidity was only observed during the first five days of incubation, with a clear peak at day 4 (not statistically significant). PLRH embryos only registered mortality on days 3 and 4 of incubation with a clear peak at day 3 (not statistically significant).



***(p<0.05)**

**Graph 36. Distribution of mortality incidence (percentage) during the incubation period for control and humidity-challenged eggs.**

## CARBON DIOXIDE-RELATED TRIAL

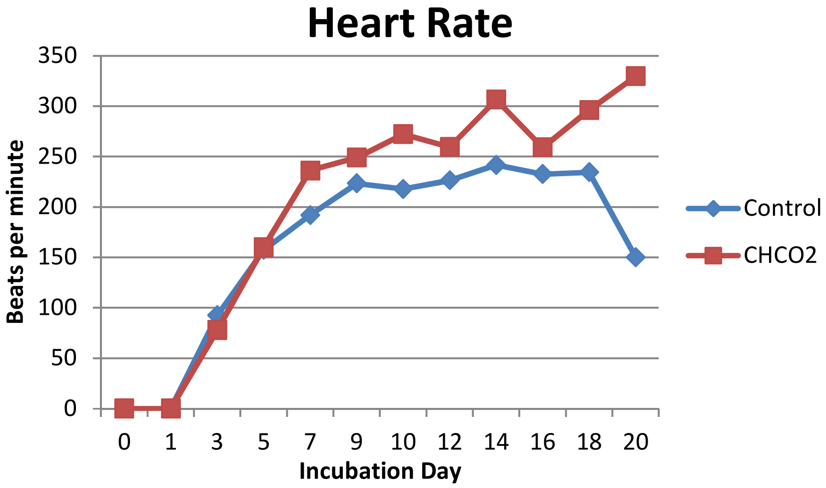
A total of 141 trial embryos were analyzed, described and catalogued, and later compared against 442 control embryos. During the four separate trials, over 3000 images were taken, over 1000 histology slides produced, and compared against 10.000 images and 2600 control-embryo histology slides. For effects of simplifying reading, all statistical data has been compiled and is present in Annex 4.

#### Ante-mortem observations

*Behavior*

***Heart rate***

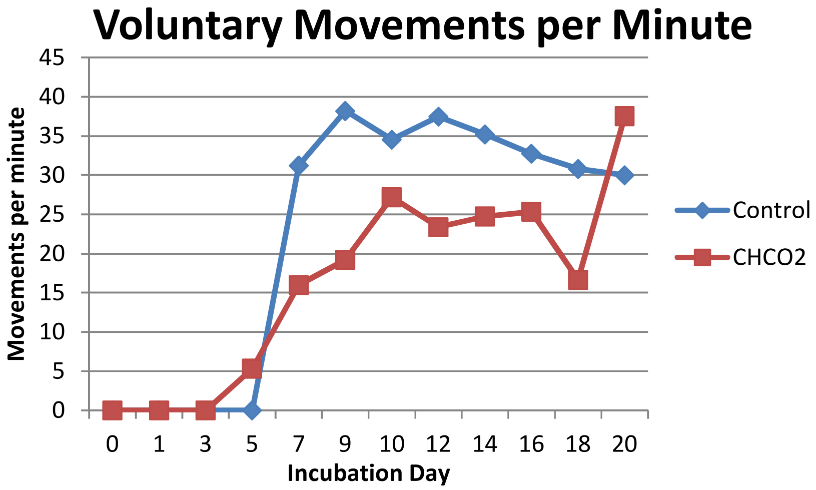
Heart rate was consistently higher than control values, particularly from day 5 onward and until the end of incubation (Graph 37).



**Graph 37. Heart rate (beats/minute) throughout incubation for control and carbon dioxide-challenged eggs.**

***Voluntary movements per minute***

Graph 38 shows that challenged embryos exhibited early onset of voluntary movement (on day 5 instead of the usual day 6-7). However, VMM values for CHCO2 embryos remained consistently below control VMM values until day 20, when challenged embryos exhibited a sudden rise in VMM, above control values.



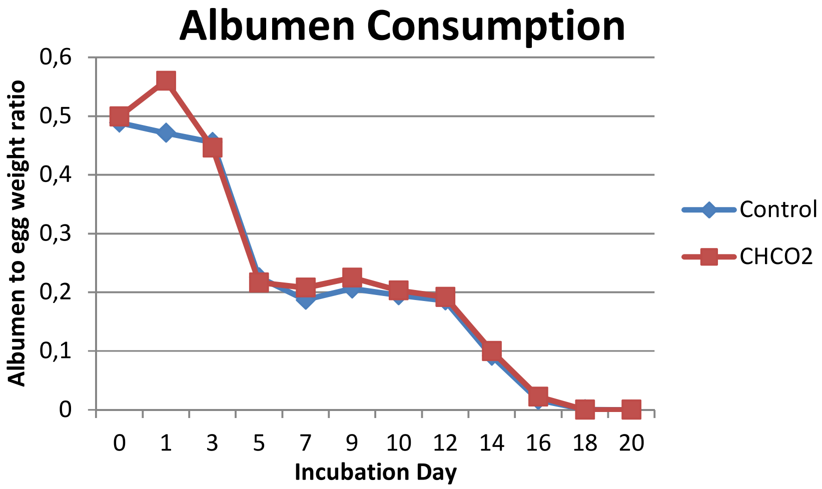
**Graph 38. Voluntary movement (movements/minute) throughout incubation for control and carbon dioxide-challenged eggs.**

**Post-mortem observations**

*Nutrient reserves usage*

***Albumen consumption***

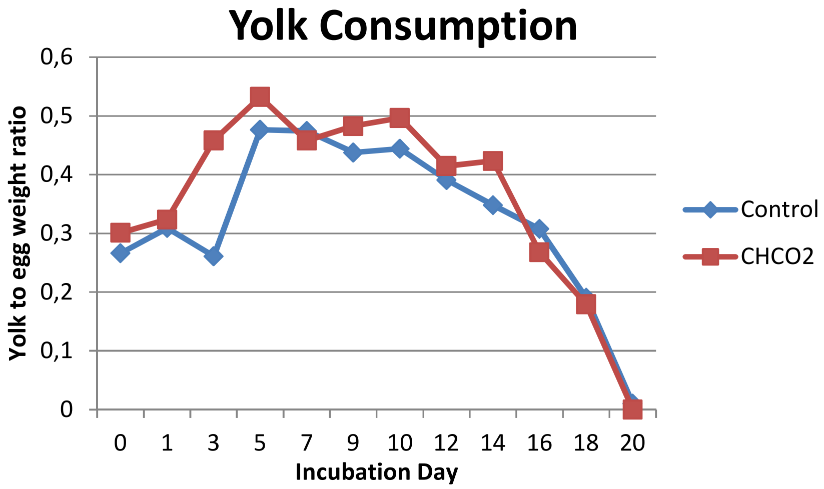
No statistically significant differences were found in albumen consumption for embryos challenged with constant high carbon dioxide levels except for a sudden rise in AR at day 1 (Graph 39).



**Graph 39. Albumen consumption (albumen weight to egg weight ratio) throughout incubation for control and carbon dioxide-challenged eggs.**

### *Yolk consumption*

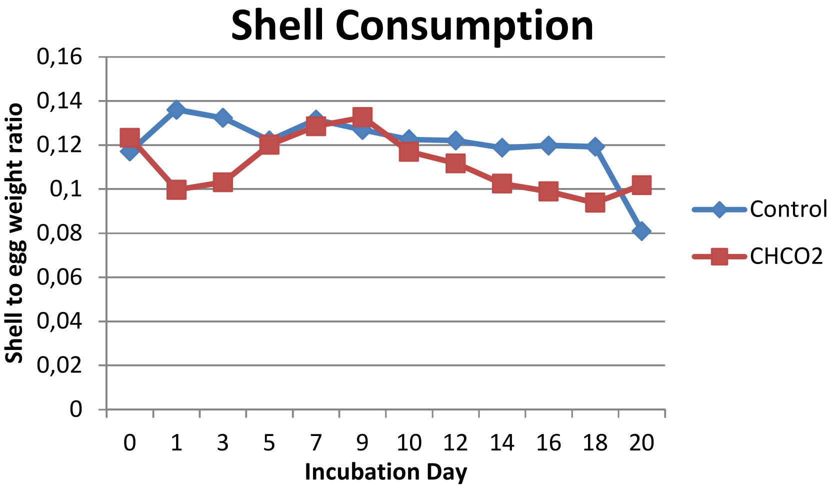
No statistically significant differences were found in yolk consumption for embryos challenged with constant high carbon dioxide levels except for the time period between days 1 and 5, when YR values registered above control YR values (Graph 40).



**Graph 40. Yolk consumption (yolk weight to egg weight ratio) throughout incubation for control and carbon dioxide-challenged eggs.**

### *Shell consumption*

Values of SR for challenged embryos differed from those of control eggs, dropping more abruptly than the former between day 10 and day 18 (Graph 41). At day 20 of incubation, SR values registered for CHCO2 embryos seemed to rise, but with no statistical significance.

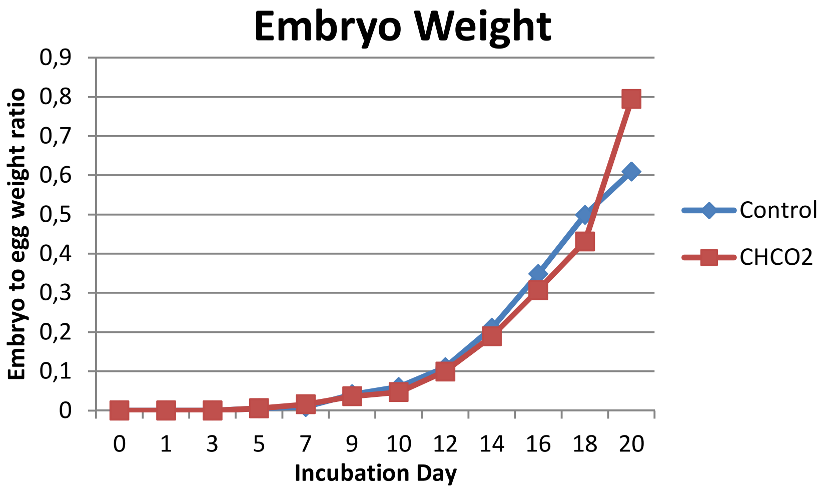


**Graph 41. Shell consumption (shell weight to egg weight ratio) throughout incubation for control and carbon dioxide-challenged eggs.**

*Embryonic development*

***Embryo weight gain***

Manipulating $CO_2$ concentration levels had limited effects on ER, as shown in Graph 42. The only differences registered between CHCO2 and control embryos occurred at day 20 incubation, with the former exhibiting higher ER values when compared to the latter.



**Graph 42. Embryo growth (embryo weight to egg weight ratio) throughout incubation for control and carbon dioxide-challenged eggs.**

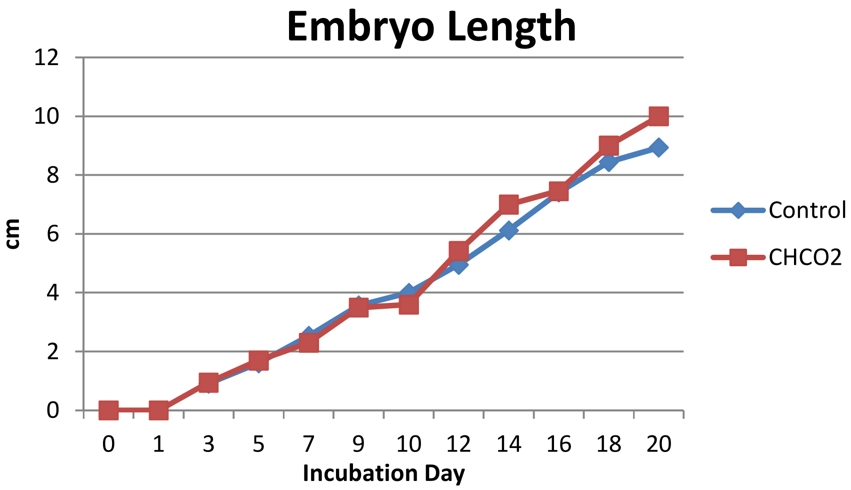
***Morphological indicators of embryo growth***

Most of the morphometric indicators measured showed no statistically significant changes when compared to controls. These were namely Embryo length (Graph 43), Wing length (Graph 44), Leg length (Graph 45), Comb length (Graph 47), Feather Length (Graph 49) and Eye Diameter (Graph 48).

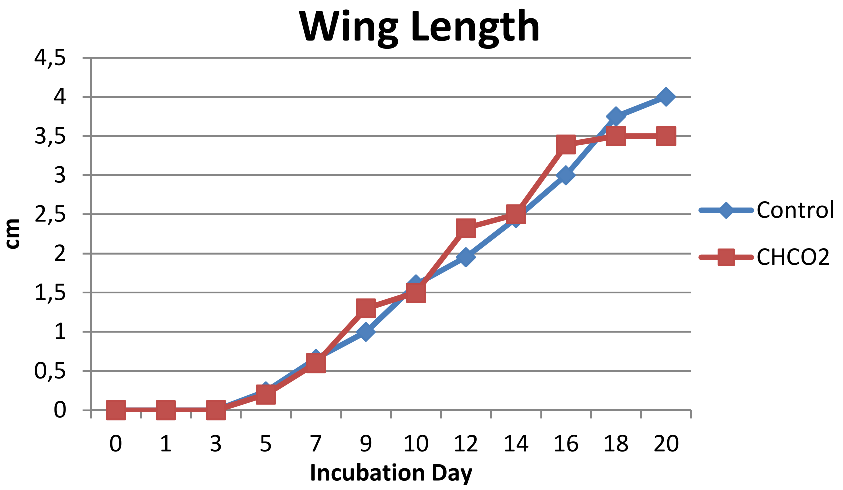
Beak Length presented with measurements consistently higher than controls, with departure from control values occurring at day 9, as is visible in Graph 46.

Toe Length was mostly unaffected, except for a period between days 10 and 12, when CHCO2 embryos registered higher toe lengths than the control group embryos (Graph 50). This difference disappeared by day 16 of incubation and was non-significant by day 20.

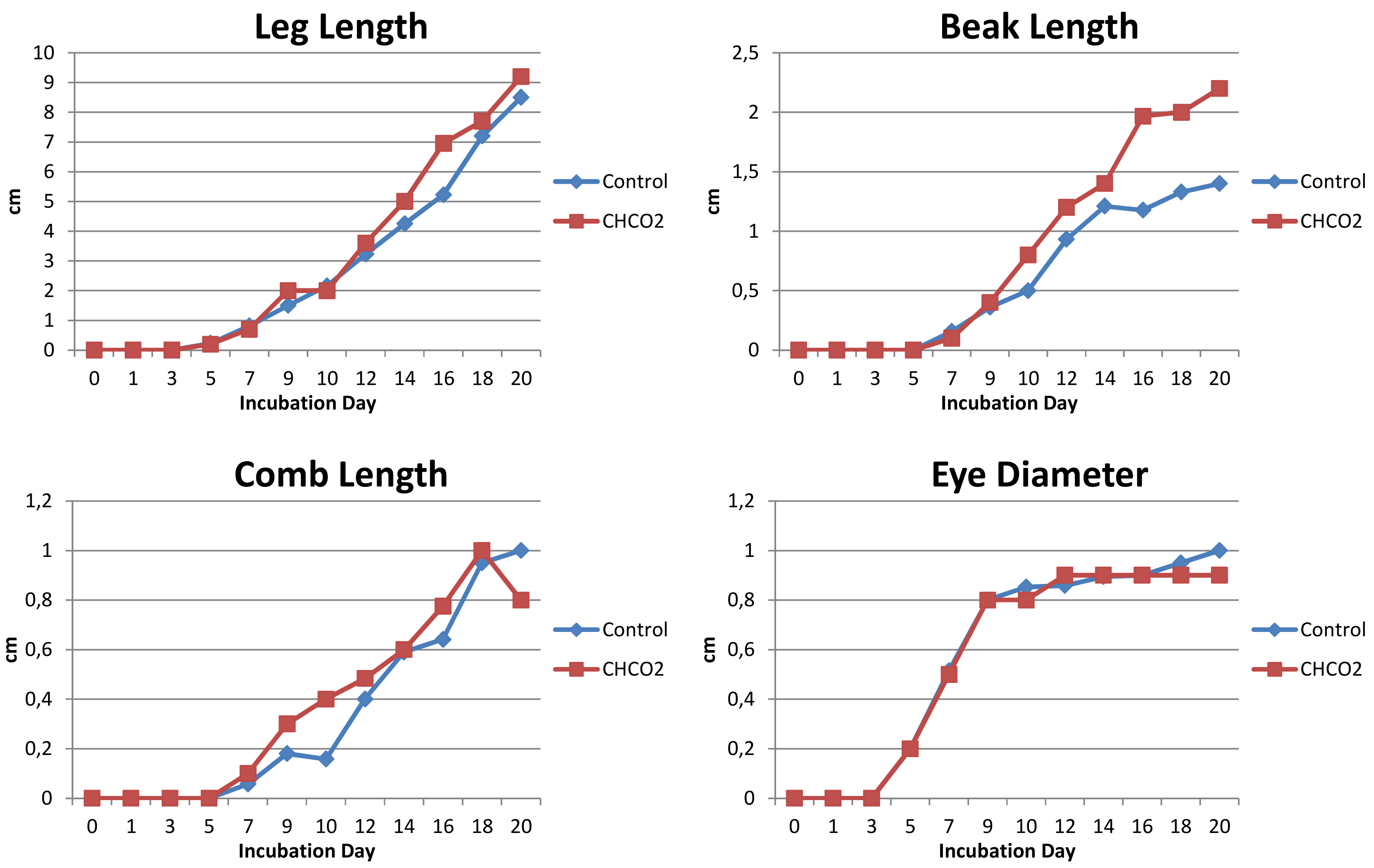
Although CHCO2 embryos exhibited nail formation early, by day 9, the speed of nail growth was slower until day 14. Final NL was also lower when compared to control embryos (Graph 51).



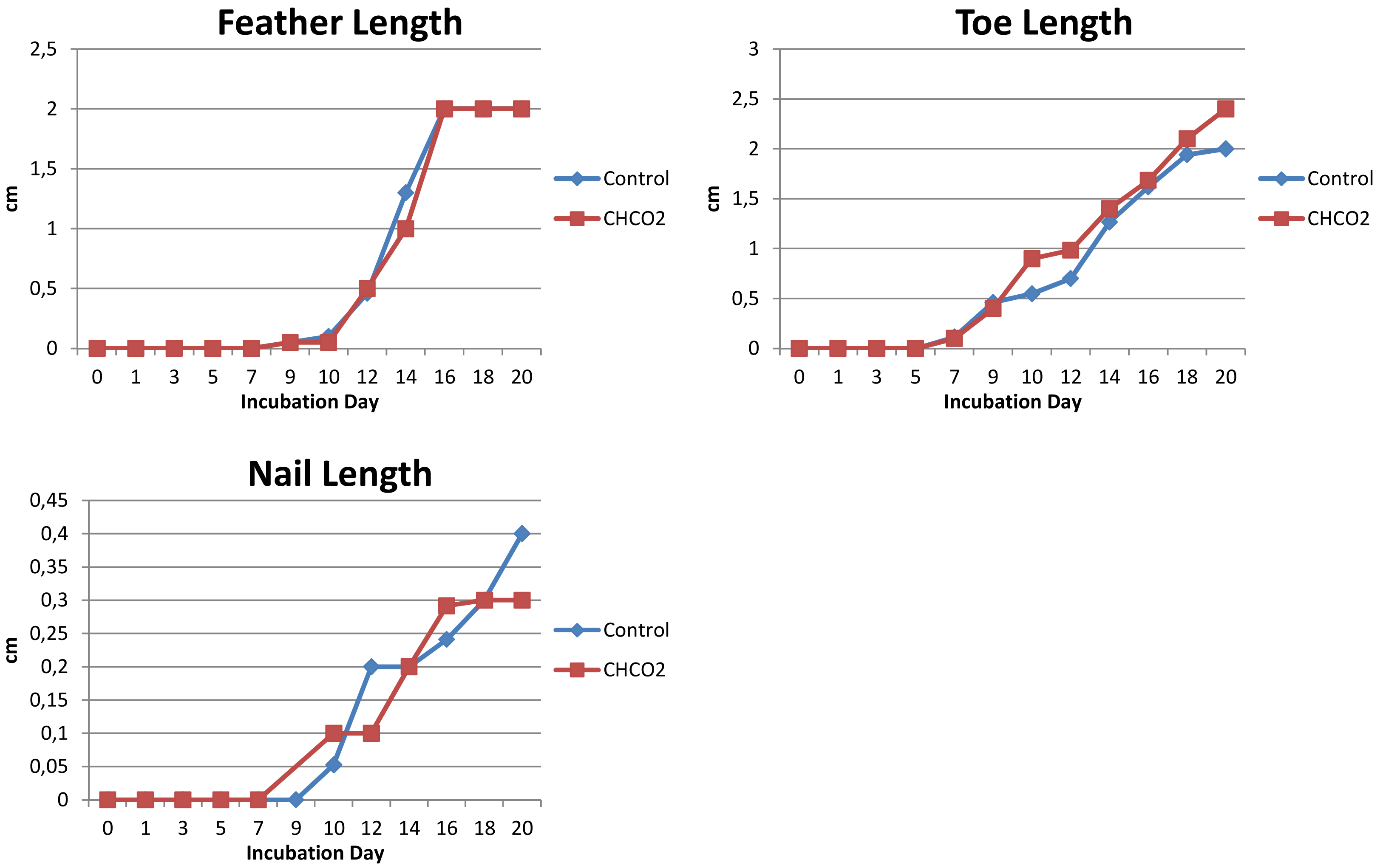
**Graph 43. Embryo growth (embryo length) throughout incubation for control and carbon dioxide-challenged eggs.**



**Graph 44. Embryo growth (wing length) throughout incubation for control and carbon dioxide-challenged eggs.**



**Graphs 45 - 48. Embryo growth throughout incubation for control and carbon dioxide-challenged eggs.**
**Upper Left – Leg Length; Upper Right – Beak Length; Lower Left – Comb Length; Lower Right – Eye Diameter.**



**Graphs 49 - 51. Embryo growth throughout incubation for control and carbon dioxide-challenged eggs. Upper Left – Feather Length; Upper Right – Toe Length; Lower Left – Nail Length.**

***Histological analysis***

No significant lesions or differences were found in all organs and tissues analyzed between challenged and control embryos.

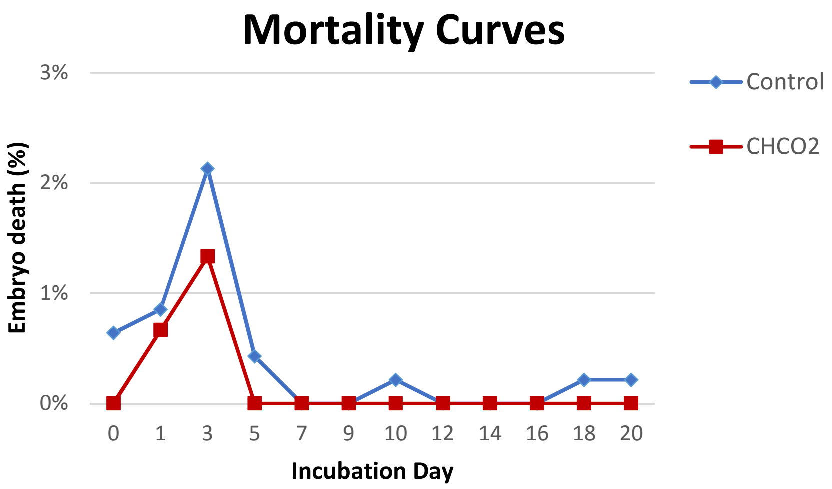
*Embryonic viability*

***Malformation rates and types***

No malformations were recorded for CHCO2 embryos.

***Mortality rates***

Mortality was not statistically difference from controls, no greater than 2% for CHCO2 embryos and restricted to the first 3 days of incubation, as shown in Graph 52.



**Graph 52. Distribution of mortality incidence (percentage) during the incubation period for control and carbon dioxide-challenged eggs.**

# DISCUSSION

It is not by mere chance that birds, along with many other vertebrates, have relied for so long on the production of eggs for their successful propagation to all of Earth's continents (Tian et al., 2010). As discussed before, the avian egg contains all the raw materials necessary for the formation of the embryo, apart from oxygen (Bellairs & Osmond, 2005; Needham, 1926a, 1926b, 1926c; Joseph Needham, 1927a, 1927b; J. Needham, 1927). Not only that but its shape and structure provide a sturdy defense against mechanical shock, dehydration and microbiological contamination (Chien et al., 2009; Hunton, 2005; Jonchere et al., 2010). In sum, the egg encloses a safe environment in which the embryo can develop at its species' own more-or-less narrowly regulated rhythm, protected from the adversities of the environment outside the shell and free from most vulnerabilities (Tian et al., 2010; Yalcin & Siegel, 2003). In the confinements of its egg, the embryo will have its development dictated primarily by its genetic programming, influenced by a relatively limited number of external factors (Tian et al., 2010).

The most important of these are, undoubtedly, related to the physical environment in which incubation takes place, with three major key factors already identified: temperature, relative humidity and carbon dioxide concentration (Bruzual et al., 2000a; Lourens, 2008; Mortola, 2009).

Before moving into a more detailed discussion of the various findings, it is important to note that, regardless of the variable in question, pinpoint treatments always seemed to produce limited, temporary effects on embryonic development that were simply compensated and erased before growth is completed and hatching takes place. On the contrary, continuous treatments generally produce more visible and long lasting effects. Consequently, discussion will mainly focus on the latter as they provide the most valuable insights in this study.

## Embryonic behavior

Heart rate and embryonic movement (here expressed as movements per minute) were used as behavioral markers, as studies have shown their usefulness in assessing embryonic distress in other species, humans included (Chung et al., 2001; DiPietro, Costigan, & Gurewitsch, 2003). Studies available in other species, as well as in the chicken, state that embryos respond to stressful conditions with an increase in heart rate - as is typical of the fright, flight or fight reaction for adults - as well as with a decrease in movement, in an effort to conserve oxygen and energy (Chung et al., 2001; DiPietro et al., 2003; M. Nechaeva, Vladimirova, & Alexeeva, 2010; M. V. Nechaeva, 2011).

In chicken embryos, heart rate is seldom studied throughout the whole of the incubation process and embryonic movement even less so. Studies available about these two parameters are generally connected to embryonic resistance and adaptation to hypoxia or hypothermia (Mortola & Labbe, 2005; Mortola, Marinescu, Pierre, & Artman, 2012; Mortola, Wills, Trippenbach, & Al Awam, 2010; M. Nechaeva et al., 2010). These studies indicate that embryos respond to the lack of oxygen or heat (the energy that the embryo so desperately requires for metabolism) by lowering their heart rate and movement in an effort to preserve their reserves until incubation conditions improve (Andrewartha et al., 2011; Mortola et al., 2012). A single study points to a biphasic response by avian embryos to hyperthermia, with early increase in heart rate during the ectothermic period - in which the embryo is completely dependent on external heat sources - and late decrease in heart rate as the embryo progresses further and further into homeothermy (Piestun et al., 2009).

All treatments applied in this study resulted in elevated heart rate and decreased embryo movement. However, when analyzing how heart rates and voluntary movements vary throughout incubation in the different trials, no obvious differential patterns can be discerned to characterize the specific response to each manipulated variable. Even the early onset of voluntary movement at day 5 of incubation, observed for CHRH, CLRH, PHRH and CHCO2 embryos, cannot be safely interpreted as a significant change. Tong et al (2013) have reported it to be a normal variation, found in healthy embryos not subjected to any type of stressor, meaning that it may be of no particular significance or consequence for the results of this study. Thus, where HR and VMM are concerned, the embryonic responses registered seem to follow the trend observed for other species. The infrequent, fragmentary data found in other studies about chicken embryos is not enough to establish a definitive agreement or disagreement with this theory. Interpreted alone, the results suggest a non-specific response to distress and to a less than ideal incubation environment. This observation merits further study, particularly focused on the utilization of heart rate as an indicator of distress in sample embryos in industrial incubators, as is already done with temperature and shock detectors placed in the trays and connected to mobile devices. Unlike the complex apparatuses or laboratory preparations used to detect heart beat in other studies (of which only three constitute an exception in poultry science) (Lierz, Gooss, & Hafez, 2006; Piestun et al., 2009; Shinder et al., 2011), the Buddy™ cardiac monitor has proved easy to use and, if adapted, potentially applicable to a higher number of eggs in non-laboratorial environments. It may provide the necessary clues to the making of a future, industrial model that would allow for a closer monitoring of embryonic distress.

## Embryonic metabolism

The albumen, yolk and, to a much smaller and narrower extent, the eggshell are the only three sources of nutrients available to the avian embryo throughout its development (Bellairs & Osmond, 2005; Patten, 1971). Without access to any other reserves for its nourishment than those provided by the egg and its contents, the embryo has to incorporate and metabolize the organic and inorganic components of the egg and its shell for its own development and growth (Lourens et al., 2011; Needham, 1926b, 1926c; Joseph Needham, 1927a; J. Needham, 1927). It is for this reason that for the purposes of this study AR, YR and SR were used as monitors of nutrient consumption during embryonic development.

The most significant changes found regarding the rate of embryonic consumption of albumen had to do with relatively consistently raised AR values, in comparison to control embryos. Although some of trial groups had a higher day 1 AR to start with, when compared with control embryos, this disparity was always resolved during the first days of incubation (generally at day 4-5). From that point on, the most significant changes in AR values observed involved delay and reduction in the expected drop in AR, with consistently higher values (and therefore consistently lower albumen consumption) for embryos challenged with temperature (and especially with higher temperatures) and relative humidity treatments. CHCO2 embryos appear to be unaffected in their metabolism of albumen by the challenge conditions.

Similarly to albumen, yolk consumption rates have shown a general trend of lowered metabolism for temperature and RH-challenged embryos. Again, CHCO2 embryos did not show significant changes.

As a side note, a sudden rise in YR can be seen in all groups (control included) between days 3 and 5 of incubation. This may seem unexpected at first, as the embryo has no ability to produce yolk and the egg has no source from which to acquire it. During early embryonic metabolism, water, which constitutes 88.5% of total albumen weight, is displaced from the albumen into the yolk (Bellairs & Osmond, 2005; K. Mann, 2007). This is due, among other reasons, to changes in pH caused by embryonic metabolism of albumen-derived glucose, as well as to the growth of semi-permeable extra-embryonic membranes, that promote osmotic changes and lead to the accumulation of water in the yolk just below the embryo and embryo-related tissues (Babiker & Baggott, 1995; Bellairs & Osmond, 2005; D. C. Deeming, 1989; Pellet-Many & Baggott, 2005). The early rise in YR was, therefore, attributed to this migration of water from the albumen to the yolk.

Shell to egg weight ratios were seemingly unaffected by temperature or RH manipulation. In spite of differing from control, SR values obtained for CHCO2 embryos appear erratic throughout incubation and do not allow for the establishment of an obvious pattern. For this reason, no further considerations were made regarding shell to egg ratios and they were discarded as a significant marker for changes in embryonic metabolism.

Thus, considering the dynamics of embryonic nutrient consumption and the composition of both albumen and yolk, the differences observed between control (lower) and challenge (higher) AR and YR values can be interpreted in combination, indicating lowered embryonic metabolism in challenged embryos, in response to the stress caused by changes in incubation temperature or relative humidity. As previously observed for behavioral markers, no specific patterns arise to make possible the distinction between responses to specific stressors, again indicating a generalist response to a set of given stressors, in this case temperature and humidity. As thermal or hydric stress is registered, the embryo responds by lowering its metabolism (and henceforth its consumption of both yolk and albumen) in an attempt to compensate for the limiting and harsh environmental conditions. Similar observations have been communicated by Uni et al. (2014) and supported by other published works (Hulet et al., 2007; Leksrisompong et al., 2007; Molenaar et al., 2011).

## Embryonic growth

#### Gross changes

A number of parameters can be used to monitor embryonic growth without the aid of expensive, specialized equipment. Total embryonic weight, organ weight, body dimension, are the most readily accessible of these parameters, with the first two being most commonly used in the literature (Leksrisompong et al., 2007; Maatjens et al., 2014; Reijrink et al., 2010; Yahav, Collin, Shinder, & Picard, 2004).

Organ weight was discarded as a parameter of embryonic growth. This was done in order to avoid removing the organs from their correct anatomic positions and/or damaging them, which could have had a negative effect on the subsequent histological analysis of the embryos. Additionally, this study sought to explore the third parameter mentioned, since morphometric parameters are rarely used and the allometric growth of the various embryonic body parts is commonly ignored when studying embryonic response to physical challenge in the incubation environment.

Embryo to egg-weight ratio, one of the parameters chosen to monitor embryonic growth, follows a tendency for lower embryo weights in temperature-challenged embryos, when compared to controls. CHT embryos only showed this tendency in the final third of the incubation period; CLT embryos, in turn, presented lower ER values much earlier, on day 7 of incubation. Contrarily, continuous changes in RH in either direction, as well as rises in $CO_2$ concentration, have resulted in higher late-incubation ER values for challenged embryos.

How can ER be higher for these treatments if metabolism is slower? To answer this it is essential to point out that, for the purposes of this study, embryonic body weight refers to the full weight of the embryo proper as well as to that of the internalized yolk sac. Therefore, an elevated final weight is not always directly correlated with a larger, heavier embryo but can, instead, be due to the internalization of a larger, less depleted yolk sac. As stated before, embryos respond to stress by lowering metabolism, which in turn results in a lower consumption of nutrients (Uni et al., 2014). Since the dynamics of nutrient consumption have the albumen being fully consumed first and the yolk only partially consumed during incubation, it is in the latter egg compartment that the consequences of this lower metabolism will become more evident (Moran, 2007). It is in the yolk sac that the unused nutrients will accumulate (Bellairs & Osmond, 2005). If more yolk is left in the yolk sac at the end of incubation, more yolk is internalized with the yolk sac, to serve as a portable nutrient reserve for the first days of the hatchling's life (Moran, 2007).

Taking this fact into consideration, the apparently inconsistent elevation in final ER values found for RH and $CO_2$ treatments (when compared to controls) now seems more compatible with a reduced embryonic metabolism in response to chronic stress. These embryos may, thus, have simply internalized a larger yolk sac, which naturally increases their final weight. In the case of RH-related treatments, YR values contribute even further to support this theory.

However, the question of how to explain the lower ER values obtained for temperature treatments now poses itself. If this non-specific defense mechanism of reducing metabolism to save energy was the only factor in play, all treatments inducing chronic stress, regardless of their nature, would be expected to yield heavier embryos. This is not apparently the case in this study, since both continuously elevated and continuously lower temperatures have resulted in lower final embryo weight.

Exposure to constantly high incubation temperatures is known to increase embryonic metabolism (Lourens, 2008). It, then, would be logical to expect a final result of embryos with smaller internalized yolk sacs, and, if growth is not affected, generally lighter hatchlings. This is, in fact, documented in previously published works, in which poor uniformity in bodyweight has also been reported for batches subjected to high incubation temperatures (Hulet et al., 2007; Leksrisompong et al., 2007).

This increase in metabolism appears, however, to be biphasic, with metabolic acceleration being restricted to the first half of incubation (until day 9), during which heat production by the embryo is minimal (Lourens, 2008; Sozcu & Ipek, 2015). From then on, the embryo is no longer just absorbing the environmental heat but also emitting it itself. The progressively increasing embryonic metabolism, results in the production of an important by-product: heat (Barri, 2008). No differently from carbon dioxide and water vapor, this by-product must be eliminated through the eggshell and into the environment directly around the egg. This causes the real temperature around and inside the egg to increase beyond that registered by the incubator’s thermal sensors (Joseph, Lourens, & Moran, 2006). It also means that the temperature registered in the incubator is often lower than the temperature that the embryo experiences (Lourens, 2008; Lourens et al., 2011).

This fact is especially evident for high-performance breeds, whose genetically metabolically-accelerated embryos seem to overheat even when incubated under the accepted ideal 37.8ºC, and for which lower average incubation temperatures have been recommended (Janke, Tzschentke, & Boerjan, 2004; Oviedo-Rondón et al., 2009). This is also the reason why many studies now rely on measuring eggshell temperature rather than average in-incubator temperature (Lourens, 2008). However, the technology necessary to use this thermal indicator is yet to be made accessible for industrial settings (where the sheer dimensions of the incubator cause temperature to vary depending on where in the room the sensors are placed (Hulet et al., 2007)), deeming it necessary to opt for an average one-size-fits-all in-room temperature setting. This, paired with the relatively small size of the incubator used for this study, is the main reason behind why eggshell temperature was not used to monitor temperature.

Therefore, even if an embryo responds to a high energy (temperature) input with an increase in its metabolic rate (and thus nutrient usage) during the first half of incubation (Lourens, van den Brand, Heetkamp, Meijerhof, & Kemp, 2007), maintaining this response during the second half soon becomes unsustainable. The higher the metabolism, the more heat is produced and dissipated into the environment, the more the embryo overheats

(Lourens, 2008; Meijerhof & van Beek, 1993). This creates a vicious cycle in which the embryo contributes, with its activity, to increase and maintain its own stress.

A significant drop in metabolic activity would, therefore, be expected to occur during the second half of incubation, delaying embryonic growth and yolk consumption, and resulting in a scenario in everything similar to that present in both RH and $CO_2$ treatments.

Why, then, were CHT embryos not heavier, but lighter than controls at the end of incubation? The answer can be found in Graph 4, where CHT embryos clearly have a much higher YR at the end of incubation than control embryos. Because of the stress imposed on them by the higher temperatures, the CHT embryos appear to have suffered a delay in the chronological chain of events prior to hatching. Unlike controls, most CHT embryos had not yet begun to incorporate the yolk sac when break-outs were performed on day 20. In fact, unlike control embryos, which commonly pipped and hatched by day 20 (making them fall beyond the scope of this study and therefore preventing their usage as experimental subjects), only one CHT embryo managed to successfully hatch. Weak from birth, this chick never demonstrated pecking behavior and eventually died, three days later, due to an unhealed navel complicated by secondary omphalitis.

Because the yolk sac was still outside the body by day 20, the yolk found in it was registered as part of the YR rather than a part of the embryonic weight. The final ER for these embryos was, therefore, a sole measure of the weight of the embryos *per se*, making them appear lighter than controls.

Therefore, it is not possible to agree or disagree with the previously published works mentioned above, and carried out by other authors, on whether embryos subjected to overheating are lighter or heavier at hatch than those incubated under ideal conditions. It is, however, important to underline the disturbance of the normal hatching timetable created by thermal stress.

If higher temperatures increase early *in ovo* metabolism, then a lower energy (i.e., temperature) input during early incubation should, in theory, lower it, delaying embryonic growth, as well as the onset of embryonic heat production (Black & Burggren, 2004a). ER values collected from CLT embryos illustrate this tendency. In spite of the very discreet changes in AR and YR values during early incubation (Graphs 3 and 4), when compared to controls, ER values for these embryos were already lower than controls as early as day 10, even if very subtly so.

ER values then rose at a steady rate for CLT embryos, from this day onward. However, they remained below control values (and, for the most part, below ER values for other temperature treatments) until day 20, in spite of the incorporation of the yolk sac.

It becomes obvious that, although unable to grow and acquire as much weight as controls, CLT embryos were, nevertheless able to develop, particularly from day 12 onward. If a comparison is made between YR values for CLT and CHT treatments, visible in Graph 4, it becomes apparent that, before day 12, the YR values for both treatments are virtually indistinguishable. Conversely, from day 12 onward, they begin to drop for CLT embryos at a rate much more similar to that of controls than of CHT embryos. This could indicate that, for some reason, CLT embryos seem to be under less thermal stress during the last third of incubation than CHT embryos, and that their metabolism may not be as gravely affected when compared to that of the latter.

As mentioned before, it is between days 9 and 10 that the embryo starts producing significant amounts of heat (Hulet et al., 2007; Tong et al., 2013). From this point on, as embryo-derived metabolic heat is produced, the temperature around the egg begins to rise above the average temperature in the room (Hulet et al., 2007). This could, in theory, be enough to further boost metabolism, bringing it up to “standard” values and partially or completely compensating the earlier reduced embryonic growth. From the observations stated above, the turning point seems to have occurred around day 12 of incubation for CLT embryos. It could, thus, be suggested that the final lower embryo weight is due to a partial and insufficient late compensation of whatever delays in growth were caused by the carryover effects of an apparently discreet decrease in early embryonic metabolism.

Regarding morphometric indicators of embryo growth, it is noticeable that manipulating incubation conditions has little to no effect in final embryonic dimensions, as well as in the normal, allometric growth of a number of structures/organs such as the wings, legs, toes, comb and eyes. Of all gross morphometric parameters measured, only Feather Length, Beak Length and Nail Length showed consistent changes for one or more type of treatment applied.

If interpreted as a continuous series of measurements throughout incubation, Feather Length can be (and was, for the purposes of this study) used to evaluate the speed of feathering in both normal and challenged embryos. Thus, it becomes apparent that certain treatments, namely temperature-related treatments affect the onset and speed of feather formation and growth. Continuously high temperatures appear to induce faster feathering, while lower temperatures slow down the feathering process. Interestingly, PHT embryos also seemed to suffer a delay in feathering, with the first feathers erupting late in the incubation process.

Beak length was found to be higher for all challenged embryos than for control embryos, regardless of the treatment applied, but relatively similar between treatments. There seems to be no biological reason behind this divergence in BL values, and no major differences in EL were observed, indicating that this is not the likely cause for such blatantly different BL values. The same occurs for Nail Length values, with all groups reaching lower final nail lengths than control embryos, in spite of the apparently precocious nail formation occurring in most RH- and in $CO_2$ related treatments. With no other explanation becoming evident, the differences in BL and NL can possibly be attributed to early differences in the anatomical landmarks used in the measuring of these structures and the results presented here should be interpreted with a level of prudence.

The information obtained from gross, external morphometric parameters is, therefore, limited as most structures (with the exception of the feathers) seem to adhere strictly to their pre-programmed timelines of development and growth while others do not offer enough of a pattern of response to changes in incubation conditions to constitute reliable markers of environmental manipulation. Still, this highly predictable behavior reinforces the value of using such parameters in establishing the period of embryonic death when examining dead-in-shell embryos since they are not bound to be influenced by the physical conditions of incubation.

#### Microscopic changes

Studies regarding the influence of temperature in embryonic development very rarely go as far as to analyze the embryos on a microscopic level to search for deeper understanding of how the organs respond to thermal stress. Yet, a number of surprising observations can be made if such analytical techniques are used.

The changes observed in the bursa of Fabricius in response to temperature manipulation are a good example of this. When incubation temperature is chronically elevated, lymphoid follicle formation seems to be stimulated, at least until day 14 of embryonic development.

A single report about the effects of thermal stress on bursal development was found in the literature. In it, Oznurlu et al. (2010), describe a significant delay in the normal embryonic development of the thymus and bursa of Fabricius of a broiler strain, evident from day 13 of incubation. In spite of the differences often found between layer and broiler strains, even during embryonic development (e.g., time of hatch, yolk consumption, oxygen consumption) (Everaert et al., 2008; Janke et al., 2004; Ohta, Yoshida, & Tsushima, 2004), bursal response in the layer strain used for this study to chronically stressful factors would not be expected to be so diametrically opposed to that found by Oznurlu et al. (2010) in broilers.

In fact, subsequent observations made in older embryos revealed a scenario much more compatible with Oznurlu et al.'s report, albeit with some differences. From day 14 onward, the seemingly faster follicle development appeared to slow down considerably and, eventually, stagnate. By day 16, the lymphoid follicles in the bursa of CHT embryos were no larger or more abundant than in control bursas. By day 18, the follicles were not only similar to controls in number and size, but much poorer in lymphocytes than the latter. Most follicles barely had lymphocytes at all, indicating that what at first looked like a quickening in bursal development had, in fact, given place to an immature, depleted organ. Oznurlu et al. (2010) reported a significantly lower number in bursal follicles after embryonic exposure to chronically high temperatures, which the authors attributed to the immunosuppressant effects of chronically elevated corticosteroid levels, generated by chronic thermal stress. Moreover, these authors described these follicles as being smaller in size than those of control embryos (Oznurlu et al., 2010).

No such differences were found in this study. In spite of this, the bursal lymphoid population of day 18 CHT embryos indicates a lower immune status for these embryos, when compared to controls, similar to the one described by Oznurlu et al. (2010) . No significant differences were found in the embryonic development of the thymus in this study.

Conversely, CLT embryos suffered a severe delay in bursal follicle formation, evident from the first stages of bursal differentiation (day 12), with a persistently lower follicular density, smaller follicles and scarcer lymphoid populations found in each follicle when compared to control bursae. However, this difference in lymphoid cellular density for each follicle was not as marked as the one found for CHT embryos. In the case of CLT embryos, the hindering in immune function caused by stress seems to rest more strongly on the lower number and size of follicles than on reduced follicle colonization by lymphoid cells. No literature was found on the effects of sub-optimal temperatures on the embryonic development of avian lymphoid organs, by which the present study may be considered pioneer. Data available regarding the effects of cold stress in hatched chicks indicates only that this may have a suppressant effect on immunity due to chronic circulation of high levels of corticosteroids (Hangalapura, Nieuwland, Buyse, Kemp, & Parmentier, 2004).

This lack of information is most probably due to the greater likelihood of embryo overheating than of cold stress.

The findings stated above should be carefully taken into consideration for their important practical effects. It is well known that post-hatch immune status is vital to chick response to vaccines administered to day-old chicks (Butcher & Yegani, 2008). However, with the progressively more generalized development and use of *in ovo* vaccines at day 18 of incubation (C. S. Li, Wang, & Chou, 2005), the matter of embryonic immunity gains even greater relevance. Based on the observation that chicken embryos possess immunocompetent cells from day 13 onward, the practice of administering live *in ovo* vaccines relies on the existence of a healthy and normally developed population of both T and B lymphocytes for its effective use, thus making any stressor capable of delaying bursal development all the more critical (Bellairs & Osmond, 2005; Jankovic et al., 1975; Kajiwara, Shigeta, Horiuchi, Matsuda, & Furusawa, 2003; Schijns, van de Zande, Lupiani, Reddy, & Kaiser, 2014). If a scant, less than competent immune cell population is present by day 18, the embryo's immune system will not respond properly to the priming effects of the vaccine and production of embryonic antibodies will likely be inadequate. Moreover, the administration of vaccines significantly lowers maternal antibody titters (Michell et al., 2009). These two effects combined will result in the hatching chicks being left more susceptible to disease after vaccination than if the vaccine had not been administered in the first place, with subsequent reduction in chick viability and post-hatch performance.

Another example of the influence of incubation temperature on embryonic development is found in the gizzard of embryos incubated under continuously high temperatures.

On day 12 of incubation, the time point at which the secretory glands of the gizzard begin to form, the gizzard of these embryos presented cystic cavities in the developing mucosal lining, caused by the accumulation of basophilic material in large cytoplasmic vacuoles, with distension and rupture of the affected cells. By day 18 of incubation, the cavities had given place to large vesicles, often evidencing detachment of the more superficial sections of the mucosa from the underlying glandular tissue. When not detached, these layers of the mucosa also appeared thickened and foamy due to the presence of large vacuoles in the cytoplasm of the cells. Moreover, the regular, ridge-like architecture of the gizzard mucosa, commonly found in control embryos of the same age, was distorted and disorganized in CHT embryos, appearing poorer in the cuboidal cells that line the tubular, glandular epithelium of this organ.

These changes could very likely lead to losses in the performance of hatchlings and, later, chicks due to a severe hindering of the digestive process at gizzard level. A review article published by Gjevre et al. (2013) on gizzard erosion and ulceration syndrome mentions that lesions of the koilin layer and gizzard mucosa have been found in newly-hatched chicks and even in embryos, indicating a possible, congenital background as an element in play in the multifactorial syndrome of gizzard ulceration in poultry. Among the suspected causes of gizzard erosion/ulceration in newly hatched chicks are breeder diet, breeder age and capillary fragility combined with a transient increase in blood pressure during hatching (Gjevre et al., 2013). The lesions found in this study indicate that changes caused by elevated incubation temperatures may also play a fundamental role in this syndrome, by leading to progressive dystrophy, with fragility and irregularity of the mucosal surface and subsequent changes in the secretion and deposition of the koilin layer. Because chicks may start ingesting feed in the first twenty four hours after hatching, the combination of a fragile koilin layer with the input of the low-pH digesta from the proventriculus, as well as the presence of feed particles, could be enough to increase susceptibility to gizzard erosion/ulceration (Gjevre et al., 2013; Uni & Ferket, 2004). This would subsequently lead to reduced feed intake, growth, weight gain and overall performance of the newly hatched flock.

Some of the control embryos in this study presented mucosal/submucosal cavities similar to those described for CHT embryos (albeit more discreet, in much smaller number and only at an early stage, with no dystrophy observed at day 18). This indicates that temperature may not be the only factor involved in the appearance of such dystrophies, but possibly acts as a facilitator or exacerbating stimulus for the development of gizzard lesions.

The liver changes observed in both CHRH and CLRH embryos between day 7 and day 9, with delay in the formation of hepatic plates, seems to be a transient situation, later compensated and apparently obliterated, with no obvious long-term consequences in performance or metabolism. Since no breakouts are usually performed at this early embryonic age in an industrial setting, and considering that embryos that die during this period suffer autolysis throughout the following 12-14 days spent in the incubator/hatcher at relatively elevated temperatures, these changes may easily go unnoticed.

In fact, all of the changes described above will go unnoticed at an industrial level, since no sampling for histological analysis is routinely performed as a method for incubation and chick-quality monitorization. As previously stated, literature available on this subject is scarce and done only at laboratorial, experimental level, allowing no possible comparison with the reality in the field. However, the findings described in this study reveal that histological analysis is a tool of evident value in monitoring embryonic development, explaining some of the issues/challenges that may appear in a nascent flock and, finally, in understanding why hatchlings do not always perform as they were expected to.

Analysis of dead-in-shell embryos has yielded no significant differences in lesion patterns between challenged and control embryos. Due to the accelerated autolytic changes promoted by the incubation environment, cause of death is not always easy or possible to establish. This also prevents the establishment of any patterns that could help in diagnosing a specific causative agent for embryo loss.

## Embryonic viability

Whenever gross lesions were found, these pertained to congenital malformations in a single or in multiple organs/structures. During incubation, as each organ and system develops, many opportunities for the occurrence of spontaneous malformations arise and, even in an ideal setting where embryonic stress is reduced to a minimum, malformations are expected to occur (Romanoff & Romanoff, 1972). These can be due to a number of different causes (e.g., chromosome anomalies). Some malformations may merely carry aesthetic impact, while others are completely incompatible with survival (Cobb, 2008; Romanoff & Romanoff, 1972; Tullett, 2008).

In an industrial production setting, in which standardization of the final “product” is desirable and any gross deviations/aberrations are deemed repugnant by the consumer, the presence of even an uncomplicated, aesthetical malformation is reason enough for the elimination of an embryo/chick to prevent wasting resources in raising a bird that the consumer will not buy. Thus, the higher the prevalence of malformations, the greater the number of dead or culled birds and the lower the performance of each incubated lot.

From the results obtained in this study, it is clear that only continuous changes in incubation temperature (in either direction) seem to have a significant effect on the incidence of spontaneous malformations. Malformation incidence rates were highest for CHT embryos and mostly affected the abdominal wall, cranial vault, limbs and skin. CLT embryos exhibited malformations of the abdominal wall, eyes, cranial vault, beak and limbs. The abdominal wall and skull/cranial vault were the two structures most commonly affected, despite the treatment applied or even in the absence of treatment, suggesting a particular susceptibility of these structures to develop abnormally.

In spite of the low percentage of each specific type of malformation observed, some of the findings in this study warrant further discussion. For instance, in every case of duplication of ocular structures, the left eye was always the one affected, and always with seemingly complete, if at times deformed, eyes. To the best of the author's knowledge, this was also the first time that such malformation was described for the species. Such asymmetry in preference was not found when one of the eyes was hypotrophied or absent.

Similarly, the beak demonstrated asymmetrical susceptibility in the occurrence of malformation. Here, the upper (or maxillary) portion of the beak was always found to be the one affected.

Interestingly, and contrarily to a number of accounts in literature available on teratogenesis in other species, in which exencephaly appears as a relatively rare condition, considered to be a stepping stone in the development of anencephaly (Carlson, 2004; Drews, 1995; K. L. Moore & Persaud, 2008; Romanoff & Romanoff, 1972; Sadler, 2011), only one in the six cases of skull malformation leading to brain exposure found in this study was classified as anencephaly, while all the others were deemed to be of exencephaly.

A study performed in 1996 by (Peterka, Peterkova, & Likovsky) on the teratogenic and lethal effects caused by exposure to either hyperthermia or hypothermia between days 3 and 9 of incubation similarly describes only exencephaly cases but since no histological analysis was performed, this classification cannot be confirmed. Similarly, Romanoff&Romanoff (1972) mention exencephaly as a naturally occurring malformation, defining it as the "exposure of the whole or a part of an imperfect brain" but lacking in histological confirmation and leaving no references in poultry science literature against which to compare the results obtained in this study. On a side note, care should be taken to avoid confusion when consulting the same study, in which anencephaly is defined as the absence of the brain altogether.

The hallmark of anencephaly is the appearance of exposed brain tissue as a disorganized degenerated/necrotic mass. Conversely, in exencephaly, nervous tissue appears relatively healthy, if poorly organized due to the absence of the shaping effects of the limiting/constricting structure that is the cranial vault (K. L. Moore & Persaud, 2008). Authors claim that exposure of the brain to the amniotic environment, patent in cases of exencephaly, determines its degeneration and necrosis, leading to anencephaly and, subsequently, embryonic death (Carlson, 2004; Drews, 1995; K. L. Moore & Persaud, 2008; Sadler, 2011). However, in most cases found throughout this study, the brain tissue appeared healthy, or only marginally affected, even in cases where the embryo survived until the later break-out points of the study (days 18-20). In most of the exencephaly cases identified, in fact, exposure of the brain tissue was not the immediate cause of death of the affected embryos, which showed signs of embryonic vitality very similar to their cohorts, at the time of break-out. The findings of this study indicate that, for some reason, exposure of the embryonic brain to amniotic fluid did not seem to be as harmful in the chicken as is described for other species.

Although most malformations found were simple (a single organ affected), compound or complex malformations (several organs concomitantly affected) were a relatively common event found in both control and temperature-challenged embryos (with no significant differences between these groups). This suggests a possible, naturally occurring genetic/chromosomal cause to such mutations. Unfortunately, since no genetic analysis was made, the results obtained in this study do not allow for confirmation or dismissal of this theory.

Although no examples of malformation were found in embryos challenged with high $CO_2$ concentrations, other studies exist that mention varying degrees of facial malformation after exposure to hypoxia (Smith et al., 2013). Although exposed to elevated concentrations in carbon dioxide, embryos in this study may not have suffered hypoxia in levels significant enough to cause visible changes.

The most important marker of embryonic viability is, undoubtedly, embryonic mortality (Romanoff & Romanoff, 1972). Application of continuously low temperatures was the only treatment which caused a dramatic increase in total mortality due to an elevation in early embryonic mortality.

However, not only is overall mortality an important indicator to be studied, but the time of embryonic death has proved just as important in this study. For most treatments, including control and the CLT treatment mentioned above time of death was clustered around the first four days of embryonic development. These observations are in agreement with previously published works (Lourens, 2008; Lourens et al., 2005; van der Pol et al., 2013). It is during this period that the foundations for normal organic metabolism and development are being laid, in the form of vital organs and systems (e.g., chorioallantoic membrane, heart, central nervous system), each of which with the potential to threaten embryonic viability should a developmental anomaly or failure in function occur (Bellairs & Osmond, 2005; Romanoff & Romanoff, 1972). On the contrary, exposure to continuously high relative humidity did not significantly increase overall mortality but instead result in a sudden spike in mortality at the end of incubation. This could have been caused by overhydration and/or overall embryonic weakness, possibly related to lower metabolism, with lower energy availability.

Therefore, as with the other observations made in this study, the systematic analysis of embryonic mortality would be important in the industrial setting, benefitting from the significantly larger number of eggs and embryos available and allowing for the establishment of more definitive patterns of embryonic response to different sources and types of stress.

In summary, all the observations made during this study have made evident the remarkable ability of chick embryos to adapt to unfavorable incubation conditions in order to survive, grow and, eventually, hatch. However, they have also made obvious that such flexibility is neither unlimited nor without a price. Whether it is a short-lived delay of no obvious consequence or a long-lasting change that could put future performance at risk, there is a cost to incubating eggs under less than ideal conditions. Improving performance will require a definite knowledge of that cost as well as the understanding that, ultimately, embryonic development is not merely a fixed, pre-programmed chain of events by which a single cell multiplies and transforms into a fully-formed chick. Instead, it is a dynamic process, continuously shaped by the interaction of the embryo with its environment and oriented toward a single, all-important purpose: survival.

# PART TWO - *IN OVO* INFECTION WITH PATHOGENIC AGENTS UNDER LABORATORY CONDITIONS

## INTRODUCTION

Poultry can become infected with a variety of pathogenic bacteria and viruses that can be transmitted to the next generation of birds through a number of different routes (Dominguez, Gomez, & Zumalacarregui, 2002; Pattison, 2008). Prior to eggshell deposition, forming eggs can be subjected to descending infections from colonized ovarian tissue, ascending infections from colonized vaginal and cloacal tissues, and lateral infections from colonized upper oviduct tissues (De Buck, Van Immerseel, Haesebrouck, & Ducatelle, 2004).

*Salmonella enterica* is notoriously capable of infecting both ovary and oviduct, whether independently from each other, at the same time or one after the other (De Buck et al., 2004; Keller, Benson, Krotec, & Eckroade, 1995). Salmonella Enteritidis has caused greater frequencies of egg contamination than other serovars in numerous experimental infection studies, perhaps related to an enhanced ability to adhere to reproductive tract mucosa (De Buck et al., 2004; Guard-Petter, 2001). *Salmonella* Enteritidis strains can differ significantly from each other in their abilities to invade reproductive organs and contaminate eggs, although reproductive organ colonization at high frequencies or involving large numbers of *Salmonella* cells does not always predict a high frequency of associated egg contamination (De Buck et al., 2004; Humphrey, Whitehead, Gawler, Henley, & Rowe, 1991).

A systemic *S. enterica* var. Enteritidis infection in laying hens can lead to colonization of the ovary, where the bacterium can interact with the cellular components of the preovulatory follicle (namely, the granulosa cells), and invade and multiply in these cells (Cox, Berrang, & Cason, 2000; De Buck et al., 2004; Schoeni, Glass, McDermott, & Wong, 1995). *S*. Enteritidis is then able to penetrate the vitelline membrane and multiply within the interior yolk contents after deposition onto the exterior surface of intact egg yolks (Gast et al., 2010; Guan, Grenier, & Brooks, 2006).

Although *Salmonella* serovars have been isolated from both the yolk and the albumen, according to most authors, the albumen is most frequently contaminated, pointing to the oviduct tissue as the colonization site (De Buck et al., 2004; Gast et al., 2010; Humphrey et al., 1991). The oviduct can be invaded and colonized by the bacterium and *Salmonella* bacteria (most commonly *S*. Enteritidis) have been found on the mucosal surface and within epithelial cells, lining the oviduct in naturally infected hens (Guard-Petter, 2001). This was previously interpreted as the sole result of an ascending infection from the cloaca (Keller et al., 1995). However, intravaginal infection tends to ascend only to the lower parts of the oviduct, but can cause the contamination of forming eggs. The subsequent contamination of the egg following intravaginal infection takes place in the isthmus, uterus, vagina or cloaca and rarely in the upper oviduct and ovaries. Once present in the lumen of the oviduct, *Salmonella* Enteritidis can contaminate forming eggs and establish a long-lasting colonization of the oviduct tissue (De Buck et al., 2004).

Infected hens lay contaminated eggs in a clustered and intermittent way, possibly caused by the occasional reappearance of bacteria from the infected tissue into the lumen of the oviduct (Guard-Petter, 2001; Humphrey, Baskerville, Mawer, Rowe, & Hopper, 1989; Humphrey et al., 1991). This egression might be induced by stress, hormonal variations or fluctuations in the immunological protection or other unknown factors (Humphrey et al., 1991).

Of the *Salmonella enterica* serovars known to infect chickens, *S.* Enteritidis, Typhimurium and Pullorum are the only serovars reportedly capable of colonizing the reproductive organs, with S. Enteritidis being the predominant serotype associated with laying hens and eggs. However, the biological reason for this is still unclear. A possible explanation is that *S.* Enteritidis might have a higher tropism and affinity for the reproductive organs of the hen than other serovars (De Buck et al., 2004). In a study by Gantois et al. (2008), strains of the *Salmonella* serotypes Enteritidis and Typhimurium were observed to colonize the reproductive organs better than strains belonging to the serotypes Heidelberg, Virchow and Hadar.

Another possible explanation for the greater efficiency with which *S.* Enteritidis is vertically transmitted is that isolates or serovars of *Salmonella* that are less aggressive in the reproductive tract might have an advantage over more aggressive ones to end up contaminating eggs (De Buck et al., 2004). For instance, challenge with *S.* Typhimurium causes pathological lesions in layers, including atrophy or shrinkage of the oviduct, and, in some cases, total cessation of egg production (De Buck et al., 2004; Pattison, 2008). In contrast, *S.* Enteritidis does not cause grossly visible pathological lesions (Guard-Petter, 2001; Moussa, Gassem, Al-Doss, S., & Abdel Mawgood, 2010).

Gantois et al (2008) also observed that low numbers bacteria of the strains of *Salmonella* Enteritidis, *Salmonella* Typhimurium and *Salmonella* Heidelberg were capable of surviving in the egg albumen during egg formation while ones belonging to the serotypes Virchow and Hadar were killed much faster. This could mean that, in natural infections, when the bacterial concentrations in forming eggs are lower, *Salmonella* Virchow strains and *Salmonella* Hadar strains could be killed before oviposition and thus provide an explanation why *Salmonella* strains belonging to the serotypes Virchow and Hadar are nearly never isolated from intact eggs (Gantois et al., 2008).

Colonization of the reproductive tract is not the only means of bacterial transmission from chicken to egg, as *Salmonella* bacteria can penetrate eggshells, and it can be difficult to distinguish between contamination during formation of the egg or after oviposition (Cox et al., 2000; De Reu et al., 2006). The major extrinsic factors identified as being important to the transmission of Salmonella through the shell are: bacterial strain, temperature differential, humidity, number of organisms present on the eggshell, storage conditions and, most importantly, the presence of shell defects, such as cracks (De Reu et al., 2006; Messens, Grijspeerdt, & Herman, 2006).

Apart from contamination within the hen reproductive system after formation of the shell due to contamination of the oviduct itself, shell contamination most likely depends on either contact with feces containing *Salmonella* during expulsion of the egg from the hen, or following oviposition, when the shell acquires contamination from all surfaces with which it makes contact (Chousalkar, Flynn, Sutherland, Roberts, & Cheetham, 2010; Messens et al., 2006). Fecal contamination is, however, unlikely to occur during oviposition in a healthy laying hen, since the egg itself forces the vagina to stretch beyond the alimentary tract as the egg is laid, thus protecting it from fecal contamination. In addition, the stretching of the cloacal lining effectively makes the intestinal tract somewhat slit-like, further reducing the opportunity for contamination of eggshell. This is why most eggshells in healthy birds are not covered in feces at oviposition. For this reason, fecal contamination is more likely to occur after oviposition, through contact with feces or the environment, making the hygiene in the chicken house and during egg handling and processing critical (De Buck et al., 2004).

Penetration of eggshell by *S.* Enteritidis, as well as *S.* Typhimurium and other serovars has repeatedly been described under experimental conditions (Cox et al., 2000; De Reu et al., 2006). The primordial route for bacteria to penetrate intact eggs is the pores with diameters in the range of 6–65μm, far above the bacterial dimensions (Cook, Beissinger, Toranzos, Rodriguez, & Arendt, 2003; De Reu et al., 2006). No correlation seems to exist between the number of pores and the bacterial eggshell penetration, nor between the loss of weight at the pores and the whole egg contamination. This could be because not all pores extend through the thickness of the shell. In fact, some end abruptly, barring entrance to the bacteria. Additionally, cuticular capping (the defense provided by the plugging of eggshell pores by the outer, cuticular layer) may also prevent microbial penetration, although this mechanism of protection has been questioned (De Reu et al., 2006).

The eggshell appears to be more easily penetrated immediately after the egg is laid. It is suggested that for the first minutes after oviposition, the cuticle is immature and some pores may therefore still be open. Furthermore, when the egg is exposed to an environment cooler than the chicken's body temperature (42.1ºC), negative pressure may develop, especially in the presence of high environmental RH, facilitating the migration of bacteria through the eggshell and membranes (Keller et al., 1995). In addition, *Salmonella* organisms on eggshells can die rapidly during storage. Survival is, however, enhanced by low temperatures, especially when relative humidity is low, probably due to the slower metabolism induced by the disadvantageous conditions on the dry eggshell surface (Messens et al., 2006). As storage periods increase and the eggs "age", the cuticle ages as well, becoming dehydrated and shrinking so that the pores become more exposed to bacterial penetration (Svobodová & Tůmová, 2015).

Although various studies have been carried out on the effects of contamination with *Salmonella* in bird and human health, most poultry-related works are focused on detecting Salmonella in carcasses and foodstuffs (Cheung & Kam, 2011; Salehi, Mahzounieh, & Saeedzadeh, 2005), decontaminating eggs with and without breaking the egg (Davies & Breslin, 2003; Lasagabaster, Arboleya, & de Marañón, 2011; Rodriguez-Romo & Yousef, 2005) and on clinical signs in the parent or nascent flocks (Parsons, Crayford, Humphrey, & Wigley, 2013; Pattison, 2008; Setta, Barrow, Kaiser, & Jones, 2012). Not on the avian embryo.

For this reason, this study was aimed at observing the effects of vertical infection with *Salmonella enterica* serovars Enteritidis and Typhimurium from the embryo's point of view.

# MATERIALS AND METHODS

## Optimization of PCR protocol for detection of *Salmonella* spp. in eggs

### 1.1 Bacterial cultures

Two *Salmonella* reference strains were chosen for infection trials and, hence, for PCR optimization. Both were obtained from the Spanish Type Culture Collection (CECT), University of Valencia, Spain. The first was a *S.enterica* var. Enteritidis reference strain (CECT-4300) and the second was a *S.enterica* var. Typhimurium reference strain (CECT-443).

Strains were grown overnight in Brain Heart Infusion medium (BHI) with Fetal Calf Serum (FCS), at 37ºC, and later inoculated into Blood Agar and grown overnight at 37ºC, for further enrichment. Culture pureness was assessed by inoculation in MacConkey Agar, Hektoen Enteric Agar, Brilliant Green Agar and Kligler Iron Agar selective mediums and overnight incubation at 37ºC (Day, Basavanna, & Sharma, 2009; Malorny, Bunge, & Helmuth, 2007). An API-20E® (bioMérieux) test kit for the identification of enteric bacteria was then used for species identification.

Culture samples of each strain were taken for quantification studies. The samples were diluted in sterile physiological serum (NaCl, 0.9%) in 1:10 serial dilutions ranging from $10^{-1}$ to $10^{-7}$. The optical density of dilutions $10^{-1}$ through $10^{-4}$ was assessed with a spectrophotometer and the values recorded. Dilutions $10^{-3}$ through $10^{-7}$ were then inoculated in duplicate into Plate Count Agar (Liofilchem®) and incubated overnight at 37ºC. After incubation, the colonies in both Petri dishes corresponding to the lowest dilution with a suitable number of colonies (15 to 150) for each strain were counted and the number of colony forming units (CFU) per milliliter (ml) determined (Baker & Breach, 1967; Collins & Lyne, 1984; Csordas, Barak, & Delwiche, 2004). The optical density values previously recorded and the established CFU/ml were then used to build a regression graph and equation for future trials.

### 1.2 PCR optimization from reference culture samples

Reference cultures were grown overnight in Blood Agar, at 37ºC, and extraction performed by boiling for each reference strain. Briefly, 5 colony samples of either strain were suspended in a 1.5 ml Eppendorf® tube containing 100 µl of Tris-EDTA (TE), pH 8, and boiled for 10 minutes, at 100ºC. The samples were then centrifuged at 14000 rpm (16654 G) for 2 minutes and the supernatant transferred to a new tube to be used as DNA template.

Three sets of primer pairs were used; the first was specific for the invA gene located in the salmonella pathogenicity island 1 which is highly conserved in *Salmonella* species and encodes a type III secretion system that exports proteins in response to bacterial contact with epithelial cells, stimulating endocytosis (Galan, Ginocchio, & Costeas, 1992; Rahn et al., 1992; Salehi et al., 2005); the second was specific for the sefA gene, corresponding to SEF14, a structural subunit of the fimbriae found in *S.* Enteritidis (Clouthier, Muller, Doran, Collinson, & Kay, 1993; Doran et al., 1996). Finally, the third was specific for the fliC gene which encodes for flagelin, the main component of the flagellum found in *S.* Typhimurium (Aldridge, Gnerer, Karlinsey, & Hughes, 2006; Soumet et al., 1999). The sequences, target genes and product size for each of the primer pairs used is presented in Table 2. All primers were obtained lyophilized from a commercial supplier (STAB VIDA laboratories) and re-suspended according to manufacturer’s instructions.

**Table 3. Primers used for amplification of Salmonella species and Enteritidis and Typhimurium serovars.**

| Specificity | Target gene | Primer sequence (5’-3’) | | Product size |
|---|---|---|---|---|
| *Salmonella* spp. | invA | FW | GTG AAA TTA TCG CCA CGT TCG GGC AA | 284bp |
| | | RV | TCA TCG CAC CGT CAA AGG AAC C | |
| *S.* Enteritidis | sefA | FW | GAT ACT GCT GAA CGT AGA AGG | 488bp |
| | | RV | GCG TAA ATC AGC ATC TGC AGT AGC | |
| *S.* Typhimurium | fliC | FW | CGG TGT TGC CCA GGT TGG TAA T | 620bp |
| | | RV | ACT GGT AAA GATGGC T’ | |

PCR amplifications were performed in a final volume of 25 µl in micro-amplification tubes (PCR tubes). The reaction mixtures consisted of 1 µl of the DNA template, 12.5 µl of a commercial master mix (Supreme NZYTaq® 2x Green Master Mix, NZYTech), 1 µl of the forward and reverse primers of each primer pair and the volume of the reaction mixture was completed to 25 µl using deionized distilled water (DDW). The thermal cycler was adjusted as follows: Initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min. and extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min and the PCR products were stored in the thermal cycler at 4°C until they were collected. The PCR products were tested for positive amplification by electrophoresis in a 1% agarose gel stained with GelRed nucleic acid gel stain using suitable molecular weight markers (NZYDNA® Ladder VI, NZYTech)(Moussa et al., 2010).

### 1.3 Inoculation of eggs with *Salmonella*

Ten free-range eggs were collected from a *Salmonella*-free backyard flock and used to test the flock for infection with *Salmonella* spp. Egg breakout was performed in a laminar flow chamber. Briefly, each egg's shell was disinfected with an iodine solution and then broken over the air chamber with a sharp blow of a closed pair of sterile scissors. The shell over the air chamber was then removed as well as the inner shell membrane. The albumen was removed first, with a 25 ml pipette, into a 50 ml Falcon tube. The yolk was later removed with a pipette into a Falcon tube. A 5 ml sample of albumen (or a 10 ml sample of yolk) was then inoculated into 200 ml of Buffered Peptone Water (BPW). All samples were incubated overnight, at 37ºC. All samples with positive bacterial growth were then inoculated into Rappaport Vassiliadis medium (RVS) (200 µl of sample in BPW/20 ml RVS) and left to incubate overnight at 42ºC.

After absence of bacterial growth in the first batch of eggs was confirmed, a second batch of six free range eggs was later obtained and inoculated with one of two inoculum concentrations (3 eggs per inoculum) of *S. enterica* var. Enteritidis reference strain (CECT-4300). Briefly, the reference culture was grown overnight in Blood Agar, at 37ºC. The inoculum was prepared by suspending a culture sample in sterile NaCl (0.9%) and adjusting optical density to obtain two different concentration inocula: a high concentration inoculum ($1x10^5$ CFU/ml) and a low concentration inoculum ($1x10^2$ CFU/ml). Both inocula were stained with Brilliant Blue food dye in a 10 µl dye/3 ml inocula concentration. All eggs were inoculated in a laminar flow chamber. The shell over the inoculation point and on the blunt pole of the egg, over the air cell (located by candling), was disinfected with an iodine solution.

To reduce pressure inside the egg and prevent leakage of contents during inoculation, a small opening into the air cell was first created, using a blunt egg punch. A second opening was then created at the inoculation site. In 4 of the eggs, the inoculum was then deposited near the yolk with a 1 ml syringe equipped with a 26 G needle. In the two remaining eggs, the inoculum was deposited at the narrow pole of the egg, as far away from the yolk as possible. Both openings were closed with histological grade paraffin, first the inoculation site opening and later the air chamber opening. The eggs were left to incubate overnight in vertical position (blunt pole up), without turning, at 37.8ºC, to allow for inoculum spread and any possible bacterial growth to occur.

### 1.4 DNA extraction and PCR detection of Salmonella from inoculated egg samples

After incubation, the eggs were broken out and samples taken as described above.

A 2.5 ml sample of each egg's albumen and yolk was collected for direct DNA extraction. The sample was suspended in 7.5 ml of TE and homogenized. The sample was then centrifuged at 5000 rpm (4640 G) for 5 minutes and the supernatant discarded. The pellet was washed once with 2.5 ml phosphate buffered saline (PBS) and later with 600 µl of TE. The pellet was then ressuspended in 1000 µl TE and boiled for 10 minutes, at 100ºC. The DNA sample was obtained after centrifugation at 14000 rpm (16654 G), for 2 minutes.

A 1 ml sample of either albumen or yolk was also inoculated into 9 ml of Tryptic Soy Broth (TSB) as a pre-enrichment step, and left to incubate overnight at 37ºC. The samples were then centrifuged at 3000 rpm (1670 G) for 5 minutes and the supernatant discarded. The bacterial pellet was washed twice with 600 µl PBS and ressuspended in 500 µl TE. Boiling was performed as described above.

PCR analysis was performed as described for pure reference strains (Figure 55).

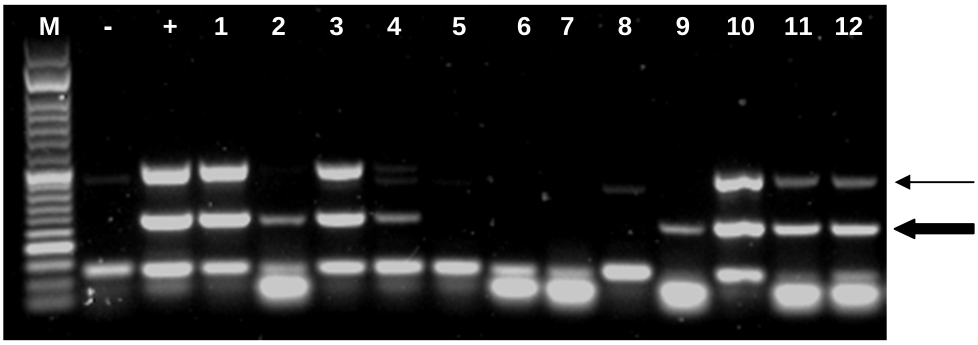


**Figure 55. Multiplex PCR for *Salmonella* Enteritidis.**
**Note the double bar pattern, where the lower bar (thick arrow) corresponds to amplification of the sefA gene and the higher bar (thin arrow) corresponds to amplification of the invA gene.**

## Experimental Infection of SPF Eggs with Infectious Agents

### Trial 1 – Experimental Infection with a High Dose Inoculum of *Salmonella enterica* var. Enteritidis reference strain (SE 10^5)

A high concentration inoculum ($10^5$ CFU/ml) of *S. Enterica* var. Enteritidis (CECT 4300) was prepared according to the procedures described above. The dye was omitted in order to reduce the presence of any possible inhibitors of bacterial growth to a minimum.

One hundred forty three specific-pathogen free eggs were obtained and randomly divided in two groups: one negative control (inoculated with NaCl 0.9%) of 66 eggs and one challenge group (inoculated with *Salmonella*) of 77 eggs. Egg weights ranged between

54.98 g and 66.68 g (average weight 60.54 g ± 2.73 g). All eggs were inoculated as described above.

The eggs were stored for less than 7 days, at 14-16ºC, 75-80% relative humidity (RH), and pre-warmed at 22-24ºC for 12 h before incubation. An OVA-EASY® Advance 380 (Brinsea) cabinet incubator with a maximum capacity of 384 eggs was used to incubate the eggs. Incubation conditions were as follows: from day 0 to day 18, eggs were incubated at 37.8ºC, RH 50-55% (turning – 1x/hour); from day 18 onwards the eggs were set on the hatcher trays (in horizontal position) and incubated at 37.8ºC, RH 60-65% (no turning). The incubator was calibrated (±0.1ºC) previously to the trials.

Every 48 hours, a sample of eggs was taken from the incubator, candled, weighed and subjected to euthanasia procedures similar to those described in the previous chapter. After euthanasia, break-out was performed in a laminar air flow chamber and samples of albumen and yolk taken aseptically for DNA extraction and PCR analysis to confirm presence/absence of *Salmonella*. Extraction and PCR protocols have been previously described. The embryos were then fixed in 10% buffered formalin and allowed to fixate before any further manipulation was undertaken. All fixed embryos were measured, weighed and photographed and taken *in toto* for routine histopathology processing.

#### Trial 2 – Experimental Infection with a Low Dose Inoculum of *Salmonella enterica* var. Enteritidis reference strain (SE 10^2)

A low concentration inoculum ($10^2$ CFU/ml) of *S. enterica* var. Enteritidis (CECT 4300) was prepared according to the procedures described for Trial 1.

One hundred forty three specific pathogen-free eggs were obtained and randomly divided in two groups: one negative control (inoculated with NaCl 0.9%) and one challenge group (inoculated with *Salmonella*). All eggs were inoculated as described above. Both groups of eggs were then incubated and processed as described for Trial 1. Egg weights ranged between 50.6 g and 64.1 g (average weight 58.3 g ± 2.5 g).

#### Trial 3 – Experimental Infection with a High Dose Inoculum of *Salmonella enterica* var. Typhimurium reference strain (ST 10^5)

A high concentration inoculum ($10^5$ CFU/ml) of *S. enterica* var. Typhimurium (CECT 443) was prepared according to the procedures described for Trial 1.

One hundred forty three specific pathogen-free eggs were obtained and randomly divided in two groups: one negative control (inoculated with NaCl 0.9%) and one challenge group (inoculated with *Salmonella*). All eggs were inoculated as described above. Both groups of eggs were then incubated and processed as described for Trial 1. Egg weights ranged between 44.4 g and 55.3 g (average weight 50.6 g ± 1.8 g).

#### Trial 4 – Experimental Infection with a Low Dose Inoculum of *Salmonella enterica* var. Typhimurium reference strain (ST 10^2)

A low concentration inoculum ($10^2$ CFU/ml) of *S. enterica* var. Typhimurium (CECT 443) was prepared according to the procedures described for Trial 1.

One hundred forty three specific pathogen-free eggs were obtained and randomly divided in two groups: one negative control (inoculated with NaCl 0.9%) and one challenge group (inoculated with *Salmonella*). All eggs were inoculated as described above. Both groups of eggs were then incubated and processed as described for Trial 1. Egg weights ranged between 47.5 g and 62.0 g (average weight 53.1 g ± 1.9 g).

A summary of all trial conditions is presented in Table 4 for easier reading.

**Table 4. Summary of the trials and treatments applied.**

| Trial | Group | Inoculum | Strain |
|---|---|---|---|
| 1 | Control | 0.9% NaCl | - |
| SE 10^5 | Trial | $10^5$ CFU/ml 0.9% NaCl | *S. enterica* var. Enteritidis (CECT 4300) |
| 2 | Control | 0.9% NaCl | - |
| SE 10^2 | Trial | $10^2$ CFU/ml 0.9% NaCl | *S. enterica* var. Enteritidis (CECT 4300) |
| 3 | Control | 0.9% NaCl | - |
| ST 10^5 | Trial | $10^5$ CFU/ml0.9% NaCl | *S. enterica* var. Typhimurium (CECT 443) |
| 4 | Control | 0.9% NaCl | - |
| ST 10^2 | Trial | $10^2$ CFU/ml0.9% NaCl | *S. enterica* var. Typhimurium (CECT 443) |

## Data Analysis

Data was subjected to the Student t-test, and to Chi-square test where appropriate, by means of the SPSS Statistics 19 software (IBM. 2013).

# RESULTS

## PRE-INOCULATION OBSERVATIONS

During the first inoculation and DNA extraction attempts, an unexpected finding was observed on pre-break out candling of the eggs which had been inoculated at the narrow pole of the egg, away from the yolk (and not re-positioned since). In these eggs, the inoculum, stained with Brilliant Blue dye, was no longer in the albumen but located near the yolk, above and around the embryonic disc, after overnight incubation (Figure 56). The stained inoculum did not float freely, moving instead with the embryo and yolk whenever the egg was manipulated and re-positioned.

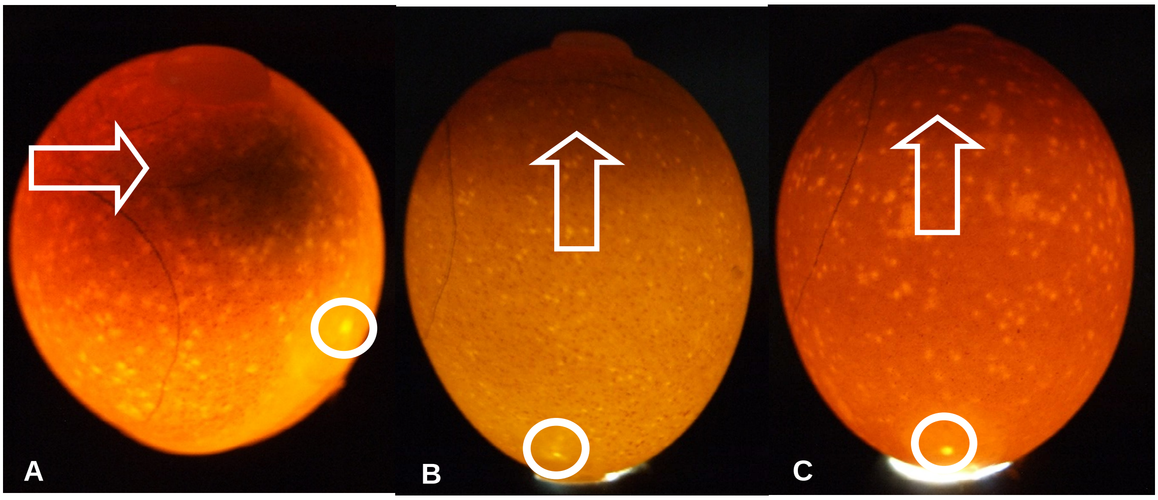


**Figure 56. Eggs inoculated with *S.* Enteridis after incubation overnight.**
**Egg A was inoculated near the yolk and eggs B and C on the opposite pole. Note the final inoculum location (arrow) and the point of inoculation (circle).**

## *SALMONELLA ENTERICA* VAR. ENTERITIDIS TRIALS

### HIGH INOCULUM ($10^5$ CFU/ML)

Ante-mortem, nutrient usage and embryonic growth parameters could not be compared between controls and trial eggs due to high and precocious embryonic death of the inoculated embryos in the latter group. Therefore, only embryonic viability parameters will be reported and later discussed. For effects of simplifying reading, all statistical data has been compiled and is present in Annex 5.

## *Embryonic viability*

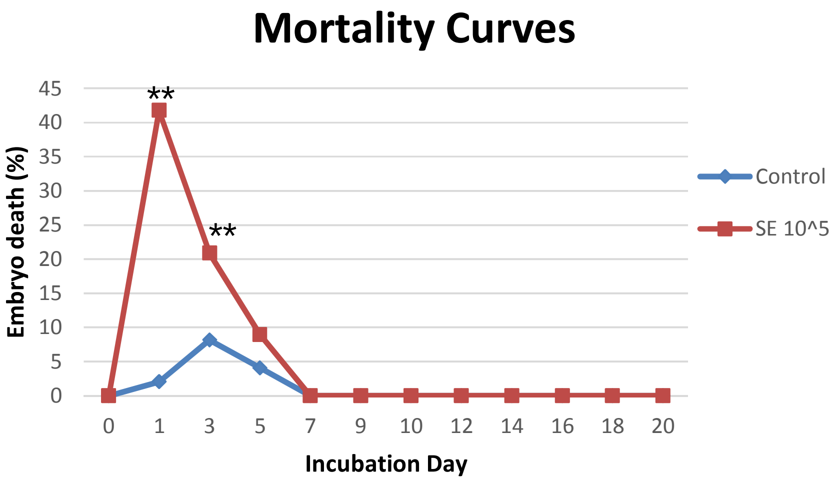
### ***Malformation rates and types***

No malformations were detected on challenged or control embryos.

### ***Mortality rates***

Significantly higher (*p*<0.01) mortality rates were found for the trial group, with 71.6% mortality for inoculated embryos against 14.3% for sham-inoculated embryos (Graph 53). Mortality was restricted to the first seven days of incubation, with most deaths occurring at day 1 (41.8%) and day 3 (20.9%) for challenged embryos. Only a single inoculated embryo survived until day 18 of incubation.

Control embryo mortality rates peaked at day 3 with 8.2% mortality.



*******p*<0.01**

**Graph 53. Distribution of mortality incidence (percentage) during the incubation period for control and *S.* Enteritidis $10^5$ CFU/ml challenged eggs.**

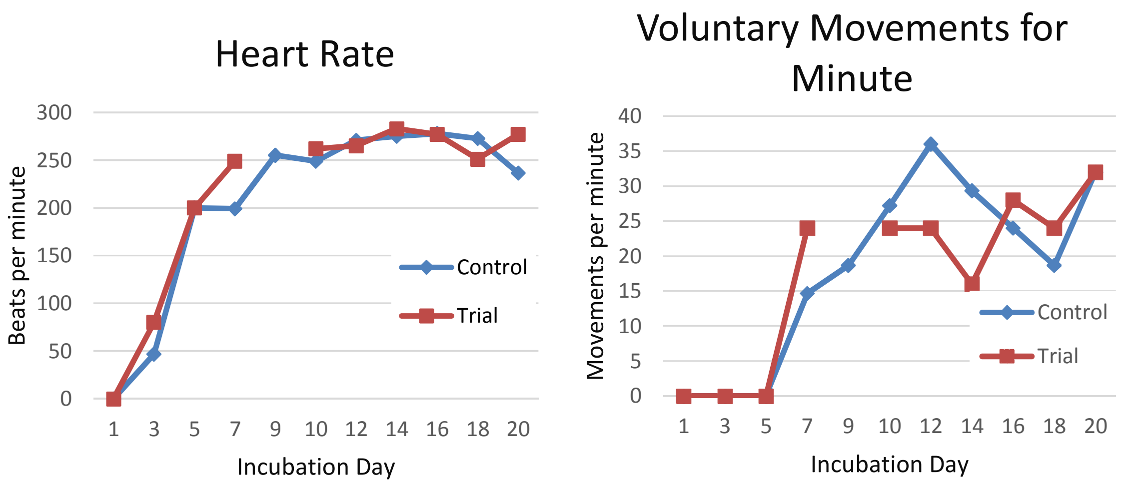
## LOW INOCULUM ($10^2$ CFU/ML)

For effects of simplifying reading, all statistical data has been compiled and is present in Annex 5.

**Ante-mortem observations**

*Behavior*

No statistically significant differences were found between control and challenged embryos, regarding behavioral markers (Graphs 54, 55). The gap in information shown for *Salmonella*-inoculated embryos on day 9 of incubation resulted from no viable embryos having been found in the random sample taken from the trial group on that day.

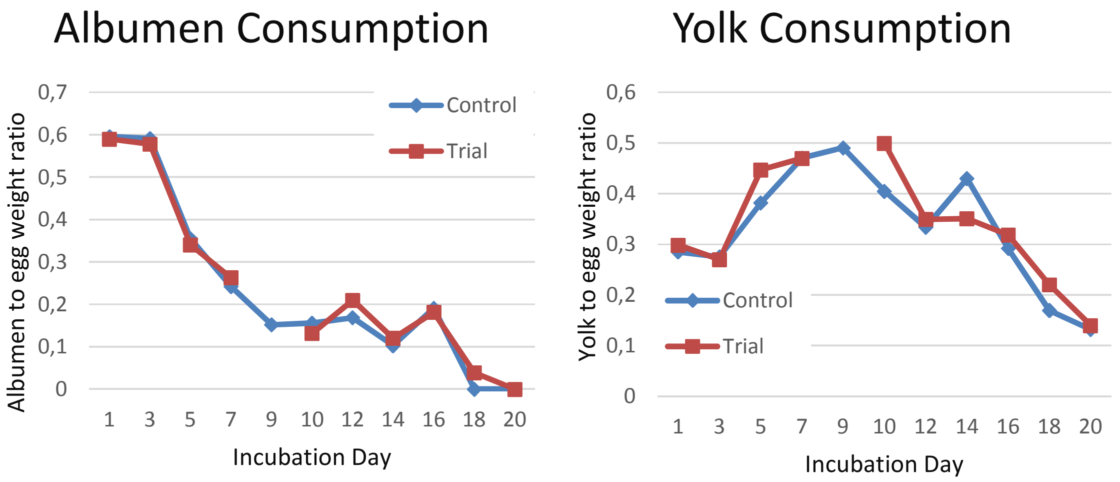


**Graphs 54 and 55. Heart rate (beats/minute) and Voluntary Movements per Minute (movements/min) throughout incubation for control and *S.* Enteritidis $10^2$ CFU/ml challenged eggs.**

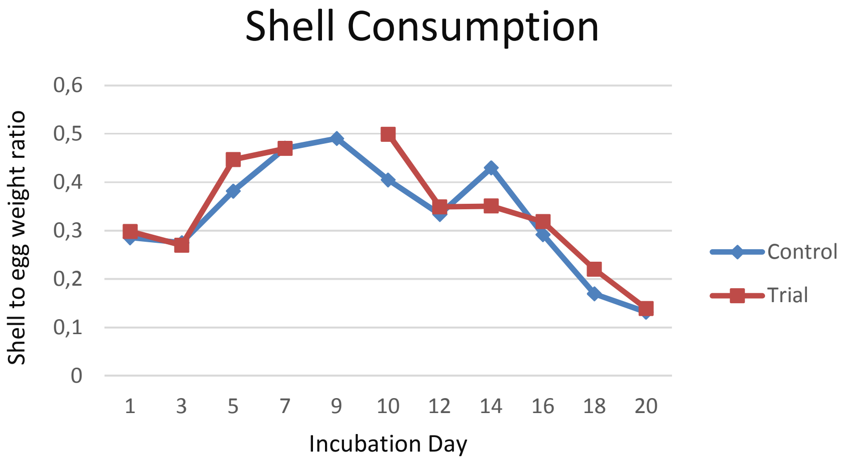
**Post-mortem observations**

*Nutrient reserves usage*

No statistically significant differences were found between control and challenged embryos, regarding nutrient usage markers (Graphs 56-58).



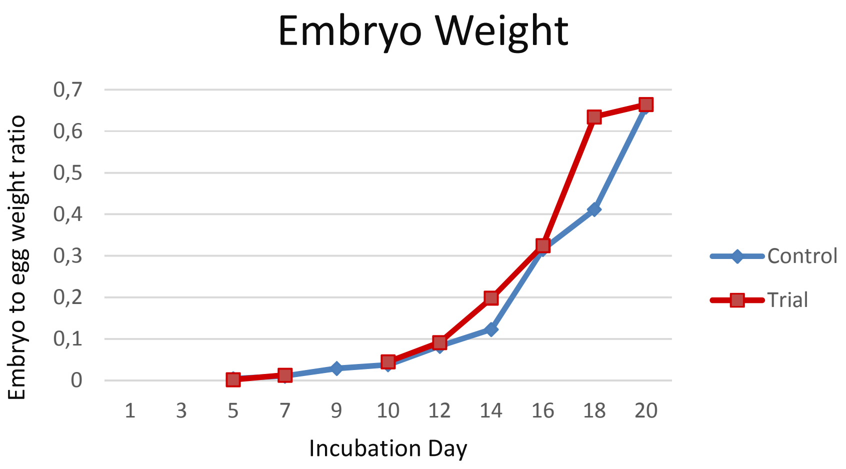
**Graphs 56 and 57. Albumen consumption (albumen weight to egg weight ratio) and Yolk consumption (yolk weight to egg weight ratio) throughout incubation for control and *S.* Enteritidis $10^2$ CFU/ml challenged eggs.**



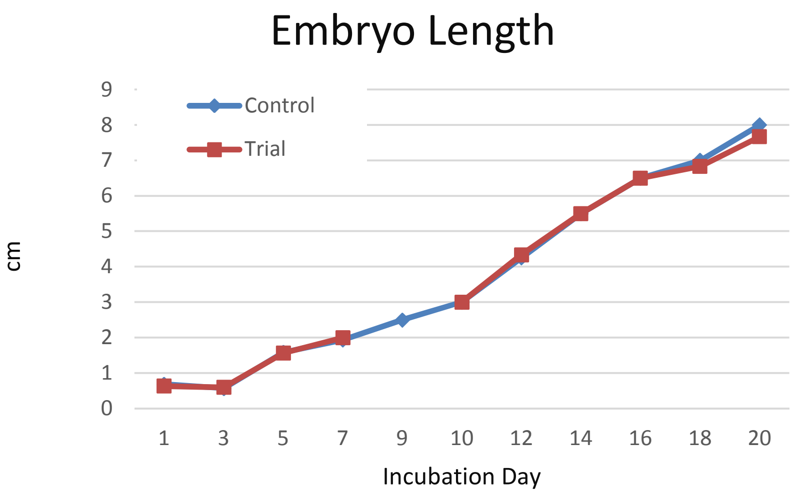
**Graph 58. Shell consumption (shell weight to egg weight ratio) throughout incubation for control and *S.* Enteritidis $10^2$ CFU/ml challenged eggs.**

*Embryonic development*

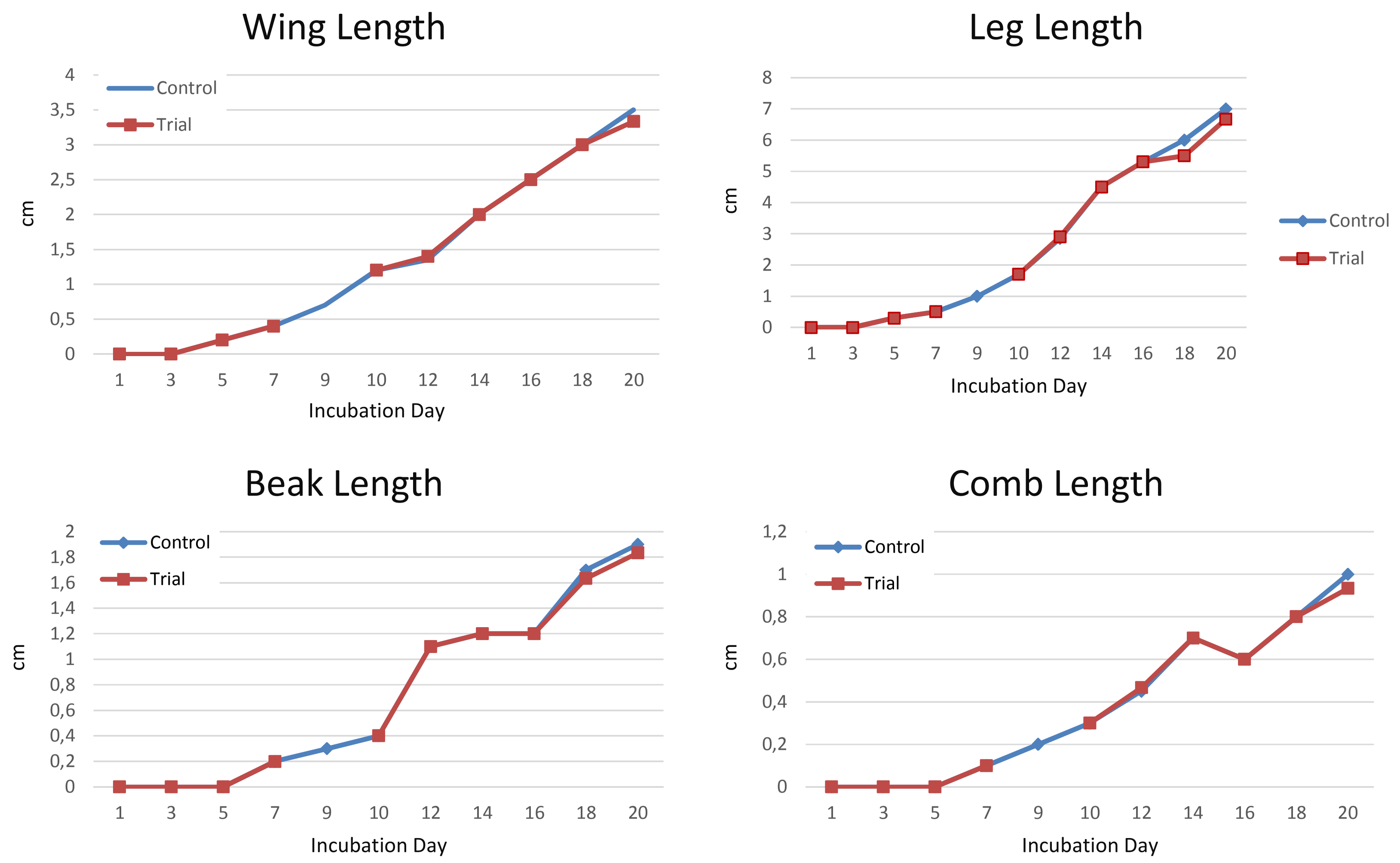
No statistically significant differences were found in the embryonic development and growth of challenged embryos (Graphs 59-68).



**Graph 59. Embryo growth (embryo weight to egg weight ratio) throughout incubation for control and *S*. Enteritidis $10^2$ CFU/ml challenged eggs.**



**Graph 60. Embryo growth (embryo length) throughout incubation for control and *S*. Enteritidis $10^2$ CFU/ml challenged eggs.**

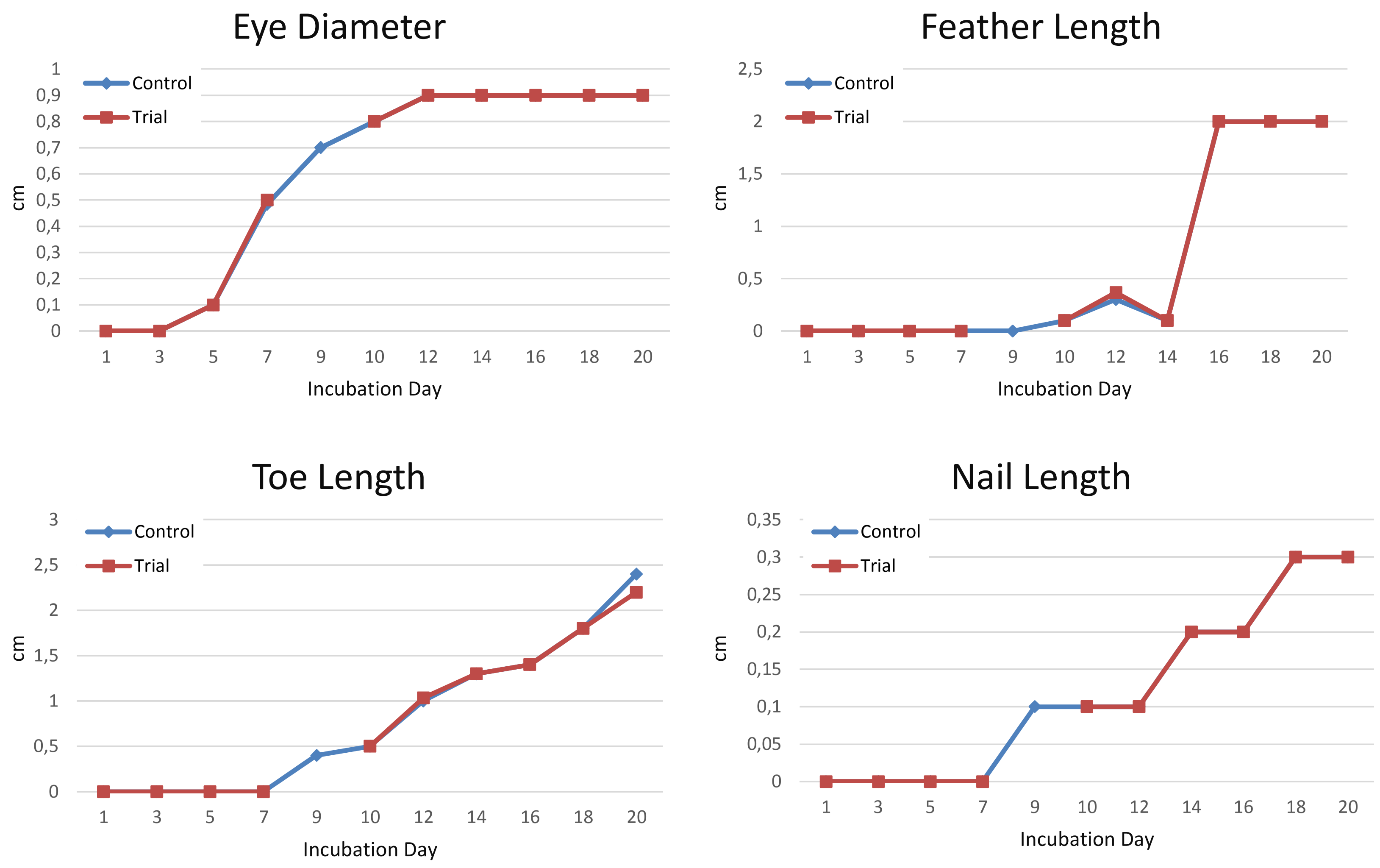

Wing Length

Leg Length

Beak Length

Comb Length

**Graphs 61 - 64. Embryo growth throughout incubation for control and *S*. Enteritidis $10^2$ CFU/ml challenged eggs. Upper Left – Wing Length; Upper Right – Leg Length; Lower Left – Beak Length; Lower Right – Comb Length**



**Graphs 65 - 68. Embryo growth throughout incubation for control and *S.* Enteritidis $10^2$ CFU/ml challenged eggs. Upper Left – Eye Diameter; Upper Right – Feather Length; Lower Left – Toe Length; Lower Right – Nail Length**

*Embryonic viability*

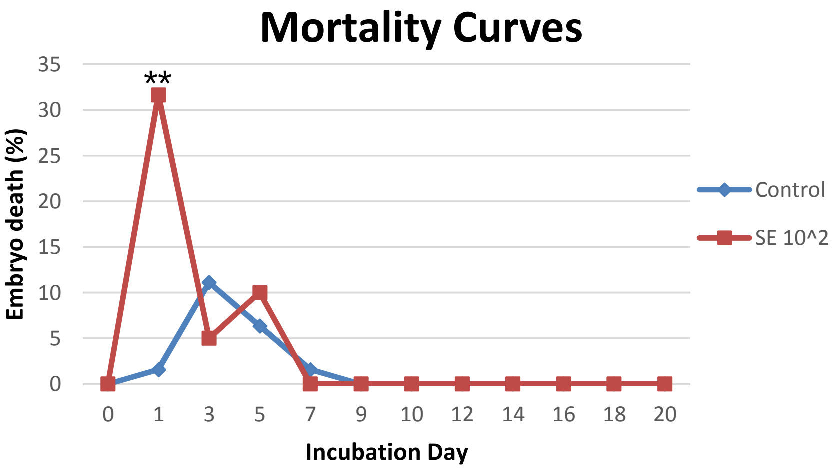
### Malformation rates and types

A single case (2%) of compound malformation was found in the control group, affecting the eyes (bilateral anophthalmia), beak (maxillary hypoplasia) and cranial vault (exencephaly). Two cases (3%) of malformation were found in the challenged group, one of which a simple malformation affecting the eyes (unilateral microphthalmia), and the other a compound malformation affecting the eyes (unilateral anophthalmia) and beak (dysplasia with crossed beak).

### Mortality rates

Significantly higher ($p$<0.01) mortality rates were found for the trial group, with 46.67% deaths of inoculated embryos against 20.6% of sham-inoculated embryos (Graph 69). Mortality was restricted to the first five days of incubation for challenged embryos, with most deaths occurring at day 1 (31.8%), then at day 5 (10%) and day 3 (5%). Half of the eggs open at day 20 contained viable embryos.

Control embryo mortality occurred during the first seven days of incubation, with a peak at day 3 (11.1%).



**$^{**}p$<0.01**

**Graph 69. Distribution of mortality incidence (percentage) during the incubation period for control and *S.* Enteritidis $10^2$ CFU/ml challenged eggs.**

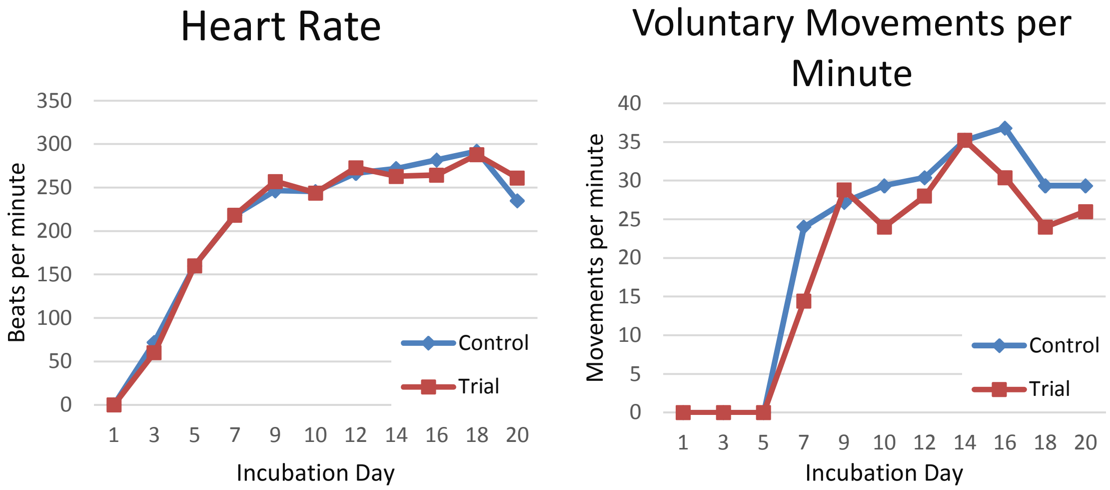
## *SALMONELLA ENTERICA* VAR. TYPHIMURIUM TRIALS
## HIGH INOCULUM ($10^5$ CFU/ML)

For effects of simplifying reading, all statistical data has been compiled and is present in Annex 5.

#### Ante-mortem observations

*Behavior*

No statistically significant differences were found between control and challenged embryos, regarding behavioral markers (Graphs 70, 71).

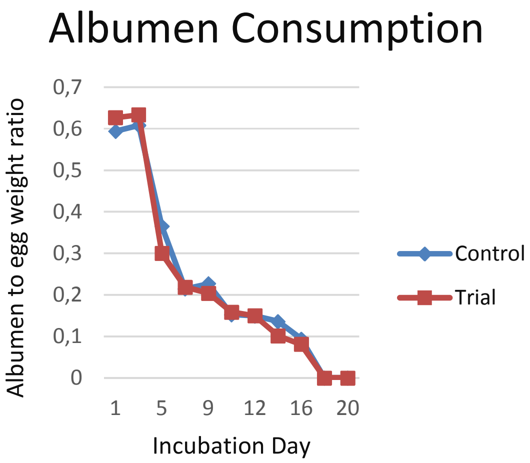


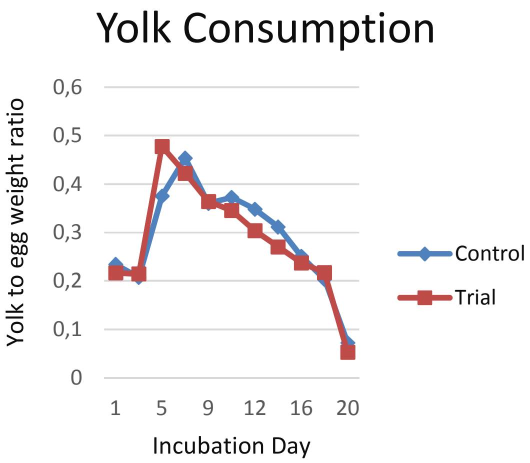
**Graphs 70 and 71. Heart rate (beats/minute) and Voluntary Movements per Minute (movements/min) throughout incubation for control and *S.* Typhimurium $10^5$ CFU/ml challenged eggs.**

#### Post-mortem observations

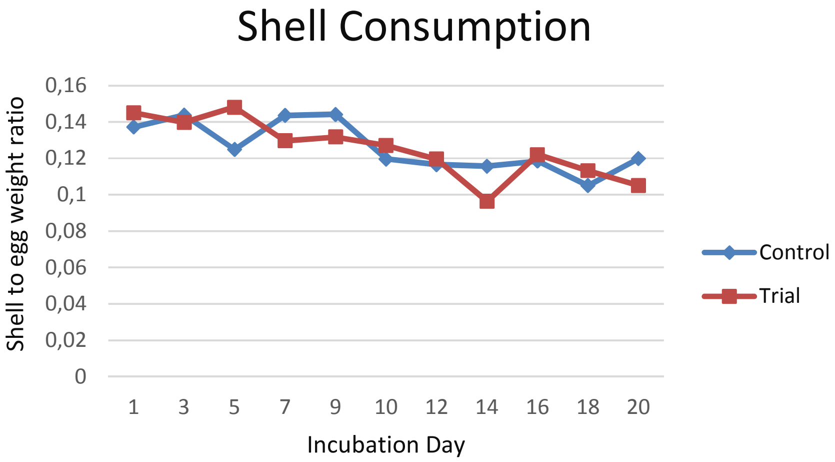
*Nutrient reserves usage*

No statistically significant differences were found between control and challenged embryos, regarding nutrient usage markers (Graphs 72-74).





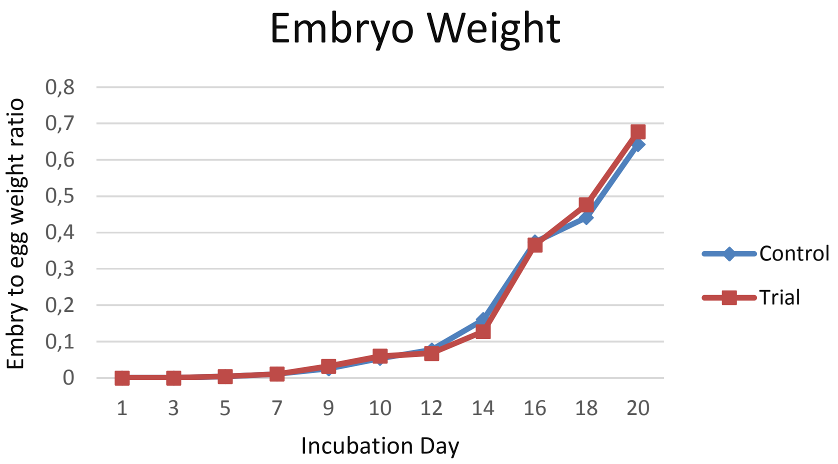
**Graphs 72 and 73. Albumen consumption (albumen weight to egg weight ratio) and Yolk consumption (yolk weight to egg weight ratio) throughout incubation for control and *S.* Typhimurium 10$^5$ CFU/ml challenged eggs.**



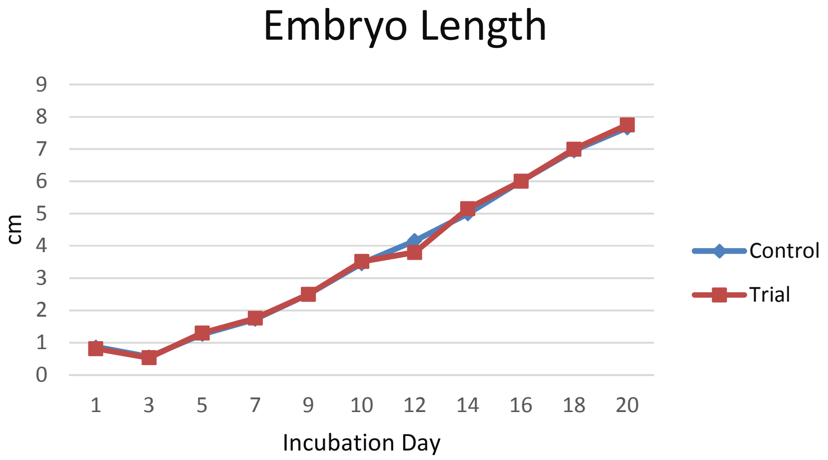
**Graph 74. Shell consumption (shell weight to egg weight ratio) throughout incubation for control and *S.* Typhimurium 10$^5$ CFU/ml challenged eggs.**

*Embryonic development*

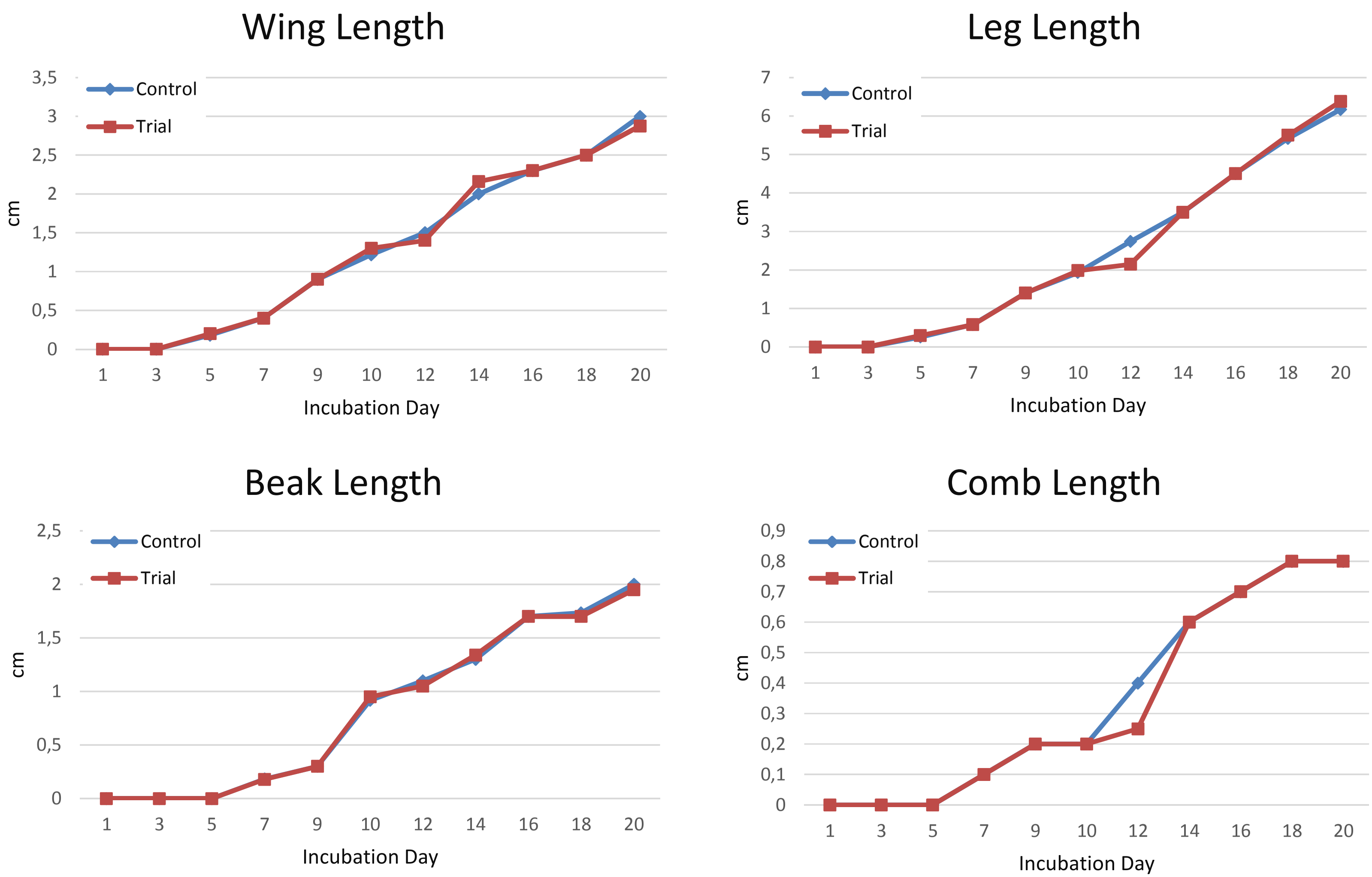
No statistically significant differences were found in the embryonic development and growth of challenged embryos (Graphs 75-84).



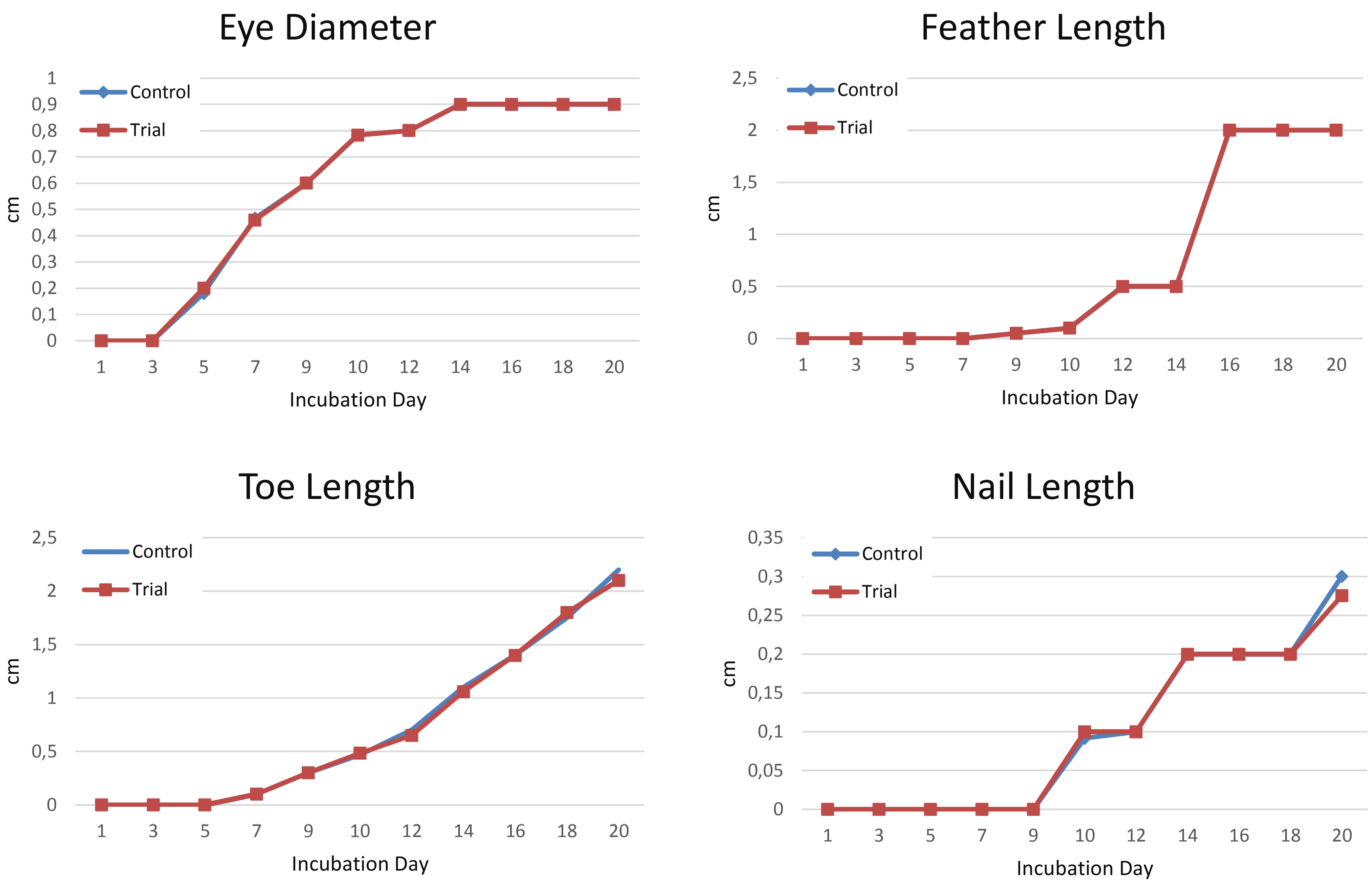
**Graph 75. Embryo growth (embryo weight to egg weight ratio) throughout incubation for control and *S.* Typhimurium $10^5$ CFU/ml challenged eggs.**



**Graph 76. Embryo growth (embryo length) throughout incubation for control and *S.* Typhimurium $10^5$ CFU/ml challenged eggs.**



**Graphs 77 - 80. Embryo growth throughout incubation for control and *S.* Typhimurium $10^5$ CFU/ml challenged eggs. Upper Left – Wing Length; Upper Right – Leg Length; Lower Left – Beak Length; Lower Right – Comb Length**



**Graphs 81 - 84. Embryo growth throughout incubation for control and S. Typhimurium $10^5$ CFU/ml challenged eggs. Upper Left – Eye Diameter; Upper Right – Feather Length; Lower Left – Toe Length; Lower Right – Nail Length**

## *Embryonic viability*

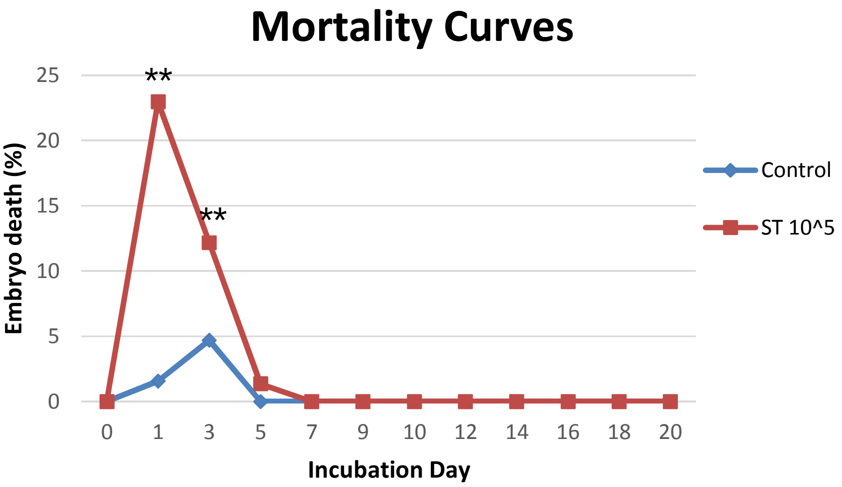
### ***Malformation rates and types***

No statistically significant differences were found between malformation rates for control (2%) and challenged (4%) embryos. Malformations mostly pertained to the head (acrania, anencephaly), eyes (bilateral anophthalmia), and the thoracic wall and heart (ectopia cordis).

### ***Mortality rates***

Significantly higher (*p*<0.01) mortality rates were found for the trial group, with 35.1% deaths of inoculated embryos against 6.3% of sham-inoculated embryos (Graph 85). Mortality was restricted to the first five days of incubation, with most deaths occurring at day 1 (23.0%) and day 3 (12.2%) for challenged embryos.

Control embryo mortality was restricted to the first three days of incubation and peaked at day 3 with 4.7% mortality.



****$p$<0.01**

**Graph 85. Distribution of mortality incidence (percentage) during the incubation period for control and *S.* Typhimurium $10^5$ CFU/ml challenged eggs.**

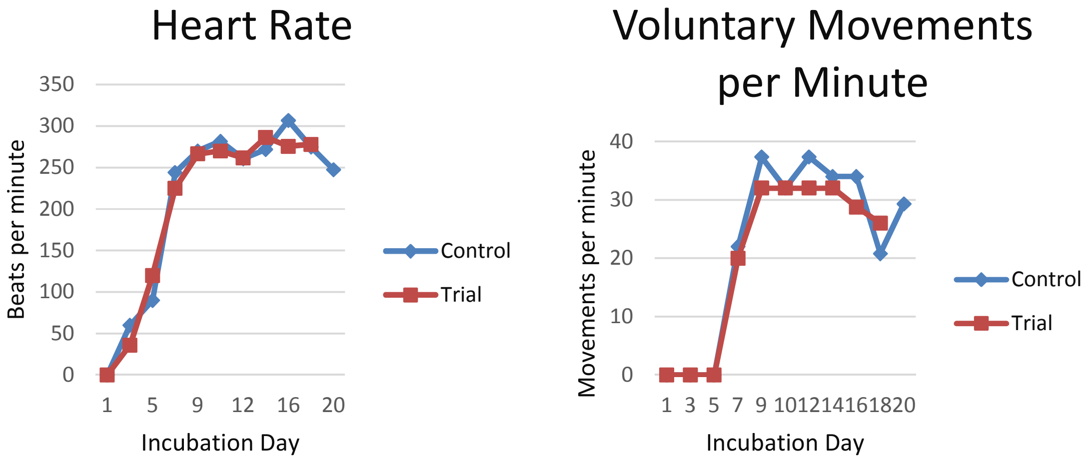
## LOW INOCULUM ($10^2$ CFU/ML)

For effects of simplifying reading, all statistical data has been compiled and is present in Annex 5.

### Ante-mortem observations

*Behavior*

No statistically significant differences were found between control and challenged embryos, regarding behavioral markers (Graphs 86, 87).

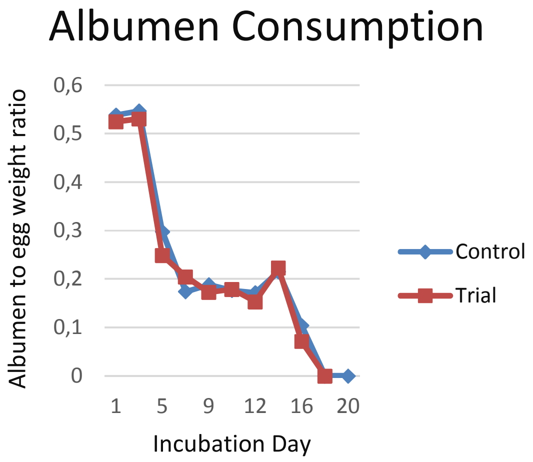


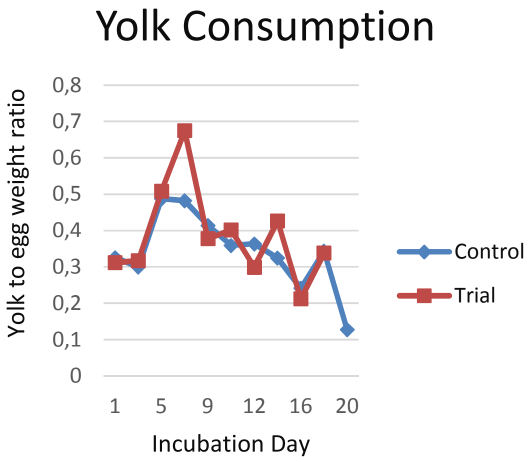
**Graphs 86 and 87. Heart rate (beats/minute) and Voluntary Movements per Minute (movements/min) throughout incubation for control and *S.* Typhimurium $10^2$ CFU/ml challenged eggs.**

### Post-mortem observations

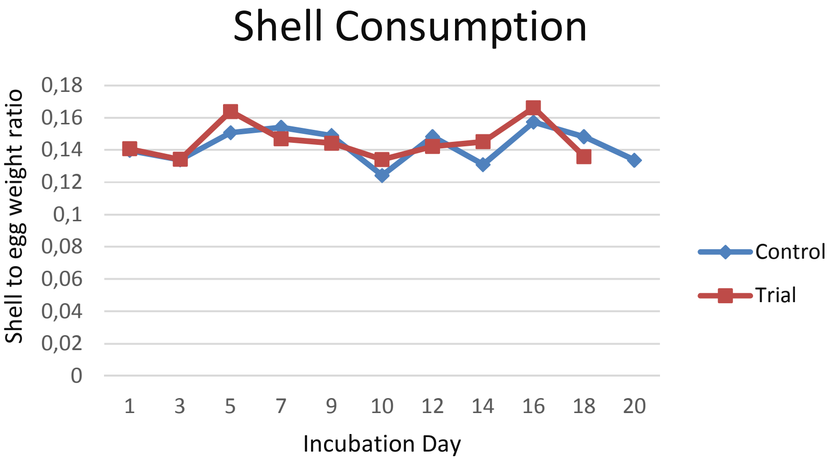
*Nutrient reserves usage*

No statistically significant differences were found between control and challenged embryos, regarding nutrient usage markers (Graphs 88-90).





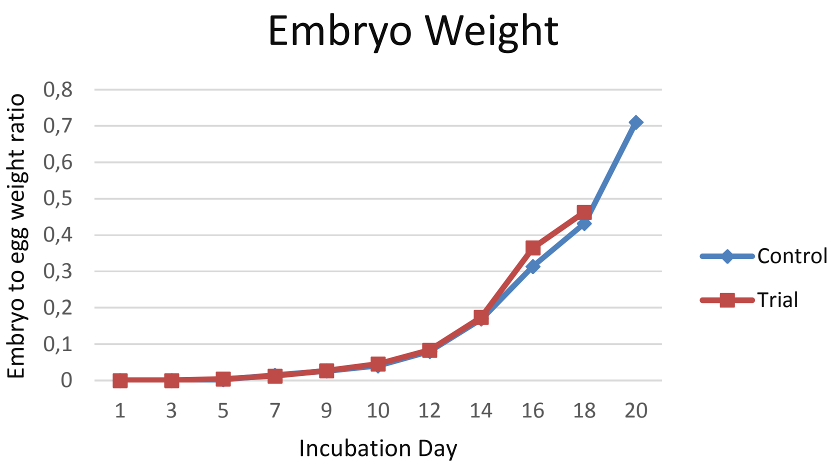
**Graphs 88 and 89. Albumen consumption (albumen weight to egg weight ratio) and Yolk consumption (yolk weight to egg weight ratio) throughout incubation for control and *S.* Typhimurium 10$^2$ CFU/ml challenged eggs.**



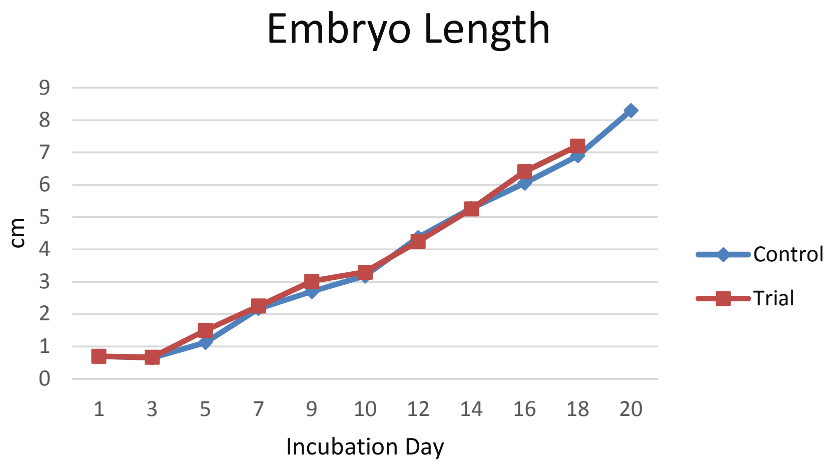
**Graph 90. Shell consumption (shell weight to egg weight ratio) throughout incubation for control and *S.* Typhimurium 10$^2$ CFU/ml challenged eggs.**

*Embryonic development*

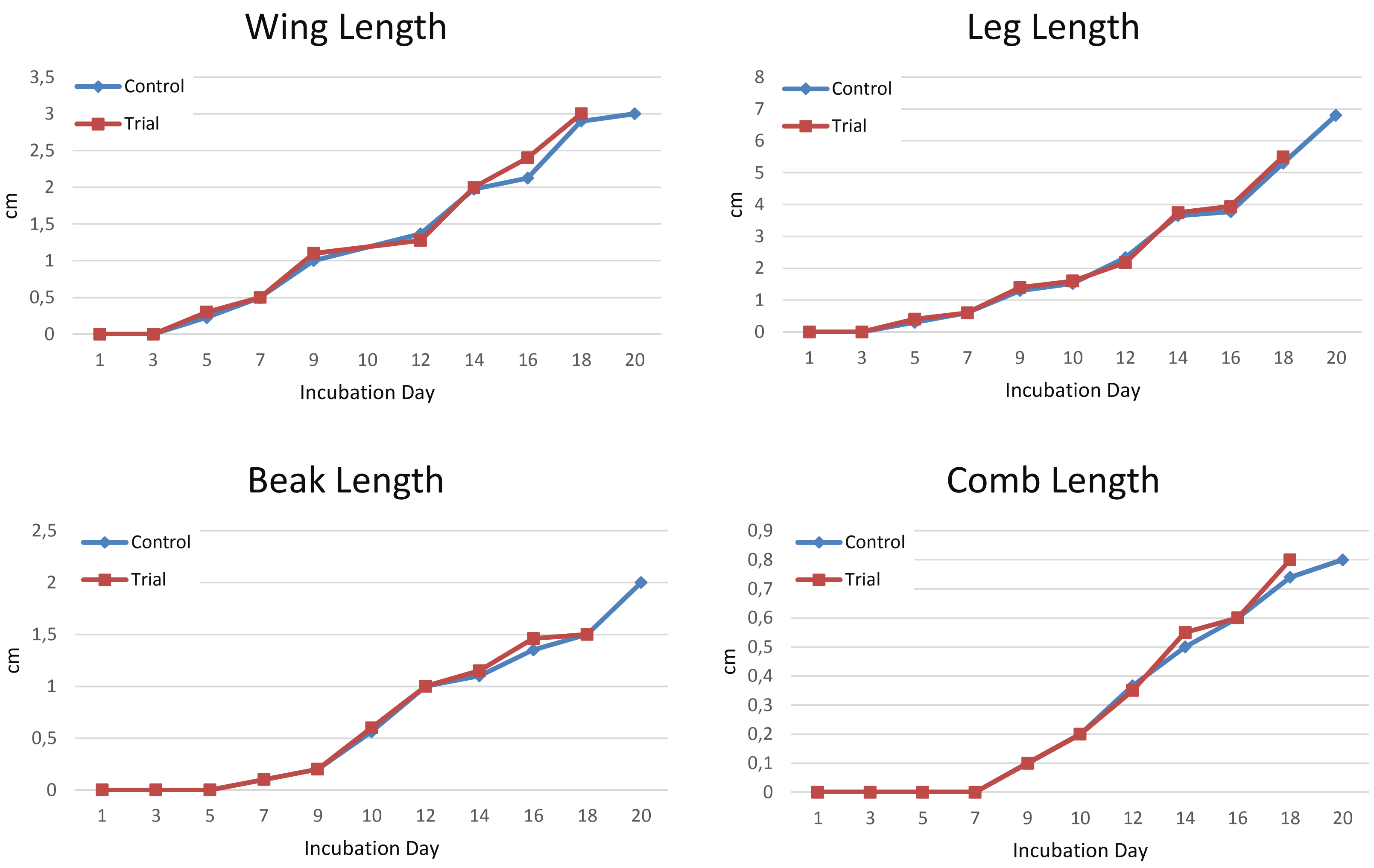
No statistically significant differences were found in the embryonic development and growth of challenged embryos (Graphs 91-100).



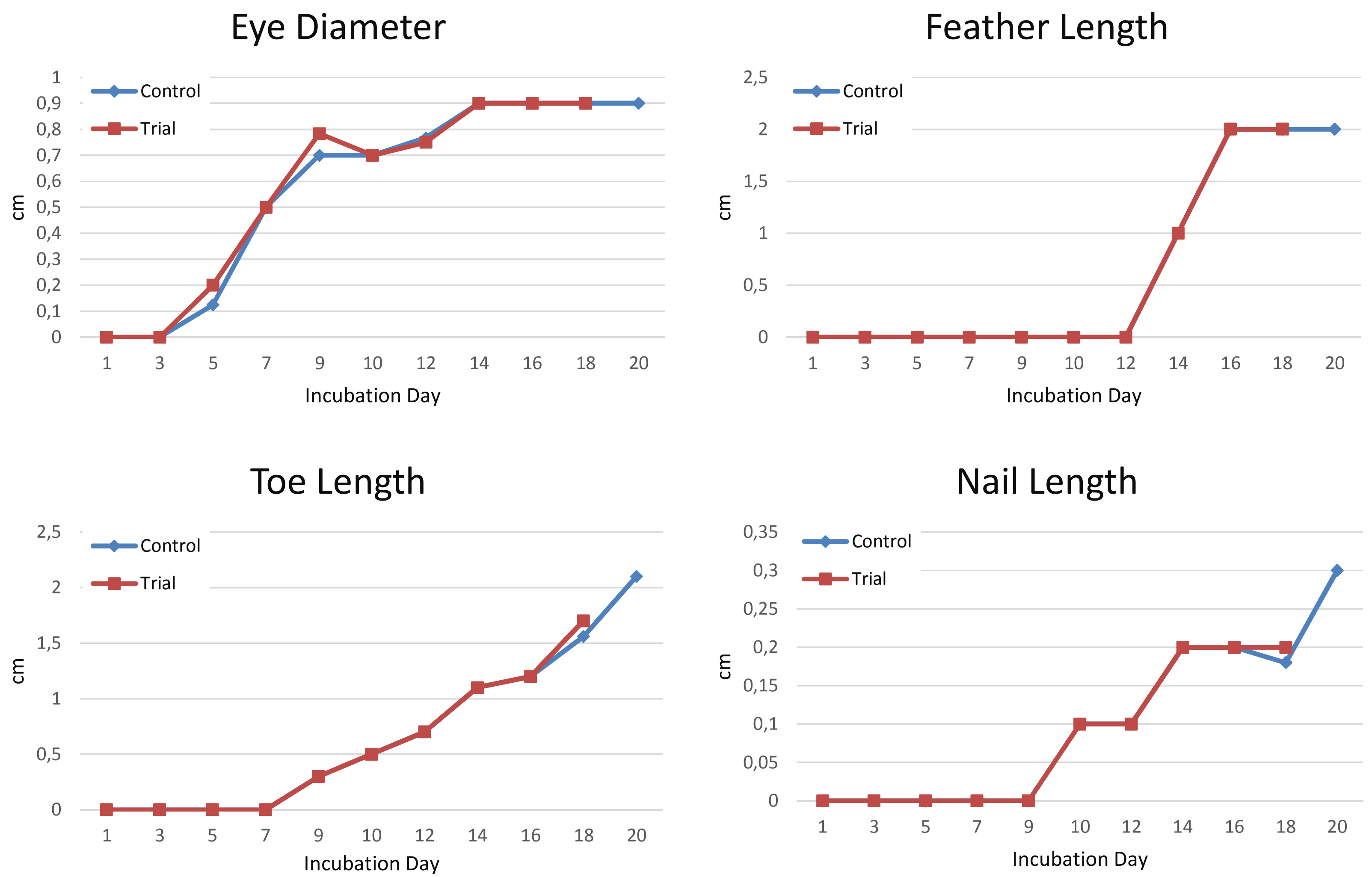
**Graph 91. Embryo growth (embryo weight to egg weight ratio) throughout incubation for control and *S.* Typhimurium $10^2$ CFU/ml challenged eggs.**



**Graph 92. Embryo growth (embryo length) throughout incubation for control and *S.* Typhimurium $10^2$ CFU/ml challenged eggs.**



**Graphs 93 - 96. Embryo growth throughout incubation for control and *S.* Typhimurium $10^2$ CFU/ml challenged eggs. Upper Left – Wing Length; Upper Right – Leg Length; Lower Left – Beak Length; Lower Right – Comb Length**



**Graphs 97 - 100. Embryo growth throughout incubation for control and S. Typhimurium $10^2$ CFU/ml challenged eggs. Upper Left – Eye Diameter; Upper Right – Feather Length; Lower Left – Toe Length; Lower Right – Nail Length**

*Embryonic viability*

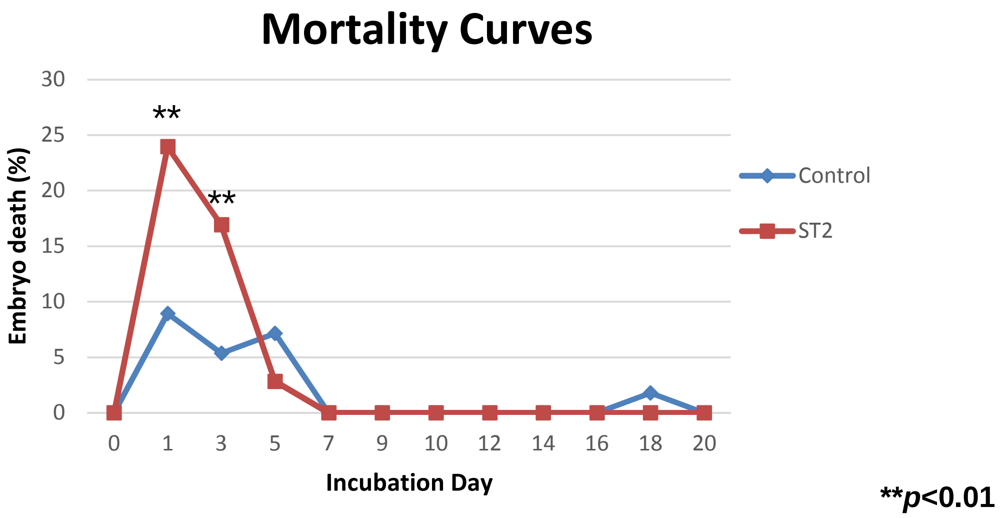
### ***Malformation rates and types***

No malformations were detected on challenged or control embryos.

### ***Mortality rates***

Significantly higher (*p*<0.01) mortality rates were found for the trial group, with 39.7% mortality for inoculated embryos against 24.1% for sham-inoculated embryos (Graph 101). Mortality was restricted to the first five days of incubation, with most deaths occurring at day 1 (27.4%) and day 3 (11.0%) for challenged embryos.

Control embryo mortality occurred in two peaks, a greater one at day 1 (19.0%) and a smaller one at day 5 (5.2%).



**Graph 101. Distribution of mortality incidence (percentage) during the incubation period for control and *S.* Enteritidis $10^2$ CFU/ml challenged eggs.**

## GENERAL CONSIDERATIONS

In none of the trials were there any lesions or histological changes found in viable, *Salmonella*-positive embryos. No microscopic differences were detected between these embryos and control embryos.

Lesions found in non-viable embryos were, similarly, not significantly different from those found in dead-in-shell control embryos. However, trial embryos found dead in the egg consistently presented with more advanced decomposition, with loss of affinity for nuclear stains and generalized acidophilic staining (Figure 57).

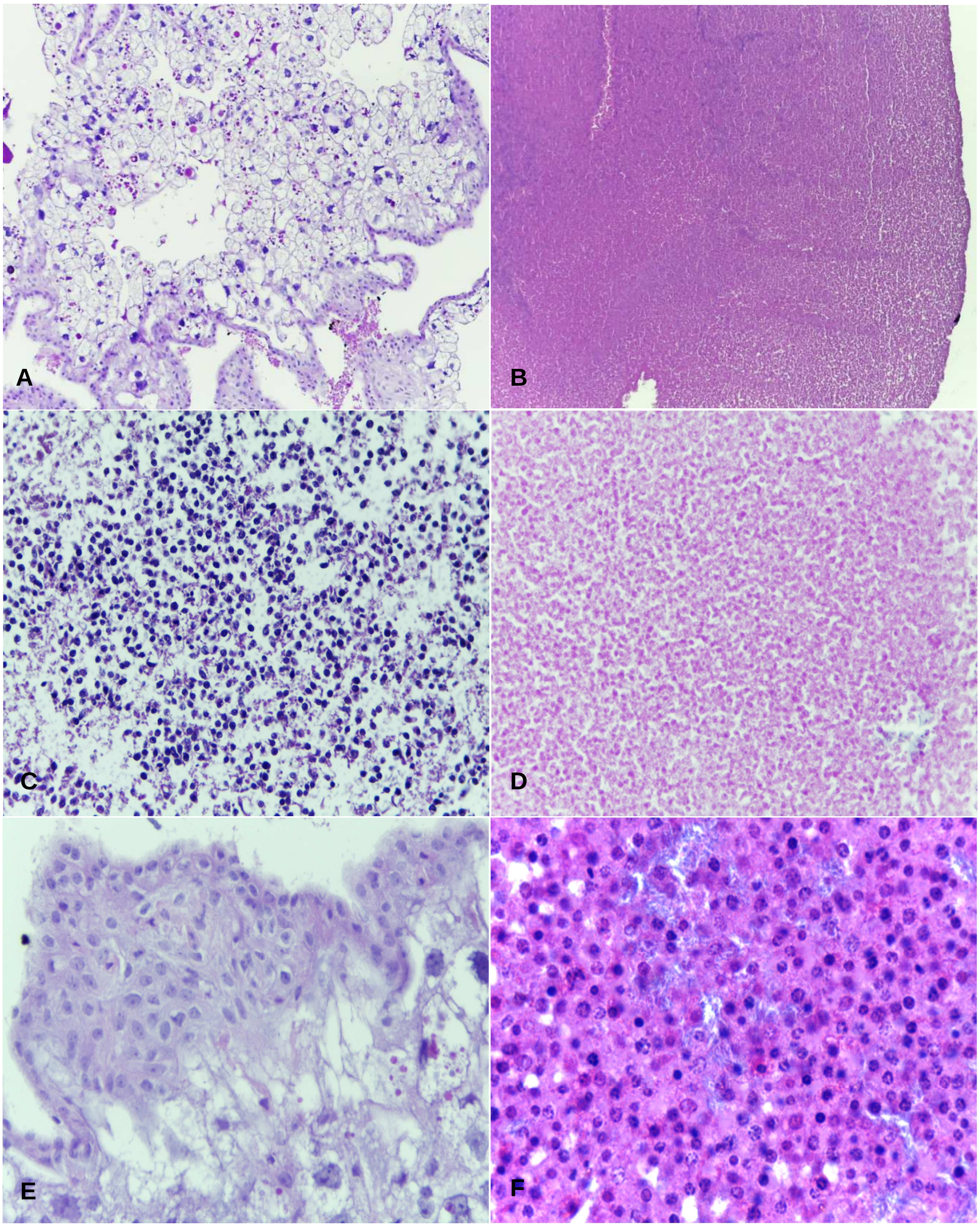


**Figure 57 (A-F). Microscopy images of dead in-shell embryos.**
**A – Control embryo, extra-embryonic membranes (H&E, 100x); B – *Salmonella*-positive embryo dead and found at the same timepoint as embryo A. Note the predominantly acidophilic staining (H&E, 40x); C (control) and D (trial) – embryos dead at day 5 of incubation. Note the loss of nuclear staining in image D (H&E, 400x); E – Control embryo. Note the still visible cellular limits and lightly acidophilic cytoplasm (H&E, 1000x); F – Trial embryo. Note the heavily acidophilic cytoplasm, loss of cellular limits and bacterial aggregates (H&E, 1000x).**

## DISCUSSION

In spite of having been scientifically demonstrated to occur, contamination of egg contents through penetration of the eggshell after fecal contamination is not likely to be the major route of contamination, as indicated by studies performed using a variety of *Salmonella* serotypes and non-*Salmonella* species where *S.* Enteritidis was the only bacterium isolated from egg contents in spite of various other bacteria having been isolated from eggshells (De Buck et al., 2004). Added to the comparatively higher prevalence of *S.* Enteritidis - the most efficient of *Salmonella* serovars to colonize the chicken reproductive tract - results point to internal contamination being more likely to occur during formation of the egg than after lay (De Buck et al., 2004; Guard-Bouldin & Buhr, 2006; Guard-Petter, 2001).

In this study, bacteria were inoculated directly into the egg, in close proximity of the yolk, bypassing the various layers of the albumen in order to mimic vertical transmission originating from infection of the ovarian follicles after the spread of Salmonella through the layer’s body. Moreover, experimentally infected laying hens have often been reported to deposit *Salmonella* Enteritidis on the vitelline membrane (Gast & Holt, 2001). It is interesting to note, however, the curious results obtained during the pre-trial inoculation experiments, in which bacteria of both serovars of Salmonella tested were isolated from the yolk (and the embryonic disc) after deposition of the inoculum in the albumen, at the most distant point possible from the yolk, and overnight incubation.

Both *S.* Enteritidis and Typhimurium are flagellate, mobile bacteria with the ability to penetrate mucosae and eggshells and membranes in order to disseminate (Prescott, Harley, & Klein, 2005). It is believed that *Salmonella* cells that are deposited in the albumen are able to migrate to and penetrate through the vitelline membrane in the egg post-lay, in order to reach the yolk and thus gain access to the pool of nutrients therein (Gast et al., 2010). Flagella are thought to be necessary components for bacterial migration towards the vitelline membrane in whole eggs and non-motile mutants and serovars do not exhibit relevant bacterial growth in egg contents (Gantois et al., 2009; Gantois et al., 2008). As the egg ages, the vitelline membrane gradually deteriorates, resulting in the release of nutrients into the albumen, possibly attracting bacteria that can penetrate the vitelline membrane and multiply in the nutrient-rich yolk (Gantois et al., 2009). Gantois et al. (2009) have hypothesized that leakage out of the yolk into the albumen would generate a gradient of amino acids, sugars or other yolk components, triggering a chemotactic movement towards the vitelline membrane. This hypothesis is consistent with the fact that *S*. Enteritidis grows rapidly in eggs only after 28 days of storage at room temperature (Gantois et al., 2009).

In this study, however, the bacterial inoculum not only managed to migrate through the albumen toward the yolk, along the longer axis of the egg, but also seemed to become located exclusively above and around the embryonic disc, remaining in close relation to it even when the egg was manipulated and re-positioned. This highly specified location of the inoculum indicates that, in what fertilized, incubating eggs are concerned, *Salmonella* may have a strong positive tropism not just toward the yolk, but toward the embryo proper, as well as the necessary biological tools to cross egg compartments and reach its main target. Although the storage periods to which the eggs in this study were subjected were far shorter than those reported by Gantois et al., results may still coadunate with Gantois' theory regarding the leakage of nutrients through the vitelline membrane as a chemotactic factor in the migration of *Salmonella* toward the yolk if the following is considered: compared to the rest of the yolk, the embryonic disc is an area of intense metabolism, cell migration and nutrient and osmotic flow, especially during the first two days of incubation.

As mentioned above, during this period, the albumen penetrates through the vitelline membrane and accumulates distal to the outer area of the blastoderm (Babiker & Baggott, 1995; Bellairs & Osmond, 2005). Additionally, the ectoderm of the embryo seems to control sodium transport across the vitelline membrane (Babiker & Baggott, 1995). This ionic and nutrient flow through such a specifically located area of the yolk membrane may serve as enough of a chemotactic stimulus for *Salmonella*, guiding the bacteria present in the albumen not just toward the yolk, but specifically toward the embryonic disc, where nutrients are more readily available than across the remainder of what is still an integral yolk membrane (Gantois et al., 2009). This early tropism toward the embryo could have an additional biological meaning, since *Salmonella* is also known to infect embryos, producing persistently infected, asymptomatic chicks that will later serve as a reservoir of the bacteria for the whole nascent flock (Pattison, 2008).

In fact, none of the viable embryos from eggs inoculated with either of the *Salmonella* serotypes used in this study exhibited any significant changes regarding behavior, nutrient usage or embryonic growth, indicating that infection with *Salmonella* may have no effect on overall embryonic development and performance if the embryo is capable of surviving its coexistence with the bacterium. The absence of histological lesions or changes of any kind in inoculated embryos further strengthens this theory. Indeed, no bacterial aggregates were detected on microscopy observation of slides produced from inoculated embryos which tested positive for the presence of *Salmonella* on PCR analysis. Many serovars of *Salmonella enterica* behave as facultative intracellular parasites capable of surviving destruction in phagocytic cells, such as macrophages, and even proliferating in membrane-bound cytoplasmic vacuoles, to a greater or lesser extent depending on the environment provided by each different type of host cell. It is in this fashion that *Salmonella* is thought to disseminate, using the host's own immune network to its advantage. Experimental and natural infection studies performed in several species point to the Peyer's Patches and spleen as permissive sites for *Salmonella* intracellular proliferation and dissemination through the reticuloendothelial system (Garcia-del Portillo, 2001). Further molecular studies to detect how and where in the embryo *Salmonella* tends to proliferate are necessary if any further interpretations are to be made from the results obtained here but the absence of a visibly active, infection-related stress response underlines how easily the presence of *Salmonella* in a flock can go undetected if only hatchling performance is taken into account. Moreover, it underlines how highly adapted the bacterium is to this specific host.

However, vertical infection with *Salmonella* is not without its clinical signs, non-specific as they may be. The most striking consequence of the vertical infection induced in this study was a sudden increase in embryonic mortality, most prominently limited to the first five days of incubation and embryonic development. Note that for the *S.* Enteritidis $10^5$ trial group, only two of over seventy embryos inoculated survived past day 7 of incubation, while only a little over 50% of all embryos inoculated were found to be viable on break-out at their designated timepoints for the *S.* Enteritidis $10^2$ group. While concentrations of $10^5$ CFU/ml are an exaggeration when compared to the 10 colony forming units reported for naturally infected eggs, the $10^2$ CFU/ml (with a 0.1 ml inoculum) used for the *S.* Enteritidis $10^2$ group should closely mimic naturally occurring infection, the same occurring for the *S.* Typhimurium $10^5$ and *S.* Typhimurium $10^2$ groups. It is interesting to remark that for both serovars tested total embryonic mortality was approximately five times higher than controls in the high inoculum challenge groups, and twice as high as control-embryo mortality rates for the low inoculum challenge groups.

An argument may be made that the first five days constitute the critical the critical make-it-or-break-it period, during which the embryo, exposed to the bacterium, must either adapt to its presence (and vice versa) or perish, leaving the nutrient-rich yolk available for the microbe to consume without any competition from the now deceased embryo. In effect, while none of the viable embryos showed any visible signs of presence of the bacterium on histology, all of the samples taken from embryos found dead had frequent and evident bacterial aggregates, easily spotted among the cellular sheets of quickly decomposing embryonic tissue. Whether embryonic death was the result of early, intense bacterial proliferation or if the bacteria proliferated only after the embryo had expired is impossible to determine.

It is also noteworthy that mortality curves for both challenged and control embryos were similar in shape, with embryonic death being mostly limited to the first five days of incubation for sham-inoculated embryos. This somewhat conflicts with the above stated as it shows a clear susceptibility of embryos during this stage, to changes that are incompatible with life, regardless of the presence or absence of a pathogen. However, the amplitude of the difference between mortality rates makes obvious the impact that such a challenge as infection with *Salmonella* presents to embryos.

Elevation in mortality rates was evident for both serotypes tested but comparatively more pronounced after challenge with *S.* Enteritidis. This could mean that, although vertical infection with *S.* Enteritidis is far more likely to occur (De Buck et al., 2004), it is also more likely to lead to embryonic death than infection with *S.* Typhimurium, possibly due to the former's greater adaptation for survival and proliferation in the egg. Further studies are necessary to confirm this.

Either way, even though infection with *Salmonella* does not seem to actively interfere with embryonic performance by delaying growth or otherwise altering normal metabolic behavior in viable, growing embryos (as, for instance, incubation temperature manipulations do), it interferes with the overall hatcher's performance by reducing hatchability (i.e., the number of chicks hatched per number of eggs incubated) due to an increase in early embryonic mortality. *Salmonella* should, therefore, be considered a prime suspect whenever drops in overall hatchery performance appear to be connected to elevated early mortality, especially if no other changes in embryo/chick performance are detected.

# PART THREE - APPLICATION OF LABORATORY INFORMATION TO A FIELD SCREENING

## INTRODUCTION

In the previous chapters, an experimental approach was taken to study the effects on embryonic development and performance of unfavorable incubation temperature, relative humidity and carbon dioxide conditions. The effects of vertical infection with *Salmonella enterica* serovars Enteritidis and Typhimurium were likewise studied.

However, experimental, laboratory work is only as meaningful to field operations as it is able to properly mirror field conditions and, in a highly controlled environment, provide information that can be applicable to the reality of the setting it aims to study.

For this purpose, in this chapter the information previously gathered from embryos in incubation trials led under carefully monitored, aseptic conditions, was applied to the real-life scenario of a field screening of samples collected from an industrial hatchery. This was made possible by collaboration with a major industrial hatchery, which kindly provided a random sample of eggs that, after 21 days of incubation in a regular, high-capacity incubator, had completely failed to hatch.

These samples were analyzed as thoroughly as possible, keeping in mind that any issues detected might not necessarily relate to changes in the variables previously studied. The eggs were not only analyzed according to the formerly described procedures for the laboratory trials but also examined as thoroughly as possible, in the spirit of true hatchery practices and performance evaluation.

# MATERIALS AND METHODS

Fifty one eggs (Figure 58) were obtained from a commercial hatchery. All eggs originated from the same flock and lot of 45 week-old Ross 300 breeder hens, and then been set to incubate in trays in a ChickMaster incubator following the incubation protocol available in Annex 6. Fertility rates were estimated at 98% via random sampling. Eggs had then moved to the hatcher at day 18 of incubation and set to hatch in baskets following the protocol available in Annex 2 Table 2. Hatching rates were calculated at 87.2%.

**Figure 58. Eggs prior to processing.**

Once in the laboratory, the eggs were weighed, evaluated for shell integrity and contamination with either excreta or blood, and then aseptically opened in the laminar air flow chamber. A sample of albumen and/or yolk was aseptically taken from each egg for classical bacterial culture and DNA extraction. The embryos were then fixed in 10% buffered formalin and allowed to fixate before any further manipulation was undertaken to reduce the risk of microbiological spread to the operator. All fixed embryos were measured, weighed and photographed and taken *in toto* for routine histopathology processing.

Samples were streaked onto Blood Agar and MacConkey Agar and inoculated into BHI medium. After incubation for 24 hours at 37ºC, samples with growth were again streaked onto Blood Agar and incubated overnight at 37ºC. Isolates negative on Gram staining were then tested for oxidase activity and further streaked onto an indole-methyl red-Voges-Proskauer-citrate (IMViC) test kit if found negative. All oxidase-positive isolates were further tested with an API20NE® (bioMérieux) test kit. Isolates positive on Gram staining were tested for catalase activity.

To detect the presence of Salmonella strains, samples were pre-enriched by inoculation in BPW and incubated overnight at 37ºC. Samples with positive bacterial growth were then inoculated into RVS and left to incubate overnight at 42ºC. Further inoculation into Hektoen Enteric Agar, Xylose Lysine Deoxycholate Agar (XLD) and Triple Sugar Iron Agar (TSI) mediums was performed and incubation carried out at 37ºC for 24 hours. An API-20E® (bioMérieux) test kit for the identification of enteric bacteria was subsequently used for species identification.

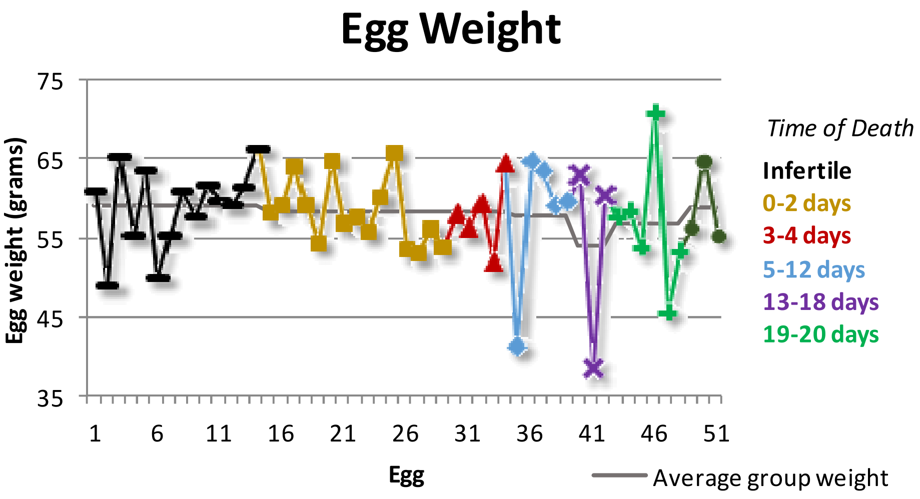
Direct DNA extraction and subsequent Multiplex PCR testing of egg content samples for the presence of *Salmonella* strains was performed according to the protocol described in the previous, *Salmonella*-related chapter.

# RESULTS

## Gross analysis

### Egg weight

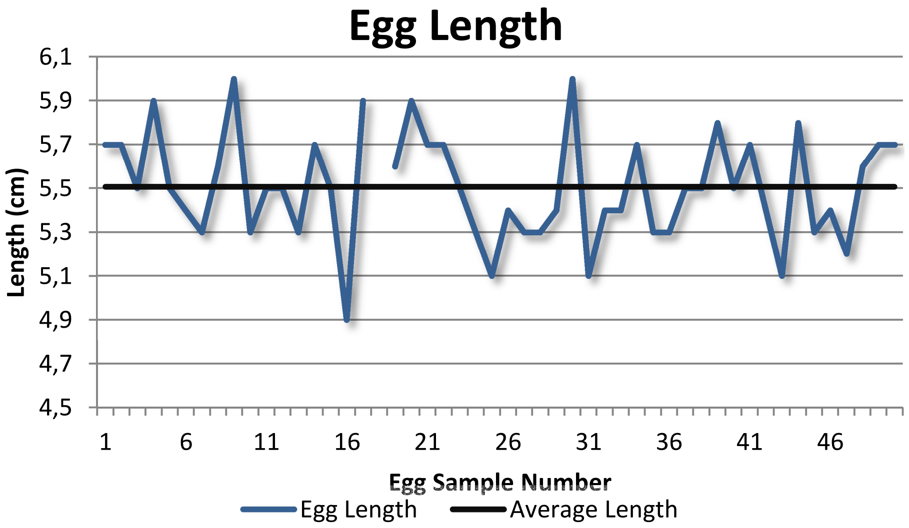
Egg weights ranged between 38.7 g and 71.16 g (average weight 58.1 g ± 6.1 g). Once the eggs had been opened and fertility and time of death estimated, their respective egg weights were organized in seven groups: infertile eggs (average weight 59.2 g ± 8.5 g), eggs with embryos deceased between days 0 and 2 of incubation (average weight 58.3 g ± 4.1 g), eggs with embryos between 3 and 4 days of development (average weight 58.3 g ± 4.6 g), eggs with embryos dead between days 5 and 12 of incubation (average weight 57.9 g ± 9.4 g), eggs with embryos between days 13 and 18 of embryonic development (average weight 54.1 g ± 13.4 g), eggs with 19 to 20 day-old embryos (average weight 56.9 g ± 8.3 g) and, finally, eggs with embryos in the 21st (or hatching) day of incubation (average weight 59.0 g ± 5.1 g). Average egg weights did not vary significantly between groups but varied greatly within groups, as is visible in Graph 102. The smallest egg weight amplitude was found in the 21 day group, with a difference of 9.4 g between the lightest and the heaviest eggs. The 19 to 20 day group exhibited the highest weight amplitude with variations as high as 25.2 g between eggs.



**Graph 102. Registered egg weights according to fertility and time of death.
It is noticeable how egg weights vary greatly within groups, but not between them.**

### Egg dimensions and shape

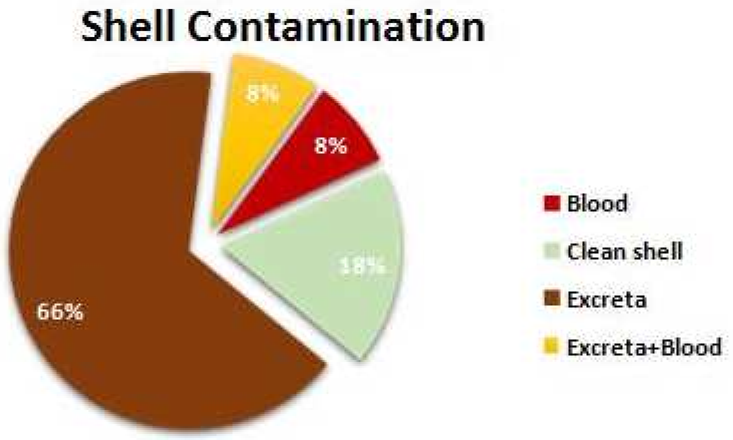
Egg shape varied greatly, from nearly round to ellipsoid. Average egg length (long axis length) was 5.5 cm ± 0.3 cm, with minimum length registered at 4.9 cm and maximum registered egg length of 6 cm (Graph 103).



**Graph 103. Egg dimensions recorded according to length of the longer axis.**

**Shell hygiene and contamination**

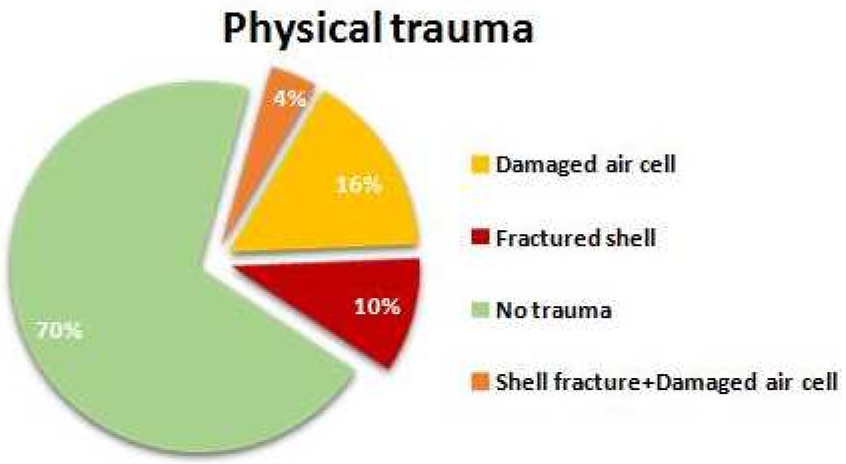
Of the 51 eggs analyzed, only 9 eggs (18%) exhibited a clean or negligibly soiled shell. Of the remaining eggs, 76.5% (n=39) had shells moderately to heavily contaminated with excreta, 8% (n=4) with blood and 8% (n=4) with both (Graph 104).



**Graph 104. Distribution of eggs according to presence and type of contamination.**

**Shell integrity and trauma**

A large majority (70%) of eggs did not present any signs of trauma (Graph 105). Signs of trauma detected were mainly fractures in the shell proper and rupture (or dislocation) of the air cell. The former were detected in five eggs (10%) and the latter in 8 eggs (16%). An additional 2 eggs (4%) presented both.



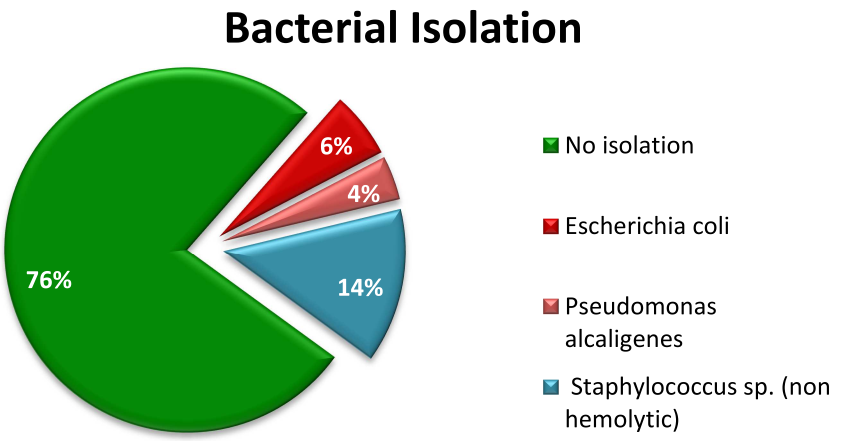
**Graph 105. Egg distribution according to shell and air cell integrity.**

## Bacterial isolation

Thirty eight (76%) of the 51 samples analyzed were negative on classical bacterial isolation (Graph 106). Seven (14%) eggs presented growth of *Staphylococcus* sp. (non-hemolytic). *Escherichia coli* was present in 3 eggs (6%) and *Pseudomonas alcaligenes* in 2 (4%). No mixed bacterial populations were found and no *Salmonella* species were isolated from any of the samples or detected on PCR analysis.

Of the 12 eggs with positive bacterial growth, 9 presented shells contaminated with either excreta or blood. Six of these eggs also presented signs of trauma but only 2 had fractured shells. *E. coli* and *Staphylococcus sp.*, respectively, were isolated from these two eggs.

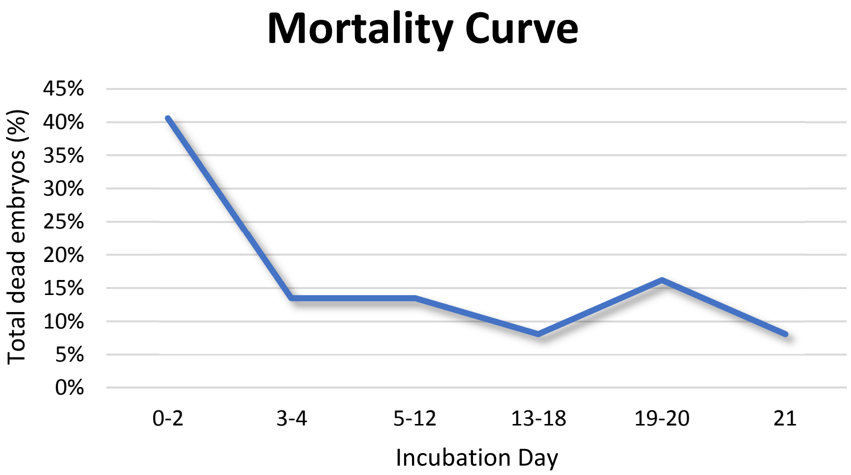
In two eggs, bacterial aggregates were seen on histology but not isolated from the respective collected samples.



**Graph 106. Distribution of eggs according to presence or and type of bacterial contamination/infection.**

## Time of embryonic death

Fourteen (27. 5%) of the fifty one samples were found to be infertile eggs. The embryos found in the remaining, fertile eggs, were divided into the following groups, according to estimated embryonic age at the time of death: embryos deceased between days 0 and 2 of incubation (40.5% of fertile eggs), embryos between 3 and 4 days of development (13.5% of fertile eggs), embryos dead between days 5 and 12 of incubation (13.5% of fertile eggs), embryos between days 13 and 18 of embryonic development (8,1% of fertile eggs), 19 to 20 day-old embryos (16.2% of fertile eggs) and embryos in the 21st day of incubation (deceased in the process of hatching) (8.1% of fertile eggs). The mortality curve for the analyzed sample can be found in Graph 107. Time of death was estimated by gross exam and confirmed/corrected after microscopic examination of the embryos.



**Graph 107. Distribution of embryonic mortality according to estimated time of death.**

## Gross and microscopic lesions

### 0-2 days of incubation

In all cases in which time of death was placed between the beginning and the second day of incubation, no histological structures compatible with a complete embryo were found in spite of the presence of both extra-embryonic membranes and, in 13% of cases, hematic pigment was noted. In two of these cases, however, random mesodermal tissues were present, such as fibrous tissue and cartilage.

In three of the eggs, bacteria were found, belonging to the genera *Pseudomonas*, *Escherichia* and *Staphylococcus*, respectively. Only the first case presented signs of trauma, in the form of a damaged air cell. In all cases, the shell of the affected eggs was contaminated with excreta.

#### 3-4 days of incubation

In four of the five cases placed in this category there was no embryo found. Instead, only a mass of disorganized cells was present, sometimes arranged into more complex tissues (e.g., embryonic renal structures) but without a functional anatomical connection to the rest of the body. Moreover, in one of these cases, there was cystic malformation of the embryonic and extra-embryonic structures (Figure 59A). *Staphylococcus sp* bacteria were isolated from the yolk/albumen sample collected. The eggshell in this case presented no signs of trauma but was contaminated with excreta. The only normally developed embryo exhibited an underdeveloped heart, disproportionate in size to the embryonic body and incapable of ensuring normal blood flow.

#### 5-12 days of incubation

Most of the embryos deceased during this time period were too decomposed to allow for an accurate diagnosis of the cause of death. In one of these embryos, however, massive intra-abdominal hemorrhage was found. The shell of the respective egg was fractured. No direct source of the bleeding was identified. One other embryo presented gross malformation of the beak (maxillary hypoplasia), the cranial vault (exencephaly) and the eyes (bilateral anophthalmia). In one of the remaining cases, *Staphylococcus sp*. bacteria were isolated from the yolk/albumen sample collected. The eggshell was clean and not damaged in this case, and no direct link could be established between the presence of the bacteria and the cause of death.

#### 13-18 days of incubation

All embryos in this category were too decomposed to allow for an accurate diagnosis of the cause of death. However, one of the embryos exhibited gross malformation of the beak (dysplasia/crossed beak) (Figure 59B) and the keel (sinuous keel). Another embryo was positive for the presence of *Pseudomonas alcaligenes* bacteria. The eggshell was contaminated with excreta but intact, as was the air cell.

#### 19-20 days of incubation

The embryos in this category were fully developed but presented no signs of lung inflation with air. Of the six embryos, one presented absence of the cranial vault, with exposure of the brain and hemorrhage, possibly associated with local trauma (Figure 59C). This was deemed to be the cause of death.

Another embryo was found to have a malformation of the amniotic membrane (Figure 59F). This structure was wrapped around the embryo’s neck, causing it to narrow and curve just above the crop. This embryo presented generalized congestion of the internal organs and embryonic tissues.

A third embryo exhibited a minor abdominal wall defect (Figure 59D), with protrusion of the already internalized yolk sac through the abdominal wall (gastroschisis). Upon histological analysis, the embryo also presented changes in the gizzard, which had a very thin, or locally non-existent, koilin layer (when compared with same-age embryos), with blood and autolyzed tissue present in the lumen of the organ. Additionally, the respective egg was found to have a ruptured air cell.

One of the eggs containing 19-20 day-old embryos presented a ruptured air cell membrane and yolk sac, the latter appearing congested and having its contents spilled around the embryo. Additionally, the intestine of this embryo was likewise congested, the gizzard flaccid and distended, and the crop filled with brownish liquid. On microscopic analysis, the whole digestive tract was distended, from crop to intestine, with contents consistent with yolk. The lungs were congested, moderately collapsed and the bronchi filled with acidophilic, amorphous contents. Decomposition was advanced. *E. coli* bacteria were isolated from the egg contents in pure culture. The eggshell was contaminated with both excreta and blood in this case.

One of the embryos was too decomposed to allow for a diagnosis of the cause of death. The shell of the respective egg was fractured and the air cell membrane dislocated to the narrow pole of the egg.

Finally, the sixth embryo did not present any malformations or lesions indicative of the cause of death. In spite of being clean, the eggshell was fractured and *Staphylococcus sp*. isolated from the yolk sample collected.

#### 21 days of incubation

Two of the three embryos/chicks is this group were still alive at the time of egg collection and died during transport to the laboratory. The first had not yet pipped neither through the air cell membrane nor through the eggshell, albeit having completed internalization of the yolk sac. Histologically, this embryo presented generalized congestion, pulmonary edema and only partial inflation of the lung tissue (25% of available alveoli) with air. Death was attributed to congestive heart failure secondary to cooling of the egg and embryo after removal from the hatcher.

In the second case, the death of the chick occurred after a failed attempt to hatch. The chick presented similar lesions to the first case described in this category. Likewise, generalized congestion, pulmonary edema and only partial inflation of the lung tissue (25% of available alveoli) with air were found. Death was attributed to the same causes of the previous case.

The single case of malposition present in this screening was found in the third embryo of this group. The embryo in question had its head in the narrow pole of the egg, appearing flaccid and edematous (anasarca and intramuscular edema). The lung tissue showed no signs of inflation with air. *Escherichia coli* was isolated from yolk sample collected. Fractures were also detected in the shell of the egg harboring this embryo, which was otherwise clean.

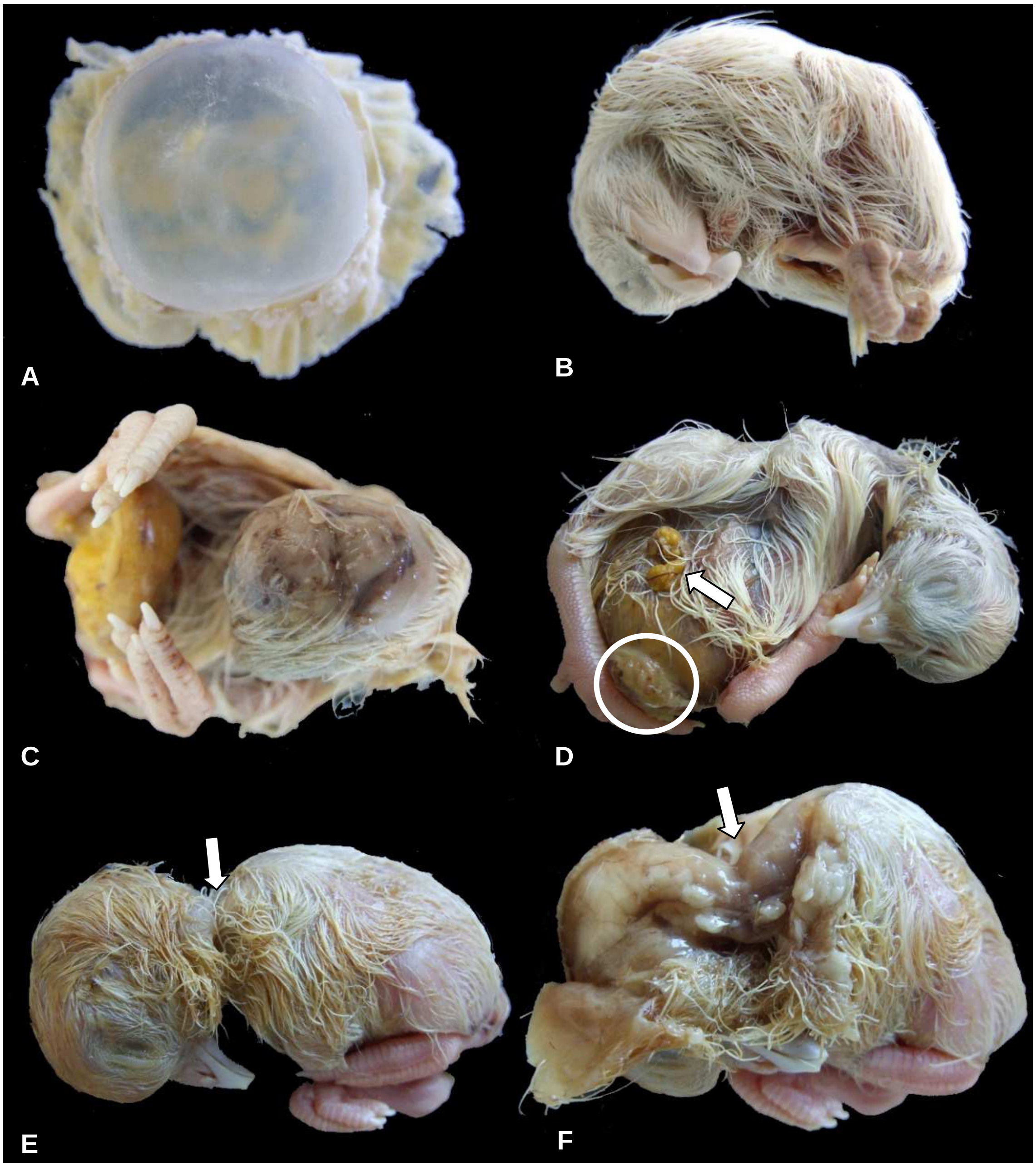


**Figure 59. Examples of malformations found.**
**A) Cystic embryo; B) Crossed beak. Note that the upper beak does present the same longitudinal axis as the lower beak; C) Exencephaly. Note the complete absence of bone and skin coverage over the brain; D) Gastroschisis. Note the protrusion of the yolk sac through the abdominal wall (arrow). Circle indicates the umbilicus; E) Neck compression caused by amniotic band. Note the abrupt narrowing of the neck (arrow) and swollen appearance of the head; F) Same embryo as in E, after partial skinning. Note the compression band (arrow) caused by the malformed amnion and subsequent distortion of the spine.**

# DISCUSSION

When analyzing hatchery performance, a panoply of factors that can influence incubation and hatchability should be taken into account. These can be divided into: i) breeder farm-related factors, such as flock fertility, age, management, health and nutrition status (Christensen, 2001; Elibol, Peak, & Brake, 2002; Peebles et al., 2001; Wilson, 1997); ii) egg-related factors, such as egg weight and uniformity of the egg batch (Elibol & Brake, 2008b); iii) egg transport and storage factors, such as packaging, egg trauma during transport to the hatchery, length of storage periods and storage environment conditions (Elibol et al., 2002; Fasenko, 2007; Ruiz & Lunam, 2002; Seydim & Dawson, 1999; Wilson, 2004); iv) incubation environment factors, such as incubator temperature, relative humidity, ventilation, carbon dioxide concentration, egg position, egg turning intervals and angles, and pre-incubation warming cycles (Bruzual et al., 2000a; Elibol & Brake, 2008a; Elibol et al., 2002; Lourens et al., 2007; Lourens et al., 2005; Molenaar et al., 2013; Noiva et al., 2014; Peebles et al., 2001; Tullett, 2008); v) infectious factors, such as vertical infection with *Salmonella enterica*, *Ornithobacterium rhinotracheale*, *Mycoplasma gallisepticum*, Infectious Bronchitis Virus, Avian Leukosis Virus and Reovirus (Pattison, 2008); vi) and handling factors, such as trauma or shell contamination secondary to the manual or automated handling of the eggs during setting and transport inside the hatchery (Cobb, 2008; Tullett, 2008).

From this simple example of a factor list, it is clear that any investigation of hatchery performance merits a careful, thorough and multidisciplinary approach, and that the opportunities for mistakes (and for improvement) can be many and varied, even in the case of an industry that is not necessarily performing below standard. The following discussion of the results obtained will be oriented, as closely as possible, according to this list.

## Breeder farm-related factors

For the purposes of this study, the only information available regarding the parent stock had to do with flock strain and age. However, even in the absence of more information, observations made at the hatchery can signal problems originating at the breeder farm. For example, if a flock presents a low fertility rate (which is not the case in this study) poor health conditions or faulty management practices may be to blame (Ernst, Bradley, Delany, Abbott, & Craig, 1999; Pattison, 2008).

Eggshell hygiene is another indicator of good management practices. From the gross analysis of the eggs in the sample in study, it is apparent that shell hygiene is less than ideal, since approximately 82% of all eggs had contaminated shells with either excreta, blood or both. An excessive number of eggs contaminated with excreta can result from one or more issues at the breeder farm, such as improper nest and/or collection system hygiene, infrequent egg collection, insufficient nests or nest unavailability, rejection of unsuitable (or unsuitably placed) nests by the birds, aggressive behavior secondary to overcrowding or unsuitable feeding periods that compete with laying behavior. Feed contamination with toxins (such as mycotoxins) or high dietary fiber contents may also increase the number of eggs soiled with excreta (Aviagen, 2013).

Shell contamination with blood, present in 8% of the eggs, may be the result of aggression secondary to overcrowding or inappropriate male-female ratios, cloacal damage due to sexual immaturity (not applicable for the flock studied here) or overstimulation (Aviagen, 2013).

Additionally, the presence of such contaminated eggs indicates that these are not being rejected for hatching, neither at the breeder farm nor later, at the hatchery.

Embryonic death can also signal issues with breeder nutrition. For instance, embryonic death between days 13 and 17 of incubation is uncommon, as embryos that reach this stage tend to survive this period of rapid growth (Tullett, 2008). The 8% mortality rate found for embryos in this category could signal nutritional deficiencies.

The poor uniformity in egg weight is another flag for possible issues with flock nutrition. This will be further discussed in the following paragraphs.

## Egg-related factors

Weighing the eggs has made apparent that there was great variation in egg weight in the sample collected, with weight differences between the lighter and heavier eggs varying no less than 9 g and as much as 25 g, within the various established groups. Considering that this was a randomly collected sample, there is little reason to assume that such variation was not present in the whole batch and that egg calibration prior to incubation was not performed. Since embryonic development naturally leads to a 12% egg weight loss between days 0 and 18 of incubation (due to albumen and yolk metabolism with evaporation of water vapor as a by-product) (Peebles et al., 2001), it is essential to take time of death into consideration when interpreting weight values, so as to not establish wrong comparisons between eggs with poorly developed embryos and eggs at the end of incubation.

Egg weight affects incubation and hatchability. Lighter, smaller eggs tend to hatch earlier and yield smaller, weaker chicks, while larger, heavier eggs take longer to hatch (Cobb, 2008). Because hatching windows (the period of time necessary for most of the eggs to hatch) tend to be as narrow as possible in order to avoid chick dehydration (the longer the newly-hatched chicks remain in the hatcher, the greater the dehydration they suffer) and allow for more efficient operations (Tullett, 2008), incubating eggs that are too different in weight will either broaden the hatch window or (if the hatch window is fixed) reduce the number of successfully hatched chicks and, therefore, hatching performance. Thus, guaranteeing as uniform an egg batch possible through calibration and selection prior to incubation will increase hatching performance.

The poor uniformity in the egg batch can also act as an indicator of poor breeder-flock uniformity, since hen weight and egg weight have been shown to be positively correlated (Di Masso, Dottavio, Canet, & Font, 1998). Differences in nutritional status or presence of infectious diseases could similarly result in such different egg weights (Pattison, 2008). If close communication exists between farm and hatchery, as is desirable for integrated systems, then such observations could result in management improvements, with obvious positive consequences.

Similarly to egg weight, performance can be affected by egg size and shape. For instance, round or ellipsoid eggs tend to present lower hatchability than oval-shaped eggs (Tullett, 2008; Wilson, 2004). In the sample studied, egg shape varied greatly, as did egg size, again indicating a less than discriminating pre-incubation selection and calibration of the eggs. These characteristics also influence egg handling in the hatchery during setting and transfer processes and will thus be discussed later, in the Handling Factors section.

## Egg transport and storage factors

Eggshell fractures are the more conspicuous signs of rough transport and handling (Aviagen, 2013; Tullett, 2008; Wilson, 2004). Inappropriate packaging or accommodation in the transport vehicle, trepidation and incorrect speed may cause the shells to crack to a greater or lesser extent, with spillage of egg contents or just subtle hairline fractures (Hamilton, Hollands, Voisey, & Grunder, 1979; Seydim & Dawson, 1999). Such eggs should be rejected for incubation, since they present an increased risk for contamination and are unlikely to yield a hatchling (Ernst, Bradley, Abbott, & Craig, 2004; Tullett, 2008). Another, less obvious sign of trauma is the rupture, loosening or dislocation of the air cell that sits at the blunt end of the egg (Knox & Olsen, 1936). Unlike a fracture, which can be caused by simple compression or short vertical drops, dislocation of the air cell may require greater force, more prolonged exposure to trauma and/or the introduction of air into the egg (e.g., as in the case of an associated shell fracture). Although the relatively high percentage of damaged air cells (20%) may be skewed by the fact that only non-viable eggs were analyzed, such observations should be registered and these values regularly monitored as a secondary indicator of the quality of egg transport.

High incidence of cystic embryos has also been associated with pre-incubation trauma (Ernst et al., 1999) but the single case found in this sample does not allow for any conclusions to be drawn on this matter.

## Incubation environment factors

The observations made and results obtained do not put in evidence any issues with the incubation environment *per se*. Although the high incidence of malformations (≈11%) could raise suspicion regarding temperature settings (as demonstrated in the previous chapter relative to changes in the physical incubation environment), the fact that this value refers to the incidence of malformations in a sample of non-viable eggs (and not in the whole batch of incubated eggs) makes it very likely that it is artificially elevated and that a direct, literal interpretation would lead to wrong assumptions. However, if routinely monitored, this parameter can be a precious source of information in performance investigations.

## Infectious factors

Over 70% of eggs analyzed did not render any positive results on either classic microbiological culture or molecular analysis. The remaining 30% were infected/contaminated with coagulase-negative *Staphylococcus* species, *Pseudomonas alcaligenes* or *Escherichia coli*.

Coagulase-negative staphylococci, including many species such as *Staphylococcus hyicus*, *Staphylococcus gallinarum*, *Staphylococcus arlettae*, *Staphylococcus chromogenes*, *Staphylococcus xylosus*, and *S. epidermidis*, have commonly been isolated from the nares, skin, upper alimentary and intestinal tract, and urogenital tract of chickens (K. Bhargava & Zhang, 2012; Kawano et al., 1996). Although most cases of *Staphylococcus*-induced disease in chickens are due to *Staphylococcus aureus* (which is coagulase positive), coagulase-negative species such as *S. hyicus*, *S. sciuri*, *S. simulans*, *S. epidermidis*, and so on, have occasionally been isolated from sick birds and implicated as causative agents of tenosynovitis and dermatitis (K. Bhargava & Zhang, 2012; McNamee & Smyth, 2000). However, the fairly low number of such cases suggests that coagulase-negative staphylococcal infections in chickens tend to be opportunistic in nature and that the relationship between bacterium and bird is mostly a peaceful one (Aarestrup et al., 2000; Awan & Matsumoto, 1998).

*Pseudomonas alcaligenes*, on the contrary, is not a known commensal organism of the avian body. Ubiquitous and environmental, this bacterium is usually isolated from water and soil samples (Suzuki et al., 2013) and has not so far been implicated in poultry disease.

Of the three species of bacteria found contaminating the eggs in the sample in study, only *E. coli* has been proven to be vertically transmitted (Giovanardi et al., 2005; Petersen, Christensen, Kuhnert, Bisgaard, & Olsen, 2006). This bacterium, a normal inhabitant of the intestine of most animals, where it performs an important role of competitive exclusion of potentially pathogenic bacteria such as *Salmonella*, is a common agent of disease in poultry (Pattison, 2008). *Escherichia coli* infections in poultry include a variety of localized and systemic infections, in which *E. coli* may be involved as a primary or secondary infectious agent (Petersen et al., 2006). Bird-to-bird transmission is usually oral-fecal, but transmission from chicken to egg to hatchling is also common and can be responsible for high chick mortality (e.g., due to omphalitis/yolk sac infection) (Giovanardi et al., 2005; Pattison, 2008). However, unlike *Salmonella*, infection of the oviduct with *E. coli* is not the primary mode of transmission to the egg. In fact, the most important source of egg infection seems to be fecal contamination of the egg surface, with subsequent penetration of the shell and membranes by the bacterium (Pattison, 2008).

Considering that (i) *Staphyloccocus* and *P. alcaligenes* similarly do not naturally colonize the oviduct, (ii) that most of the infected/contaminated eggs had shells contaminated with excreta, and (iii) that only one *Staphylococcus*-positive egg (and none of the *Pseudomonas*-positive eggs) presented eggshell fractures, the reason behind the presence of these three types of bacteria inside the eggs may very well be the same. In fact, the presence of these bacteria on the eggshell could have led to its subsequent penetration and migration of the organisms into the egg. Eggshell contamination with excreta provides a probable cause for the origin of the staphylococci. Regarding *Pseudomonas*, poor nest or floor hygiene (assuming that floor eggs are being incubated) or contamination of the water used to regulate relative humidity levels inside the incubator could be to blame.

As previously discussed in the *Salmonella* trial chapter, the shell cuticle, as well as pore conformation, should prevent bacterial penetration of the eggshell (De Reu et al., 2006). However, if the egg is exposed to an environment cooler than its internal structure, paired with high relative humidity, condensation takes place over the shell and the usual inside-to-outside movement of gases and water vapor can suffer a switch in the opposite direction (outside-to-inside), facilitating the migration of bacteria through the eggshell and membranes (Keller et al., 1995). Therefore, not only does the isolation of intestinal and environmental bacteria from the eggs indicate possible deficiencies with farm and egg hygiene, it may also indicate that the eggs are suffering periods of cooling and condensation throughout the their journey to and through the hatchery.

The fact that embryos deceased at all stages of development were found in infected eggs makes it impossible to ascertain the role of the bacteria in embryonic death (i.e., whether death occurred prior to or as a consequence of bacterial proliferation).

## Handling factors

Handling during hatchery operations, such as egg setting in the incubator trays, transfer to the hatcher and (optional) *in ovo* vaccination at day 18 of the incubation process, and setting in the hatching baskets, creates a number of opportunities for egg damage, shell (and subsequent egg) contamination and other occurrences with the potential to reduce the number of hatched chicks (Cobb, 2008; Tullett, 2008). Nowadays, egg setting and transfer tends to be mechanical and automated in order to minimize such issues (Cobb, 2008). These systems generally rely on vacuum applied by row upon row of rubber/silicon precision sucker heads, which lift the eggs by the blunt end and carefully position them in their individual slots in the setter trays (Petersime, 2013a, 2014). However, such systems require the eggs to be similar in size and shape in order to operate correctly. If the eggs are too spherical, the likelihood that they will be set in the tray wrong side up increases drastically (Tullett, 2008). If the eggs are too narrow, the sucker heads may not be able to grip them firmly enough. If they are too short, the eggs will be dropped into their slot in the tray instead of being gently placed there. If they are too long, they will be crushed against the tray. If they are too wide or round, they may not fit in the tray slots at all. Because egg trays are rigid (Petersime, 2013b), eggs that do not fit properly into their slots may also oscillate violently and break when the trays are tilted during incubation.

Therefore, the likelihood that trauma will occur increases as egg uniformity decreases. The great variability in egg shape (from round to ellipsoid) and size (with differences of over 1 cm in length between the longest and shortest eggs) poses yet another issue with potential detrimental effects in performance.

Proper calibration of the eggs prior to incubation could, thus, reduce the percentage of fractured eggs found (12%) and the number of embryonic deaths associated with trauma. Eggshell fractures have deadly consequences for the embryo (Tullett, 2008). The case of the fractured egg presenting a 5-12 day-old embryo with massive internal bleeding is a good example of this. Moreover, shell fractures facilitate bacterial penetration and propagation. The infection of the 19-20 day-old embryo found positive for *Staphylococcus sp*. could have been secondary to the fracture found on the eggshell of its respective egg.

Again, it should be pointed out that the percentage of fractured shells may be inflated here by the fact that the sampling was applied at the end of hatch, on non-viable eggs.

However, the fact that four out of six embryos found to have died between days 19 and 20 of incubation presented internal and or external signs of trauma - in one case so severe that the yolk sac ruptured, flooding the embryo's gastrointestinal and respiratory tracts with yolk – can also serve as indication of rough handling during transfer between the incubator and the hatcher, at day 18 of incubation.

## Final considerations

All the eggs studied in this chapter originated from the same 45-week-old Aviagen Ross 308 breeder hen flock, with an estimated fertility of 98%. Although standard fertility rates are not provided by Aviagen, performance objectives available for this breeder strain and age indicate a standard hatchability (number of hatched chicks per number of eggs incubated) of 87.5% (Aviagen, 2011). The hatching rate for the batch of eggs from which this sample was taken was estimated by the hatchery staff at 87.2%, just 0.3% below standard. These values seem to indicate an already good performance, even in the presence of possible underlying faults in transport, pre-incubation sorting, and in handling.

But are they enough to assess the health and operation of a hatchery and of the verticalized system in which it is integrated? How detrimental are these faults? How much does 0.3% mean in poultry production?

The hatchery from which the sample was collected uses ChickMaster™ incubators. This brand offers incubation units that range between 10,000 and 133,000 eggs in capacity. Thus, a 0.3% loss in performance can mean a loss of anywhere between 30 and 399 hatching chicks per incubation cycle. Considering a hypothetical scenario in which a hatchery is equipped with only two 133,000-egg incubators, incubating a maximum of 266,000 eggs monthly, this loss will have equated to a full batch of 10,000 birds by the end of a single year.

It is a well-established truth that not every fertile egg will hatch and that not every chick that hatches will survive to and through production age. However, in a business of thousands of birds, even a small percentage of deaths can mean a significant loss in profit. As is patent in this study, the hatchery can function as a veritable nexus in performance monitoring and improvement, both upstream and downstream in the production line, providing valuable information, signaling issues and conditioning the chick health from the very beginning.

In summary, although the results obtained in this case do not point to any issues related to the variables studied in previous chapters, they add to the valuable information obtained in those chapters and make the case a good example of a number of factors that should be considered when investigating drops in hatchery performance or elaborating strategies to increase hatchability, chick quality and, ultimately, profit.

# CONCLUSIONS

This project aimed at collecting and interpreting essential information regarding embryonal response to both infectious and physical challenge that could serve to aid and refine available techniques of embryodiagnosis and ultimately determine the real potential of this technique in the monitoring of both flock and hatchery health. For this purpose, eggs were incubated under different, controlled environments or after infection with a vertically transmitted pathogen, and an extensive list of parameters used to monitor subsequent changes in embryonic behavior, growth and viability.

The observations made in this study put in evidence the impact that the incubation environmental conditions can have on embryonic development. Overall, the effects of manipulating temperature were more prominent than those of manipulating humidity and carbon dioxide, which closely mimic each other. Temporary changes in the incubation environment produced likewise temporary effects, while continuous changes in temperature and relative humidity affected organ development during at least part of the embryonic development period. Changes were most conspicuous for temperature-related treatments, affecting lymphoid follicle formation in the Bursa of Fabricius and causing disruption of the gizzard mucosa, both with potentially important post-hatch effects but both likely to go unnoticed at an industrial level if no sampling is routinely performed. Lesions in dead embryos did not allow for diagnosis of cause of death. However, mortality and malformation rates and patterns exhibited some level of association with the parameter being manipulated.

Additionally, this study investigated the effects exerted by infection with Salmonella serovar Enteritidis and Typhimurium over embryonic development. The results obtained suggested that no changes occur in performance in viable embryos throughout incubation, in spite of the ongoing infection, highlighting the ability of these two *Salmonella* serovars in generating asymptomatic carriers. However, vertical infection with *Salmonella* was associated with higher embryonic mortality rates, resulting in an overall reduction in incubation performance. The lower mortality rates found in the S. Typhimurium trials also suggested that although *S.* Enteritidis is more commonly vertically transmitted), infection with *S.* Typhimurium may be less likely to induce embryonic mortality, and therefore more easily result in the hatching of asymptomatic carriers. Moreover, a final observation regarding the migration of bacteria inside the egg strongly indicated that *Salmonella* exhibits a strong positive tropism toward the embryo, successfully crossing egg compartments to lodge near the embryonic disc even after inoculation in the antimicrobial environment of the albumen.

In a final trial, this study applied knowledge generated during experimental manipulation to a field survey using post-hatch samples from a commercial hatchery. Although the results obtained did not point to any issues related to the variables studied, they nonetheless put in evidence the importance that systematic monitorization of hatching performance and chick quality can have in the industrial setting, where profit and sustainability depend on the hatchery’s ability to produce in quality and in quantity.

Moreover, the results obtained throughout this study reveal the wealth of information that can be obtained when what is considered a waste product is repurposed as an indicator of hatchery health, making the most out of a sample which has little to no market value.

# REFERENCES

Aarestrup, F. M., Agersø, Y., Ahrens, P., Jørgensen, J. C. Ø., Madsen, M., & Jensen, L. B. (2000). Antimicrobial susceptibility and presence of resistance genes in staphylococci from poultry. *Veterinary Microbiology, 74*(4), 353-364.

Abd-Elhakim, A., Thieme, O., Schwabenbauer, K., & S. Ahmed, Z. (2009). *Mapping traditional poultry hatcheries in Egypt.* Rome: FAOo. Document Number)

Aldridge, P., Gnerer, J., Karlinsey, J. E., & Hughes, K. T. (2006). Transcriptional and translational control of the Salmonella fliC gene. *J Bacteriol, 188*(12), 4487-4496.

Aleksandrova, A., Czirok, A., Szabo, A., Filla, M. B., Hossain, M. J., Whelan, P. F., et al. (2012). Convective tissue movements play a major role in avian endocardial morphogenesis. *Dev Biol, 363*(2), 348-361.

Allenspach, A. L. (1966). The reopening process of the esophagus in the normal chick and the crookedneck dwarf mutant. *Development, 15*(1), 67-76.

Anderson-Berry, A., O'Brien, E. A., Bleyl, S. B., Lawson, A., Gundersen, N., Ryssman, D., et al. (2005). Vasculogenesis drives pulmonary vascular growth in the developing chick embryo. *Developmental Dynamics, 233*(1), 145-153.

Andrewartha, S. J., Tazawa, H., & Burggren, W. W. (2011). Embryonic control of heart rate: examining developmental patterns and temperature and oxygenation influences using embryonic avian models. *Respir Physiol Neurobiol, 178*(1), 84-96.

Anonymous. (1882, 12 August 1882). Poultry Notes. *The Queenslander (Brisbane, Qld. : 1866 - 1939)*. Retrieved 16 May 2012, from
http://nla.gov.au/nla.news-article19785867

Anonymous. (1901, 29 March 1901). Poultry. *Euroa Advertiser (Vic. : 1884 - 1920)*. Retrieved 10 May 2012, from
http://nla.gov.au/nla.news-article65535612

Anonymous. (1903, 21 November 1903). Incubator Marvel. *Examiner (Launceston, Tas. : 1900 - 1954)*. Retrieved 10 May 2012, from
http://nla.gov.au/nla.news-article35572302

Anonymous. (1923, 23 January 1923). The Electric Hen. *The Register (Adelaide, SA : 1901 - 1929)*. Retrieved 10 May 2012, from
http://nla.gov.au/nla.news-article63826389

Anonymous. (1925a, 20 May 1925). Automatic Incubator. *The Northern Miner (Charters Towers, Qld. : 1874 - 1954)*. Retrieved 9 May 2012, from
http://nla.gov.au/nla.news-article80571120

Anonymous. (1925b, 17 September 1925). Poultry Notes. *Western Mail (Perth, WA : 1885 - 1954)*. Retrieved 11 May 2012, from
http://nla.gov.au/nla.news-article37647534

Anonymous. (1929, November 19th 1929). Ancient Egyptians Had Egg Incubators of Mud. *The Reading Eagle,* p. 9, from http://news.google.com/newspapers?id=RUcxAAAAIBAJ&sjid=VuEFAAAAIBAJ&hl=pt-PT&pg=4185%2C4157151

Anonymous. (1930, February 14th 1930). Incubators Used in Ancient Egypt. *The Milwaukee Journal,* p. 25, from http://news.google.com/newspapers?id=KqBQAAAAIBAJ&sjid=NyIEAAAAIBAJ&hl=pt-PT&pg=6006%2C2995472

Anonymous. (1932, 7 April 1932). Farm And Field. *The Central Queensland Herald (Rockhampton, Qld. : 1930 - 1956)*. Retrieved 10 May 2012, from
http://nla.gov.au/nla.news-article70552464

Anonymous. (1933a, 27 December 1933). Successful Poultry Farming. No. 8--Artificial Incubation. *Advocate (Burnie, Tas. : 1890 - 1954)*. Retrieved 10 May 2012, from http://nla.gov.au/nla.news-article68065762

Anonymous. (1933b, 30 June 1933). To Hatch 16,000 Eggs At Once. *The Advertiser (Adelaide, SA : 1931 - 1954)*. Retrieved 10 May 2012, from http://nla.gov.au/nla.news-article46984163

Anonymous. (1936a, 19 September 1936). Artificial Hatching Of Hens' Eggs. *The Courier-Mail (Brisbane, Qld. : 1933 - 1954)*. Retrieved 10 May 2012, from http://nla.gov.au/nla.news-article37027755

Anonymous. (1936b, 28 October 1936). Poultry For Profit X-Ray For Eggs. *Advocate (Burnie, Tas. : 1890 - 1954)*. Retrieved 11 May 2012, from http://nla.gov.au/nla.news-article68090422

Anonymous. (1937, 23 July 1937). Electric Incubator Installed. *Morning Bulletin (Rockhampton, Qld. : 1878 - 1954)*. Retrieved 10 May 2012, from http://nla.gov.au/nla.news-article57630464

Anonymous. (2010a). Company Profile - Jamesway Incubator Company. from http://www.jamesway.com/en/about-us.html

Anonymous. (2010b). History of Innovation - Jamesway Incubator Company. from http://www.jamesway.com/en/about-us/history-of-innovation.html

Anonymous. (2012). About Pas Reform. Retrieved June, 19th, 2012, from http://www.pasreform.com/about-pas-reform.html

Arazi, H., Yoselewitz, I., Malka, Y., Kelner, Y., Genin, O., & Pines, M. (2009). Osteopontin and calbindin gene expression in the eggshell gland as related to eggshell abnormalities. *Poult Sci, 88*(3), 647-653.

Arendt, D., & Nubler-Jung, K. (1999). Rearranging gastrulation in the name of yolk: evolution of gastrulation in yolk-rich amniote eggs. *Mech Dev, 81*(1-2), 3-22.

Argüello, C., Alanís, J., Pantoja, O., & Valenzuela, B. (1986). Electrophysiological and ultrastructural study of the atrioventricular canal during the development of the chick embryo. *Journal of Molecular and Cellular Cardiology, 18*(5), 499-510.

Aviagen. (2011). *Ross 308 Parent Stock- Performance Objectives*: Aviagen.

Aviagen. (2013). *Hatching Egg Shell Quality - Assessing Abnormalities*: Aviagen.

AVMA. (2007). *AVMA Guidelines on Euthanasia*.

Awan, M. A., & Matsumoto, M. (1998). Heterogeneity of staphylococci and other bacteria isolated from six-week-old broiler chickens. *Poultry Science, 77*(7), 944-949.

Babiker, E. M., & Baggott, G. K. (1995). The role of ion transport in the formation of sub-embryonic fluid by the embryo of the Japanese quail. *Br Poult Sci, 36*(3), 371-383.

Bachvarova, R. F. (1999). Establishment of anterior-posterior polarity in avian embryos. *Curr Opin Genet Dev, 9*(4), 411-416.

Baker, F. J., & Breach, M. R. (1967). *Handbook of bacteriological technique* (2nd ed.). London,: Butterworths.

Baldwin, H. S. (1996). Early embryonic vascular development. *Cardiovascular research, 31*(supp1), E34-E45.

Banerjee, P., Keener, K. M., & Lukito, V. D. (2011). Influence of carbon dioxide on the activity of chicken egg white lysozyme. *Poult Sci, 90*(4), 889-895.

Barnwell, R. (2007). Bio-response incubation for better hatch and post-hatch performance. *World Poultry, 23*(1), 20-21.

Barri, A. (2008). *Effects of incubation temperature and transportation stress on yolk utilization, small intestine development, and post-hatch performance of high-yield broiler chicks.* Faculty of the Virginia Polytechnic Institute and State University, Blacksburg.

Bellairs, R. (1961). The structure of the yolk of the hen's egg as studied by electron microscopy. I. The yolk of the unincubated egg. *J Biophys Biochem Cytol, 11*, 207-225.

Bellairs, R., & Boyde, A. (1969). Scanning electron microscopy of the shell membranes of the hen's egg. *Z Zellforsch Mikrosk Anat, 96*(2), 237-249.

Bellairs, R., & Osmond, M. (2005). *The Atlas Of Chick Development*: Elsevier Science & Tech.

Bellairs, R., & Veini, M. (1984). Experimental analysis of control mechanisms in somite segmentation in avian embryos. II. Reduction of material in the gastrula stages of the chick. *J Embryol Exp Morphol, 79*, 183-200.

Bennett, T., & Cobb, J. L. S. (1969). Studies on the avian gizzard: The development of the gizzard and its innervation. *Zeitschrift für Zellforschung und Mikroskopische Anatomie, 98*(4), 599-621.

Benton, C. E., Jr., Walsh, T. J., & Brake, J. (2001). Effects of presence of a blastoderm on albumen height and pH of broiler hatching eggs. *Poult Sci, 80*(7), 955-957.

Bhargava, K., & Zhang, Y. (2012). Multidrug-resistant coagulase-negative Staphylococci in food animals. *J Appl Microbiol, 113*(5), 1027-1036.

Bhargava, S. (2007). Role of nerve growth factor and its receptor in the morphogenesis of neural tube in early chick embryo. *Gen Comp Endocrinol, 153*(1-3), 141-146.

Black, J. L., & Burggren, W. W. (2004a). Acclimation to hypothermic incubation in developing chicken embryos (Gallus domesticus): I. Developmental effects and chronic and acute metabolic adjustments. *J Exp Biol, 207*(Pt 9), 1543-1552.

Black, J. L., & Burggren, W. W. (2004b). Acclimation to hypothermic incubation in developing chicken embryos (Gallus domesticus): II. Hematology and blood O2 transport. *J Exp Biol, 207*(Pt 9), 1553-1561.

Blount, J. D., Houston, D. C., & Møller, A. P. (2000). Why egg yolk is yellow. *Trends in Ecology & Evolution, 15*(2), 47-49.

Boerjan, M. (2004). Single stage incubation is the most natural choice. *World Poultry, 20*(7), 18-20.

Boerjan, M. (2012). The benefits of single-stage incubation to food safety. Retrieved June, 25th, 2012, from http://www.pasreform.com/academy/frequently-asked-questions/incubation/19-the-benefits-of-single-stage-incubation-to-food-safety.html

Braun, E. J., & Scanes, C. G. (2015). Chapter 12 - Osmoregulatory Systems of Birds. In *Sturkie's Avian Physiology (Sixth Edition)* (pp. 285-300). San Diego: Academic Press.

Bruggeman, V., Van As, P., & Decuypere, E. (2002). Developmental endocrinology of the reproductive axis in the chicken embryo. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 131*(4), 839-846.

Bruggeman, V., Witters, A., De Smit, L., Debonne, M., Everaert, N., Kamers, B., et al. (2007). Acid-base balance in chicken embryos (Gallus domesticus) incubated under high CO2 concentrations during the first 10 days of incubation. *Respir Physiol Neurobiol, 159*(2), 147-154.

Bruzual, J. J., Peak, S. D., Brake, J., & Peebles, E. D. (2000a). Effects of relative humidity during incubation on hatchability and body weight of broiler chicks from young breeder flocks. *Poult Sci, 79*(6), 827-830.

Bruzual, J. J., Peak, S. D., Brake, J., & Peebles, E. D. (2000b). Effects of relative humidity during the last five days of incubation and brooding temperature on performance of broiler chicks from young broiler breeders. *Poult Sci, 79*(10), 1385-1391.

Buckeye Incubator, C., & Newtown Giant Incubator Co, S. O. (1926). *Successful hatchery operation*.

Burnham, M. R., Peebles, E. D., Gardner, C. W., Brake, J., Bruzual, J. J., & Gerard, P. D. (2001). Effects of incubator humidity and hen age on yolk composition in broiler hatching eggs from young breeders. *Poult Sci, 80*(10), 1444-1450.

Butcher, G. D., & Yegani, M. (2008). Investigating Vaccination Failure in Poultry Flocks
Florida: University of Florida Institute of Food and Agricultural Sciences.

Caplan, A. I., & Koutroupas, S. (1973). The control of muscle and cartilage development in the chick limb: the role of differential vascularization. *Development, 29*(3), 571-583.

Carlson, B. M. (2004). *Human Embryology and Developmental Biology*: Mosby.

Catala, M., Teillet, M. A., De Robertis, E. M., & Le Douarin, M. L. (1996). A spinal cord fate map in the avian embryo: while regressing, Hensen's node lays down the notochord and floor plate thus joining the spinal cord lateral walls. *Development, 122*(9), 2599-2610.

Cater, D. B., & Lawrie, N. R. (1951). A histochemical study of the developing preen glands of chicks from fourteenth day of incubation until fourteen days after hatching. *The Journal of Physiology, 112*(3-4), 405-419.

Chapple, S. (2012). About Chick Master - Company History. In R. Noiva (Ed.).

Cheung, P.-Y., & Kam, K. M. (2011). Salmonella in food surveillance: PCR, immunoassays, and other rapid detection and quantification methods. *Food Research International*.

Chiba, Y., Khandoker, A. H., Nobuta, M., Moriya, K., Akiyama, R., & Tazawa, H. (2002). Development of respiratory rhythms in perinatal chick embryos. *Comp Biochem Physiol A Mol Integr Physiol, 131*(4), 817-824.

ChickMaster. (2006). The case for single stage incubation. *World Poultry, 22*(6), 16-17.

ChickMaster. (2012). Avida S Insert Brochure (pp. 1-2).

Chien, Y. C., Hincke, M. T., & McKee, M. D. (2009). Ultrastructure of avian eggshell during resorption following egg fertilization. *J Struct Biol, 168*(3), 527-538.

Chien, Y. C., Hincke, M. T., Vali, H., & McKee, M. D. (2008). Ultrastructural matrix-mineral relationships in avian eggshell, and effects of osteopontin on calcite growth in vitro. *J Struct Biol, 163*(1), 84-99.

Chousalkar, K. K., Flynn, P., Sutherland, M., Roberts, J. R., & Cheetham, B. F. (2010). Recovery of Salmonella and Escherichia coli from commercial egg shells and effect of translucency on bacterial penetration in eggs. *Int J Food Microbiol, 142*(1-2), 207-213.

Christensen, V. L. (2001). Factors associated with early embryonic mortality. *World's Poultry Science Journal, 57*(04), 359-372.

Chung, D. Y., Sim, Y. B., Park, K. T., Yi, S. H., Shin, J. C., & Kim, S. P. (2001). Spectral analysis of fetal heart rate variability as a predictor of intrapartum fetal distress. *Int J Gynaecol Obstet, 73*(2), 109-116.

Clouthier, S. C., Muller, K. H., Doran, J. L., Collinson, S. K., & Kay, W. W. (1993). Characterization of three fimbrial genes, sefABC, of Salmonella enteritidis. *J Bacteriol, 175*(9), 2523-2533.

Cobb. (2008). *Hatchery Managment Guide*. Colchester, Essex: Cobb-Vantress Inc.

Collins, C. H., & Lyne, P. M. (1984). *Microbiological methods* (5th ed.). London ; Boston: Butterworths.

Comission Regulation (EC) No 589/2008 of 23 June 2008 laying down detailed rules for implementing Council Regulation (EC) No 1234/2007 as regards marketing standards for eggs, (2008).

Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes, (1986).

Conway, C. J., & Martin, T. E. (2000). Effects of ambient temperature on avian incubation behavior. *Behavioral Ecology, 11*(2), 178-188.

Cook, M. I., Beissinger, S. R., Toranzos, G. A., Rodriguez, R. A., & Arendt, W. J. (2003). Trans-shell infection by pathogenic micro-organisms reduces the shelf life of non-incubated bird's eggs: a constraint on the onset of incubation? *Proc Biol Sci, 270*(1530), 2233-2240.

Coulombre, A. J., Coulombre, J. L., & Mehta, H. (1962). The skeleton of the eye: I. Conjunctival papillae and scleral ossicles. *Developmental Biology, 5*(3), 382-401.

Cox, N. A., Berrang, M. E., & Cason, J. A. (2000). Salmonella penetration of egg shells and proliferation in broiler hatching eggs--a review. *Poult Sci, 79*(11), 1571-1574.

Csordas, A. T., Barak, J. D., & Delwiche, M. J. (2004). Comparison of primers for the detection of Salmonella enterica serovars using real-time PCR. *Lett Appl Microbiol, 39*(2), 187-193.

Cyphers Incubator, C. (1912). *Poultry Growers Guide for 1912*: Cyphers Incubator Company.

Davies, R. H., & Breslin, M. (2003). Investigation of Salmonella contamination and disinfection in farm egg-packing plants. *J Appl Microbiol, 94*(2), 191-196.

Day, J. B., Basavanna, U., & Sharma, S. K. (2009). Development of a cell culture method to isolate and enrich Salmonella enterica serotype enteritidis from shell eggs for subsequent detection by real-time PCR. *Appl Environ Microbiol, 75*(16), 5321-5327.

De Buck, J., Van Immerseel, F., Haesebrouck, F., & Ducatelle, R. (2004). Colonization of the chicken reproductive tract and egg contamination by Salmonella. *J Appl Microbiol, 97*(2), 233-245.

de Jong, F., Geerts, W. J., Lamers, W. H., Los, J. A., & Moorman, A. F. (1987). Isomyosin expression patterns in tubular stages of chicken heart development: a 3-D immunohistochemical analysis. *Anat Embryol (Berl), 177*(1), 81-90.

De Reu, K., Grijspeerdt, K., Messens, W., Heyndrickx, M., Uyttendaele, M., Debevere, J., et al. (2006). Eggshell factors influencing eggshell penetration and whole egg contamination by different bacteria, including Salmonella enteritidis. *Int J Food Microbiol, 112*(3), 253-260.

De Smit, L., Bruggeman, V., Debonne, M., Tona, J. K., Kamers, B., Everaert, N., et al. (2008). The effect of nonventilation during early incubation on the embryonic development of chicks of two commercial broiler strains differing in ascites susceptibility. *Poult Sci, 87*(3), 551-560.

Deeming, D. C. (1989). Importance of sub-embryonic fluid and albumen in the embryo's response to turning of the egg during incubation. *Br Poult Sci, 30*(3), 591-606.

Deeming, D. C. (1998). Vulnerability of the Embry to Hatching Egg Disinfection. *World Poultry, 14*(9), 29-30.

Deeming, D. C. (2008). Avian brood patch temperature: Relationships with female body mass, incubation period, developmental maturity and phylogeny. *Journal of Thermal Biology, 33*, 345- 354.

DeFouw, D. O., Rizzo, V. J., Steinfeld, R., & Feinberg, R. N. (1989). Mapping of the microcirculation in the chick chorioallantoic membrane during normal angiogenesis. *Microvascular Research, 38*(2), 136-147.

Degraeve, P. (2009). Bio-response incubation to maximise hatchability and chick performance. *International Hatchery Practice, 23*(8), 11.

Di Masso, R. J., Dottavio, A. M., Canet, Z. E., & Font, M. T. (1998). Body weight and egg weight dynamics in layers. *Poultry Science, 77*(6), 791-796.

Dieterlen-Lievre, F. (1975). On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *J Embryol Exp Morphol, 33*(3), 607-619.

Dieterlen-Lievre, F., & Le Douarin, N. M. (2004). From the hemangioblast to self-tolerance: a series of innovations gained from studies on the avian embryo. *Mech Dev, 121*(9), 1117-1128.

Diodorus, S., & Mayer, B. (1933). The Library of History, Book I, *Loeb Classical Library edition* (Vol. 1).

DiPietro, J. A., Costigan, K. A., & Gurewitsch, E. D. (2003). Fetal response to induced maternal stress. *Early Hum Dev, 74*(2), 125-138.

Dominguez, C., Gomez, I., & Zumalacarregui, J. (2002). Prevalence of Salmonella and Campylobacter in retail chicken meat in Spain. *Int J Food Microbiol, 72*(1-2), 165-168.

Doran, J. L., Collinson, K. S., Clouthier, S. C., Cebula, T. A., Koch, W. H., Burian, J., et al. (1996). Diagnostic potential ofsefADNA probes toSalmonella enteritidisand certain other O-serogroup D1Salmonellaserovars. *Molecular and Cellular Probes, 10*(4), 233-246.

Drapkin, P. T., & Silverman, A.-J. (1999). Development of the chick olfactory nerve. *Developmental Dynamics, 214*(4), 349-360.

Drews, U. (1995). *Color Atlas of Embryology*: G,. Thieme Verlag.

Duband, J. L., Volberg, T., Sabanay, I., Thiery, J. P., & Geiger, B. (1988). Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. *Development, 103*(2), 325-344.

Elibol, O., & Brake, J. (2008a). Effect of egg position during three and fourteen days of storage and turning frequency during subsequent incubation on hatchability of broiler hatching eggs. *Poult Sci, 87*(6), 1237-1241.

Elibol, O., & Brake, J. (2008b). Effect of Egg Weight and Position Relative to Incubator Fan on Broiler Hatchability and Chick Quality. *Poultry Science, 87*(9), 1913-1918.

Elibol, O., Peak, S. D., & Brake, J. (2002). Effect of flock age, length of egg storage, and frequency of turning during storage on hatchability of broiler hatching eggs. *Poult Sci, 81*(7), 945-950.

Ernst, R. A., Bradley, F. A., Abbott, U. K., & Craig, R. M. (2004). *Egg Candling and Breakout Analysis*. Oakland: University of Californiao. Document Number)

Ernst, R. A., Bradley, F. A., Delany, M. E., Abbott, U. K., & Craig, R. M. (1999). *Common Incubation Problems: Causes and Remedies*. Retrieved. from.

Everaert, N., Willemsen, H., De Smit, L., Witters, A., De Baerdemaeker, J., Decuypere, E., et al. (2008). Comparison of a modern broiler and layer strain during embryonic development and the hatching process. *Br Poult Sci, 49*(5), 574-582.

Farner, D. S., King, J. R., & Parkes, K. C. (2013). *Avian Biology*: Elsevier Science.

Fasenko, G. M. (2007). Egg storage and the embryo. *Poult Sci, 86*(5), 1020-1024.

Feast, M., Noble, R. C., Speake, B. K., & Ferguson, M. W. (1998). The effect of temporary reductions in incubation temperature on growth characteristics and lipid utilisation in the chick embryo. *J Anat, 193 ( Pt 3)*, 383-390.

Feeney, R. E., Rhodes, M. B., & Anderson, J. S. (1960). The distribution and role of sialic acid in chicken egg white. *J Biol Chem, 235*, 2633-2637.

Fernandez, M. S., Araya, M., & Arias, J. L. (1997). Eggshells are shaped by a precise spatio-temporal arrangement of sequentially deposited macromolecules. *Matrix Biol, 16*(1), 13-20.

Fernandez, M. S., Escobar, C., Lavelin, I., Pines, M., & Arias, J. L. (2003). Localization of osteopontin in oviduct tissue and eggshell during different stages of the avian egg laying cycle. *J Struct Biol, 143*(3), 171-180.

Franz-Odendaal, T. A. (2008). Toward understanding the development of scleral ossicles in the chicken, Gallus gallus. *Developmental Dynamics, 237*(11), 3240-3251.

French, N. A. (1997). Modeling incubation temperature: the effects of incubator design, embryonic development, and egg size. *Poult Sci, 76*(1), 124-133.

Friebová-Zemanová, Z. (1981). Formation of the chick mesonephros. *Anatomy and Embryology, 161*(3), 341-354.

Galan, J. E., Ginocchio, C., & Costeas, P. (1992). Molecular and functional characterization of the Salmonella invasion gene invA: homology of InvA to members of a new protein family. *J Bacteriol, 174*(13), 4338-4349.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouck, F., Gast, R., Humphrey, T. J., et al. (2009). Mechanisms of egg contamination by Salmonella Enteritidis. *FEMS Microbiol Rev, 33*(4), 718-738.

Gantois, I., Eeckhaut, V., Pasmans, F., Haesebrouck, F., Ducatelle, R., & Van Immerseel, F. (2008). A comparative study on the pathogenesis of egg contamination by different serotypes of Salmonella. *Avian Pathol, 37*(4), 399-406.

Garcia-del Portillo, F. (2001). Salmonella intracellular proliferation: where, when and how? *Microbes and Infection, 3*(14-15), 1305-1311.

Garriock, R. J., & Mikawa, T. (2011). Early arterial differentiation and patterning in the avian embryo model. *Semin Cell Dev Biol, 22*(9), 985-992.

Gasc, J.-M., & Stumpf, W. E. (1981). Sexual differentiation of the urogenital tract in the chicken embryo: autoradiographic localization of sex-steroid target cells during development. *Development, 63*(1), 207-223.

Gast, R. K., & Holt, P. S. (2001). Assessing the Frequency and Consequences of Salmonella Enteritidis Deposition on the Egg Yolk Membrane. *Poultry Science, 80*(7), 997-1002.

Gast, R. K., Jones, D. R., Anderson, K. E., Guraya, R., Guard, J., & Holt, P. S. (2010). In vitro penetration of Salmonella Enteritidis through yolk membranes of eggs from 6 genetically distinct commercial lines of laying hens. *Poultry Science, 89*(8), 1732-1736.

Gautron, J., Hincke, M. T., Mann, K., Panheleux, M., Bain, M., McKee, M. D., et al. (2001). Ovocalyxin-32, a novel chicken eggshell matrix protein. isolation, amino acid sequencing, cloning, and immunocytochemical localization. *J Biol Chem, 276*(42), 39243-39252.

Gautron, J., Murayama, E., Vignal, A., Morisson, M., McKee, M. D., Rehault, S., et al. (2007). Cloning of ovocalyxin-36, a novel chicken eggshell protein related to lipopolysaccharide-binding proteins, bactericidal permeability-increasing proteins, and plunc family proteins. *J Biol Chem, 282*(8), 5273-5286.

Gerhardt, H., Liebner, S., & Wolburg, H. (1996). The pecten oculi of the chicken as a new in vivo model of the blood-brain barrier. *Cell and Tissue Research, 285*(1), 91-100.

Giamborne, J. J. (1997). Vaccination Methods in the Hatchery. *World Poultry, 13*(7), 19-23.

Giansanti, F., Leboffe, L., Pitari, G., Ippoliti, R., & Antonini, G. (2012). Physiological roles of ovotransferrin. *Biochim Biophys Acta, 1820*(3), 218-225.

Gilbert, A. B., Perry, M. M., Waddington, D., & Hardie, M. A. (1983). Role of atresia in establishing the follicular hierarchy in the ovary of the domestic hen (Gallus domesticus). *J Reprod Fertil, 69*(1), 221-227.

Giovanardi, D., Campagnari, E., Ruffoni, L. S., Pesente, P., Ortali, G., & Furlattini, V. (2005). Avian pathogenic Escherichia coli transmission from broiler breeders to their progeny in an integrated poultry production chain. *Avian Pathology, 34*(4), 313-318.

Gjevre, A. G., Kaldhusdal, M., & Eriksen, G. S. (2013). Gizzard erosion and ulceration syndrome in chickens and turkeys: a review of causal or predisposing factors. *Avian Pathol, 42*(4), 297-303.

Gong, D., Wilson, P. W., Bain, M. M., McDade, K., Kalina, J., Herve-Grepinet, V., et al. (2010). Gallin; an antimicrobial peptide member of a new avian defensin family, the ovodefensins, has been subject to recent gene duplication. *BMC Immunol, 11*, 12.

Goodall, N., Kisiswa, L., Prashar, A., Faulkner, S., Tokarczuk, P., Singh, K., et al. (2009). 3-Dimensional modelling of chick embryo eye development and growth using high resolution magnetic resonance imaging. *Exp Eye Res, 89*(4), 511-521.

Graham, A. (2001). The development and evolution of the pharyngeal arches. *Journal of Anatomy, 199*(1-2), 133-141.

Grapin-Botton, A., & Melton, D. A. (2000). Endoderm development: from patterning to organogenesis. *Trends Genet, 16*(3), 124-130.

Grevellec, A., & Tucker, A. S. (2010). The pharyngeal pouches and clefts: Development, evolution, structure and derivatives. *Seminars in Cell & Developmental Biology, 21*(3), 325-332.

Greyner, H., & Dzialowski, E. M. (2008). Mechanisms mediating the oxygen-induced vasoreactivity of the ductus arteriosus in the chicken embryo. *Am J Physiol Regul Integr Comp Physiol, 295*(5), R1647-1659.

Guan, J., Grenier, C., & Brooks, B. W. (2006). In Vitro Study of Salmonella enteritidis and Salmonella typhimurium Definitive Type 104: Survival in Egg Albumen and Penetration through the Vitelline Membrane. *Poultry Science, 85*(9), 1678-1681.

Guard-Bouldin, J., & Buhr, R. J. (2006). Evaluation of Eggshell Quality of Hens Infected with Salmonella enteritidis by Application of Compression. *Poultry Science, 85*(1), 129-135.

Guard-Petter, J. (2001). The chicken, the egg and Salmonella enteritidis. *Environ Microbiol, 3*(7), 421-430.

Guerin-Dubiard, C., Pasco, M., Molle, D., Desert, C., Croguennec, T., & Nau, F. (2006). Proteomic analysis of hen egg white. *J Agric Food Chem, 54*(11), 3901-3910.

Ha, Y. W., Son, M. J., Yun, K. S., & Kim, Y. S. (2007). Relationship between eggshell strength and keratan sulfate of eggshell membranes. *Comp Biochem Physiol A Mol Integr Physiol, 147*(4), 1109-1115.

Hallonet, M. E., Teillet, M. A., & Le Douarin, N. M. (1990). A new approach to the development of the cerebellum provided by the quail-chick marker system. *Development, 108*(1), 19-31.

Halsted, A. M. (1884). *Artificial incubation and incubators. A treatise on raising poultry by artificial means with descriptions and illustrations of every incubator or hatching apparatus worthy of notice, in ancient or modern times*. [Rye? N.Y.].

Hamal, K. R., Burgess, S. C., Pevzner, I. Y., & Erf, G. F. (2006). Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. *Poult Sci, 85*(8), 1364-1372.

Hamburger, V., & Hamilton, H. L. (1992). A series of normal stages in the development of the chick embryo. *Dev Dyn, 195*(4), 231-272.

Hamilton, R. M. J., Hollands, K. G., Voisey, P. W., & Grunder, A. A. (1979). Relationship between egg shell quality and shell breakage and factors that affect shell breakage in the fieldâ€“A review. *World's Poultry Science Journal, 35*(03), 177-190.

Hammershoj, M., & Qvist, K. B. (2001). Research Note: Importance of Hen Age and Egg Storage Time for Egg Albumen Foaming. *LWT - Food Science and Technology, 34*(2), 118-120.

Han, H., Li, X., Yu, K., & Lian, Z. (2011). Effects of Different CO, Levels on Chicken Embryonic Development During Early Stage of Incubation. *Journal of Animal and Veterinary Advances, 10*(20), 2624-2630.

Hangalapura, B. N., Nieuwland, M. G., Buyse, J., Kemp, B., & Parmentier, H. K. (2004). Effect of duration of cold stress on plasma adrenal and thyroid hormone levels and immune responses in chicken lines divergently selected for antibody responses. *Poult Sci, 83*(10), 1644-1649.

Hernandez-Hernandez, A., Gomez-Morales, J., Rodriguez-Navarro, A. B., Gautron, J., Nys, Y., & Garcia-Ruiz, J. M. (2008). Identification of Some Active Proteins in the Process of Hen Eggshell Formation. *Crystal Growth & Design, 8*(12), 4330-4339.

Hilfer, S. R. (1979). Follicle formation in the embryonic chick thyroid. III. Initiation of follicle formation. *Tissue and Cell, 11*(4), 727-740.

Hill, D. (1998). Practical Hatchery Sanitation Guidelines to Assure Quality. *World Poultry, 14*(10), 30-32.

Hincke, M. T., Chien, Y. C., Gerstenfeld, L. C., & McKee, M. D. (2008). Colloidal-gold immunocytochemical localization of osteopontin in avian eggshell gland and eggshell. *J Histochem Cytochem, 56*(5), 467-476.

Hincke, M. T., Gautron, J., Panheleux, M., Garcia-Ruiz, J., McKee, M. D., & Nys, Y. (2000). Identification and localization of lysozyme as a component of eggshell membranes and eggshell matrix. *Matrix Biology, 19*(5), 443-453.

Hincke, M. T., Nys, Y., & Gautron, J. (2010). The Role of Matrix Proteins in Eggshell Formation. *The Journal of Poultry Science, 47*(3), 208-219.

Hiruma, T., & Nakamura, H. (2003). Origin and development of the pronephros in the chick embryo. *Journal of Anatomy, 203*(6), 539-552.

Hoad, D. C. (1983). *Transportation of Day Old Chicks*. Paper presented at the Poultry Veterinary Study Group - Spring Meeting.

Hooley, W. (1919). *Will Hooley's Practical Poultry Culture: A Complete and Most Practical Treatise on Modern Poultry Culture ... with Special Chapter on "the Intensive System"*: Poultry Press, Limited.

Hopkins, M. L. (1935). Development of the thyroid gland in the chick embryo. *Journal of Morphology, 58*(2), 585-613.

Hsu, L., & Jeng, A. Y. (1995). Growth-promoting effects of endothelin-1 and endothelin-3 on chick embryos. *Brain Res, 703*(1-2), 72-78.

Hulet, R., Gladys, G., Hill, D., Meijerhof, R., & El-Shiekh, T. (2007). Influence of egg shell embryonic incubation temperature and broiler breeder flock age on posthatch growth performance and carcass characteristics. *Poult Sci, 86*(2), 408-412.

Humphrey, T. J., Baskerville, A., Mawer, S., Rowe, B., & Hopper, S. (1989). Salmonella enteritidis phage type 4 from the contents of intact eggs: a study involving naturally infected hens. *Epidemiol Infect, 103*(3), 415-423.

Humphrey, T. J., Whitehead, A., Gawler, A. H., Henley, A., & Rowe, B. (1991). Numbers of Salmonella enteritidis in the contents of naturally contaminated hens' eggs. *Epidemiol Infect, 106*(3), 489-496.

Hunton, P. (2005). Research on eggshell structure and quality: an historical overview. *Revista Brasileira de Ciência Avícola, 7*, 67-71.

Iowa State, U. (2013). Poultry Industry Manual. In U. S. D. o. Agriculture (Ed.), *The Foreign Animal Disease Preparedness and Response Plan (FAD PReP)/National Animal Health Emergency Management System (NAHEMS) Guidelines*. Ames: United States Department of Agriculture.

ISA. (2009). From Egg to Chicken, *Hatchery Manual*. The Netherlands: Institut de Sélection Animale BV.

Jackson, H. W. (1919). *Artificial Incubating and Brooding: A Complete Guide to the Successful Hatching and Rearing of Chicks by Modern Artificial Methods*: Reliable Poultry Journal Publishing Company.

Jaffredo, T., Gautier, R., Brajeul, V., & Dieterlen-Lievre, F. (2000). Tracing the progeny of the aortic hemangioblast in the avian embryo. *Dev Biol, 224*(2), 204-214.

James, R. G., & Schultheiss, T. M. (2003). Patterning of the avian intermediate mesoderm by lateral plate and axial tissues. *Dev Biol, 253*(1), 109-124.

Janke, O., Tzschentke, B., & Boerjan, M. (2004). Comparative Investigations of Heat Production and Body Temperature in Embryos of Modern Chicken Breeds. *Avian and Poultry Biology Reviews, 15*(3-4), 191-196.

Janke, O., Tzschentke, B., Hochel, J., & Nichelmann, M. (2002). Metabolic responses of chicken and muscovy duck embryos to high incubation temperatures. *Comp Biochem Physiol A Mol Integr Physiol, 131*(4), 741-750.

Jankovic, B. D., Isakovic, K., Lukic, M. L., Vujanovic, N. L., Petrovic, S., & Markovic, B. M. (1975). Immunological capacity of the chicken embryo. I. Relationship between the maturation of lymphoid tissues and the occurrence of cell-mediated immunity in the developing chicken embryo. *Immunology, 29*(3), 497-508.

Jiang, B., & Mine, Y. (2001). Phosphopeptides derived from hen egg yolk phosvitin: effect of molecular size on the calcium-binding properties. *Biosci Biotechnol Biochem, 65*(5), 1187-1190.

Jonchere, V., Rehault-Godbert, S., Hennequet-Antier, C., Cabau, C., Sibut, V., Cogburn, L. A., et al. (2010). Gene expression profiling to identify eggshell proteins involved in physical defense of the chicken egg. *BMC Genomics, 11*, 57.

Jones, M. P., Pierce, K. E., & Ward, D. (2007). Avian Vision: A Review of Form and Function with Special Consideration to Birds of Prey. *Journal of Exotic Pet Medicine, 16*(2), 69-87.

Jones, R. (1970). Manejo de Huevos Para Inbación. In Shaver (Ed.): Shaver Poultry Breeding Farms Limited.

Joseph, N. S., Lourens, A., & Moran, E. T., Jr. (2006). The effects of suboptimal eggshell temperature during incubation on broiler chick quality, live performance, and further processing yield. *Poult Sci, 85*(5), 932-938.

Kajiwara, E., Shigeta, A., Horiuchi, H., Matsuda, H., & Furusawa, S. (2003). Development of Peyer's Patch and Cecal Tonsil in Gut-Associated Lymphoid Tissues in the Chicken Embryo. *Journal of Veterinary Medical Science, 65*(5), 607-614.

Kane, J. N., Anzovin, S., & Podell, J. (1997). *Famous first facts : a record of first happenings, discoveries, and inventions in American history / Joseph Nathan Kane, Steven Anzovin & Janet Podell*. New York :: H.W. Wilson.

Karadas, F., Pappas, A. C., Surai, P. F., & Speake, B. K. (2005). Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 141*(2), 244-251.

Kardon, G. (1998). Muscle and tendon morphogenesis in the avian hind limb. *Development, 125*(20), 4019-4032.

Karlsson, O., & Lilja, C. (2008). Eggshell structure, mode of development and growth rate in birds. *Zoology (Jena), 111*(6), 494-502.

Kawano, J., Shimizu, A., Saitoh, Y., Yagi, M., Saito, T., & Okamoto, R. (1996). Isolation of methicillin-resistant coagulase-negative staphylococci from chickens. *Journal of Clinical Microbiology, 34*(9), 2072-2077.

Keller, L. H., Benson, C. E., Krotec, K., & Eckroade, R. J. (1995). Salmonella enteritidis colonization of the reproductive tract and forming and freshly laid eggs of chickens. *Infect Immun, 63*(7), 2443-2449.

Kemps, B. J., Bamelis, F. R., Mertens, K., Decuypere, E. M., De Baerdemaeker, J. G., & De Ketelaere, B. (2010). The assessment of viscosity measurements on the albumen of consumption eggs as an indicator for freshness. *Poult Sci, 89*(12), 2699-2703.

Kieny, M., & Chevallier, A. (1979). Autonomy of tendon development in the embryonic chick wing. *Development, 49*(1), 153-165.

Kim, S. K., Hebrok, M., & Melton, D. A. (1997). Pancreas Development in the Chick Embryo. *Cold Spring Harbor Symposia on Quantitative Biology, 62*, 377-383.

Knox, C. W., & Olsen, M. W. (1936). The Effect of Tremulous Air Cells Upon the Hatchability of Eggs. *Poultry Science, 15*(4), 345-348.

Kodali, V. K., Gannon, S. A., Paramasivam, S., Raje, S., Polenova, T., & Thorpe, C. (2011). A novel disulfide-rich protein motif from avian eggshell membranes. *PLoS One, 6*(3), e18187.

Kulesa, P. M., Lefcort, F., & Kasemeier-Kulesa, J. C. (2009). The migration of autonomic precursor cells in the embryo. *Auton Neurosci, 151*(1), 3-9.

Kuratani, S., Tanaka, S., Ishikawa, Y., & Zukeran, C. (1988). Early development of the hypoglossal nerve in the chick embryo as observed by the whole-mount nerve staining method. *American Journal of Anatomy, 182*(2), 155-168.

Laane, H. M. (1979). The septation of the arterial pole of the heart in the chick embryo. III. Development of the truncus arteriosus of the heart of chick embryos from 5 1/2 to 7 days of incubation. *Acta morphologica Neerlando-Scandinavica, 17*(1), 1-20.

Lapao, C., Gama, L. T., & Soares, M. C. (1999). Effects of broiler breeder age and length of egg storage on albumen characteristics and hatchability. *Poult Sci, 78*(5), 640-645.

Lasagabaster, A., Arboleya, J. C., & de Marañón, I. M. (2011). Pulsed light technology for surface decontamination of eggs: Impact on Salmonella inactivation and egg quality. *Innovative Food Science & Emerging Technologies, 12*(2), 124-128.

Lavelin, I., Meiri, N., Genina, O., Alexiev, R., & Pines, M. (2001). Na(+)-K(+)-ATPase gene expression in the avian eggshell gland: distinct regulation in different cell types. *Am J Physiol Regul Integr Comp Physiol, 281*(4), R1169-1176.

Lavelin, I., Meiri, N., & Pines, M. (2000). New insight in eggshell formation. *Poult Sci, 79*(7), 1014-1017.

Lavelin, I., Yarden, N., Ben-Bassat, S., Bar, A., & Pines, M. (1998). Regulation of osteopontin gene expression during egg shell formation in the laying hen by mechanical strain. *Matrix Biol, 17*(8-9), 615-623.

Le Douarin, N., Fontaine, J., & Le Liévre, C. (1974). New studies on the neural crest origin of the avian ultimobranchial glandular cells: Interspecific combinations and cytochemical characterization of C cells based on the uptake of biogenic amine precursors. *Histochemistry, 38*(4), 297-305.

Le Douarin, N. M., & Teillet, M.-A. e. (1973). The migration of neural crest cells to the wall of the digestive tract in avian embryo. *Development, 30*(1), 31-48.

Le Douarin, N. M., & Teillet, M. A. (1973). The migration of neural crest cells to the wall of the digestive tract in avian embryo. *J Embryol Exp Morphol, 30*(1), 31-48.

Le Douarin, N. M., Teillet, M. A., Ziller, C., & Smith, J. (1978). Adrenergic differentiation of cells of the cholinergic ciliary and Remak ganglia in avian embryo after in vivo transplantation. *Proc Natl Acad Sci U S A, 75*(4), 2030-2034.

Leeson, S., & Caston, L. J. (1997). A problem with characteristics of the thin albumen in laying hens. *Poult Sci, 76*(10), 1332-1336.

Leksrisompong, N., Romero-Sanchez, H., Plumstead, P. W., Brannan, K. E., & Brake, J. (2007). Broiler incubation. 1. Effect of elevated temperature during late incubation on body weight and organs of chicks. *Poult Sci, 86*(12), 2685-2691.

Lemaire, L., & Kessel, M. (1997). Gastrulation and homeobox genes in chick embryos. *Mech Dev, 67*(1), 3-16.

Lewis, H. R. (1913). *Productive poultry husbandry : a complete text dealing with the principles and practices involved in the management of poultry / by Harry R. Lewis*. London :: J.B. Lippincott.

Li, C. S., Wang, L. Y., & Chou, C. C. (2005). Field Evaluation of Flock Production Performance of In ovo Injection of Infectious Bursal Disease Virus Immune Complex Vaccine in Commercial Broiler Farms. *The Journal of Applied Poultry Research, 14*(2), 338-344.

Li, X., Nakano, T., Sunwoo, H. H., Paek, B. H., Chae, H. S., & Sim, J. S. (1998). Effects of egg and yolk weights on yolk antibody (IgY) production in laying chickens. *Poult Sci, 77*(2), 266-270.

Lierz, M., Gooss, O., & Hafez, H. M. (2006). Noninvasive Heart Rate Measurement Using a Digital Egg Monitor in Chicken and Turkey Embryos. *Journal of Avian Medicine and Surgery, 20*(3), 141-146.

Lipar, J. L., Ketterson, E. D., Nolan, V., Jr., & Casto, J. M. (1999). Egg yolk layers vary in the concentration of steroid hormones in two avian species. *Gen Comp Endocrinol, 115*(2), 220-227.

Lippincott, W. A., & Card, L. E. (1921). *Poultry production* (3d ed.). Philadelphia,: Lea & Febiger.

Lourens, A. (2008). *Embryo temperature during incubation: practice and theory.* Wageningen University.

Lourens, A., Meijerhof, R., Kemp, B., & van den Brand, H. (2011). Energy partitioning during incubation and consequences for embryo temperature: a theoretical approach. *Poult Sci, 90*(2), 516-523.

Lourens, A., van den Brand, H., Heetkamp, M. J., Meijerhof, R., & Kemp, B. (2007). Effects of eggshell temperature and oxygen concentration on embryo growth and metabolism during incubation. *Poult Sci, 86*(10), 2194-2199.

Lourens, A., van den Brand, H., Meijerhof, R., & Kemp, B. (2005). Effect of eggshell temperature during incubation on embryo development, hatchability, and posthatch development. *Poult Sci, 84*(6), 914-920.

Maatjens, C. M., Reijrink, I. A., van den Anker, I., Molenaar, R., van der Pol, C. W., Kemp, B., et al. (2014). Temperature and CO2 during the hatching phase. II. Effects on chicken embryo physiology. *Poult Sci, 93*(3), 655-663.

Mahfudz, L. D. (2015). Hatchery Design Poultry Production Laboratory Faculty of Animal and Agriculture Science Diponegoro University.

Maina, J. N. (2003a). Developmental dynamics of the bronchial (airway) and air sac systems of the avian respiratory system from dayÂ 3 to day 26 of life: a scanning electron microscopic study of the domestic fowl, Gallus gallus variant domesticus. *Anatomy and Embryology, 207*(2), 119-134.

Maina, J. N. (2003b). A systematic study of the development of the airway (bronchial) system of the avian lung from days 3 to 26 of embryogenesis: a transmission electron microscopic study on the domestic fowl, Gallus gallus variant domesticus. *Tissue Cell, 35*(5), 375-391.

Maina, J. N. (2006). Development, structure, and function of a novel respiratory organ, the lung-air sac system of birds: to go where no other vertebrate has gone. *Biological Reviews, 81*(4), 545-579.

Makanya, A. N., Hlushchuk, R., & Djonov, V. (2011). The pulmonary blood-gas barrier in the avian embryo: inauguration, development and refinement. *Respir Physiol Neurobiol, 178*(1), 30-38.

Malorny, B., Bunge, C., & Helmuth, R. (2007). A real-time PCR for the detection of Salmonella Enteritidis in poultry meat and consumption eggs. *J Microbiol Methods, 70*(2), 245-251.

Maňáková, E., & Titlbach, M. (2007). Development of the Chick Pancreas with Regard to Estimation of the Relative Occurrence and Growth of Endocrine Tissue. *Anatomia, Histologia, Embryologia, 36*(2), 127-134.

Mann, K. (2007). The chicken egg white proteome. *Proteomics, 7*(19), 3558-3568.

Mann, K. (2008). Proteomic analysis of the chicken egg vitelline membrane. *Proteomics, 8*(11), 2322-2332.

Mann, K., Gautron, J. l., Nys, Y., McKee, M. D., Bajari, T., Schneider, W. J., et al. (2003). Disulfide-linked heterodimeric clusterin is a component of the chicken eggshell matrix and egg white. *Matrix Biology, 22*(5), 397-407.

Mann, K., & Mann, M. (2008). The chicken egg yolk plasma and granule proteomes. *Proteomics, 8*(1), 178-191.

Marques, V. M. M. B. (2012). Petersime's entrance in the European markets. In R. Noiva (Ed.). Lisbon.

Martin, R. D. (2001). The Development of Artificial Incubation of Eggs.

Mattila, P., Lehikoinen, K., Kiiskinen, T., & Piironen, V. (1999). Cholecalciferol and 25-hydroxycholecalciferol content of chicken egg yolk as affected by the cholecalciferol content of feed. *J Agric Food Chem, 47*(10), 4089-4092.

Mayes, F. J., & Takeballi, M. A. (1984). Storage of the Eggs of the Fowl (Gallus domesticus) before Incubation: A Review. *World's Poultry Science Journal, 40*(02), 131-140.

McIndoe, W. (1959). A lipophosphoprotein complex in hen plasma associated with yolk production. *Biochem J, 72*(1), 153-159.

McNabb, F. M. (2006). Avian thyroid development and adaptive plasticity. *Gen Comp Endocrinol, 147*(2), 93-101.

McNamee, P. T., & Smyth, J. A. (2000). Bacterial chondronecrosis with osteomyelitis ('femoral head necrosis') of broiler chickens: A review. *Avian Pathology, 29*(5), 477-495.

Meade, K. G., Higgs, R., Lloyd, A. T., Giles, S., & O'Farrelly, C. (2009). Differential antimicrobial peptide gene expression patterns during early chicken embryological development. *Dev Comp Immunol, 33*(4), 516-524.

Meijerhof, R. (1997). The Importance of Egg and Chick Transportation. *World Poultry, 13*(11), 17-18.

Meijerhof, R., & van Beek, G. (1993). Mathematical Modelling of Temperature and Moisture Loss of Hatching Eggs. *Journal of Theoretical Biology, 165*(1), 27-41.

Merida-Velasco, J. A., Espin-Ferra, J., Valdecasas, R. L., & Garcia-Garcia, J. D. (1988). Morphogenesis of the ultimobranchial body and its colonizing cells in the chick embryo. *Archives d'anatomie, d'histologie et d'embryologie normales et experimentales, 71*, 43-55.

Messens, W., Grijspeerdt, K., & Herman, L. (2006). Eggshell penetration of hen's eggs by Salmonella enterica serovar Enteritidis upon various storage conditions. *Br Poult Sci, 47*(5), 554-560.

Mey, J., & Thanos, S. (2000). Development of the visual system of the chick. I. Cell differentiation and histogenesis. *Brain Res Brain Res Rev, 32*(2-3), 343-379.

Michell, B. C., Gomes, A. D., Baião, N. C., Resende, M., Lara, L. J. C., & Martins, N. R. S. (2009). Effect of maternally-derived antibodies on the performance and immunity of broilers induced by in ovo or post-hatching immunizations with a live vaccine against infectious bursal disease. *Revista Brasileira de Ciência Avícola, 11*, 57-63.

Mikšík, I., Holáň, V., & Deyl, Z. (1996). Avian eggshell pigments and their variability. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 113*(3), 607-612.

Milinsk, M. C., Murakami, A. E., Gomes, S. T. M., Matsushita, M., & de Souza, N. E. (2003). Fatty acid profile of egg yolk lipids from hens fed diets rich in n-3 fatty acids. *Food Chemistry, 83*(2), 287-292.

Miller, J. B., & Stockdale, F. E. (1986). Developmental regulation of the multiple myogenic cell lineages of the avian embryo. *J Cell Biol, 103*(6 Pt 1), 2197-2208.

Miller, S. A., Clark, C., Cooney, R., Crary, E., & Payzant, W. (1998). Apoptosis Fenestrates Chick Cloacal Membrane and Occluded Rectal Cord and May Have a Minor Role in Removal of Pharyngeal Membranesa. *Annals of the New York Academy of Sciences, 857*(1), 268-271.

Mine, Y., & Kovacs-Nolan, J. (2004). Biologically Active Hen Egg Components in Human Health and Disease. *The Journal of Poultry Science, 41*(1), 1-29.

Molenaar, R., Hulet, R., Meijerhof, R., Maatjens, C. M., Kemp, B., & van den Brand, H. (2011). High eggshell temperatures during incubation decrease growth performance and increase the incidence of ascites in broiler chickens. *Poult Sci, 90*(3), 624-632.

Molenaar, R., Meijerhof, R., van den Anker, I., Heetkamp, M. J., van den Borne, J. J., Kemp, B., et al. (2010). Effect of eggshell temperature and oxygen concentration on survival rate and nutrient utilization in chicken embryos. *Poult Sci, 89*(9), 2010-2021.

Molenaar, R., van den Borne, J. J., Hazejager, E., Kristensen, N. B., Heetkamp, M. J., Meijerhof, R., et al. (2013). High environmental temperature increases glucose requirement in the developing chicken embryo. *PLoS One, 8*(4), e59637.

Monsoro-Burq, A. H., Stieber, A., Bontoux, M., Le Douarin, N., & Gonatas, N. K. (1998). Environmental factors modulate the size and the secretory activity of the notochord. A study of the Golgi apparatus in avian embryos. *C R Acad Sci III, 321*(8), 621-631.

Moonen, R. M., & Villamor, E. (2011). Developmental changes in mesenteric artery reactivity in embryonic and newly hatched chicks. *J Comp Physiol B, 181*(8), 1063-1073.

Moore, K. L., & Persaud, T. V. N. (2008). *Embriologia Clínica*: Saunders Elsevier.

Moore, M. A. S., & Owen, J. J. T. (1967). EXPERIMENTAL STUDIES ON THE DEVELOPMENT OF THE THYMUS. *The Journal of Experimental Medicine, 126*(4), 715-726.

Moran, E. T., Jr. (2007). Nutrition of the developing embryo and hatchling. *Poult Sci, 86*(5), 1043-1049.

Moritz, K. M., & Wintour, E. M. (1999). Functional development of the meso- and metanephros. *Pediatric Nephrology, 13*(2), 171-178.

Mortola, J. P. (2006). Metabolic response to cooling temperatures in chicken embryos and hatchlings after cold incubation. *Comp Biochem Physiol A Mol Integr Physiol, 145*(4), 441-448.

Mortola, J. P. (2009). Gas exchange in avian embryos and hatchlings. *Comp Biochem Physiol A Mol Integr Physiol, 153*(4), 359-377.

Mortola, J. P., & Labbe, K. (2005). Oxygen consumption of the chicken embryo: interaction between temperature and oxygenation. *Respir Physiol Neurobiol, 146*(1), 97-106.

Mortola, J. P., Marinescu, D. C., Pierre, A., & Artman, L. (2012). Metabolic and heart rate responses to hypoxia in early chicken embryos in the transition from diffusive to convective gas transport. *Respir Physiol Neurobiol, 181*(2), 109-117.

Mortola, J. P., Wills, K., Trippenbach, T., & Al Awam, K. (2010). Interactive effects of temperature and hypoxia on heart rate and oxygen consumption of the 3-day old chicken embryo. *Comp Biochem Physiol A Mol Integr Physiol, 155*(3), 301-308.

Moussa, I. M., Gassem, M. A., Al-Doss, A. A., S., M. W. A., & Abdel Mawgood, A. L. (2010). Using molecular techniques for rapid detection of Salmonella serovars in frozen chicken and chicken products collected from Riyadh, Saudi Arabia. *Afr J Biotech, 9*(5), 612-619.

Mueller, C. A., Burggren, W. W., Tazawa, H., & Scanes, C. G. (2015). Chapter 32 - The Physiology of the Avian Embryo. In *Sturkie's Avian Physiology (Sixth Edition)* (pp. 739-766). San Diego: Academic Press.

Muniyappa, K., & Adiga, P. R. (1979). Isolation and characterization of thiamin-binding protein from chicken egg white. *Biochem J, 177*(3), 887-894.

Nakamura, Y., Yamamoto, Y., Usui, F., Mushika, T., Ono, T., Setioko, A. R., et al. (2007). Migration and proliferation of primordial germ cells in the early chicken embryo. *Poult Sci, 86*(10), 2182-2193.

Nakano, T., Ikawa, N., & Ozimek, L. (2001). Extraction of glycosaminoglycans from chicken eggshell. *Poult Sci, 80*(5), 681-684.

Nakano, T., Ikawa, N. I., & Ozimek, L. (2003). Chemical composition of chicken eggshell and shell membranes. *Poult Sci, 82*(3), 510-514.

Nechaeva, M., Vladimirova, I., & Alexeeva, T. (2010). Effect of Acute Hypoxia on the Motor Activity and Heart Rate of the 10- and 14-Day Chick Embryo. *The Open Ornithology Journal, 3*, 127-133.

Nechaeva, M. V. (2011). Physiological responses to acute changes in temperature and oxygenation in bird and reptile embryos. *Respir Physiol Neurobiol, 178*(1), 108-117.

Needham, J. (1926a). The Energy-Sources in Ontogenesis I. The Urea Content of the Developing Avian Egg. *Journal of Experimental Biology, 3*(3), 189-205.

Needham, J. (1926b). The Energy-Sources in Ontogenesis II. The Uric Acid Content and the General Protein Metabolism of the Developing Avian Egg. *Journal of Experimental Biology, 4*(2), 114-144.

Needham, J. (1926c). The Energy-Sources in Ontogenesis III. The Ammonia Content of the Developing Avian Egg and the Theory of Recapitulation. *J Exp Biol, 4*, 145-154.

Needham, J. (1927a). The Energy-Sources in Ontogenesis V. The Carbohydrate Metabolism of the Developing Avian Egg. *Journal of Experimental Biology, 5*(1), 6-26.

Needham, J. (1927b). The Energy-Sources in Ontogenesis VI. The Relation between Combustion, Constitution, and Concentration in the Developing Avian Embryo. *Journal of Experimental Biology, 5*(1), 27-42.

Needham, J. (1927). The Energy-Sources in Ontogensis IV. The Relation Between Absorption and Combustion of Protein and Fat in the Developing Avian Egg. *J Exp Biol 4*, 258-279.

Newman, S. A., & Frisch, H. L. (1979). Dynamics of skeletal pattern formation in developing chick limb. *Science, 205*(4407), 662-668.

Nielsen, H. (1998). Hen age and fatty acid composition of egg yolk lipid. *Br Poult Sci, 39*(1), 53-56.

Nimalaratne, C., Lopes-Lutz, D., Schieber, A., & Wu, J. (2011). Free aromatic amino acids in egg yolk show antioxidant properties. *Food Chemistry, 129*(1), 155-161.

Nisson, C. (1894). *Nisson on incubation: especially adapted to the Pacific Coast*: C. Nisson.

Noiva, R. M., Menezes, A. C., & Peleteiro, M. C. (2014). Influence of temperature and humidity manipulation on chicken embryonic development. *BMC Vet Res, 10*, 234.

Nomura, O., Nakabayashi, O., Nishimori, K., Yasue, H., & Mizuno, S. (1999). Expression of five steroidogenic genes including aromatase gene at early developmental stages of chicken male and female embryos. *J Steroid Biochem Mol Biol, 71*(3-4), 103-109.

Nowicki, J. L., Takimoto, R., & Burke, A. C. (2003). The lateral somitic frontier: dorso-ventral aspects of anterio-posterior regionalization in avian embryos. *Mech Dev, 120*(2), 227-240.

Nys, Y., Gautron, J. l., Garcia-Ruiz, J. M., & Hincke, M. T. (2004). Avian eggshell mineralization: biochemical and functional characterization of matrix proteins. *Comptes Rendus Palevol, 3*(6â€“7), 549-562.

Odabas, A. Z., Miles, R. D., Balaban, M. O., & Portier, K. M. (2007). Changes in Brown Eggshell Color As the Hen Ages. *Poultry Science, 86*(2), 356-363.

Ohta, Y., Yoshida, T., & Tsushima, N. (2004). Comparison between broilers and layers for growth and protein use by embryos. *Poultry Science, 83*(5), 783-787.

Okabayashi, N., Yasuo, S., Watanabe, M., Namikawa, T., Ebihara, S., & Yoshimura, T. (2003). Ontogeny of circadian clock gene expression in the pineal and the suprachiasmatic nucleus of chick embryo. *Brain Res, 990*(1-2), 231-234.

Oviedo-Rondón, E. O., Wineland, M. J., Funderburk, S., Small, J., Cutchin, H., & Mann, M. (2009). Incubation conditions affect leg health in large, high-yield broilers. *The Journal of Applied Poultry Research, 18*(3), 640-646.

Oznurlu, Y., Celik, I., Telatar, T., & Sur, E. (2010). Histochemical and histological evaluations of the effects of high incubation temperature on embryonic development of thymus and bursa of Fabricius in broiler chickens. *British Poultry Science, 51*(1), 43-51.

Page, K. M., Maini, P. K., Monk, N. A., & Stern, C. D. (2001). A model of primitive streak initiation in the chick embryo. *J Theor Biol, 208*(4), 419-438.

Panheleux, M., Bain, M., Fernandez, M. S., Morales, I., Gautron, J., Arias, J. L., et al. (1999). Organic matrix composition and ultrastructure of eggshell: a comparative study. *Br Poult Sci, 40*(2), 240-252.

Parakkal, P. F., & Matoltsy, A. G. (1968). An electron microscopic study of developing chick skin. *Journal of Ultrastructure Research, 23*(5), 403-416.

Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, (2010).

Parsons, B. N., Crayford, G., Humphrey, T. J., & Wigley, P. (2013). Infection of chickens with antimicrobial-resistant Salmonella enterica Typhimurium DT193 and monophasic Salmonella Typhimurium-like variants: an emerging risk to the poultry industry? *Avian Pathol, 42*(5), 443-446.

PasReform. (2010). Circadian incubation$^{TM}$ - next generation design for the future-focused hatchery. 1-16.

PasReform. (2012). Future Focused Incubation (pp. 1-16).

Patten, B. M. (1971). *Early embryology of the chick* (5th ed.). New York,: McGraw-Hill.

Pattison, M. (2008). *Poultry diseases* (6th ed.). Edinburgh ; New York: Elsevier/Butterworth-Heinemann.

Peebles, E. D., Burnham, M. R., Gardner, C. W., Brake, J., Bruzual, J. J., & Gerard, P. D. (2001). Effects of incubational humidity and hen age on embryo composition in broiler hatching eggs from young breeders. *Poult Sci, 80*(9), 1299-1304.

Pellet-Many, C., & Baggott, G. K. (2005). The pH and ionic composition of the sub-embryonic fluid of the Japanese quail (Coturnix c. japonica). *Avian and Poultry Biology Reviews, 16*(4), 176-177.

Pera, E., Stein, S., & Kessel, M. (1999). Ectodermal patterning in the avian embryo: epidermis versus neural plate. *Development, 126*(1), 63-73.

Perriton, C. L., Powles, N., Chiang, C., Maconochie, M. K., & Cohn, M. J. (2002). Sonic hedgehog Signaling from the Urethral Epithelium Controls External Genital Development. *Developmental Biology, 247*(1), 26-46.

Peterka, M., Peterkova, R., & Likovsky, Z. (1996). Teratogenic and lethal effects of long-term hyperthermia and hypothermia in the chick embryo. *Reprod Toxicol, 10*(4), 327-332.

Petersen, A., Christensen, J. P., Kuhnert, P., Bisgaard, M., & Olsen, J. E. (2006). Vertical transmission of a fluoroquinolone-resistant Escherichia coli within an integrated broiler operation. *Veterinary Microbiology, 116*(1â€“3), 120-128.

Petersime. (2011). Embryo-Response Incubation$^{TM}$ - Incubation technologies for maximum economic return. 1-19.

Petersime. (2013a). Petersime Transferring Machine (PTM) Semi-automatic egg transfer table for small size operations. Belgium.

Petersime. (2013b). Tray B00568 Standard setter tray for chicken eggs. Belgium.

Petersime. (2014). Vacuum egg lifter- Lightweight vacuum egg lifter for smooth egg handling. Belgium.

Petersime, I. M. (1925). United States Patent No.

Petersime, I. M., & Petersime, R. M. (1939). United States Patent No.

Petersime, R. M. (1932). United States Patent No.

Petersime, R. M. (1941). United States Patent No.

Piestun, Y., Halevy, O., & Yahav, S. (2009). Thermal manipulations of broiler embryos--the effect on thermoregulation and development during embryogenesis. *Poult Sci, 88*(12), 2677-2688.

Pines, M. (2007). *The involvement of matrix proteins in eggshell formation.* Paper presented at the "19th Australian Poultry Science Symposium", New South Wales, Australia.

Pines, M., Knopov, V., & Bar, A. (1995). Involvement of osteopontin in egg shell formation in the laying chicken. *Matrix Biol, 14*(9), 765-771.

Poelmann, R. E., Gittenberger-de Groot, A. C., & Hierck, B. P. (2008). The development of the heart and microcirculation: role of shear stress. *Med Biol Eng Comput, 46*(5), 479-484.

Poelmann, R. E., Groot, A. C. G.-d., Vicente-Steijn, R., Wisse, L. J., Bartelings, M. M., Everts, S., et al. (2014). Evolution and Development of Ventricular Septation in the Amniote Heart. *PLoS One, 9*(9), e106569.

Prescott, l. M., Harley, J. P., & Klein, D. A. (2005). *Microbiology* (6th ed.). New York: McGraw Hill.

Pulikanti, R., Peebles, E. D., Keirs, R. W., Bennett, L. W., Keralapurath, M. M., & Gerard, P. D. (2010). Pipping muscle and liver metabolic profile changes and relationships in broiler embryos on days 15 and 19 of incubation. *Poult Sci, 89*(5), 860-865.

Qayyum, S. R., Webb, S., Anderson, R. H., Verbeek, F. J., Brown, N. A., & Richardson, M. K. (2001). Septation and valvar formation in the outflow tract of the embryonic chick heart. *The Anatomical Record, 264*(3), 273-283.

Rahn, K., De Grandis, S. A., Clarke, R. C., McEwen, S. A., Galan, J. E., Ginocchio, C., et al. (1992). Amplification of an invA gene sequence of Salmonella typhimurium by polymerase chain reaction as a specific method of detection of Salmonella. *Mol Cell Probes, 6*(4), 271-279.

Réaumur, R.-A. F. d. (1750). *The art of hatching and bringing up domestick fowls of all kinds at any time of the year. Either by means of the heat of hot-beds, or that of common fire*. London: C. Davis [etc.].

Reijntjes, S., Blentic, A., Gale, E., & Maden, M. (2005). The control of morphogen signalling: regulation of the synthesis and catabolism of retinoic acid in the developing embryo. *Dev Biol, 285*(1), 224-237.

Reijrink, I. A., Meijerhof, R., Kemp, B., & van den Brand, H. (2010). Influence of egg warming during storage and hypercapnic incubation on egg characteristics, embryonic development, hatchability, and chick quality. *Poult Sci, 89*(11), 2470-2483.

Rettenbacher, S., Mostl, E., & Groothuis, T. G. (2009). Gestagens and glucocorticoids in chicken eggs. *Gen Comp Endocrinol, 164*(2-3), 125-129.

Robertson, K., & Mason, I. (1995). Expression of ret in the chicken embryo suggests roles in regionalisation of the vagal neural tube and somites and in development of multiple neural crest and placodal lineages. *Mech Dev, 53*(3), 329-344.

Rodriguez-Gallardo, L., Sanchez-Arrones, L., Fernandez-Garre, P., & Puelles, L. (2005). Agreement and disagreement among fate maps of the chick neural plate. *Brain Res Brain Res Rev, 49*(2), 191-201.

Rodriguez-Romo, L. A., & Yousef, A. E. (2005). Inactivation of Salmonella enterica serovar Enteritidis on shell eggs by ozone and UV radiation. *J Food Prot, 68*(4), 711-717.

Romanoff, A. L., & Romanoff, A. J. (1972). *Pathogenesis of the avian embryo; an analysis of causes of malformations and prenatal death [by] Alexis L. Romanoff, with the collaboration of Anastasia J. Romanoff*. New York: Wiley-Interscience.

Ruiz, J., & Lunam, C. A. (2002). Effect of pre-incubation storage conditions on hatchability, chick weight at hatch and hatching time in broiler breeders. *British Poultry Science, 43*(3), 374-383.

Sadler, T. W. (2011). *Langman's Medical Embryology*: Wolters Kluwer Health.

Salehi, T. Z., Mahzounieh, M., & Saeedzadeh, A. (2005). Detection of InvA Gene in Isolated Salmonella from Broilers by PCR Method. *International Journal of Poultry Science, 4*(8), 557-559.

Sanders, E. J., & Wride, M. A. (1997). Roles for growth and differentiation factors in avian embryonic development. *Poult Sci, 76*(1), 111-117.

Scanes, C. G. (2015). Chapter 22 - Avian Endocrine System. In *Sturkie's Avian Physiology (Sixth Edition)* (pp. 489-496). San Diego: Academic Press.

Schijns, V. E. J. C., van de Zande, S., Lupiani, B., Reddy, S. M., & Kaiser, K. A. S. K. (2014). Chapter 20 - Practical Aspects of Poultry Vaccination. In *Avian Immunology (Second Edition)* (pp. 345-362). Boston: Academic Press.

Schlueter, J., & Brand, T. (2009). A right-sided pathway involving FGF8/Snai1 controls asymmetric development of the proepicardium in the chick embryo. *Proc Natl Acad Sci U S A, 106*(18), 7485-7490.

Schneider, R. A. (1999). Neural crest can form cartilages normally derived from mesoderm during development of the avian head skeleton. *Dev Biol, 208*(2), 441-455.

Schoeni, J. L., Glass, K. A., McDermott, J. L., & Wong, A. C. (1995). Growth and penetration of Salmonella enteritidis, Salmonella heidelberg and Salmonella typhimurium in eggs. *Int J Food Microbiol, 24*(3), 385-396.

Schowengerdt, P. F., & Colorado Agricultural College. Extension, S. (1918). *Incubation and Brooding*: Colorado Agricultural College.

Setta, A., Barrow, P. A., Kaiser, P., & Jones, M. A. (2012). Immune dynamics following infection of avian macrophages and epithelial cells with typhoidal and non-typhoidal Salmonella enterica serovars; bacterial invasion and persistence, nitric oxide and oxygen production, differential host gene expression, NF-ÎºB signalling and cell cytotoxicity. *Veterinary Immunology and Immunopathology, 146*(3â€“4), 212-224.

Seydim, A. C., & Dawson, P. L. (1999). Packaging effects on shell egg breakage rates during simulated transportation. *Poultry Science, 78*(1), 148-151.

Shafey, T. M., Al-Batshan, H. A., Ghannam, M. M., & Al-Ayed, M. S. (2005). Effect of intensity of eggshell pigment and illuminated incubation on hatchability of brown eggs. *Br Poult Sci, 46*(2), 190-198.

Shah, R. M., & Crawford, B. J. (1980). Development of the secondary palate in chick embryo: a light and electron microscopic and histochemical study. *Investigative & cell pathology, 3*(4), 319-328.

Shah, T. A. (2011). *Heat Stabilizer.* Electrical Universiti Kuala Lumpur, Kuala Lumpur.

Shamim, H., & Mason, I. (1998). Expression of Gbx-2 during early development of the chick embryo. *Mech Dev, 76*(1-2), 157-159.

Sharma, J. M., Zhang, Y., Jensen, D., Silke, R., & Yeh, H. Y. (2002). Field Trial in Commercial Broilers with a Multivalent In Ovo Vaccine Comprising a Mixture of Live Viral Vaccines Against Marek's Disease, Infectious Bursal Disease, Newcastle Disease, and Fowl Pox. *Avian Diseases, 46*(3), 613-622.

Shaver. (1980). Production & Handling of Hatching Eggs for Maximum Hatchability. Ontario: Shaver Poultry Breeding Farms Limited.

Shellswell, G. B., & Wolpert, L. (1977). The pattern of muscle and tendon development in the chick wing In D. A. Ede, J. R. Hinchliffe & M. Balls (Eds.), *Vertebrate Limb and Somite Morphogenesis, British Society for Developmental Biology Symposia, Cambridge Studies in Medieval Life and Thought* (Vol. Volume 3
pp. 72-86): CUP Archive.

Shen, X., Steyrer, E., Retzek, H., Sanders, E. J., & Schneider, W. J. (1993). Chicken oocyte growth: receptor-mediated yolk deposition. *Cell Tissue Res, 272*(3), 459-471.

Shinder, D., Ruzal, M., Giloh, M., Druyan, S., Piestun, Y., & Yahav, S. (2011). Improvement of cold resistance and performance of broilers by acute cold exposure during late embryogenesis. *Poult Sci, 90*(3), 633-641.

Silversides, F. G., & Budgell, K. (2004). The relationships among measures of egg albumen height, pH, and whipping volume. *Poult Sci, 83*(10), 1619-1623.

Smith, F., Hu, D., Young, N. M., Lainoff, A. J., Jamniczky, H. A., Maltepe, E., et al. (2013). The effect of hypoxia on facial shape variation and disease phenotypes in chicken embryos. *Dis Model Mech, 6*(4), 915-924.

Sneddon, H., Hadden, R., & Hepper, P. G. (1998). Chemosensory learning in the chicken embryo. *Physiol Behav, 64*(2), 133-139.

Soledad Fernandez, M., Moya, A., Lopez, L., & Arias, J. L. (2001). Secretion pattern, ultrastructural localization and function of extracellular matrix molecules involved in eggshell formation. *Matrix Biol, 19*(8), 793-803.

Solomon, S. E. (2010). The eggshell: strength, structure and function. *Br Poult Sci, 51 Suppl 1*, 52-59.

Soumet, C., Ermel, G., Rose, N., Rose, V., Drouin, P., Salvat, G., et al. (1999). Evaluation of a multiplex PCR assay for simultaneous identification of Salmonella sp., Salmonella enteritidis and Salmonella typhimurium from environmental swabs of poultry houses. *Lett Appl Microbiol, 28*(2), 113-117.

Sozcu, A., & Ipek, A. (2015). Acute and chronic eggshell temperature manipulations during hatching term influence hatchability, broiler performance, and ascites incidence. *Poult Sci, 94*(2), 319-327.

Speake, B. K., Murray, A. M., & Noble, R. C. (1998). Transport and transformations of yolk lipids during development of the avian embryo. *Prog Lipid Res, 37*(1), 1-32.

Stern, C. D. (2005). The chick; a great model system becomes even greater. *Dev Cell, 8*(1), 9-17.

Sugimoto, Y., Sanuki, S., Ohsako, S., Higashimoto, Y., Kondo, M., Kurawaki, J., et al. (1999). Ovalbumin in developing chicken eggs migrates from egg white to embryonic organs while changing its conformation and thermal stability. *J Biol Chem, 274*(16), 11030-11037.

Surai, P. F., Ionov, I. A., Kuklenko, T. V., Kostjuk, I. A., MacPherson, A., Speake, B. K., et al. (1998). Effect of supplementing the hen's diet with vitamin A on the accumulation of vitamins A and E, ascorbic acid and carotenoids in the egg yolk and in the embryonic liver. *Br Poult Sci, 39*(2), 257-263.

Surai, P. F., Speake, B. K., & Sparks, N. H. C. (2001). Carotenoids in Avian Nutrition and Embryonic Development. 1. Absorption, Availability and Levels in Plasma and Egg Yolk. *The Journal of Poultry Science, 38*(1), 1-27.

Surai, P. F., Speake, B. K., Wood, N. A., Blount, J. D., Bortolotti, G. R., & Sparks, N. H. (2001). Carotenoid discrimination by the avian embryo: a lesson from wild birds. *Comp Biochem Physiol B Biochem Mol Biol, 128*(4), 743-750.

Sutcliffe, J. H. (1909). *Incubation, natural and artificial, with diagrams and description of eggs in various stages of incubation, description of incubators and rearers, also notes on breeding stock and feeding and rearing chickens*. London.: "The Feathered World".

Suzuki, M., Suzuki, S., Matsui, M., Hiraki, Y., Kawano, F., & Shibayama, K. (2013). Genome Sequence of a Strain of the Human Pathogenic Bacterium Pseudomonas alcaligenes That Caused Bloodstream Infection. *Genome Announcements, 1*(5), e00919-00913.

Svobodová, J., & Tůmová, E. (2015). Factors Affecting Microbial Contamination of Market Eggs: A Review. *Scientia Agriculturae Bohemica, 45*(4), 226-237.

Takeuchi, Y., Nishimura, K., Aoki, N., Adachi, T., Sato, C., Kitajima, K., et al. (1999). A 42-kDa glycoprotein from chicken egg-envelope, an avian homolog of the ZPC family glycoproteins in mammalian Zona pellucida. Its first identification, cDNA cloning and granulosa cell-specific expression. *Eur J Biochem, 260*(3), 736-742.

Tan, C. K., Chen, T. W., Chan, H. L., & Ng, L. S. (1992). A scanning and transmission electron microscopic study of the membranes of chicken egg. *Histol Histopathol, 7*(3), 339-345.

Thanos, S., & Mey, J. r. (2001). Development of the visual system of the chick: II. Mechanisms of axonal guidance. *Brain Research Reviews, 35*(3), 205-245.

Thommes, R. C. (1987). Ontogenesis of thyroid function and regulation in the developing chick embryo. *The Journal of experimental zoology. Supplement : published under auspices of the American Society of Zoologists and the Division of Comparative Physiology and Biochemistry / the Wistar Institute of Anatomy and Biology, 1*, 273-279.

Tian, X., Gautron, J., Monget, P., & Pascal, G. (2010). What Makes an Egg Unique? Clues from Evolutionary Scenarios of Egg-Specific Genes. *Biology of Reproduction, 83*(6), 893-900.

Tong, Q., Romanini, C. E., Exadaktylos, V., Bahr, C., Berckmans, D., Bergoug, H., et al. (2013). Embryonic development and the physiological factors that coordinate hatching in domestic chickens. *Poultry Science, 92*(3), 620-628.

Tsai, W. T., Yang, J. M., Lai, C. W., Cheng, Y. H., Lin, C. C., & Yeh, C. W. (2006). Characterization and adsorption properties of eggshells and eggshell membrane. *Bioresour Technol, 97*(3), 488-493.

Tuan, R. S., & Ono, T. (1986). Regulation of extraembryonic calcium mobilization by the developing chick embryo. *Development, 97*(1), 63-74.

Tullett, S. (2008). *ROSS TECH Investigating Hatchery Practice*. Newbridge: Aviagen.

Tzschentke, B. (2008). Monitoring the development of thermoregulation in poultry embryos and its influence by incubation temperature. *Computers and Electronics in Agriculture, 64*, 61-71.

Uni, Z., & Ferket, R. P. (2004). Methods for early nutrition and their potential. *World's Poultry Science Journal, 60*(01), 101-111.

Uni, Z., Yadgary, L., & Yair, R. (2014, June, 24th). *The nutritional limitations and requirement of the breeder progeny.* Paper presented at the 14th European Poultry Conference, Stavanger.

van den Berg, G., & Moorman, A. F. (2011). Development of the pulmonary vein and the systemic venous sinus: an interactive 3D overview. *PLoS One, 6*(7), e22055.

van der Pol, C. W., van Roovert-Reijrink, I. A. M., Maatjens, C. M., van den Brand, H., & Molenaar, R. (2013). Effect of relative humidity during incubation at a set eggshell temperature and brooding temperature posthatch on embryonic mortality and chick quality. *Poultry Science, 92*(8), 2145-2155.

van der Sluis, W. (2004a). From multi- to advanced single-stage incubation in twenty years. *World Poultry, 20*(3), 18-19.

van der Sluis, W. (2004b). "We have the future of hatchery technology at our fingertips". *World Poultry, 20*(10), 24-26.

Ventura, A., do Nascimento, A. A., dos Santos, M. A. J., Vieira-Lopes, D. A., Sales, A., & Pinheiro, N. L. (2013). Histological Description of Morphogenesis of the Gastroesophageal Mucosa of Gallus gallus domesticus (Linnaeus, 1758). *International Journal of Morphology, 31*, 1331-1339.

Vieira, P. M., Vieira, A. V., Sanders, E. J., Steyrer, E., Nimpf, J., & Schneider, W. J. (1995). Chicken yolk contains bona fide high density lipoprotein particles. *J Lipid Res, 36*(3), 601-610.

von Blumroder, D., & Tonhardt, H. (2002). Influence of long-term changes in incubation temperature on catecholamine levels in plasma of chicken embryos (Gallus gallus f. domestica). *Comp Biochem Physiol A Mol Integr Physiol, 131*(4), 701-711.

von Culin, C., & von Culin, E. (1894). *The art of incubation and brooding*. Delaware City, Del.: E. & C. Von Culin.

Voronov, D. A., Alford, P. W., Xu, G., & Taber, L. A. (2004). The role of mechanical forces in dextral rotation during cardiac looping in the chick embryo. *Dev Biol, 272*(2), 339-350.

Wahl, C., & Noden, D. M. (2001). Cryptic responses to tissue manipulations in avian embryos. *Int J Dev Neurosci, 19*(2), 183-196.

Wang, X., Ford, B. C., Praul, C. A., & Leach, R. M., Jr. (2002). Collagen X expression in oviduct tissue during the different stages of the egg laying cycle. *Poult Sci, 81*(6), 805-808.

Wang, Y., Dur, O., Patrick, M. J., Tinney, J. P., Tobita, K., Keller, B. B., et al. (2009). Aortic arch morphogenesis and flow modeling in the chick embryo. *Ann Biomed Eng, 37*(6), 1069-1081.

Wellman-Labadie, O., Lakshminarayanan, R., & Hincke, M. T. (2008). Antimicrobial properties of avian eggshell-specific C-type lectin-like proteins. *FEBS Lett, 582*(5), 699-704.

Wessells, N. K. (1965). Morphology and proliferation during early feather development. *Developmental Biology, 12*(1), 131-153.

Wilkinson, J. G. (1841). *Manners and customs of the ancient Egyptions: Including their private life, government, laws, arts, manufacturers, religion, and early history : Derived from a comparison of the paintings, sculptures, and monuments still existing, with the accounts of ancient authors : Illustrated by drawings of those subjects*: J. Murray.

Wilson, H. R. (1997). Effects of maternal nutrition on hatchability. *Poultry Science, 76*(1), 134-143.

Wilson, H. R. (2004). *Hatchability problems analysis*. Retrieved. from.

Wittler, L., & Kessel, M. (2004). The acquisition of neural fate in the chick. *Mech Dev, 121*(9), 1031-1042.

Woods, J. E., Mennella, J. A., & Thommes, R. C. (1981). The hypothalamic-adenohypophyseal-gonadal axes in the developing chick embryo. *General and Comparative Endocrinology, 45*(1), 66-73.

Wright, L. (1902). *The New Book of Poultry*. London: Cassel and Company, Limited.

Wu, T. M., Rodriguez, J. P., Fink, D. J., Carrino, D. A., Blackwell, J., Caplan, A. I., et al. (1995). Crystallization studies on avian eggshell membranes: implications for the molecular factors controlling eggshell formation. *Matrix Biol, 14*(6), 507-513.

Xu, G., Kemp, P. S., Hwu, J. A., Beagley, A. M., Bayly, P. V., & Taber, L. A. (2011). Opening angles and material properties of the early embryonic chick brain. *J Biomech Eng, 132*(1), 011005.

Yahav, S., Collin, A., Shinder, D., & Picard, M. (2004). Thermal manipulations during broiler chick embryogenesis: effects of timing and temperature. *Poult Sci, 83*(12), 1959-1963.

Yalcin, S., & Siegel, P. B. (2003). Exposure to cold or heat during incubation on developmental stability of broiler embryos. *Poult Sci, 82*(9), 1388-1392.

# ADDITIONAL

# MATERIAL

## Annex 1. Summary of most significant events in embryonic development by day of incubation

| Day | Most significant events | Macroscopic observations |
|---|---|---|
| **0** | No obvious tissue development | Infertile blastodisc - small dense irregularly shaped white area about 2 mm across, surrounded by a clear, roughly circular area up to 4 mm in diameter which appears to be filled with bubbles.<br>Fertile blastoderm - large (4-5 mm diameter), uniformly round, white ring or “doughnut” with a clear center, sometimes with a small white spot in the center of the ring. Occasionally appears as a solid white, perfectly round disc. |
| **1** | Beginning of the development of the head, the neural groove and the digestive tube. | Appearance of tissue development in the form of an area occupied by the extra-embryonic membranes, about 1 centimeter in diameter. |
| **2** | Development of the neural tube, special senses, heart and first blood vessels.<br>Development and differentiation of the brain. | Tissue development is very visible. Membranes occupy almost the entire upper surface of the yolk.<br>Appearance of the first blood vessels. |
| **3** | Development of branchial clefts and arches<br>Development of the respiratory apparatus and alantoid membrane begins.<br>Appearance of the limb buds. | Well-developed circulatory system with very visible blood vessels and heartbeat.<br>The inner shell membrane looks white when the shell above the air cell is removed. |
| **4** | Development of the adnexal glands of the digestive system.<br>Development of the cloaca begins. | Eye pigmented.<br>The inner shell membrane looks white when the shell above the air cell is removed. |
| **5** | Development of arterial trunk.<br>Formation of hepatic vein.<br>Development of mesonephric kidney.<br>Development of reproductive organs begins.<br>Innervation of the esophagus.<br>Chondrification of the femur, tibia and fibula. | Appearance of elbows and knees |
| **6** | Completion of interventricular septum.<br>Development of the gizzard.<br>Pancreas differentiation begins.<br>Innervation of the eyes.<br>Larynx forms.<br>Chondrification of tibiotarsus and metatarsus. | Appearance of beak.<br>Voluntary movements begin |

| Day | Most significant events | Macroscopic observations |
|---|---|---|
| **7** | Proventricular glands form.<br>Ribs form<br>Chondrification of skeleton.<br>Differentiation of the ovary.<br>Crop bends and dilates.<br>Dorsal and ventral horns in the spinal cord formed. | Comb growth begins.<br>Egg tooth begins to appear |
| **8** | Auerbach's plexus is formed.<br>Development of bone marrow begins. | Feather tracts seen.<br>Upper and lower beak equal in length |
| **9** | Pancreas fully formed.<br>Development of syringeal cartilages and muscles begins.<br>Sternum and keel form.<br>Ossification of the skull.<br>Migration of abdominal air sacs. | Embryo starts to look bird-like.<br>Mouth opening appears |
| **10** | Expansion of abdominal air sacs begins.<br>Cornification of the beak begins.<br>Development of uropygial gland begins. | Egg tooth prominent.<br>Toe nails. |
| **11** | Kidneys begin to excrete.<br>Rotation of duodenal loop complete.<br>Development of Herderian glands begins. | Comb serrated.<br>Tail feathers apparent |
| **12** | Ossification of the scapula and ilium.<br>Müllerian ducts disappear (male).<br>Secretory glands in the gizzard.<br>Recanalization of large intestine.<br>Lymphoid tissue in the Bursa | Toes fully formed.<br>First few visible feathers |
| **13** | Differentiation into spermatogonia.<br>Pituitary-gonadal axis established.<br>Ossification of the pubis.<br>Lymphoid tissue in the Bursa | Appearance of scales.<br>Body covered lightly with feathers |

| Day | Most significant events | Macroscopic observations |
|---|---|---|
| **14** | Differentiation of Henle loops.<br>Surfactant produced in lungs.<br>Ossification of the ischium.<br>Beak hardened. | Embryo turns head towards large end of egg |
| **15** | Metanephric kidneys.<br>Abdominal air sacs fully inflated. | Gut is drawn into abdominal cavity |
| **16** | Cloacal aperture transversal.<br>Esophageal mucous glands formed | Feathers cover complete body.<br>Albumen nearly gone |
| **17** | Parathyroids fully differentiated | Amniotic fluid decreases.<br>Head is between legs. |
| **18** | | Growth of embryo nearly complete.<br>Yolk sac is still on outside of embryo.<br>Head is under the right wing.<br>Ideally, egg has lost 12% of fresh weight |
| **19** | Ossification of the synsacrum begins | Yolk sac draws into body cavity.<br>Amniotic fluid gone.<br>Embryo occupies most of space within egg (not in the air cell). |
| **20** | Yolk sac drawn completely into body.<br>Embryo becomes a chick (breathing in air cell).<br>Ductus arteriosus and arterial foramen closed. | Yolk sac drawn completely into body.<br>Internal and external pip. |
| **21** | Hatching | Hatching |

# Annex 2. Laboratory protocols

**Annex 2 Table 1. Procedures applied to each egg and embryo depending on incubation day.**

| Day | Procedure |
|---|---|
| **0** | Weighting |
| | Candling |
| | Measurement of air chamber |
| | Detection of fractures and other anomalies |
| | Euthanasia (<4ºC, 4 h) |
| | Detection and measurement of blastodisc/blastoderm |
| | Weighing of the albumen, yolk and shell |
| | Embryo fixation in 10% buffered formalin |
| **1** | Candling |
| | Measurement of air chamber |
| | Detection of embryo |
| | Euthanasia (<4ºC, 4 h) |
| | Fertility diagnosis |
| | Detection and measurement of embryo and EEM |
| | Weighing of the albumen, yolk and shell |
| | Embryo fixation in 10% buffered formalin |
| **2** | Candling |
| | Measurement of air chamber |
| | Detection of embryo |
| | Euthanasia (<4ºC, 4 h) |
| | Fertility diagnosis |
| | Detection and measurement of embryo, EEM and extra-embryonic blood vessels |
| | Weighing of the albumen, yolk and shell |
| | Embryo fixation in 10% buffered formalin |
| **3** | Candling |
| | Measurement of air chamber |
| | Detection and measurement of heartbeat |
| | Euthanasia (<4ºC, 4 h) |
| | Fertility diagnosis |
| | Detection and measurement of embryo, EEM, extra-embryonic blood vessels and heart |
| | Weighing of the albumen, yolk and shell |
| | Embryo fixation in 10% buffered formalin |

**Annex 2 Table 1. Procedures applied to each egg and embryo depending on incubation day (cont.).**

| Day | Procedure |
|---|---|
| **4** | Candling<br>Measurement of air chamber<br>Detection and measurement of heartbeat<br>Euthanasia (<4ºC, 4 h)<br>Fertility diagnosis<br>Detection and measurement of embryo, eyes, EEM, extra-embryonic blood vessels and heart<br>Weighing of the embryo, albumen, yolk and shell<br>Embryo fixation in 10% buffered formalin |
| **5** | Candling<br>Measurement of air chamber<br>Detection and measurement of heartbeat, movement<br>Euthanasia (<4ºC, 4 h)<br>Fertility diagnosis<br>Detection and measurement of embryo, heart, eyes, limbs, EEM, extra-embryonic blood vessels<br>Weighing of the embryo, albumen, yolk and shell<br>Embryo fixation in 10% buffered formalin |
| **6** | Candling<br>Measurement of air chamber<br>Detection and measurement of heartbeat, movement<br>Euthanasia (<4ºC, 4 h)<br>Fertility diagnosis<br>Detection and measurement of embryo, heart, eyes, beak, limbs, EEM, extra-embryonic blood vessels<br>Weighing of the embryo, albumen, yolk and shell<br>Embryo fixation in 10% buffered formalin |
| **7** | Candling<br>Measurement of air chamber<br>Detection and measurement of heartbeat, movement<br>Euthanasia (<4ºC, 4 h)<br>Fertility diagnosis<br>Detection and measurement of embryo, heart, eyes, beak, egg tooth, limbs, feathers, comb, EEM, extra-embryonic blood vessels<br>Weighing of the embryo, albumen, yolk and shell<br>Embryo fixation in 10% buffered formalin |

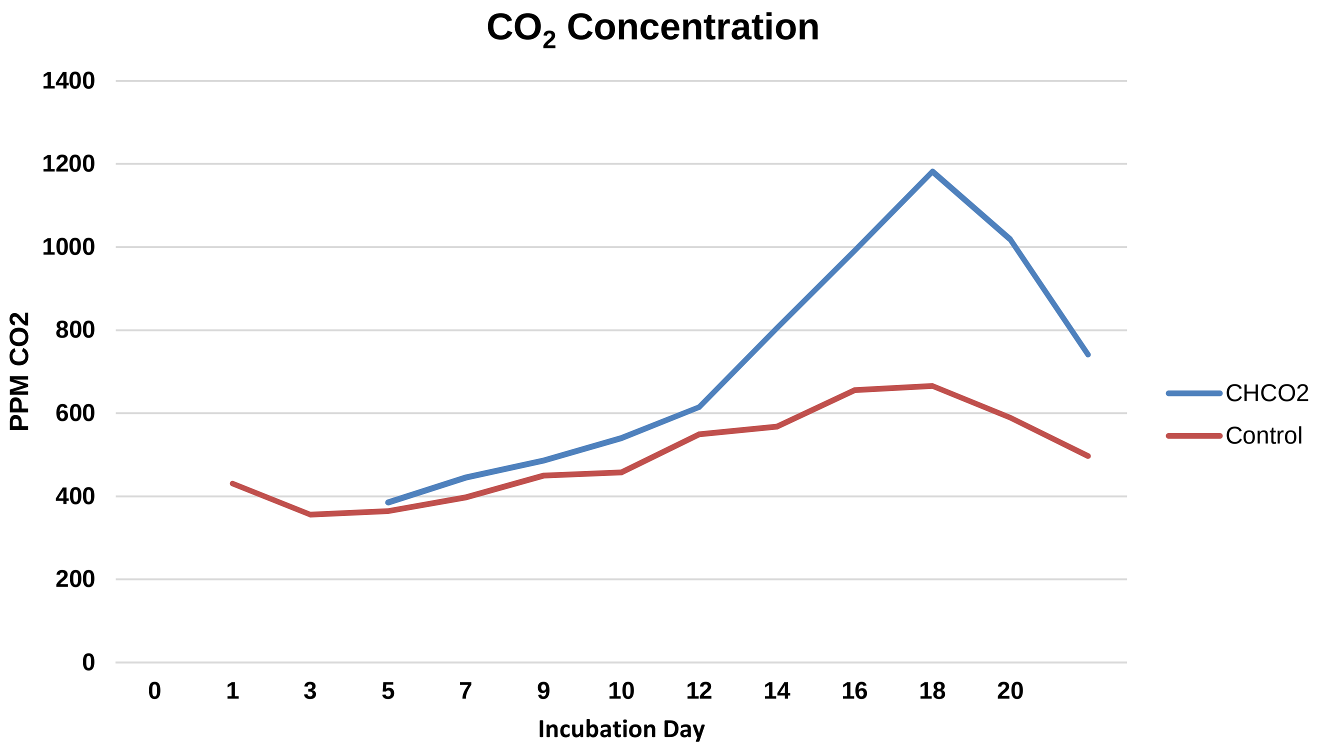
**Annex 2 Table 1. Procedures applied to each egg and embryo depending on incubation day (concl.).**

| Day | Procedure |
|---|---|
| **8** | Candling<br>Measurement of air chamber<br>Detection and measurement of heartbeat, movement<br>Euthanasia (<4ºC, 4 h)<br>Fertility diagnosis<br>Detection and measurement of embryo, heart, eyes, beak, mouth, egg tooth, limbs, feathers, comb, EEM, extra-embryonic blood vessels<br>Weighing of the embryo, albumen, yolk and shell<br>Embryo fixation in 10% buffered formalin |
| **9-14** | Candling<br>Measurement of air chamber<br>Detection and measurement of heartbeat, movement<br>Euthanasia (<4ºC, 4 h)<br>Fertility diagnosis<br>Detection and measurement of embryo, heart, eyes, beak, mouth, egg tooth, limbs, toes, nails, feathers, comb, EEM, extra-embryonic blood vessels<br>Weighing of the embryo, albumen, yolk and shell<br>Embryo fixation in 10% buffered formalin |
| **15-19** | Candling<br>Measurement of air chamber<br>Detection and measurement of heartbeat, movement<br>Euthanasia (100% $CO_2$, 20 min)<br>Fertility diagnosis<br>Detection and measurement of embryo, heart, eyes, beak, mouth, egg tooth, limbs, toes, nails, feathers, comb, EEM, extra-embryonic blood vessels<br>Weighing of the embryo, albumen, yolk and shell<br>Embryo fixation in 10% buffered formalin |
| **20** | Candling<br>Measurement of air chamber<br>Detection and measurement of heartbeat, movement<br>Euthanasia (100% $CO_2$, 20 min)<br>Fertility diagnosis<br>Note position and pip<br>Detection and measurement of embryo, heart, eyes, beak, mouth, egg tooth, limbs, toes, nails, feathers, comb, EEM, extra-embryonic blood vessels<br>Weighing of the embryo, albumen, yolk and shell<br>Embryo fixation in 10% buffered formalin |

**Annex 2 Table 2. Procedures applied to each egg and embryo depending on incubation day.**

| Location | Procedure |
|---|---|
| **Workbench** | Visual inspection<br>Egg weighing<br>Egg measuring |
| **Dark room** | Candling |
| **Laminar air flow chamber** | Aseptical breakout<br>Albumen/yolk sample collection<br>Embryo collection into formalin<br>DNA extraction for *Salmonella* PCR |
| **Workbench** | Microbiological analysis<br>*Salmonella* PCR |
| **Fume hood** | Embryo measurement<br>Embryo weighing<br>Gross exam<br>Time of Death estimation<br>Sample collection for histology |
| **Microscopy room** | Histologic exam |

# Annex 3. Carbon dioxide concentrations in the incubator during the CHCO2 trial.



# Annex 4. Mean values of the parameters monitored at each time-point, by treatment

## Annex 4 Table 1. Mean values of behavioral, nutrient utilization and growth parameters, by treatment.

| Day | Group | Shell Ratio | | Albumen Ratio | | Yolk Ratio | | Embryo Ratio | | Heart Rate (bpm) | | Voluntary Movements (mov/min) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 1 | Control | 0,14 | 0,0023 | 0,47 | 0,02422 | 0,31 | 0,02142 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 |
| | CHT | 0,14 | 0,00 | 0,57* | 0,01 | 0,26 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CLT | 0,13 | 0,00 | 0,58* | 0,01 | 0,29 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PHT | 0,14 | 0,00 | 0,56 | 0,01 | 0,30 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PLT | 0,12 | 0,00 | 0,58 | 0,01 | 0,30 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CHRH | 0,13 | 0,00 | 0,58* | 0,01 | 0,29 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CLHR | 0,14 | 0,01 | 0,56* | 0,01 | 0,29 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PHRH | 0,12 | 0,00 | 0,58 | 0,01 | 0,30 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PLRH | 0,10 | 0,00 | 0,56 | 0,01 | 0,32 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CO2 | 0,10 | 0,00 | 0,56* | 0,00 | 0,32 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 3 | Control | 0,13 | 0,00249 | 0,46 | 0,0188 | 0,26 | 0,01471 | 0,00 | 0 | 92,44 | 10,2817 | 0,00 | 0 |
| | CHT | 0,13 | 0,00 | 0,48 | 0,01 | 0,27 | 0,01 | 0,00 | 0,00 | 133,60 | 4,59 | 0,00 | 0,00 |
| | CLT | 0,12 | 0,00 | 0,55* | 0,01 | 0,32* | 0,01 | 0,00 | 0,00 | 126,25 | 13,26 | 0,00 | 0,00 |
| | PHT | 0,13 | 0,00 | 0,50 | 0,01 | 0,35 | 0,02 | 0,00 | 0,00 | 166,33 | 22,53 | 0,00 | 0,00 |
| | PLT | 0,13 | 0,01 | 0,49 | 0,01 | 0,37 | 0,01 | 0,00 | 0,00 | 136,00 | 6,47 | 0,00 | 0,00 |
| | CHRH | 0,13 | 0,00 | 0,53* | 0,01 | 0,34* | 0,01 | 0,00 | 0,00 | 78,33 | 15,07 | 0,00 | 0,00 |
| | CLHR | 0,14 | 0,00 | 0,49 | 0,01 | 0,37* | 0,01 | 0,00 | 0,00 | 88,33 | 3,86 | 0,00 | 0,00 |
| | PHRH | 0,11 | 0,00 | 0,52 | 0,02 | 0,35 | 0,01 | 0,00 | 0,00 | 86,15 | 4,74 | 0,00 | 0,00 |
| | PLRH | 0,10 | 0,00 | 0,47 | 0,02 | 0,42 | 0,02 | 0,00 | 0,00 | 89,09 | 4,95 | 0,00 | 0,00 |
| | CO2 | 0,10 | 0,00 | 0,45* | 0,02 | 0,46* | 0,03 | 0,00 | 0,00 | 78,00 | 9,17 | 0,00 | 0,00 |

**Annex 4 Table 1. Mean values of behavioral, nutrient utilization and growth parameters, by treatment (cont.).**

| Day | Group | Shell Ratio | | Albumen Ratio | | Yolk Ratio | | Embryo Ratio | | Heart Rate (bpm) | | Voluntary Movements (mov/min) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 5 | Control | 0,12 | 0,00 | 0,23 | 0,01 | 0,48 | 0,02 | 0,01 | 0,00 | 157,40 | 1,46 | 0,00 | 0,00 |
| | CHT | 0,13 | 0,01 | 0,24 | 0,02 | 0,55 | 0,02 | 0,00 | 0,00 | 133,33* | 3,77 | 0,00 | 0,00 |
| | CLT | 0,12 | 0,00 | 0,28 | 0,02 | 0,54 | 0,03 | 0,00 | 0,00 | 191,50* | 7,38 | 0,00 | 0,00 |
| | PHT | 0,12 | 0,00 | 0,25 | 0,01 | 0,57 | 0,03 | 0,00 | 0,00 | 159,27 | 1,18 | 0,00 | 0,00 |
| | PLT | 0,12 | 0,00 | 0,25 | 0,01 | 0,59 | 0,01 | 0,00 | 0,00 | 161,54 | 1,95 | 0,00 | 0,00 |
| | CHRH | 0,12 | 0,00 | 0,30* | 0,02 | 0,54 | 0,02 | 0,00 | 0,00 | 160,00 | 0,00 | 16,00* | 0,00 |
| | CLHR | 0,13 | 0,00 | 0,25 | 0,01 | 0,55 | 0,03 | 0,00 | 0,00 | 160,00 | 0,00 | 4,80* | 2,44 |
| | PHRH | 0,12 | 0,00 | 0,22 | 0,01 | 0,57 | 0,01 | 0,00 | 0,00 | 160,00 | 0,00 | 6,67* | 2,38 |
| | PLRH | 0,12 | 0,00 | 0,25 | 0,02 | 0,55 | 0,02 | 0,00 | 0,00 | 180,00 | 0,00 | 0,00 | 0,00 |
| | CO2 | 0,12 | 0,00 | 0,22 | 0,01 | 0,53 | 0,02 | 0,01 | 0,00 | 160,00 | 0,00 | 5,33* | 2,27 |
| 7 | Control | 0,13 | 0,00 | 0,19 | 0,01 | 0,47 | 0,02 | 0,01 | 0,00 | 191,90 | 6,71 | 31,24 | 1,22 |
| | CHT | 0,13 | 0,00 | 0,24 | 0,01 | 0,56* | 0,02 | 0,02 | 0,00 | 295,00* | 5,88 | 29,82 | 1,90 |
| | CLT | 0,13 | 0,00 | 0,26* | 0,02 | 0,54 | 0,02 | 0,01 | 0,00 | 185,38 | 12,23 | 18,00* | 1,31 |
| | PHT | 0,13 | 0,00 | 0,20 | 0,02 | 0,61 | 0,02 | 0,01 | 0,00 | 206,50 | 12,99 | 21,33 | 1,14 |
| | PLT | 0,13 | 0,00 | 0,26 | 0,01 | 0,54 | 0,01 | 0,01 | 0,00 | 234,67 | 4,75 | 20,67* | 1,19 |
| | CHRH | 0,12 | 0,00 | 0,22 | 0,00 | 0,54 | 0,03 | 0,01 | 0,00 | 241,36* | 7,42 | 19,64* | 1,26 |
| | CLHR | 0,13 | 0,00 | 0,22 | 0,01 | 0,53 | 0,01 | 0,02* | 0,00 | 211,92 | 20,49 | 16,67 | 1,19 |
| | PHRH | 0,12 | 0,00 | 0,19 | 0,01 | 0,53 | 0,01 | 0,02 | 0,00 | 240,83* | 6,41 | 16,67 | 2,08 |
| | PLRH | 0,12 | 0,00 | 0,19 | 0,01 | 0,57 | 0,02 | 0,01 | 0,00 | 268,91 | 4,30 | 13,82 | 1,13 |
| | CO2 | 0,13 | 0,00 | 0,21 | 0,01 | 0,46 | 0,03 | 0,02 | 0,00 | 236,08* | 4,73 | 16,00 | 0,00 |

### Annex 4 Table 1. Mean values of behavioral, nutrient utilization and growth parameters, by treatment (cont.).

| Day | Group | Shell Ratio | | Albumen Ratio | | Yolk Ratio | | Embryo Ratio | | Heart Rate (bpm) | | Voluntary Movements (mov/min) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 9 | Control | 0,13 | 0,00 | 0,21 | 0,01 | 0,44 | 0,02 | 0,04 | 0,00 | 223,35 | 7,16 | 38,20 | 1,55 |
| | CHT | 0,13 | 0,00 | 0,25* | 0,01 | 0,55* | 0,01 | 0,04 | 0,00 | 296,45* | 7,15 | 26,18* | 1,13 |
| | CLT | 0,12 | 0,00 | 0,19 | 0,01 | 0,55* | 0,02 | 0,03* | 0,00 | 233,60 | 1,63 | 16,00* | 3,58 |
| | PHT | 0,12 | 0,00 | 0,21 | 0,01 | 0,51* | 0,01 | 0,03* | 0,00 | 255,58 | 6,12 | 24,67* | 1,83 |
| | PLT | 0,12 | 0,00 | 0,23 | 0,01 | 0,52* | 0,01 | 0,03* | 0,00 | 251,54 | 8,30 | 24,00* | 1,81 |
| | CHRH | 0,12 | 0,00 | 0,21 | 0,01 | 0,53* | 0,01 | 0,03* | 0,00 | 250,42 | 6,36 | 19,00* | 1,64 |
| | CLHR | 0,12 | 0,00 | 0,22 | 0,01 | 0,50* | 0,01 | 0,03* | 0,00 | 237,36 | 24,04 | 24,00* | 4,18 |
| | PHRH | 0,12 | 0,00 | 0,21 | 0,02 | 0,52* | 0,01 | 0,03* | 0,00 | 258,92 | 9,97 | 24,00* | 0,00 |
| | PLRH | 0,11 | 0,00 | 0,21 | 0,01 | 0,51* | 0,01 | 0,02* | 0,00 | 268,70 | 4,64 | 24,00* | 0,00 |
| | CO2 | 0,13 | 0,00 | 0,22 | 0,01 | 0,48 | 0,01 | 0,04 | 0,00 | 249,00 | 9,89 | 19,20* | 1,31 |
| 10 | Control | 0,12 | 0,00 | 0,19 | 0,00 | 0,44 | 0,02 | 0,06 | 0,00 | 217,84 | 4,56 | 34,53 | 1,38 |
| | CHT | 0,12 | 0,00 | 0,25* | 0,01 | 0,55* | 0,01 | 0,06 | 0,00 | 278,11* | 6,46 | 29,33 | 1,89 |
| | CLT | 0,12 | 0,01 | 0,21 | 0,01 | 0,53* | 0,01 | 0,04* | 0,00 | 234,88 | 5,64 | 19,00* | 3,00 |
| | PHT | 0,12 | 0,00 | 0,23 | 0,01 | 0,48 | 0,01 | 0,04* | 0,00 | 244,50* | 7,51 | 22,00* | 1,74 |
| | PLT | 0,11 | 0,00 | 0,22 | 0,01 | 0,49 | 0,01 | 0,05 | 0,00 | 251,33* | 7,18 | 18,67* | 2,67 |
| | CHRH | 0,11 | 0,00 | 0,22 | 0,01 | 0,52* | 0,01 | 0,04* | 0,00 | 241,83 | 8,04 | 17,67* | 1,94 |
| | CLHR | 0,11 | 0,00 | 0,22 | 0,01 | 0,46 | 0,01 | 0,05 | 0,00 | 245,83* | 7,87 | 24,00* | 2,65 |
| | PHRH | 0,12 | 0,00 | 0,21 | 0,01 | 0,49 | 0,01 | 0,04* | 0,00 | 229,67 | 6,89 | 22,00* | 1,44 |
| | PLRH | 0,11 | 0,00 | 0,21 | 0,01 | 0,52* | 0,01 | 0,03* | 0,00 | 264,17 | 7,82 | 24,00* | 0,98 |
| | CO2 | 0,12 | 0,00 | 0,20 | 0,01 | 0,50* | 0,02 | 0,05 | 0,00 | 272,10* | 5,62 | 27,20 | 1,31 |

**Annex 4 Table 1. Mean values of behavioral, nutrient utilization and growth parameters, by treatment (cont.).**

| Day | Group | Shell Ratio | | Albumen Ratio | | Yolk Ratio | | Embryo Ratio | | Heart Rate (bpm) | | Voluntary Movements (mov/min) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 12 | Control | 0,12 | 0,00 | 0,19 | 0,01 | 0,39 | 0,01 | 0,11 | 0,00 | 226,41 | 5,47 | 37,45 | 0,81 |
| | CHT | 0,12 | 0,00 | 0,24* | 0,02 | 0,48* | 0,02 | 0,11 | 0,00 | 282,50* | 3,80 | 28,00* | 2,46 |
| | CLT | 0,11 | 0,00 | 0,20 | 0,01 | 0,49* | 0,02 | 0,07* | 0,00 | 255,57* | 4,49 | 21,71* | 2,29 |
| | PHT | 0,12 | 0,00 | 0,21 | 0,00 | 0,44* | 0,01 | 0,08* | 0,00 | 256,31* | 6,03 | 26,46* | 1,40 |
| | PLT | 0,12 | 0,00 | 0,20 | 0,00 | 0,43* | 0,01 | 0,09* | 0,00 | 291,44* | 6,61 | 24,89* | 3,38 |
| | CHRH | 0,11 | 0,00 | 0,19 | 0,00 | 0,48* | 0,01 | 0,08* | 0,00 | 263,54* | 6,28 | 21,85* | 1,61 |
| | CLHR | 0,11 | 0,00 | 0,19 | 0,01 | 0,36 | 0,01 | 0,09* | 0,00 | 261,62* | 5,58 | 22,15* | 1,90 |
| | PHRH | 0,11 | 0,00 | 0,20 | 0,01 | 0,43* | 0,01 | 0,06* | 0,00 | 286,27* | 10,49 | 29,82* | 1,13 |
| | PLRH | 0,11 | 0,00 | 0,20 | 0,01 | 0,43* | 0,01 | 0,06* | 0,00 | 286,27* | 10,49 | 29,82* | 1,13 |
| | CO2 | 0,11 | 0,00 | 0,19 | 0,01 | 0,41 | 0,01 | 0,10 | 0,00 | 259,54* | 6,95 | 23,38* | 2,63 |
| 14 | Control | 0,12 | 0,00 | 0,09 | 0,01 | 0,35 | 0,02 | 0,21 | 0,01 | 241,70 | 8,67 | 35,20 | 0,90 |
| | CHT | 0,12 | 0,00 | 0,11 | 0,01 | 0,52* | 0,01 | 0,21 | 0,01 | 273,45 | 11,14 | 23,27* | 1,69 |
| | CLT | 0,11 | 0,00 | 0,13 | 0,01 | 0,45* | 0,01 | 0,13* | 0,01 | 253,67 | 4,79 | 21,33* | 3,37 |
| | PHT | 0,11 | 0,01 | 0,10 | 0,01 | 0,35 | 0,04 | 0,15* | 0,02 | 239,30 | 27,59 | 25,60* | 3,11 |
| | PLT | 0,11 | 0,00 | 0,11 | 0,01 | 0,36 | 0,02 | 0,16* | 0,00 | 279,55 | 6,59 | 24,73* | 2,00 |
| | CHRH | 0,10 | 0,00 | 0,10 | 0,01 | 0,39 | 0,01 | 0,16* | 0,00 | 265,23 | 6,39 | 26,46* | 2,81 |
| | CLHR | 0,11 | 0,00 | 0,09 | 0,01 | 0,36 | 0,01 | 0,20 | 0,01 | 261,36 | 5,28 | 21,82* | 2,43 |
| | PHRH | 0,12 | 0,00 | 0,10 | 0,01 | 0,42* | 0,02 | 0,18 | 0,01 | 270,30 | 6,63 | 27,20 | 2,44 |
| | PLRH | 0,11 | 0,00 | 0,09 | 0,01 | 0,36 | 0,01 | 0,11* | 0,00 | 282,25 | 6,48 | 26,00* | 1,04 |
| | CO2 | 0,10 | 0,00 | 0,10 | 0,01 | 0,42* | 0,02 | 0,19 | 0,00 | 306,45 | 28,61 | 24,73* | 2,28 |

**Annex 4 Table 1. Mean values of behavioral, nutrient utilization and growth parameters, by treatment (cont.).**

| Day | Group | Shell Ratio |  | Albumen Ratio |  | Yolk Ratio |  | Embryo Ratio |  | Heart Rate (bpm) |  | Voluntary Movements (mov/min) |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 16 | Control | 0,12 | 0,00 | 0,02 | 0,00 | 0,31 | 0,02 | 0,35 | 0,01 | 232,64 | 6,39 | 32,73 | 1,04 |
|  | CHT | 0,13 | 0,01 | 0,06* | 0,01 | 0,45* | 0,02 | 0,29* | 0,01 | 311,80 | 10,69 | 28,00 | 2,46 |
|  | CLT | 0,12 | 0,00 | 0,08* | 0,01 | 0,35 | 0,02 | 0,24* | 0,01 | 271,33 | 3,73 | 32,00 | 2,92 |
|  | PHT | 0,10 | 0,00 | 0,03 | 0,01 | 0,29 | 0,01 | 0,32 | 0,01 | 252,83 | 9,22 | 28,00 | 1,84 |
|  | PLT | 0,10 | 0,00 | 0,04 | 0,00 | 0,26 | 0,01 | 0,30 | 0,01 | 295,17 | 6,10 | 20,67* | 2,50 |
|  | CHRH | 0,10 | 0,00 | 0,02 | 0,01 | 0,33 | 0,01 | 0,28* | 0,01 | 271,46 | 8,32 | 20,31* | 2,33 |
|  | CLHR | 0,11 | 0,00 | 0,02 | 0,00 | 0,24* | 0,01 | 0,32 | 0,01 | 277,75 | 8,62 | 28,00 | 1,56 |
|  | PHRH | 0,10 | 0,00 | 0,02 | 0,01 | 0,33 | 0,01 | 0,29* | 0,00 | 288,83 | 6,22 | 28,00 | 1,21 |
|  | PLRH | 0,10 | 0,00 | 0,02 | 0,00 | 0,27 | 0,01 | 0,17* | 0,00 | 286,25 | 6,01 | 23,33* | 2,30 |
|  | CO2 | 0,10 | 0,00 | 0,02 | 0,01 | 0,27 | 0,02 | 0,31 | 0,01 | 259,08 | 7,73 | 25,33 | 2,16 |
| 18 | Control | 0,12 | 0,00 | 0,00 | 0,00 | 0,19 | 0,01 | 0,50 | 0,01 | 234,40 | 8,96 | 30,80 | 0,88 |
|  | CHT | 0,13 | 0,00 | 0,02 | 0,01 | 0,39* | 0,01 | 0,42* | 0,02 | 268,09 | 9,35 | 24,73 | 2,00 |
|  | CLT | 0,10 | 0,01 | 0,01 | 0,00 | 0,29* | 0,02 | 0,31* | 0,01 | 266,83 | 5,40 | 25,33 | 3,21 |
|  | PHT | 0,10 | 0,00 | 0,04 | 0,04 | 0,22* | 0,01 | 0,47 | 0,01 | 305,90* | 9,99 | 11,20* | 3,41 |
|  | PLT | 0,10 | 0,00 | 0,01 | 0,01 | 0,22* | 0,01 | 0,46 | 0,02 | 273,55 | 8,66 | 21,09* | 2,70 |
|  | CHRH | 0,09 | 0,00 | 0,00 | 0,00 | 0,24* | 0,01 | 0,42* | 0,01 | 294,42* | 6,06 | 19,33* | 1,54 |
|  | CLHR | 0,11 | 0,00 | 0,00 | 0,00 | 0,14* | 0,01 | 0,48 | 0,00 | 278,36* | 7,65 | 21,09* | 2,70 |
|  | PHRH | 0,11 | 0,00 | 0,00 | 0,00 | 0,18 | 0,01 | 0,46 | 0,01 | 302,17* | 4,50 | 19,33* | 1,19 |
|  | PLRH | 0,10 | 0,00 | 0,00 | 0,00 | 0,21 | 0,01 | 0,23* | 0,01 | 285,54* | 5,39 | 17,85* | 1,61 |
|  | CO2 | 0,09 | 0,00 | 0,00 | 0,00 | 0,18 | 0,01 | 0,43* | 0,01 | 296,08* | 4,84 | 16,67* | 2,08 |

Annex 4 Table 1. Mean values of behavioral, nutrient utilization and growth parameters, by treatment (conc.).

| Day | Group | Shell Ratio | | Albumen Ratio | | Yolk Ratio | | Embryo Ratio | | Heart Rate (bpm) | | Voluntary Movements (mov/min) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 20 | Control | 0,08 | 0,02 | 0,00 | 0,00 | 0,01 | 0,01 | 0,57 | 0,15 | 142,86 | 36,89 | 28,57 | 7,38 |
| | CHT | 0,13 | 0,01 | 0,03 | 0,03 | 0,27* | 0,04 | 0,50 | 0,06 | 177,11 | 45,07 | 14,22 | 5,13 |
| | CLT | 0,11 | 0,00 | 0,00* | 0,00 | 0,11 | 0,02 | 0,53 | 0,01 | 236,00* | 31,34 | 21,33 | 9,61 |
| | PHT | 0,10 | 0,00 | 0,00 | 0,00 | 0,08 | 0,02 | 0,67 | 0,00 | 251,00* | 11,00 | 8,00 | 0,00 |
| | PLT | 0,10 | 0,00 | 0,00 | 0,00 | 0,04 | 0,02 | 0,73 | 0,04 | 270,25* | 8,64 | 36,00 | 2,14 |
| | CHRH | 0,11 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,80 | 0,01 | 267,75* | 7,64 | 31,00 | 3,53 |
| | CLHR | 0,10 | 0,00 | 0,00 | 0 | 0,00 | 0 | 0,82 | 0,010 | 304,44* | 3,67 | 41,78 | 1,78 |
| | PHRH | 0,10 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,73 | 0,06 | 270,46* | 5,90 | 27,69 | 2,66 |
| | PLRH | 0,10 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,42 | 0,01 | 343,09* | 17,74 | 37,82 | 2,18 |
| | CO2 | 0,10 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,79 | 0,01 | 329,69* | 12,22 | 37,54 | 1,67 |

***Significant difference found ($p<0,05$). All significant values are displayed in comparison to the mean of the Control group. Testing done with One-Way ANOVA.**

## Annex 4 Table 2. Mean values of morphometric growth parameters, by treatment.

| Day | Group | Embryo Length (cm) | | Wing Length (cm) | | Leg Length (cm) | | Beak Length (cm) | | Comb Length (cm) | | Eye Diameter (cm) | | Feather Length (cm) | | Toe Length (cm) | | Nail Length (cm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 1 | Control | 0,00 | 0,00 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 |
| | CHT | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CLT | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PHT | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PLT | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CHRH | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CLHR | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PHRH | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PLRH | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CO2 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 3 | Control | 0,92 | 0,02459 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 |
| | CHT | 0,93 | 0,04 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CLT | 0,76 | 0,02 | 0,00 | 0,00 | key>< | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PHT | 0,68 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PLT | 0,86 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CHRH | 0,76 | 0,07 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CLHR | 0,82 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PHRH | 0,80 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | PLRH | 0,78 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | CO2 | 0,95 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |

## Annex 4 Table 2. Mean values of morphometric growth parameters, by treatment (cont.).

| Day | Group | Embryo Length (cm) |  | Wing Length (cm) |  | Leg Length (cm) |  | Beak Length (cm) |  | Comb Length (cm) |  | Eye Diameter (cm) |  | Feather Length (cm) |  | Toe Length (cm) |  | Nail Length (cm) |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 5 | Control | 1,61 | 0,01 | 0,24 | 0,01 | 0,23 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
|  | CHT | 1,92 | 0,03 | 0,30 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
|  | CLT | 1,26 | 0,05 | 0,15 | 0,02 | 0,10 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,15 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
|  | PHT | 1,49 | 0,04 | 0,20 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
|  | PLT | 1,90 | 0,03 | 0,29 | 0,01 | 0,39 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,29 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
|  | CHRH | 1,95 | 0,02 | 0,30 | 0,00 | 0,40 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
|  | CLHR | 1,77 | 0,04 | 0,30 | 0,00 | 0,40 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,29 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
|  | PHRH | 1,90 | 0,00 | 0,30 | 0,00 | 0,40 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
|  | PLRH | 2,00 | 0,00 | 0,30 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
|  | CO2 | 1,70 | 0,00 | 0,20 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 7 | Control | 2,53 | 0,08 | 0,66 | 0,01 | 0,81 | 0,02 | 0,16 | 0,01 | 0,06 | 0,01 | 0,51 | 0,02 | 0,00 | 0,00 | 0,11 | 0,02 | 0,00 | 0,00 |
|  | CHT | 2,90 | 0,00 | 0,50 | 0,00 | 0,80 | 0,00 | 0,25* | 0,00 | 0,10 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 |
|  | CLT | 2,43 | 0,03 | 0,40 | 0,00 | 0,40 | 0,00 | 0,10 | 0,00 | 0,05 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |
|  | PHT | 2,43 | 0,04 | 0,40 | 0,00 | 0,50 | 0,00 | 0,20* | 0,00 | 0,10 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |
|  | PLT | 2,40 | 0,00 | 0,50 | 0,00 | 0,70 | 0,00 | 0,20* | 0,00 | 0,10 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |
|  | CHRH | 2,50 | 0,00 | 0,60 | 0,00 | 0,70 | 0,00 | 0,30* | 0,00 | 0,10 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |
|  | CLHR | 2,50 | 0,00 | 0,50 | 0,00 | 0,70 | 0,00 | 0,10 | 0,00 | 0,10 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |
|  | PHRH | 2,60 | 0,00 | 0,60 | 0,00 | 0,70 | 0,00 | 0,10 | 0,00 | 0,10 | 0,00 | 0,60 | 0,00 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |
|  | PLRH | 2,45 | 0,05 | 0,59 | 0,01 | 0,98 | 0,02 | 0,18 | 0,02 | 0,09 | 0,01 | 0,59 | 0,01 | 0,00 | 0,00 | 0,09 | 0,01 | 0,00 | 0,00 |
|  | CO2 | 2,30 | 0,00 | 0,60 | 0,00 | 0,70 | 0,00 | 0,10 | 0,00 | 0,10 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |

**Annex 4 Table 2. Mean values of morphometric growth parameters, by treatment (cont.).**

| Day | Group | Embryo Length (cm) | | Wing Length (cm) | | Leg Length (cm) | | Beak Length (cm) | | Comb Length (cm) | | Eye Diameter (cm) | | Feather Length (cm) | | Toe Length (cm) | | Nail Length (cm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 9 | Control | 3,58 | 0,04 | 1,00 | 0,00 | 1,50 | 0,00 | 0,36 | 0,01 | 0,18 | 0,03 | 0,80 | 0,00 | 0,05 | 0,00 | 0,46 | 0,01 | 0,00 | 0,00 |
| | CHT | 3,65 | 0,03 | 1,00 | 0,00 | 1,97 | 0,03 | 0,80* | 0,00 | 0,30 | 0,00 | 0,69 | 0,01 | 0,10 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 |
| | CLT | 3,06 | 0,04 | 0,80 | 0,00 | 1,00 | 0,00 | 0,40 | 0,00 | 0,30 | 0,00 | 0,70 | 0,00 | 0,05 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 |
| | PHT | 3,23 | 0,04 | 1,00 | 0,00 | 1,20 | 0,00 | 0,30 | 0,00 | 0,20 | 0,00 | 0,70 | 0,00 | 0,05 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 |
| | PLT | 3,42 | 0,03 | 1,00 | 0,00 | 1,76 | 0,01 | 0,40 | 0,00 | 0,30 | 0,00 | 0,70 | 0,00 | 0,05 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 |
| | CHRH | 3,38 | 0,03 | 1,09 | 0,01 | 1,95 | 0,03 | 0,50 | 0,00 | 0,30 | 0,00 | 0,80 | 0,00 | 0,10 | 0,00 | 0,50 | 0,00 | 0,10 | 0,00 |
| | CLHR | 3,19 | 0,17 | 1,00 | 0,00 | 1,90 | 0,00 | 0,45 | 0,05 | 0,18 | 0,02 | 0,66 | 0,04 | 0,05 | 0,00 | 0,37 | 0,03 | 0,00 | 0,00 |
| | PHRH | 3,26 | 0,04 | 1,08 | 0,02 | 1,94 | 0,06 | 0,61 | 0,01 | 0,20 | 0,00 | 0,69 | 0,01 | 0,00 | 0,00 | 0,39 | 0,01 | 0,10 | 0,00 |
| | PLRH | 3,50 | 0,00 | 1,20 | 0,00 | 1,70 | 0,00 | 0,90* | 0,00 | 0,30 | 0,00 | 0,80 | 0,00 | 0,05 | 0,00 | 0,40 | 0,00 | 0,10 | 0,00 |
| | CO2 | 3,50 | 0,00 | 1,30 | 0,00 | 2,00 | 0,00 | 0,40 | 0,00 | 0,30 | 0,00 | 0,80 | 0,00 | 0,05 | 0,00 | 0,40 | 0,00 | 0,05 | 0,00 |
| 10 | Control | 4,00 | 0,00 | 1,61 | 0,02 | 2,16 | 0,04 | 0,50 | 0,00 | 0,16 | 0,04 | 0,85 | 0,01 | 0,10 | 0,00 | 0,55 | 0,01 | 0,05 | 0,01 |
| | CHT | 3,99 | 0,05 | 1,60 | 0,00 | 2,50 | 0,00 | 1,10* | 0,00 | 0,40 | 0,00 | 0,80 | 0,00 | 0,20 | 0,00 | 0,70 | 0,00 | 0,10 | 0,00 |
| | CLT | 3,26 | 0,05 | 1,13 | 0,02 | 1,63 | 0,02 | 0,53 | 0,02 | 0,40 | 0,00 | 0,70 | 0,00 | 0,05 | 0,00 | 0,43 | 0,02 | 0,10 | 0,00 |
| | PHT | 3,45 | 0,03 | 1,20 | 0,00 | 1,82 | 0,02 | 0,50 | 0,00 | 0,30 | 0,00 | 0,71 | 0,01 | 0,05 | 0,00 | 0,40 | 0,00 | 0,10 | 0,00 |
| | PLT | 3,79 | 0,05 | 1,50 | 0,00 | 2,33 | 0,02 | 1,00* | 0,00 | 0,40 | 0,00 | 0,80 | 0,00 | 0,10 | 0,00 | 0,70 | 0,00 | 0,10 | 0,00 |
| | CHRH | 3,83 | 0,07 | 1,23 | 0,03 | 2,33 | 0,07 | 0,60 | 0,00 | 0,40 | 0,00 | 0,80 | 0,00 | 0,10 | 0,00 | 0,57 | 0,01 | 0,10 | 0,00 |
| | CLHR | 3,97 | 0,03 | 1,10 | 0,00 | 1,80 | 0,00 | 0,80 | 0,00 | 0,30 | 0,00 | 0,80 | 0,00 | 0,05 | 0,00 | 0,50 | 0,00 | 0,10 | 0,00 |
| | PHRH | 3,70 | 0,00 | 1,30 | 0,00 | 2,00 | 0,00 | 1,00* | 0,00 | 0,40 | 0,00 | 0,90 | 0,00 | 0,00 | 0,00 | 0,50 | 0,00 | 0,10 | 0,00 |
| | PLRH | 4,10 | 0,00 | 1,40 | 0,00 | 2,20 | 0,00 | 1,00* | 0,00 | 0,40 | 0,00 | 0,90 | 0,00 | 0,10 | 0,00 | 0,60 | 0,00 | 0,10 | 0,00 |
| | CO2 | 3,60 | 0,00 | 1,50 | 0,00 | 2,00 | 0,00 | 0,80 | 0,00 | 0,40 | 0,00 | 0,80 | 0,00 | 0,05 | 0,00 | 0,90 | 0,00 | 0,10 | 0,00 |

**Annex 4 Table 2. Mean values of morphometric growth parameters, by treatment (cont.).**

| Day | Group | Embryo Length (cm) |  | Wing Length (cm) |  | Leg Length (cm) |  | Beak Length (cm) |  | Comb Length (cm) |  | Eye Diameter (cm) |  | Feather Length (cm) |  | Toe Length (cm) |  | Nail Length (cm) |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 12 | Control | 4,95 | 0,03 | 1,95 | 0,03 | 3,23 | 0,09 | 0,93 | 0,04 | 0,40 | 0,00 | 0,86 | 0,04 | 0,46 | 0,01 | 0,70 | 0,00 | 0,20 | 0,00 |
|  | CHT | 5,34 | 0,05 | 2,11 | 0,02 | 3,60 | 0,05 | 1,42* | 0,01 | 0,50 | 0,00 | 0,90 | 0,00 | 1,00 | 0,00 | 1,00 | 0,00 | 0,10* | 0,00 |
|  | CLT | 4,50 | 0,19 | 1,77 | 0,07 | 2,64 | 0,09 | 1,06 | 0,02 | 0,46 | 0,02 | 0,80 | 0,00 | 0,09 | 0,01 | 0,60 | 0,04 | 0,10* | 0,00 |
|  | PHT | 4,38 | 0,11 | 1,71 | 0,05 | 2,82 | 0,12 | 1,15 | 0,04 | 0,45 | 0,04 | 0,83 | 0,07 | 0,10 | 0,00 | 0,70 | 0,00 | 0,10* | 0,00 |
|  | PLT | 5,03 | 0,17 | 1,83 | 0,05 | 3,22 | 0,09 | 1,19 | 0,04 | 0,44 | 0,02 | 0,90 | 0,00 | 0,50 | 0,00 | 0,84 | 0,02 | 0,10* | 0,00 |
|  | CHRH | 5,18 | 0,02 | 1,99 | 0,01 | 3,48 | 0,02 | 1,40* | 0,00 | 0,50 | 0,00 | 0,90 | 0,00 | 0,50 | 0,00 | 0,98 | 0,02 | 0,10* | 0,00 |
|  | CLHR | 5,02 | 0,02 | 1,85 | 0,05 | 3,15 | 0,15 | 1,02 | 0,02 | 0,43 | 0,03 | 0,81 | 0,01 | 0,54 | 0,04 | 0,82 | 0,02 | 0,11* | 0,01 |
|  | PHRH | 5,00 | 0,00 | 2,00 | 0,00 | 3,60 | 0,00 | 1,10 | 0,00 | 0,60 | 0,00 | 0,90 | 0,00 | 0,50 | 0,00 | 1,10 | 0,00 | 0,20 | 0,00 |
|  | PLRH | 5,00 | 0,00 | 2,00 | 0,00 | 3,60 | 0,00 | 1,10 | 0,00 | 0,60 | 0,00 | 0,90 | 0,00 | 0,50 | 0,00 | 1,10 | 0,00 | 0,20 | 0,00 |
|  | CO2 | 5,42 | 0,08 | 2,33 | 0,07 | 3,60 | 0,10 | 1,20 | 0,00 | 0,48 | 0,02 | 0,90 | 0,00 | 0,50 | 0,00 | 0,98 | 0,02 | 0,10* | 0,00 |
| 14 | Control | 6,13 | 0,06 | 2,45 | 0,03 | 4,25 | 0,08 | 1,21 | 0,06 | 0,59 | 0,03 | 0,90 | 0,01 | 1,30 | 0,06 | 1,27 | 0,02 | 0,20 | 0,00 |
|  | CHT | 6,28 | 0,10 | 2,65 | 0,03 | 5,32 | 0,08 | 1,62* | 0,04 | 0,67 | 0,01 | 0,90 | 0,00 | 2,00 | 0,00 | 1,18 | 0,01 | 0,18 | 0,01 |
|  | CLT | 5,58 | 0,08 | 2,42 | 0,08 | 3,92 | 0,08 | 1,40* | 0,00 | 0,60 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 0,97 | 0,03 | 0,18 | 0,02 |
|  | PHT | 5,85 | 0,11 | 2,42 | 0,06 | 4,58 | 0,09 | 1,30 | 0,00 | 0,64 | 0,02 | 0,90 | 0,00 | 1,00 | 0,00 | 1,18 | 0,01 | 0,20 | 0,00 |
|  | PLT | 6,52 | 0,06 | 2,50 | 0,00 | 4,71 | 0,03 | 1,60* | 0,01 | 0,60 | 0,00 | 0,90 | 0,00 | 1,00 | 0,00 | 1,19 | 0,01 | 0,20 | 0,00 |
|  | CHRH | 6,62 | 0,08 | 2,50 | 0,00 | 4,62 | 0,00 | 1,50* | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 1,00 | 0,00 | 1,12 | 0,01 | 0,20 | 0,00 |
|  | CLHR | 6,64 | 0,07 | 2,50 | 0,00 | 5,00 | 0,00 | 1,30 | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 1,00 | 0,00 | 1,10 | 0,00 | 0,20 | 0,00 |
|  | PHRH | 6,50 | 0,00 | 2,50 | 0,00 | 4,00 | 0,00 | 1,60* | 0,00 | 0,50 | 0,00 | 0,90 | 0,00 | 1,00 | 0,00 | 1,20 | 0,00 | 0,20 | 0,00 |
|  | PLRH | 7,00 | 0,00 | 2,70 | 0,00 | 5,00 | 0,00 | 1,70* | 0,00 | 0,60 | 0,00 | 0,90 | 0,00 | 1,00 | 0,00 | 1,40 | 0,00 | 0,20 | 0,00 |
|  | CO2 | 7,00 | 0,00 | 2,50 | 0,00 | 5,00 | 0,00 | 1,40* | 0,00 | 0,60 | 0,00 | 0,90 | 0,00 | 1,00 | 0,00 | 1,40 | 0,00 | 0,20 | 0,00 |

## Annex 4 Table 2. Mean values of morphometric growth parameters, by treatment (cont.).

| Day | Group | Embryo Length (cm) |  | Wing Length (cm) |  | Leg Length (cm) |  | Beak Length (cm) |  | Comb Length (cm) |  | Eye Diameter (cm) |  | Feather Length (cm) |  | Toe Length (cm) |  | Nail Length (cm) |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 16 | Control | 7,41 | 0,04 | 3,00 | 0,00 | 5,23 | 0,06 | 1,18 | 0,03 | 0,64 | 0,01 | 0,90 | 0,00 | 2,00 | 0,00 | 1,62 | 0,02 | 0,24 | 0,01 |
|  | CHT | 7,22 | 0,11 | 2,90 | 0,16 | 6,30 | 0,17 | 1,68 | 0,02 | 0,70 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,68 | 0,04 | 0,20 | 0,00 |
|  | CLT | 6,33 | 0,17 | 3,00 | 0,00 | 5,50 | 0,00 | 1,60 | 0,00 | 0,62 | 0,02 | 0,92 | 0,02 | 2,00 | 0,00 | 1,17 | 0,03 | 0,20 | 0,00 |
|  | PHT | 7,67 | 0,07 | 3,00 | 0,00 | 6,53 | 0,03 | 1,70 | 0,00 | 0,88 | 0,01 | 0,90 | 0,00 | 2,00 | 0,00 | 1,68 | 0,01 | 0,20 | 0,00 |
|  | PLT | 7,71 | 0,07 | 3,00 | 0,00 | 6,21 | 0,07 | 2,00* | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,74 | 0,01 | 0,30 | 0,00 |
|  | CHRH | 8,04 | 0,04 | 3,50 | 0,00 | 6,54 | 0,04 | 1,80* | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,70 | 0,00 | 0,20 | 0,00 |
|  | CLHR | 7,29 | 0,18 | 2,95 | 0,03 | 6,50 | 0,11 | 1,69 | 0,04 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,52 | 0,02 | 0,20 | 0,00 |
|  | PHRH | 8,00 | 0,00 | 3,50 | 0,00 | 7,00 | 0,00 | 2,00* | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,60 | 0,00 | 0,20 | 0,00 |
|  | PLRH | 8,00 | 0,00 | 3,00 | 0,00 | 5,50 | 0,00 | 1,80* | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,80 | 0,00 | 0,30 | 0,00 |
|  | CO2 | 7,46 | 0,04 | 3,39 | 0,11 | 6,96 | 0,04 | 1,97* | 0,03 | 0,78 | 0,03 | 0,90 | 0,00 | 2,00 | 0,00 | 1,68 | 0,02 | 0,29 | 0,01 |
| 18 | Control | 8,45 | 0,11 | 3,75 | 0,06 | 7,20 | 0,16 | 1,33 | 0,01 | 0,95 | 0,01 | 0,95 | 0,01 | 2,00 | 0,00 | 1,94 | 0,02 | 0,30 | 0,00 |
|  | CHT | 8,32 | 0,21 | 3,41 | 0,09 | 7,45 | 0,30 | 1,83* | 0,07 | 0,88 | 0,02 | 0,90 | 0,00 | 2,00 | 0,00 | 1,85 | 0,07 | 0,22* | 0,02 |
|  | CLT | 7,67 | 0,21 | 3,25 | 0,11 | 6,83 | 0,11 | 1,60 | 0,00 | 0,87 | 0,02 | 0,90 | 0,00 | 2,00 | 0,00 | 1,90 | 0,04 | 0,30 | 0,00 |
|  | PHT | 8,45 | 0,05 | 3,45 | 0,05 | 7,72 | 0,08 | 2,00* | 0,00 | 0,90 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,18 | 0,02 | 0,30 | 0,00 |
|  | PLT | 9,05 | 0,08 | 3,95 | 0,05 | 8,50 | 0,12 | 2,00* | 0,00 | 0,99 | 0,01 | 0,90 | 0,00 | 2,00 | 0,00 | 2,30 | 0,00 | 0,30 | 0,00 |
|  | CHRH | 9,00 | 0,00 | 3,70 | 0,00 | 8,00 | 0,00 | 2,20* | 0,00 | 1,00 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,10 | 0,00 | 0,30 | 0,00 |
|  | CLHR | 8,50 | 0,00 | 3,50 | 0,00 | 7,70 | 0,00 | 2,00* | 0,00 | 1,00 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,10 | 0,00 | 0,30 | 0,00 |
|  | PHRH | 9,00 | 0,00 | 3,50 | 0,00 | 8,00 | 0,00 | 2,20 | 0,00 | 0,90 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,30 | 0,00 | 0,30 | 0,00 |
|  | PLRH | 8,42 | 0,08 | 3,42 | 0,08 | 7,22 | 0,08 | 2,08* | 0,02 | 0,98 | 0,02 | 0,90 | 0,00 | 2,00 | 0,00 | 1,99 | 0,01 | 0,30 | 0,00 |
|  | CO2 | 9,00 | 0,00 | 3,50 | 0,00 | 7,70 | 0,00 | 2,00* | 0,00 | 1,00 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,10 | 0,00 | 0,30 | 0,00 |

**Annex 4 Table 2. Mean values of morphometric growth parameters, by treatment (conc.).**

| Day | Group | Embryo Length (cm) | | Wing Length (cm) | | Leg Length (cm) | | Beak Length (cm) | | Comb Length (cm) | | Eye Diameter (cm) | | Feather Length (cm) | | Toe Length (cm) | | Nail Length (cm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 20 | Control | 8,93 | 0,07 | 4,00 | 0,00 | 8,50 | 0,00 | 1,40 | 0,00 | 1,00 | 0,00 | 1,00 | 0,00 | 2,00 | 0,00 | 2,00 | 0,00 | 0,40 | 0,00 |
| | CHT | 8,62 | 0,44 | 3,89 | 0,29 | 7,59 | 0,51 | 1,87* | 0,08 | 0,84 | 0,06 | 0,89 | 0,01 | 1,89 | 0,11 | 2,17 | 0,16 | 0,28* | 0,02 |
| | CLT | 9,33 | 0,33 | 3,50 | 0,00 | 7,70 | 0,40 | 2,00* | 0,00 | 1,00 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,33 | 0,03 | 0,30 | 0,00 |
| | PHT | 9,50 | 0,00 | 4,50 | 0,00 | 8,60 | 0,00 | 2,20* | 0,00 | 0,90 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,30 | 0,00 | 0,30 | 0,00 |
| | PLT | 10,13 | 0,18 | 4,00 | 0,00 | 8,50 | 0,00 | 2,13* | 0,04 | 1,00 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,50 | 0,00 | 0,30 | 0,00 |
| | CHRH | 9,63 | 0,13 | 4,00 | 0,00 | 9,00 | 0,00 | 2,40* | 0,00 | 1,00 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,50 | 0,00 | 0,30 | 0,00 |
| | CLHR | 9,63 | 0,13 | 4,00 | 0,00 | 9,00 | 0,00 | 2,40* | 0,00 | 1,00 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,50 | 0,00 | 0,30 | 0,00 |
| | PHRH | 9,92 | 0,08 | 3,50 | 0,00 | 8,00 | 0,00 | 2,20* | 0,00 | 1,00 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,30 | 0,00 | 0,30 | 0,00 |
| | PLRH | 10,00 | 0,00 | 4,00 | 0,00 | 9,00 | 0,00 | 2,10* | 0,00 | 1,10 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,40 | 0,00 | 0,30 | 0,00 |
| | CO2 | 10,00 | 0,00 | 3,50 | 0,00 | 9,20 | 0,00 | 2,20* | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,40 | 0,00 | 0,30 | 0,00 |

***Significant difference found ($p<0,05$). All significant values are displayed in comparison to the mean of the Control group. Testing done with One-Way ANOVA.**

**Annex 4 Table 3. Mortality and malformation values, by treatment.**

| Trial | Mortality (% dead/total fertile eggs) | | | | | | | | | | | | Total mortalitye |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day 0 | Day 1 | Day 3 | Day 5 | Day 7 | Day 9 | Day 10 | Day 12 | Day 14 | Day 16 | Day 18 | Day 20 | |
| Control | 0,6% | 0,9% | 2,8% | 0,4% | 0,2% | 0,0% | 0,2% | 0,0% | 0,0% | 0,0% | 0,4% | 0,2% | 5,7% |
| CHT | 0,0% | 0,0% | 5,2% | 0,0% | 0,0% | 0,4% | 0,0% | 0,7% | 0,0% | 0,0% | 0,0% | 0,0% | 6,3% |
| CLT | 0,0% | 17,6%* | 6,6% | 2,0% | 1,3% | 0,3% | 0,0% | 0,3% | 0,0% | 0,3% | 0,0% | 0,0% | 28,6%* |
| PHT | 0,4% | 3,7% | 3,0% | 0,0% | 0,4% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 7,4% |
| PLT | 0,0% | 1,5% | 1,5% | 0,0% | 0,4% | 0,7% | 0,0% | 0,0% | 0,0% | 0,7% | 0,0% | 0,0% | 4,8% |
| CHRH | 0,0% | 1,3% | 2,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 2,7%* | 6,0% |
| CLHR | 0,0% | 1,3% | 2,7% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 4,0% |
| PHRH | 0,7% | 0,7% | 1,3% | 0,7% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 3,3% |
| PLRH | 0,0% | 0,0% | 4,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 4,0% |
| CO2 | 0,0% | 0,7% | 1,3% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% | 2,0% |

| Trial | Malformed structures/organs (% affected/total malformation cases) | | | | | | | | | Total malformation prevalence (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | Cystic | Eye | Skull | Abdomen | Skin | Limbs | Beak | Spine | Dwarf | |
| Control | 37,50% | 12,50% | 25,00% | 12,50% | 0,00% | 0,00% | 12,50% | 25,00% | 12,50% | 2% |
| CHT | 45,45% | 0,00% | 9,09% | 18,18% | 9,09% | 9,09% | 0,00% | 0,00% | 9,09% | 5%* |
| CLT | 33,33% | 11,11% | 11,11% | 22,22% | 0,00% | 22,22% | 11,11% | 11,11% | 0,00% | 3%* |
| PHT | 0,00% | 100,00% | 33,33% | 33,33% | 0,00% | 0,00% | 33,33% | 0,00% | 0,00% | 1% |
| PLT | 50,00% | 0,00% | 0,00% | 0,00% | 0,00% | 0,00% | 0,00% | 50,00% | 0,00% | 1% |
| CHRH | 0,00% | 0,00% | 50,00% | 0,00% | 0,00% | 0,00% | 0,00% | 50,00% | 0,00% | 0% |
| CLHR | 0,00% | 0,00% | 0,00% | 50,00% | 50,00% | 0,00% | 0,00% | 0,00% | 0,00% | 1% |
| PHRH | 37,50% | 12,50% | 25,00% | 12,50% | 0,00% | 0,00% | 12,50% | 25,00% | 12,50% | 1% |
| PLRH | 45,45% | 0,00% | 9,09% | 18,18% | 9,09% | 9,09% | 0,00% | 0,00% | 9,09% | 0% |
| CO2 | 33,33% | 11,11% | 11,11% | 22,22% | 0,00% | 22,22% | 11,11% | 11,11% | 0,00% | 0% |

[*] **Significant difference found ($p<0,05$). All significant values are displayed in comparison to the mean of the Control group. Testing done with Chi-square.**

# Annex 5. Mean values of the parameters monitored at each time-point for *Salmonella* trials.

**Annex 5 Table 1. Mean values of behavioral, nutrient utilization and growth parameters for the *Salmonella* Enteritidis High Inoculum Trial.**

| Day | Group | Shell Ratio |  | Albumen Ratio |  | Yolk Ratio |  | Embryo Ratio |  | Heart Rate (bpm) |  | Voluntary Movements (mov/min) |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 1 | Control | 11,94 | 0,51 | 57,30 | 0,71 | 28,91 | 1,31 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 |
|  | SE 10^5 | 12,94 | 0,39 | 59,41 | 1,43 | 26,69 | 0,79 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 3 | Control | 12,14 | 0,39 | 54,15 | 2,21 | 27,57 | 1,32 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 |
|  | SE 10^5 | 12,55 | 0,32 | 58,47 | 2,53 | 27,52 | 0,76 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 5 | Control | 12,21 | 0,20 | 29,78 | 1,91 | 48,09 | 1,52 | 0,34 | 0,04 | 140,00 | 20,00 | 0,00 | 0,00 |
|  | SE 10^5 | 12,82 | 0,31 | 32,27 | 3,17 | 52,48 | 2,61 | 0,29 | 0,01 | 53,33 | 35,28 | 0,00 | 0,00 |
| 7 | Control | 12,92 | 0,32 | 18,95 | 1,78 | 49,69 | 2,34 | 1,09 | 0,09 | 163,50 | 43,60 | 18,00 | 4,54 |
|  | SE 10^5 | 12,69 | 0,00 | 22,26 | 0,00 | 52,45 | 0,00 | 0,99 | 0,00 | 223,00 | 0,00 | 8,00 | 0,00 |
| 9 | Control | 12,91 | 0,31 | 15,35 | 1,38 | 42,50 | 3,63 | 2,82 | 0,29 | 268,50 | 8,50 | 26,00 | 2,00 |
|  | SE 10^5 | - | - | - | - | - | - | - | - | - | - | - | - |
| 10 | Control | 13,48 | 0,40 | 17,27 | 2,72 | 46,54 | 2,05 | 3,99 | 0,22 | 264,00 | 3,08 | 28,00 | 2,31 |
|  | SE 10^5 | - | - | - | - | - | - | - | - | - | - | - | - |
| 12 | Control | 13,14 | 0,62 | 13,06 | 0,42 | 37,12 | 2,59 | 8,04 | 0,30 | 283,67 | 7,17 | 26,67 | 2,67 |
|  | SE 10^5 | 14,07 | 0,00 | 15,56 | 0,00 | 38,30 | 0,00 | 9,05 | 0,00 | 279,00 | 0,00 | 24,00 | 0,00 |
| 14 | Control | 13,32 | 0,14 | 18,14 | 1,85 | 44,17 | 2,18 | 15,19 | 1,36 | 283,25 | 3,84 | 32,00 | 0,00 |
|  | SE 10^5 | - | - | - | - | - | - | - | - | - | - | - | - |
| 16 | Control | 13,41 | 0,37 | 13,28 | 2,35 | 41,05 | 2,21 | 28,60 | 0,03 | 293,00 | 1,00 | 24,00 | 0,00 |
|  | SE 10^5 | - | - | - | - | - | - | - | - | - | - | - | - |
| 18 | Control | 14,86 | 0,31 | 0,00 | 0,00 | 31,20 | 5,63 | 46,45 | 0,45 | 273,75 | 25,07 | 20,00 | 3,27 |
|  | SE 10^5 | 13,56 | 0,00 | 0,00 | 0,00 | 36,05 | 0,00 | 42,55 | 0,00 | 306,00 | 0,00 | 16,00 | 0,00 |
| 20 | Control | 14,85 | 0,85 | 0,00 | 0,00 | 23,98 | 2,05 | 59,85 | 1,34 | 247,25 | 22,54 | 26,00 | 6,00 |
|  | SE 10^5 | - | - | - | - | - | - | - | - | - | - | - | - |

**[*] Significant difference found ($p<0,05$). All significant values are displayed in comparison to the mean of the Control group. Testing done with Student T-test.**

**Annex 5 Table 1. Mean values of behavioral, nutrient utilization and growth parameters for the *S.* Enteritidis High Inoculum Trial (cont.)**

| Day | Group | Embryo Length (cm) | | Wing Length (cm) | | Leg Length (cm) | | Beak Length (cm) | | Comb Length (cm) | | Eye Diameter (cm) | | Feather Length (cm) | | Toe Length (cm) | | Nail Length (cm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 1 | Control | 0,70 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 |
| | SE 10^5 | 0,68 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 3 | Control | 0,66 | 0,04 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 |
| | SE 10^5 | 0,66 | 0,03 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 5 | Control | 1,15 | 0,03 | 0,20 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | SE 10^5 | 1,17 | 0,03 | 0,20 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 7 | Control | 2,30 | 0,03 | 0,40 | 0,00 | 0,60 | 0,00 | 0,20 | 0,00 | 0,10 | 0,00 | 0,43 | 0,02 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |
| | SE 10^5 | 2,30 | 0,00 | 0,40 | 0,00 | 0,60 | 0,00 | 0,20 | 0,00 | 0,10 | 0,00 | 0,40 | 0,00 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |
| 9 | Control | 2,90 | 0,10 | 1,00 | 0,00 | 1,55 | 0,05 | 0,43 | 0,02 | 0,23 | 0,02 | 0,73 | 0,03 | 0,05 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 |
| | SE 10^5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 10 | Control | 3,28 | 0,02 | 1,20 | 0,00 | 2,00 | 0,00 | 0,90 | 0,00 | 0,30 | 0,00 | 0,80 | 0,00 | 0,05 | 0,00 | 0,50 | 0,00 | 0,10 | 0,00 |
| | SE 10^5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 12 | Control | 4,50 | 0,00 | 1,40 | 0,00 | 2,80 | 0,00 | 1,20 | 0,00 | 0,50 | 0,00 | 0,90 | 0,00 | 0,10 | 0,00 | 0,80 | 0,00 | 0,10 | 0,00 |
| | SE 10^5 | 4,50 | 0,00 | 1,70 | 0,00 | 3,20 | 0,00 | 1,20 | 0,00 | 0,50 | 0,00 | 0,90 | 0,00 | 0,50 | 0,00 | 0,80 | 0,00 | 0,10 | 0,00 |
| 14 | Control | 5,38 | 0,13 | 1,93 | 0,08 | 3,58 | 0,13 | 1,28 | 0,03 | 0,60 | 0,00 | 0,90 | 0,00 | 0,88 | 0,13 | 0,93 | 0,07 | 0,18 | 0,03 |
| | SE 10^5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 16 | Control | 6,00 | 0,00 | 2,50 | 0,00 | 5,00 | 0,00 | 1,40 | 0,00 | 0,70 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,50 | 0,00 | 0,20 | 0,00 |
| | SE 10^5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 18 | Control | 7,20 | 0,00 | 3,00 | 0,00 | 6,00 | 0,00 | 1,80 | 0,00 | 0,90 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,10 | 0,00 | 0,20 | 0,00 |
| | SE 10^5 | 7,20 | 0,00 | 3,00 | 0,00 | 6,00 | 0,00 | 1,80 | 0,00 | 0,90 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,10 | 0,00 | 0,20 | 0,00 |
| 20 | Control | 7,88 | 0,13 | 3,00 | 0,00 | 7,50 | 0,00 | 2,00 | 0,00 | 1,00 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,93 | 0,08 | 0,30 | 0,00 |
| | SE 10^5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

***Significant difference found ($p<0,05$). All significant values are displayed in comparison to the mean of the Control group. Testing done with Student T-test.**

**Annex 5 Table 2. Mean values of behavioral, nutrient utilization and growth parameters for the *Salmonella* Enteritidis Low Inoculum Trial.**

| Day | Group | Shell Ratio | | Albumen Ratio | | Yolk Ratio | | Embryo Ratio | | Heart Rate (bpm) | | Voluntary Movements (mov/min) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 1 | Control | 0,13 | 0,00 | 0,60 | 0,01 | 0,29 | 0,01 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 |
| | SE 10^2 | 0,14 | 0,00 | 0,59 | 0,01 | 0,30 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 3 | Control | 0,13 | 0,00 | 0,59 | 0,02 | 0,28 | 0,01 | 0,00 | 0 | 46,67 | 21,71 | 0,00 | 0 |
| | SE 10^2 | 0,12 | 0,00 | 0,58 | 0,06 | 0,27 | 0,02 | 0,00 | 0,00 | 80,00 | 36,33 | 0,00 | 0,00 |
| 5 | Control | 0,14 | 0,00 | 0,36 | 0,01 | 0,38 | 0,02 | 0,00 | 0,00 | 200,00 | 0,00 | 0,00 | 0,00 |
| | SE 10^2 | 0,14 | 0,00 | 0,34 | 0,01 | 0,45 | 0,01 | 0,00 | 0,00 | 200,00 | 0,00 | 0,00 | 0,00 |
| 7 | Control | 0,13 | 0,00 | 0,24 | 0,01 | 0,47 | 0,02 | 0,01 | 0,00 | 199,17 | 40,21 | 14,67 | 3,21 |
| | SE 10^2 | 0,14 | 0,00 | 0,26 | 0,03 | 0,47 | 0,01 | 0,01 | 0,00 | 249,00 | 2,00 | 24,00 | 0,00 |
| 9 | Control | 0,10 | 0,00 | 0,15 | 0,01 | 0,49 | 0,03 | 0,03 | 0,00 | 255,33 | 11,84 | 18,67 | 2,67 |
| | SE 10^2 | - | - | - | - | - | - | - | - | - | - | - | - |
| 10 | Control | 0,12 | 0,00 | 0,16 | 0,01 | 0,40 | 0,01 | 0,04 | 0,00 | 249,00 | 4,70 | 27,20 | 1,96 |
| | SE 10^2 | 0,14 | 0,01 | 0,13 | 0,00 | 0,50 | 0,06 | 0,02 | 0,02 | 262,00 | 15,00 | 24,00 | 8,00 |
| 12 | Control | 0,11 | 0,01 | 0,17 | 0,03 | 0,33 | 0,01 | 0,08 | 0,02 | 271,00 | 1,00 | 36,00 | 4,00 |
| | SE 10^2 | 0,13 | 0,00 | 0,21 | 0,01 | 0,35 | 0,03 | 0,09 | 0,00 | 265,00 | 3,79 | 24,00 | 8,00 |
| 14 | Control | 0,11 | 0,00 | 0,10 | 0,01 | 0,43 | 0,03 | 0,12 | 0,06 | 275,00 | 7,55 | 29,33 | 2,67 |
| | SE 10^2 | 0,12 | 0,00 | 0,12 | 0,00 | 0,35 | 0,00 | 0,20 | 0,00 | 283,00 | 0,00 | 16,00 | 0,00 |
| 16 | Control | 0,13 | 0,01 | 0,19 | 0,04 | 0,29 | 0,05 | 0,32 | 0,01 | 278,00 | 9,70 | 24,00 | 3,27 |
| | SE 10^2 | 0,13 | 0,01 | 0,18 | 0,00 | 0,32 | 0,03 | 0,32 | 0,03 | 277,00 | 14,00 | 28,00 | 12,00 |
| 18 | Control | 0,14 | 0,01 | 0,00 | 0,00 | 0,17 | 0,01 | 0,41 | 0,01 | 272,67 | 13,59 | 18,67 | 2,67 |
| | SE 10^2 | 0,14 | 0,01 | 0,04 | 0,04 | 0,22 | 0,08 | 0,63 | 0,05 | 251,00 | 6,66 | 24,00 | 4,62 |
| 20 | Control | 0,14 | 0,01 | 0,00 | 0,00 | 0,13 | 0,01 | 0,66 | 0,01 | 236,60 | 8,23 | 32,00 | 4,38 |
| | SE 10^2 | 0,15 | 0,02 | 0,00 | 0,00 | 0,14 | 0,06 | 0,66 | 0,07 | 277,00 | 4,16 | 32,00 | 8,00 |

[*] **Significant difference found ($p<0,05$). All significant values are displayed in comparison to the mean of the Control group. Testing done with Student T-test.**

**Annex 5 Table 2. Mean values of behavioral, nutrient utilization and growth parameters for the *S.* Enteritidis Low Inoculum Trial (cont.).**

| Day | Group | Embryo Length (cm) | | Wing Length (cm) | | Leg Length (cm) | | Beak Length (cm) | | Comb Length (cm) | | Eye Diameter (cm) | | Feather Length (cm) | | Toe Length (cm) | | Nail Length (cm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 1 | Control | 0,68 | 0,05426 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 |
| | SE 10^2 | 0,63 | 0,03 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 3 | Control | 0,57 | 0,02108 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 | 0,00 | 0 |
| | SE 10^2 | 0,60 | 0,20 | 0,00 | 0,04 | 0,00 | 0,07 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 5 | Control | 1,58 | 0,05 | 0,20 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | SE 10^2 | 1,57 | 0,07 | 0,20 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 7 | Control | 1,93 | 0,04 | 0,40 | 0,00 | 0,50 | 0,00 | 0,20 | 0,00 | 0,10 | 0,00 | 0,48 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | SE 10^2 | 2,00 | 0,00 | 0,40 | 0,00 | 0,50 | 0,00 | 0,20 | 0,00 | 0,10 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 9 | Control | 2,50 | 0,00 | 0,70 | 0,00 | 1,00 | 0,00 | 0,30 | 0,00 | 0,20 | 0,00 | 0,70 | 0,00 | 0,00 | 0,00 | 0,40 | 0,00 | 0,10 | 0,00 |
| | SE 10^2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 10 | Control | 3,00 | 0,00 | 1,20 | 0,00 | 1,70 | 0,00 | 0,40 | 0,00 | 0,30 | 0,00 | 0,80 | 0,00 | 0,10 | 0,00 | 0,50 | 0,00 | 0,10 | 0,00 |
| | SE 10^2 | 3,00 | 0,00 | 1,20 | 0,00 | 1,70 | 0,00 | 0,40 | 0,00 | 0,30 | 0,00 | 0,80 | 0,00 | 0,10 | 0,00 | 0,50 | 0,00 | 0,10 | 0,00 |
| 12 | Control | 4,25 | 0,25 | 1,35 | 0,15 | 2,85 | 0,15 | 1,10 | 0,00 | 0,45 | 0,05 | 0,90 | 0,00 | 0,30 | 0,20 | 1,00 | 0,10 | 0,10 | 0,00 |
| | SE 10^2 | 4,33 | 0,17 | 1,40 | 0,10 | 2,90 | 0,10 | 1,10 | 0,00 | 0,47 | 0,03 | 0,90 | 0,00 | 0,37 | 0,13 | 1,03 | 0,07 | 0,10 | 0,00 |
| 14 | Control | 5,50 | 0,00 | 2,00 | 0,00 | 4,50 | 0,00 | 1,20 | 0,00 | 0,70 | 0,00 | 0,90 | 0,00 | 0,10 | 0,00 | 1,30 | 0,00 | 0,20 | 0,00 |
| | SE 10^2 | 5,50 | 0,00 | 2,00 | 0,00 | 4,50 | 0,00 | 1,20 | 0,00 | 0,70 | 0,00 | 0,90 | 0,00 | 0,10 | 0,00 | 1,30 | 0,00 | 0,20 | 0,00 |
| 16 | Control | 6,50 | 0,00 | 2,50 | 0,00 | 5,30 | 0,00 | 1,20 | 0,00 | 0,60 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,40 | 0,00 | 0,20 | 0,00 |
| | SE 10^2 | 6,50 | 0,00 | 2,50 | 0,00 | 5,30 | 0,00 | 1,20 | 0,00 | 0,60 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,40 | 0,00 | 0,20 | 0,00 |
| 18 | Control | 7,00 | 0,00 | 3,00 | 0,00 | 6,00 | 0,00 | 1,70 | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,80 | 0,00 | 0,30 | 0,00 |
| | SE 10^2 | 6,83 | 0,17 | 3,00 | 0,00 | 5,50 | 0,50 | 1,63 | 0,07 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,80 | 0,00 | 0,30 | 0,00 |
| 20 | Control | 8,00 | 0,00 | 3,50 | 0,00 | 7,00 | 0,00 | 1,90 | 0,00 | 1,00 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,40 | 0,00 | 0,30 | 0,00 |
| | SE 10^2 | 7,67 | 0,33 | 3,33 | 0,17 | 6,67 | 0,33 | 1,83 | 0,07 | 0,93 | 0,07 | 0,90 | 0,00 | 2,00 | 0,00 | 2,20 | 0,20 | 0,30 | 0,00 |

***Significant difference found ($p<0,05$). All significant values are displayed in comparison to the mean of the Control group. Testing done with Student T-test.**

**Annex 5 Table 3. Mean values of behavioral, nutrient utilization and growth parameters for the *Salmonella* Typhimurium High Inoculum Trial.**

| Day | Group | Shell Ratio | | Albumen Ratio | | Yolk Ratio | | Embryo Ratio | | Heart Rate (bpm) | | Voluntary Movements (mov/min) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 1 | Control | 0,14 | 0,00 | 0,59 | 0,02 | 0,23 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | ST 10^5 | 0,15 | 0,00 | 0,63 | 0,02 | 0,22 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 3 | Control | 0,14 | 0,00 | 0,61 | 0,02 | 0,21 | 0,01 | 0,00 | 0,00 | 72,00 | 12,00 | 0,00 | 0,00 |
| | ST 10^5 | 0,14 | 0,00 | 0,62 | 0,03 | 0,21 | 0,01 | 0,00 | 0,00 | 60,00 | 0,00 | 0,00 | 0,00 |
| 5 | Control | 0,12 | 0,00 | 0,36 | 0,05 | 0,38 | 0,06 | 0,00 | 0,00 | 160,00 | 0,00 | 0,00 | 0,00 |
| | ST 10^5 | 0,15 | 0,00 | 0,30 | 0,04 | 0,48 | 0,01 | 0,00 | 0,00 | 160,00 | 0,00 | 0,00 | 0,00 |
| 7 | Control | 0,14 | 0,01 | 0,21 | 0,01 | 0,45 | 0,01 | 0,01 | 0,00 | 218,50 | 11,45 | 24,00 | 0,00 |
| | ST 10^5 | 0,13 | 0,01 | 0,22 | 0,01 | 0,42 | 0,01 | 0,01 | 0,00 | 218,40 | 10,80 | 14,40 | 5,88 |
| 9 | Control | 0,14 | 0,01 | 0,23 | 0,02 | 0,36 | 0,01 | 0,03 | 0,00 | 246,40 | 6,93 | 27,20 | 1,96 |
| | ST 10^5 | 0,13 | 0,00 | 0,20 | 0,01 | 0,36 | 0,01 | 0,03 | 0,00 | 257,20 | 7,45 | 28,80 | 1,96 |
| 10 | Control | 0,12 | 0,00 | 0,15 | 0,00 | 0,37 | 0,02 | 0,05 | 0,00 | 245,50 | 9,47 | 29,33 | 1,69 |
| | ST 10^5 | 0,13 | 0,01 | 0,16 | 0,01 | 0,35 | 0,01 | 0,06 | 0,00 | 244,00 | 5,91 | 24,00 | 5,06 |
| 12 | Control | 0,12 | 0,00 | 0,15 | 0,01 | 0,35 | 0,01 | 0,08 | 0,00 | 266,60 | 12,22 | 30,40 | 2,99 |
| | ST 10^5 | 0,12 | 0,00 | 0,15 | 0,02 | 0,30 | 0,00 | 0,07 | 0,01 | 273,00 | 0,00 | 28,00 | 4,00 |
| 14 | Control | 0,12 | 0,00 | 0,14 | 0,01 | 0,31 | 0,01 | 0,16 | 0,00 | 272,20 | 5,44 | 35,20 | 1,96 |
| | ST 10^5 | 0,10 | 0,02 | 0,10 | 0,03 | 0,27 | 0,07 | 0,13 | 0,03 | 263,00 | 3,87 | 35,20 | 1,96 |
| 16 | Control | 0,12 | 0,00 | 0,09 | 0,01 | 0,25 | 0,02 | 0,37 | 0,02 | 281,80 | 9,39 | 36,80 | 3,20 |
| | ST 10^5 | 0,12 | 0,01 | 0,08 | 0,01 | 0,24 | 0,02 | 0,37 | 0,02 | 264,40 | 5,16 | 30,40 | 2,99 |
| 18 | Control | 0,10 | 0,00 | 0,00 | 0,00 | 0,20 | 0,01 | 0,44 | 0,02 | 291,83 | 9,53 | 29,33 | 2,67 |
| | ST 10^5 | 0,11 | 0,00 | 0,00 | 0,00 | 0,22 | 0,00 | 0,48 | 0,00 | 288,00 | 0,00 | 24,00 | 0,00 |
| 20 | Control | 0,12 | 0,00 | 0,00 | 0,00 | 0,07 | 0,03 | 0,64 | 0,05 | 234,83 | 7,89 | 29,33 | 4,92 |
| | ST 10^5 | 0,11 | 0,00 | 0,00 | 0,00 | 0,05 | 0,02 | 0,68 | 0,05 | 261,25 | 15,22 | 26,00 | 6,00 |

**[*] Significant difference found ($p<0,05$). All significant values are displayed in comparison to the mean of the Control group. Testing done with Student T-test.**

**Annex 5 Table 3. Mean values of behavioral, nutrient utilization and growth parameters for the *S.* Typhimurium High Inoculum Trial (cont.).**

| Day | Group | Embryo Length (cm) | | Wing Length (cm) | | Leg Length (cm) | | Beak Length (cm) | | Comb Length (cm) | | Eye Diameter (cm) | | Feather Length (cm) | | Toe Length (cm) | | Nail Length (cm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 1 | Control | 0,87 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | ST 10^5 | 0,82 | 0,05 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 3 | Control | 0,56 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | ST 10^5 | 0,53 | 0,03 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 5 | Control | 1,26 | 0,04 | 0,18 | 0,02 | 0,26 | 0,04 | 0,00 | 0,00 | 0,00 | 0,00 | 0,18 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | ST 10^5 | 1,30 | 0,00 | 0,20 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 7 | Control | 1,73 | 0,11 | 0,40 | 0,00 | 0,58 | 0,02 | 0,18 | 0,02 | 0,10 | 0,00 | 0,47 | 0,03 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |
| | ST 10^5 | 1,76 | 0,15 | 0,40 | 0,00 | 0,58 | 0,02 | 0,18 | 0,02 | 0,10 | 0,00 | 0,46 | 0,04 | 0,00 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 |
| 9 | Control | 2,50 | 0,00 | 0,90 | 0,00 | 1,40 | 0,00 | 0,30 | 0,00 | 0,20 | 0,00 | 0,60 | 0,00 | 0,05 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 |
| | ST 10^5 | 2,50 | 0,00 | 0,90 | 0,00 | 1,40 | 0,00 | 0,30 | 0,00 | 0,20 | 0,00 | 0,60 | 0,00 | 0,05 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 |
| 10 | Control | 3,45 | 0,17 | 1,22 | 0,08 | 1,93 | 0,04 | 0,92 | 0,07 | 0,20 | 0,00 | 0,78 | 0,02 | 0,10 | 0,00 | 0,47 | 0,03 | 0,09 | 0,01 |
| | ST 10^5 | 3,52 | 0,07 | 1,30 | 0,04 | 1,98 | 0,07 | 0,95 | 0,05 | 0,20 | 0,00 | 0,78 | 0,02 | 0,10 | 0,00 | 0,48 | 0,02 | 0,10 | 0,00 |
| 12 | Control | 4,16 | 0,04 | 1,50 | 0,00 | 2,74 | 0,06 | 1,10 | 0,00 | 0,40 | 0,00 | 0,80 | 0,00 | 0,50 | 0,00 | 0,70 | 0,00 | 0,10 | 0,00 |
| | ST 10^5 | 3,80 | 0,40 | 1,40 | 0,00 | 2,15 | 0,25 | 1,05 | 0,05 | 0,25 | 0,15 | 0,80 | 0,00 | 0,50 | 0,00 | 0,65 | 0,05 | 0,10 | 0,00 |
| 14 | Control | 5,00 | 0,00 | 2,00 | 0,00 | 3,50 | 0,00 | 1,30 | 0,00 | 0,60 | 0,00 | 0,90 | 0,00 | 0,50 | 0,00 | 1,10 | 0,00 | 0,20 | 0,00 |
| | ST 10^5 | 5,16 | 0,14 | 2,16 | 0,14 | 3,50 | 0,00 | 1,34 | 0,02 | 0,60 | 0,00 | 0,90 | 0,00 | 0,50 | 0,00 | 1,06 | 0,04 | 0,20 | 0,00 |
| 16 | Control | 6,00 | 0,00 | 2,30 | 0,00 | 4,50 | 0,00 | 1,70 | 0,00 | 0,70 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,40 | 0,00 | 0,20 | 0,00 |
| | ST 10^5 | 6,00 | 0,00 | 2,30 | 0,00 | 4,50 | 0,00 | 1,70 | 0,00 | 0,70 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,40 | 0,00 | 0,20 | 0,00 |
| 18 | Control | 6,95 | 0,05 | 2,50 | 0,00 | 5,42 | 0,08 | 1,73 | 0,02 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,75 | 0,03 | 0,20 | 0,00 |
| | ST 10^5 | 7,00 | 0,00 | 2,50 | 0,00 | 5,50 | 0,00 | 1,70 | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,80 | 0,00 | 0,20 | 0,00 |
| 20 | Control | 7,67 | 0,11 | 3,00 | 0,00 | 6,17 | 0,11 | 2,00 | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,20 | 0,00 | 0,30 | 0,00 |
| | ST 10^5 | 7,75 | 0,25 | 2,88 | 0,13 | 6,38 | 0,13 | 1,95 | 0,05 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,10 | 0,10 | 0,28 | 0,02 |

***Significant difference found ($p<0,05$). All significant values are displayed in comparison to the mean of the Control group. Testing done with Student T-test.**

**Annex 5 Table 4. Mean values of behavioral, nutrient utilization and growth parameters for the *Salmonella* Typhimurium Low Inoculum Trial.**

| Day | Group | Shell Ratio |  | Albumen Ratio |  | Yolk Ratio |  | Embryo Ratio |  | Heart Rate (bpm) |  | Voluntary Movements (mov/min) |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  |  | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 1 | Control | 0,14 | 0,00 | 0,54 | 0,01 | 0,33 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
|  | ST 10^2 | 0,14 | 0,00 | 0,52 | 0,02 | 0,31 | 0,01 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 3 | Control | 0,13 | 0,00 | 0,55 | 0,01 | 0,30 | 0,00 | 0,00 | 0,00 | 60,00 | 0,00 | 0,00 | 0,00 |
|  | ST 10^2 | 0,13 | 0,00 | 0,53 | 0,01 | 0,32 | 0,01 | 0,00 | 0,00 | 36,00 | 14,70 | 0,00 | 0,00 |
| 5 | Control | 0,15 | 0,00 | 0,30 | 0,06 | 0,49 | 0,07 | 0,00 | 0,00 | 90,00 | 30,00 | 0,00 | 0,00 |
|  | ST 10^2 | 0,16 | 0,01 | 0,25 | 0,03 | 0,51 | 0,01 | 0,00 | 0,00 | 120,00 | 0,00 | 0,00 | 0,00 |
| 7 | Control | 0,15 | 0,02 | 0,17 | 0,00 | 0,48 | 0,01 | 0,01 | 0,00 | 244,00 | 8,83 | 22,00 | 2,00 |
|  | ST 10^2 | 0,15 | 0,01 | 0,20 | 0,00 | 0,67 | 0,10 | 0,01 | 0,00 | 225,00 | 7,00 | 20,00 | 4,00 |
| 9 | Control | 0,15 | 0,00 | 0,19 | 0,02 | 0,41 | 0,02 | 0,03 | 0,00 | 270,00 | 7,51 | 37,33 | 2,67 |
|  | ST 10^2 | 0,14 | 0,01 | 0,17 | 0,01 | 0,38 | 0,04 | 0,03 | 0,00 | 266,33 | 2,53 | 32,00 | 2,07 |
| 10 | Control | 0,12 | 0,00 | 0,18 | 0,01 | 0,36 | 0,01 | 0,04 | 0,00 | 281,60 | 4,13 | 32,00 | 2,53 |
|  | ST 10^2 | 0,13 | 0,01 | 0,18 | 0,01 | 0,40 | 0,02 | 0,05 | 0,01 | 270,00 | 7,40 | 32,00 | 5,66 |
| 12 | Control | 0,15 | 0,00 | 0,17 | 0,02 | 0,36 | 0,03 | 0,08 | 0,01 | 260,67 | 1,86 | 37,33 | 2,67 |
|  | ST 10^2 | 0,14 | 0,01 | 0,15 | 0,01 | 0,30 | 0,01 | 0,08 | 0,01 | 261,50 | 4,35 | 32,00 | 0,00 |
| 14 | Control | 0,13 | 0,01 | 0,22 | 0,08 | 0,32 | 0,05 | 0,17 | 0,00 | 271,75 | 7,33 | 34,00 | 2,00 |
|  | ST 10^2 | 0,15 | 0,00 | 0,22 | 0,07 | 0,43 | 0,01 | 0,17 | 0,04 | 286,50 | 5,50 | 32,00 | 0,00 |
| 16 | Control | 0,16 | 0,00 | 0,10 | 0,04 | 0,24 | 0,03 | 0,31 | 0,03 | 306,75 | 26,79 | 34,00 | 2,00 |
|  | ST 10^2 | 0,17 | 0,01 | 0,07 | 0,02 | 0,21 | 0,02 | 0,36 | 0,02 | 275,60 | 7,22 | 28,80 | 1,96 |
| 18 | Control | 0,15 | 0,02 | 0,00 | 0,00 | 0,34 | 0,01 | 0,43 | 0,02 | 274,80 | 14,63 | 20,80 | 1,96 |
|  | ST 10^2 | 0,14 | 0,00 | 0,00 | 0,00 | 0,34 | 0,01 | 0,46 | 0,01 | 277,75 | 7,16 | 26,00 | 2,00 |
| 20 | Control | 0,13 | 0,00 | 0,00 | 0,00 | 0,13 | 0,06 | 0,71 | 0,02 | 247,33 | 19,22 | 29,33 | 2,67 |
|  | ST 10^2 | - | - | - | - | - | - | - | - | - | - | - | - |

**[*]Significant difference found ($p<0,05$). All significant values are displayed in comparison to the mean of the Control group. Testing done with Student T-test.**

**Annex 5 Table 4. Mean values of behavioral, nutrient utilization and growth parameters for the *S.* Typhimurium Low Inoculum Trial (cont.)**

| Day | Group | Embryo Length (cm) | | Wing Length (cm) | | Leg Length (cm) | | Beak Length (cm) | | Comb Length (cm) | | Eye Diameter (cm) | | Feather Length (cm) | | Toe Length (cm) | | Nail Length (cm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM | Mean | SEM |
| 1 | Control | 0,70 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | ST 10^2 | 0,70 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 3 | Control | 0,65 | 0,05 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | ST 10^2 | 0,66 | 0,02 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 5 | Control | 1,13 | 0,10 | 0,23 | 0,05 | 0,30 | 0,07 | 0,00 | 0,00 | 0,00 | 0,00 | 0,13 | 0,05 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | ST 10^2 | 1,50 | 0,00 | 0,30 | 0,00 | 0,40 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,20 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 7 | Control | 2,18 | 0,05 | 0,50 | 0,00 | 0,60 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| | ST 10^2 | 2,25 | 0,05 | 0,50 | 0,00 | 0,60 | 0,00 | 0,10 | 0,00 | 0,00 | 0,00 | 0,50 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 | 0,00 |
| 9 | Control | 2,70 | 0,17 | 1,00 | 0,10 | 1,30 | 0,10 | 0,20 | 0,00 | 0,10 | 0,00 | 0,70 | 0,06 | 0,00 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 |
| | ST 10^2 | 3,02 | 0,16 | 1,10 | 0,00 | 1,40 | 0,00 | 0,20 | 0,00 | 0,10 | 0,00 | 0,78 | 0,02 | 0,00 | 0,00 | 0,30 | 0,00 | 0,00 | 0,00 |
| 10 | Control | 3,18 | 0,07 | 1,16 | 0,02 | 1,52 | 0,05 | 0,56 | 0,02 | 0,20 | 0,00 | 0,70 | 0,00 | 0,00 | 0,00 | 0,50 | 0,00 | 0,10 | 0,00 |
| | ST 10^2 | 3,30 | 0,00 | 1,20 | 0,00 | 1,60 | 0,00 | 0,60 | 0,00 | 0,20 | 0,00 | 0,70 | 0,00 | 0,00 | 0,00 | 0,50 | 0,00 | 0,10 | 0,00 |
| 12 | Control | 4,37 | 0,19 | 1,37 | 0,13 | 2,33 | 0,17 | 1,00 | 0,00 | 0,37 | 0,03 | 0,77 | 0,03 | 0,00 | 0,00 | 0,70 | 0,00 | 0,10 | 0,00 |
| | ST 10^2 | 4,25 | 0,10 | 1,28 | 0,10 | 2,18 | 0,12 | 1,00 | 0,00 | 0,35 | 0,03 | 0,75 | 0,03 | 0,00 | 0,00 | 0,70 | 0,00 | 0,10 | 0,00 |
| 14 | Control | 5,28 | 0,02 | 1,98 | 0,03 | 3,65 | 0,05 | 1,10 | 0,00 | 0,50 | 0,00 | 0,90 | 0,00 | 1,00 | 0,00 | 1,10 | 0,00 | 0,20 | 0,00 |
| | ST 10^2 | 5,25 | 0,25 | 2,00 | 0,00 | 3,75 | 0,05 | 1,15 | 0,05 | 0,55 | 0,05 | 0,90 | 0,00 | 1,00 | 0,00 | 1,10 | 0,00 | 0,20 | 0,00 |
| 16 | Control | 6,05 | 0,18 | 2,13 | 0,13 | 3,78 | 0,08 | 1,35 | 0,05 | 0,60 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,20 | 0,00 | 0,20 | 0,00 |
| | ST 10^2 | 6,40 | 0,10 | 2,40 | 0,10 | 3,94 | 0,06 | 1,46 | 0,04 | 0,60 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,20 | 0,00 | 0,20 | 0,00 |
| 18 | Control | 6,90 | 0,30 | 2,90 | 0,10 | 5,30 | 0,20 | 1,50 | 0,00 | 0,74 | 0,06 | 0,90 | 0,00 | 2,00 | 0,00 | 1,56 | 0,14 | 0,18 | 0,02 |
| | ST 10^2 | 7,20 | 0,00 | 3,00 | 0,00 | 5,50 | 0,00 | 1,50 | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 1,70 | 0,00 | 0,20 | 0,00 |
| 20 | Control | 8,30 | 0,00 | 3,00 | 0,00 | 6,80 | 0,00 | 2,00 | 0,00 | 0,80 | 0,00 | 0,90 | 0,00 | 2,00 | 0,00 | 2,10 | 0,00 | 0,30 | 0,00 |
| | ST 10^2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

***Significant difference found (p<0,05). All significant values are displayed in comparison to the mean of the Control group. Testing done with Student T-test.**

**Annex 5 Table 5. Mortality and malformation values for *Salmonella* trials.**

| Trial | Group | Mortality (% dead/total fertile eggs) | | | | | | | | | | | | Malformation prevalence |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Day 0 | Day 1 | Day 3 | Day 5 | Day 7 | Day 9 | Day 10 | Day 12 | Day 14 | Day 16 | Day 18 | Day 20 | |
| 1 | Control | 0% | 2% | 8% | 4% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 0% |
| | SE 10^5 | 0% | 42% | 21% | 9% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 0% |
| 2 | Control | 0% | 2% | 11% | 6% | 2% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 2% |
| | SE 10^2 | 0% | 32% | 5% | 10% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 3% |
| 3 | Control | 0% | 2% | 5% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 2% |
| | ST 10^5 | 0% | 23% | 12% | 1% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 4% |
| 4 | Control | 0% | 19% | 0% | 5% | 0% | 0% | 0% | 0% | 0% | 0% | 2% | 0% | 0% |
| | ST 10^2 | 0% | 27% | 11% | 7% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 0% | 0% |

[*] **Significant difference found ($p<0,01$). All significant values are displayed in comparison to the mean of the Control group. Testing done with Chi-square.**

## Annex 6 Incubation protocol provided by the commercial hatchery screened.

| | Incubator Protocol | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Procedure sequence** | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| **Time (hours)** | 5 | 6 | 18 | 36 | 48 | 24 | 48 | 48 | 48 | 48 | 48 | 30 |
| **Temperature (ºC)** | 25 | 38 | 38 | 37.9 | 37.8 | 37.8 | 37.7 | 37.6 | 37.4 | 37.2 | 37.1 | 36.9 |
| **Highest T allowed (ºC)** | 25.5 | 38.3 | 38.3 | 38.2 | 38.1 | 38.1 | 38 | 37.9 | 37.8 | 37.6 | 37.4 | 37.4 |
| **Lowest T allowed (ºC)** | 16 | 24 | 37.5 | 37.5 | 37.4 | 36.8 | 36.7 | 36.6 | 36.4 | 36.2 | 36.1 | 36.1 |
| **Relative Humidity (%)** | 50 | 60 | 60 | 60 | 60 | 65 | 55 | 50 | 35 | 35 | 25 | 20 |
| **Highest RH allowed (%)** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 50 | 50 | 50 |
| **Lowest RH allowed (%)** | 20 | 20 | 20 | 20 | 20 | 20 | 20 | 15 | 15 | 15 | 15 | 15 |
| **$CO_2$ (%)** | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 | 0.3 |
| **Highest $CO_2$ allowed (%)** | 1 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1 | 1 | 0.8 | 0.6 | 0.6 | 0.6 |
| **Lowest $CO_2$ allowed (%)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Turning intervals (min)** | 45 | 45 | 45 | 45 | 45 | 60 | 60 | 60 | 60 | 60 | 60 | 60 |

| | Hatcher Protocol | | | | | | |
|---|---|---|---|---|---|---|---|
| **Time (hours)** | 12 | 12 | 8 | 6 | 6 | 10 | 10 |
| **Temperature (ºC)** | 36.9 | 36.8 | 36.7 | 36.6 | 36.5 | 36.5 | 36,1 |
| **Highest T allowed (ºC)** | 37.7 | 37.7 | 37.7 | 37.6 | 37.6 | 37.5 | 37.5 |
| **Lowest T allowed (ºC)** | 36.1 | 36.1 | 36.1 | 36.1 | 36.1 | 35.6 | 35.6 |
| **Relative Humidity (%)** | 28.5 | 28.5 | 28.5 | 28.5 | 28.5 | 28.5 | 27.9 |
| **Highest RH allowed (%)** | 32.2 | 33.3 | 33.9 | 34 | 34 | 33.9 | 32.5 |
| **Lowest RH allowed (%)** | 25 | 27.5 | 27.5 | 27.5 | 27.5 | 27.5 | 27.5 |
| **$CO_2$ (%)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Highest $CO_2$ allowed (%)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Lowest $CO_2$ allowed (%)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Turning intervals (min)** | 0 | 0 | 0 | 0 | 0 | 0 | 0 |