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Maria Rita de Carvalho Godinho Macedo Pegado

Dissertação
Mestrado em Ecologia Marinha

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Orientador: Doutor Rui Rosa
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Abstract

Sharks occupy high trophic levels in marine habitats and play a key role in the structure, function and health of marine ecosystems. Sharks are also one of the most threatened groups of marine animals worldwide, mostly due to overfishing and habitat degradation or loss. Although sharks have evolved to fill many ecological niches across a wide range of habitats, they have limited capability to rapidly adapt to human-induced changes in their environments. Until now, ocean acidification was not considered as a direct climate-related threat to elasmobranchs.

In the present study we show, for the first time, that a long-term acclimation process of a tropical shark (*Chiloscyllium punctatum*) to the projected scenarios of ocean acidification (ΔpH 0.5) and warming (+4 °C) for 2100, elicited significant deleterious effects on juvenile shark's fitness and survival. During embryogenesis, none of the parameters measured (survival, development time, yolk consumption and specific growth rate) was significantly affected by hypercapnia, with the exception of routine metabolic rates at intermediate and pre-hatching stages. However, temperature warming exposure significantly affected the embryos. The effects caused by environmental conditions, experienced throughout embryogenesis, seem to have been transmitted to the next developmental stages (juvenile sharks). This carry-over effect may play a critical role in the reductions in the fitness of sharks under climate change. Thus, it is critical to directly assess the risk and vulnerability of sharks to ocean acidification and global warming so that managers and policy-makers can proactively target the most endangered shark species.

Resumo

Os tubarões ocupam altos níveis tróficos nos ecossistemas marinhos e têm um papel fundamental na estrutura, função e saúde desses mesmos ecossistemas. São um dos grupos de animais marinhos mais ameaçados a nível mundial, maioritariamente devido ao excesso de pesca e perda ou degradação do seu habitat. Apesar de os tubarões terem evoluído de forma a preencher vários nichos ecológicos numa ampla gama de habitats, têm uma capacidade limitada de se adaptar rapidamente às alterações ambientais que têm vindo a ser introduzidas pelo Homem. Essas alterações, resultantes das actividades humanas, incluem o aumento significativo da concentração de dióxido de carbono presente na atmosfera, o qual tem vindo a aumentar desde a época industrial. Consequentemente, o aumento deste gás provoca alterações químicas nos oceanos, um processo designado de hipercapnia, i.e. elevadas concentrações de dióxido de carbono nos oceanos, causando acidificação e aumento de temperatura dos mesmos. Ainda que, inicialmente, a hipercapnia tenha sido identificada como uma ameaça para corais e organismos calcificadores, foi recentemente reconhecida como prejudicial para os organismos marinhos em geral.

Apesar da relevância dos tubarões como predadores de topo no meio marinho, as informações sobre a sua ecofisiologia relativamente às alterações climáticas é ainda escassa, e até ao momento a acidificação dos oceanos não era considerada uma ameaça directa para os elasmobrânquios. Sabe-se no entanto que, pelo facto de serem animais de evolução lenta e plasticidade fenotípica baixa, os tubarões correm um risco relativamente elevado perante estas alterações.

Neste estudo, e pela primeira vez, mostrou-se que tubarões tropicais (*Chiloscyllium punctatum*) expostos a um processo de aclimatação a longo prazo, relativamente ao cenário previsto para 2100 de acidificação (ΔpH 0.5) e de aumento de temperatura dos oceanos (+4 °C), apresentaram efeitos deletérios significativos na "fitness" e na sobrevivência. Este estudo foi conduzido durante o início da ontogenia destes tubarões pois estados ontogénicos iniciais são previsivelmente os mais vulneráveis a tais mudanças climáticas. Verificou-se que durante a embriogénese nenhum dos parâmetros medidos (sobrevivência, tempo de desenvolvimento, consumo do vitelo e taxa de

crescimento) foi significativamente afectado pelos efeitos da hipercapnia, com excepção do metabolismo basal nos estádios intermédios e finais. No entanto, a exposição a uma temperatura mais elevada afectou significativamente os embriões. Observou-se que os efeitos causados pelas condições ambientais, a que foram sujeitos durante a embriogénese, foram transmitidos ao estádio de desenvolvimento seguinte (tubarões juvenis). Estes efeitos "carry over" não tinham sido descritos para este grupo de animais. Contudo, deviam ser reconhecidos pelo papel importante que podem ter na "fitness" dos tubarões perante as alterações climáticas. Assim, é importante avaliar diretamente o risco e a vulnerabilidade dos tubarões relativamente à acidificação e aumento de temperatura dos oceanos, para que os gestores e políticos possam reconhecer as espécies de tubarões mais ameaçadas de extinção.

Keywords: Climate change, *Chiloscyllium punctatum*, tropical sharks, embryogenesis, metabolism

1. Introduction

1.1. The role of oceans on Earth's climate

Oceans cover about two thirds of the earth's surface and play a vital role, both in the biogeochemical cycles and also by contributing to the planet's biodiversity (The Royal Society, 2005). Interactions between the ocean and the atmosphere regulate atmospheric composition, and therefore the climate. Oceans provide the surface temperature boundary condition for over 70% of the global atmosphere due to exchanges, absorption and emission of important gases (Bigg *et al.*, 2003). In addition, they are large reservoirs of nutrients and gases, such as carbon dioxide (CO_2) (Siegenthaler and Sarmiento, 1993), which is taken up from the atmosphere by the oceans as a result of both high wind speeds and lower CO_2 content in water masses (Sabine *et al.*, 2004).

1.2. Ocean acidification and warming

CO_2 is one of the most important gases that constitute the atmosphere of our planet, affecting both the radiative heat balance of the Earth and the calcium carbonate equilibrium in the oceans (Petit *et al.*, 1999). Over the anthropocene, CO_2 has been emitted into the atmosphere as a result of the burning of fossil fuels and cement production, and the ocean has constituted the only true net sink for this gas over the past 200 years (Sabine *et al.*, 2004). In accordance with Henry's law, when atmospheric CO_2 partial pressures rise, the quantities of CO_2 dissolved in water increase, leading to levels similar to those in air (Pörtner *et al.*, 2004a). Thus, some of the CO_2 released to the atmosphere will eventually be absorbed by the ocean, with potentially adverse consequences for marine biota (Caldeira and Wickett, 2003).

Atmospheric CO_2 concentrations have increased by 40% from pre-industrial levels of 280 ppm to more than 380 ppm today; levels that now far exceed those of the past one million years (Petit *et al.*, 1999; Houghton *et al.*, 2001). Unfortunately, human activity will continue to produce changes in the chemistry of the atmosphere over forthcoming decades and hence in the Earth's climate (Houghton *et al.*, 2001). In fact, it is predicted that continued release of CO_2 into the atmosphere will increase

the average surface temperature by 2-5 °C until 2100 (Houghton *et al.*, 2001; Meehl *et al.*, 2007; Joos and Spahni, 2007).

Chemically, CO_2 reacts with water to produce carbonic acid (H_2CO_3), which then dissociates to form bicarbonate ions (HCO_3^-) and protons (H^+) (Fig. 1). Therefore the amount of CO_2 that dissolves in seawater has a strong influence on the resultant pH of the oceans (The Royal Society, 2005). Nonetheless, it is worth noting that the term "ocean acidification" (Caldeira and Wickett, 2003) wrongly assumes that oceans will become acidic; in reality, they are becoming less basic, whereas pH is not expected to fall below 7.0 (Kleypas *et al.*, 2006).

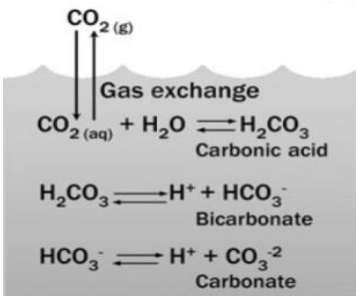

Figure 1. Carbonate system (Fabry *et al.*, 2008).

1.3. Impact of ocean warming on marine biota

Temperature is the most prevalent climate-related influence on biological function and response (Brierley and Kingsford, 2009). Also, higher temperature conditions can cause increased oxygen demands on basal metabolism. This will cause a reduction on aerobic scope and less energy to be devoted to feeding, growth and reproduction (Nilsson *et al.*, 2009).

Increasing temperatures will have specific responses in fish (Baumann *et al.*, 2011), while some species might demonstrate some physiological plasticity in their tolerances to environmental conditions, others are expected to shift their distributions to areas favorable to maintaining physiological optima (Pörtner and Farrell, 2008).

Marine tropical biota will be more sensitive to future warming than the sub-tropical and temperate ones, as they evolved in a relatively thermally stable environment (Tewksbury *et al.*, 2008; Nilsson *et al.*, 2009). Moreover, future ocean warming is expected to negatively impact the performance and survival of several tropical organisms that already live close to their thermal tolerance limits (Hoegh-Gulberg *et al.*, 2007). Yet, developmental and transgenerational plasticity might also occur, which might allow some species to adjust to future ocean conditions (Donelson *et al.*, 2011).

1.4. Impact of ocean acidification on marine biota

1.4.1. Calcification processes

Elevated partial pressure of CO_2 (pCO_2) in seawater, a process called hypercapnia (Fabry *et al.*, 2008), will cause shifts in marine chemistry. More specifically, when CO_2 reacts with seawater, it reduces the availability of carbonate ions (CO_3^{2-}) that are necessary for marine calcifying organisms to produce their calcium carbonate ($CaCO_3$) shells and skeletons (Royal Society, 2005; Kleypas *et al.*, 2006; Fabry *et al.*, 2008). The effect on these marine organisms depends on the $CaCO_3$ saturation state (Ω) which is the product of the concentrations of Ca^{2+} and CO_3^{2-}, divided by the apparent stoichiometric solubility product (K^*_{sp}) for either aragonite or calcite (which are the two types of $CaCO_3$ often secreted by marine organisms): $\Omega = [Ca^{2+}][CO_3^{2-}]/K^*_{sp}$ (Feely *et al.*, 2004). Evidence suggests that $CaCO_3$ saturation state, proportional to Ca^{2+} and CO_3^{2-} concentrations, is the key component of the seawater carbonate system that controls calcification rates (Langdon *et al.*, 2003; Marubini *et al.*, 2003; Leclercq *et al.*, 2002; Ohde and Hossain, 2004; Langdon and Atkinson, 2005; Schneider and Erez, 2006; Silverman *et al.*, 2007). Moreover, the majority of calcifying organisms investigated show a reduction of calcification in response to hypercapnic conditions, such as increases in pCO_2 and decreases in pH, CO_3^{2-} concentration and $CaCO_3$ saturation state (Gattuso *et al.*, 1998; Riebesell *et al.*, 2000; Langdon *et al.*, 2003). Similarly, some species compensate for short- and long-term hypercapnia by the dissolution of its shell (Lindinger *et al.*, 1984; Michaelidis *et al.*, 2005). Even though environmental hypercapnia has been initially identified as a major threat to corals and some calciferous organisms (Orr *et al.*, 2005), it has also been shown to promote detrimental effects on marine organisms in a more generalized way (Rosa and Seibel, 2008; Rosa *et al.*, 2013; Rosa *et al.*, in press). For instance, calcium minerals can be used by marine organisms that possess statoliths, statocysts, gastroliths or statocontia (Lowenstam and Weiner, 1989).

1.4.2. Metabolism and acid-base regulation

Oxygen (O_2) uptake and CO_2 excretion is essential for sustaining an aerobic metabolism, where O_2 is used by an organism for maximal aerobic conversion of food to energy (Carlson *et al.*, 2004).

Marine organisms can be affected by predicted future changes, such as lower pH conditions. Thereby, when these organisms are exposed to hypercapnic conditions they can decrease their oxygen consumption rate (Michaelidis *et al.*, 2005; Nilsson *et al.*, 2009).

Hypercapnia can also impact marine organisms via acid–base disturbances (Fabry *et al.*, 2008; Pörtner *et al.*, 2004a). However, if compensation of acid–base imbalance is not achieved in hypercapnic conditions, some species may show metabolism suppression as an adaptive strategy to survive (Hand, 1991; Pörtner and Reipschläger, 1996; Guppy and Withers, 1999). This mechanism is usually achieved by shutting down expensive processes, such as protein synthesis (Hand, 1991). Despite that, under persistent elevated CO_2 concentrations metabolic suppression is no longer an advantage (Langenbuch and Pörtner, 2004).

Acid–base disturbances, caused by hipercapnia, arise because when CO_2 enters an organism it equilibrates between all body compartments and, as in seawater, it reacts with internal body fluids increasing H^+ and decreasing the pH. This process will cause acidosis, affecting important mechanisms such as osmoregulation and ion regulation (Pörtner *et al.*, 2004a; Fabry *et al.*, 2008). Hence, in order to compensate a lower pH, organisms use acid-base regulation by intracellular accumulation of HCO_3^- and excretion of excess $CaCO_3$ and H^+ ions into the ambient water, which will consequently elevate the pH in the organism (Heisler, 1993; Walsh and Milligan, 1989; Pörtner and Reipschläger, 1996; Ishimatsu *et al.*, 2008; Wilson *et al.*, 2009).

Fish appear most tolerant among marine animals when exposed to high concentrations of CO_2, being able to compensate completely both blood plasma pH and intracellular pH (Michaelidis *et al.*, 2007). Also, tolerant species exhibit greater bicarbonate accumulation and, therefore, compensate more effectively for acidosis (Spicer *et al.*, 2007). However, there may be an apex beyond which acid–base regulation starts to compromise ionic balance (Cameron and Iwama, 1987). For instance, recent studies have shown that some species are unable to compensate acidosis, for they present incomplete pH compensation of coelomic fluid, despite elevated bicarbonate levels (Burnett *et al.*, 2002; Miles *et al.*, 2007).

1.5 Sharks and climate change

Despite the ecological relevance of sharks as top predators, information on physiological impacts of climate change on them is still scarce. Although the metabolism of most sharks is known to be determined by ambient temperatures (Carlson *et al.*, 2004) it is somehow puzzling that, to date, no experiments have been conducted to assess the potential impact that ocean warming and acidification may have on these cartilaginous fish. Sharks are at a relatively high risk to climate change effects due to their slow rates of evolution and low phenotypic plasticity. In other words, their tendency toward late age maturity, long lifespan and low fecundity may impair a prompt adaptive response to rapidly changing environmental conditions, especially in tropical regions (Field *et al.*, 2009; Chin *et al.*, 2010). Nonetheless, it has been argued through "Ecological Risk Assessments" (ERAs) for climate change that ocean acidification will not directly affect sharks; these studies hypothesize that sharks may only be indirectly affected through changes in their habitat, marine community structure or prey availability (Chin *et al.*, 2010).

Under this context, the aim of this study was to investigate, for the first time, the impact of ocean warming and acidification on sharks, namely on the early ontogeny (embryos and recently-hatched juveniles) of the tropical bamboo shark *Chiloscyllium punctatum* (Müller and Henle, 1838). This is a small (up to 1 m), bottom-dwelling and oviparous shark, that inhabits intertidal areas of the Indo-West Pacific, from sandy and rocky substrates to tide pools, sea grass beds and coral reefs (Compagno, 2001; Jagadis and Ignatius, 2003; Harahush *et al.*, 2007). It is worth noting that the Indo-West Pacific region has been warming at a relatively rapid rate compared to several other world regions (Vecchi *et al.*, 2006; Luo *et al.*, 2012). We conducted this study during the early ontogeny of *C. punctatum* because early ontogenetic stages are expected to be the most vulnerable to such climatic shifts (Byrne, 2011). This higher vulnerability may ultimately become a serious bottleneck for species survival.

2. Objectives

The aim of this study was to investigate the impact of future warming (+ 4^0C) and ocean acidification (ΔpH 0.5) on early ontogeny, embryos and recently-hatched juveniles, of bamboo shark (*Chiloscyllium punctatum*). Under this context, we evaluated the effect of both warming and ocean acidification on the: i) survival rates (%), ii) development time (days), iii) yolk consumption ($cm^3\ day^{-1}$), iv) specific growth rates (% day^{-1}), v) metabolic rates of early, intermediate and pre-hatching embryos (μmol O_2 h^{-1} per embryo), vi) Fulton condition of newly-hatched juveniles (K) and vii) ventilation rates (number of breaths min^{-1}).

3. Materials and Methods

3.1. Egg collection and incubation

Forty recently-spawned embryos of bamboo shark (*Chiloscyllium punctatum*) were collected by hand by local fishermen between April and July 2013 in the area of Lungsod Ng Cebu (Philippines; around 10°11′N 123°58′E) and transported by Tropical Marine Centre UK, a marine aquarium wholesaler recognized for its efforts on the sustainable fishing of reef organisms and promotion of animal welfare. At arrival to the aquaculture facilities of Laboratório Marítimo da Guia (LMG – Cascais, Portugal), all embryos were still irregular in shape, colour- and motionless (Harahush *et al.*, 2007). The shark embryos were incubated (suspended 5 cm below the water surface with strings to ensure good aeration) within four 400 L incubation systems (n=10 per system). To understand the development and physiological mechanisms by which shark early ontogenetic stages may (or may not) be able to withstand future ocean changes, the embryos were acclimated at: i) rising pCO_2 (ΔpH 0.5; 0.14% CO_2), and ii) ocean warming expected in 2100 (Meehl *et al.*, 2007), 4 °C above the average ambient temperature (26 °C) of the bamboo shark. More specifically, the embryos were reared at: i) 26 °C pH 8.0 (temperature and pH control), ii) 26 °C pH 7.5 (temperature control and hypercapnic scenario), iii) 30 °C pH 8.0 (warming scenario with pH control), and iv) 30 °C pH 7.5 (warming and hypercapnic scenario).

The life support systems were replenished daily with 100L of new seawater to maintain total alkalinity and dissolved inorganic carbon speciation due to bacterial activity (i.e. nitrifiers, denitrifiers) and acidification of the treatments (2 out of 4). The semi-closed systems were filled with 1-µm filtered, UV-irradiated seawater, with the tanks being illuminated from above with white fluorescent lamps under a photoperiod of 14-h light:10-h dark. Water quality was ensured using wet-dry filters (bioballs), protein skimmers (Schuran, Jülich, Germany) and 30W UV-sterilizers (TMC, Chorleywood, UK). Ammonia and nitrite were monitored regularly and kept below detectable levels. pH was adjusted automatically, via solenoid valves, with the Profilux controlling system (Kaiserslautern, Germany) connected to individual pH probes. pH values were monitored

every 2 seconds and lowered by injection of a certified CO_2 gas mixture (Air Liquid, Portugal) via air stones or upregulated by aerating the tanks with CO_2 filtered (using soda lime, Sigma-Aldrich) air. Seawater carbonate system speciation was calculated weekly from total alkalinity according to Sarazin *et al.* (1999) (spectrophometrically at 595nm) and pH measurements. All seawater physicochemical parameters of the different experimental (temperature and pH) setups are shown in Table 1. pH was quantified via a Metrohm pH meter (826 pH mobile, Metrohm, Filderstadt, Germany) connected to a glass electrode (Schott IoLine, SI analytics, ± 0.001) and calibrated against the seawater buffers TRIS-HCl (TRIS) and 2-aminopyridine-HCl (AMP) (Mare, Liège, Belgium) according to Dickson *et al.* (2007). Measurements were performed under temperature controlled conditions using a water bath (Lauda, Germany, ± 0.1 °C). Bicarbonate and pCO_2 values were calculated using the CO_2SYS software (Lewis and Wallace, 1998), with dissociation constants from Mehrbach *et al.* (1973) as refitted by Dickson and Millero (1987).

Table 1 - Seawater physiochemical parameters in all experimental (temperature and pH) setups. Salinity and temperature were measured daily and averaged per replicate aquarium over the whole experimental period. The combination of total alkalinity (AT) and pHT (pH total scale) was used to calculate carbonate system parameters pCO_2, CT (total carbon) and ΩAr (aragonite). Values are represented as means ± standard deviation.

	26 °C				30 °C			
	8.0		7.5		8.0		7.5	
measured								
Temperature (°C)	25.9	± 0.7	26.2	± 0.5	29.9	± 0.2	30.1	± 0.5
Salinity	35.4	± 0.9	34.9	± 0.8	35.7	± 1.1	35.8	± 0.8
pH_T	8.02	± 0.05	7.52	± 0.04	8.00	± 0.07	7.49	± 0.03
A_T (µmol kg^{-1} SW)	2050.20	± 26.64	2024.43	± 16.22	2046.90	± 27.13	2030.27	± 36.55
calculated								
pCO_2 (ppm)	371.86	± 51.93	1383.81	± 113.55	382.16	± 87.53	1481.10	± 107.72
C_T (µmol kg^{-1} SW)	1772.68	± 24.63	1956.90	± 11.58	1740.78	± 71.98	1952.46	± 11.07
Ω Ar	3.01	± 0.34	1.12	± 0.08	3.35	± 0.43	1.24	± 0.20

3.2. Survival and development time

Upon arrival to our facilities, the age of each shark was estimated according to the embryonic descriptions of Ballard *et al.* (1993) and Harahush *et al.* (2007). The forty encapsulated embryos were then individually tagged in the egg case (with colored zip ties), and the opaque external fibrous layer of the case was scraped off to enable visual and photographic analyses. As a result, the developing embryos could be easily surveyed inside their capsules when placed in front of a light source. This procedure was conducted every day to check for survival throughout embryogenesis.

Figure 2. Bamboo shark embryo in front of a light source.

3.3. Yolk consumption and specific growth rates

Embryos were left untouched for two weeks for acclimation. After that, photographs were taken on a weekly basis throughout the embryonic development. Each embryo was analyzed through the use of the image processing software, Image J, to determine the yolk-sac length and width, and embryo's total length (TL). The yolk measurements were then used to calculate the yolk volume using the formulae: $V = 1/6 (\pi w^2 L)$ (V = volume, w = width, L = length) (Rosa *et al.*, 2007).

The embryo specific growth rate was also determined, by using the formulae:
$[(\ln \text{embryo TL } (T_2) - \ln \text{embryo TL } (T_1)) / \text{number of days elapsed between } T_1 \text{ and } T_2] \times 100$ (with T_1 being the previous (week) measuring date and T_2 being the latter measuring date).

3.4. Fulton condition after hatching

Immediately after hatching, each juvenile bamboo shark was collected from the respective incubation system, weighed, measured (TL) and tagged beneath the dorsal fin (Floy Tag & Mfg, Inc.). Fulton condition was then calculated using the following formulae: $K = (\text{weight}/TL^3) \times 100$. Afterwards, the juvenile sharks were placed back in the same incubation systems and fed with mysid shrimps *ad libitum* on a daily basis.

3.5. Routine metabolic rates, thermal sensitivity and ventilation rates

Oxygen consumption rates, here used as a proxy of routine metabolic rates (RMR) were determined according to Rosa and Seibel (Rosa and Seibel, 2008; Rosa and Seibel, 2010). RMR of the embryos was analyzed at three different stages of development: i) early – body still somewhat transparent and presence of external filamentous gills; equivalent to stages 25-29 in Ballard *et al.* (1993); ii) intermediate – with more or less half the initial yolk volume already consumed and dark brown brands present over a light pink body; equivalent to stages 30-32 in Ballard *et al.* (1993); and iii) late/pre-hatching – embryo occupying the entire area of the circular pouch, with no visible yolk; equivalent to stages 33-34 in Ballard *et al.* (1993). Embryonic and juvenile (30 days post-hatching) stages (n=5 per stage) were placed within an intermittent and a full flow-through respirometry set-up (Loligo Systems, Denmark), respectively, connected to the different incubation systems (i.e. sharing the same seawater carbonate characteristics of the respective thermal and CO_2 conditions). To avoid bacterial contaminations, the seawater from the incubation systems was filtered (0.2 μm) and UV-irradiated before reaching the respirometers. The water was pumped at a constant flow rate (between 5 and 50 mL min^{-1}, for early embryos and juveniles, respectively) through the respirometers with the use of peristaltic pumps (Cole-Parmer, USA).

Oxygen concentrations were recorded at the entrance and exit of each respirometer chamber with a Clarke-type O_2 electrode connected to a Strathkelvin Instruments 929 Oxygen Interface. Before and after each run, the experimental setup was calibrated using air and nitrogen-saturated seawater and checked for electrode drift and microbial oxygen consumption. Each experiment was 6 h long: 2 h for acclimation and the following 4 h for oxygen measurement. All runs were conducted in dark conditions because this species seems to exhibit daily changes in activity, with higher activity levels during night-time (Chapman *et al.*, 2011).

Thermal sensitivity (Q_{10}) was determined using the standard equation:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)}$$

where R_2 and R_1 represent the oxygen consumption rates at temperatures T_2 and T_1, respectively.

After 30 days post-hatching, each juvenile shark was observed within the incubation systems in order to register the ventilation rates (number of breaths per minute). This procedure was repeated 3 times per individual (each with 5 min duration). Bamboo sharks were observed in the afternoon before they were fed (to exclude any potential bias caused by feeding on the respiration).

3.6 Statistical analyses

Two-way ANOVAs (using pH and temperatures as variables) were conducted to detect significant differences in survival, development time, yolk consumption, specific growth rates (SGR), Fulton condition, juvenile RMR and ventilation rates. The differences in embryos' RMR were investigated with three-way ANOVAs (i.e. with pH, temperature and life stages as variables). Normality and homogeneity of variances were verified by Kolmogorov – Smirnov and Bartlett tests, respectively. Subsequently, post-hoc tests (Tukey HSD and unequal N HSD) were performed. All statistical analyses were performed for a significance level of 0.05, using Statistica 10.0 software (StatSoft Inc., Tulsa, USA).

4. Results

4.1. Embryonic survival

Survival of bamboo shark embryos at the present-day thermal scenario (26 °C) was 100% both at normocapnic and hypercapnic conditions (Fig. 3A, 3B). However, under future ocean warming (+4 °C), survival significantly decreased (two-way ANOVA, $p<0.05$), ranging between 80% and 89% at normocapnic and hypercapnic conditions, respectively. No significant effect of pH on embryonic survival was observed ($p>0.05$; Table 2).

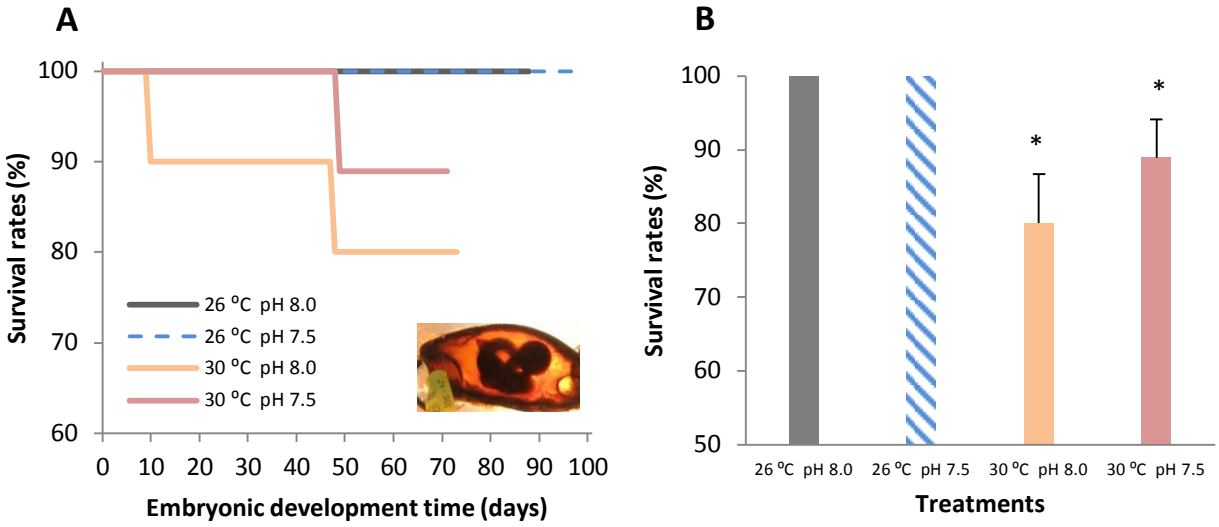

Figure 3. Impact of ocean acidification (ΔpH 0.5) and warming (+ 4 °C) on: A) survival rates during embryonic development time (%) and B) embryos' survival rates (%) of developing bamboo shark embryos (*Chiloscyllium punctatum*). Asterisks represent significant differences between temperature treatments at the same pH condition. For more statistical details also see Table 2.

4.2. Development time

The duration of the embryonic period was shortened with increasing temperature in both pH treatments (Fig. 4, two-way ANOVA, $p<0.05$; Table 2). Under normocapnia, at present-day temperature, embryogenesis lasted 98 ± 9 days, while warming up to 30 °C caused embryos to hatch after 79 ± 11 days. Under hypercapnia, embryogenesis at 26 °C lasted 102 ± 12 days, while under the future warming it was completed in 81 ± 6 days. The increase in development time at lower pH (7.5) under both thermal treatments was not significant ($p>0.05$; Table 2).

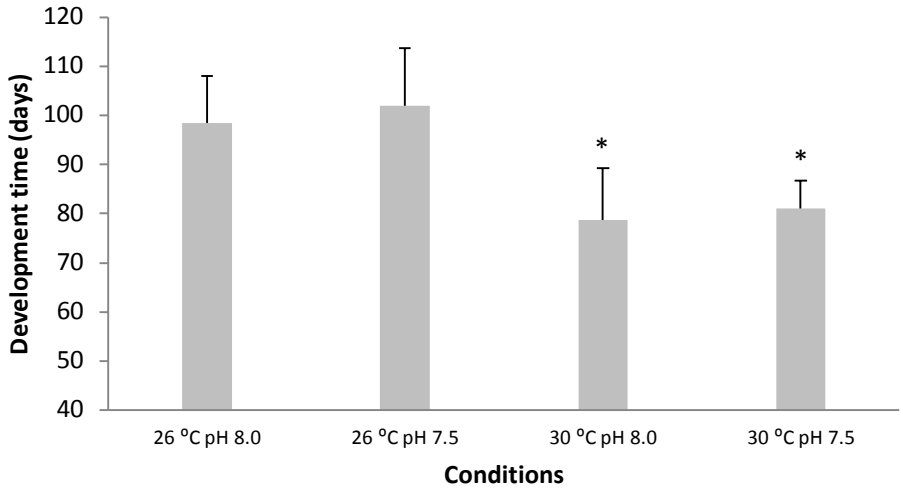

Figure 4. Impact of ocean acidification (ΔpH 0.5) and warming (+ 4 °C) on development time of embryo bamboo sharks (*Chiloscyllium punctatum*). Values represent mean ± SD. Asterisks represent significant differences between temperature treatments at the same pH condition. For more statistical details also see Table 2.

4.3. Yolk consumption

Yolk consumption rates were similar between normocapnia and hypercapnia at both thermal scenarios (Fig. 5, two-way ANOVA, $p>0.05$). Yet, yolk was consumed at significantly higher rate at 30 °C (about 0.16 cm^3 day^{-1}) than at 26 °C (around 0.12 cm^3 day^{-1}) ($p<0.05$; Table 2).

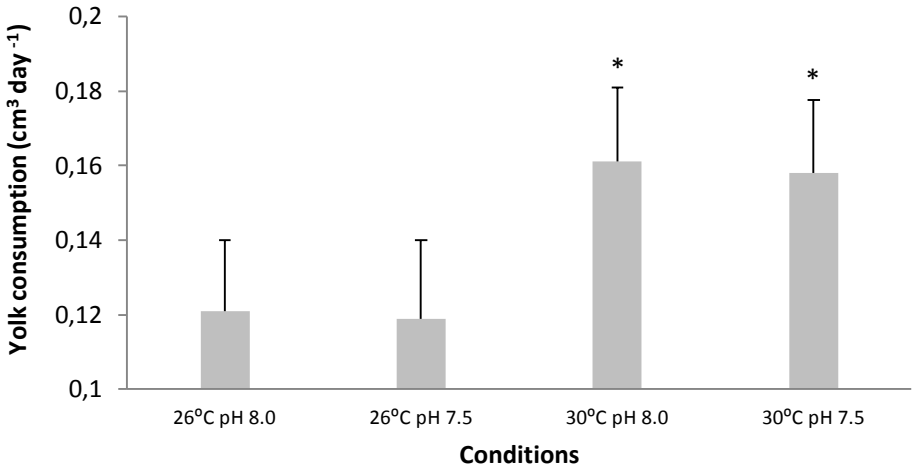

Figure 5. Impact of ocean acidification (ΔpH 0.5) and warming (+ 4 °C) on yolk consumption (cm^3 day^{-1}) of developing bamboo shark (*Chiloscyllium punctatum*) embryos. Values represent mean ± SD. Asterisks represent significant differences between temperature treatments at the same pH condition. For more statistical details also see Table 2.

4. 4. Specific growth rates

Specific growth rates (SGR) showed similar trends in treatment-specific differences, i.e. while pH did not elicit any significant change (Fig. 6, two-way ANOVA, $p>0.05$), the future warming scenario significantly increased embryonic growth (about 1.8 % day^{-1}; $p<0.05$; Table 2) as compared to the present-day thermal scenario (around 1.3 % day^{-1}).

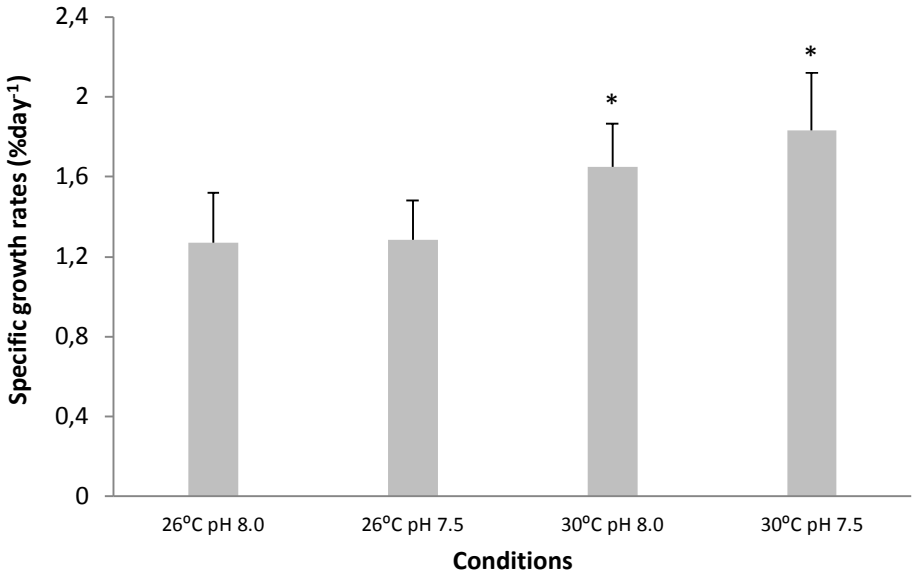

Figure 6. Impact of ocean acidification (ΔpH 0.5) and warming (+ 4 °C) on specific growth rate (% day^{-1}) of developing bamboo shark (*Chiloscyllium punctatum*) embryos. Values represent mean ± SD. Asterisks represent significant differences between temperature treatments at the same pH condition. For more statistical details also see Table 2.

4.5. Embryos' metabolic rates

Routine metabolic rates (RMR) were significantly affected by temperature, pH and embryonic stage (early, intermediate and late/pre-hatching embryos) (Fig. 7, three-way ANOVA, $p<0.05$; Table 3). At all three embryonic stages, hypercapnia led to a significant decrease in RMR under the warming scenario ($p<0.05$). RMR values ranged between 6.33 ± 3.53 (in early stages under 26 °C and pH 7.5) and 127.01 ± 11.31 μmol O_2 h^{-1} per embryo (pre-hatching stage under 30 °C and pH 8.0).

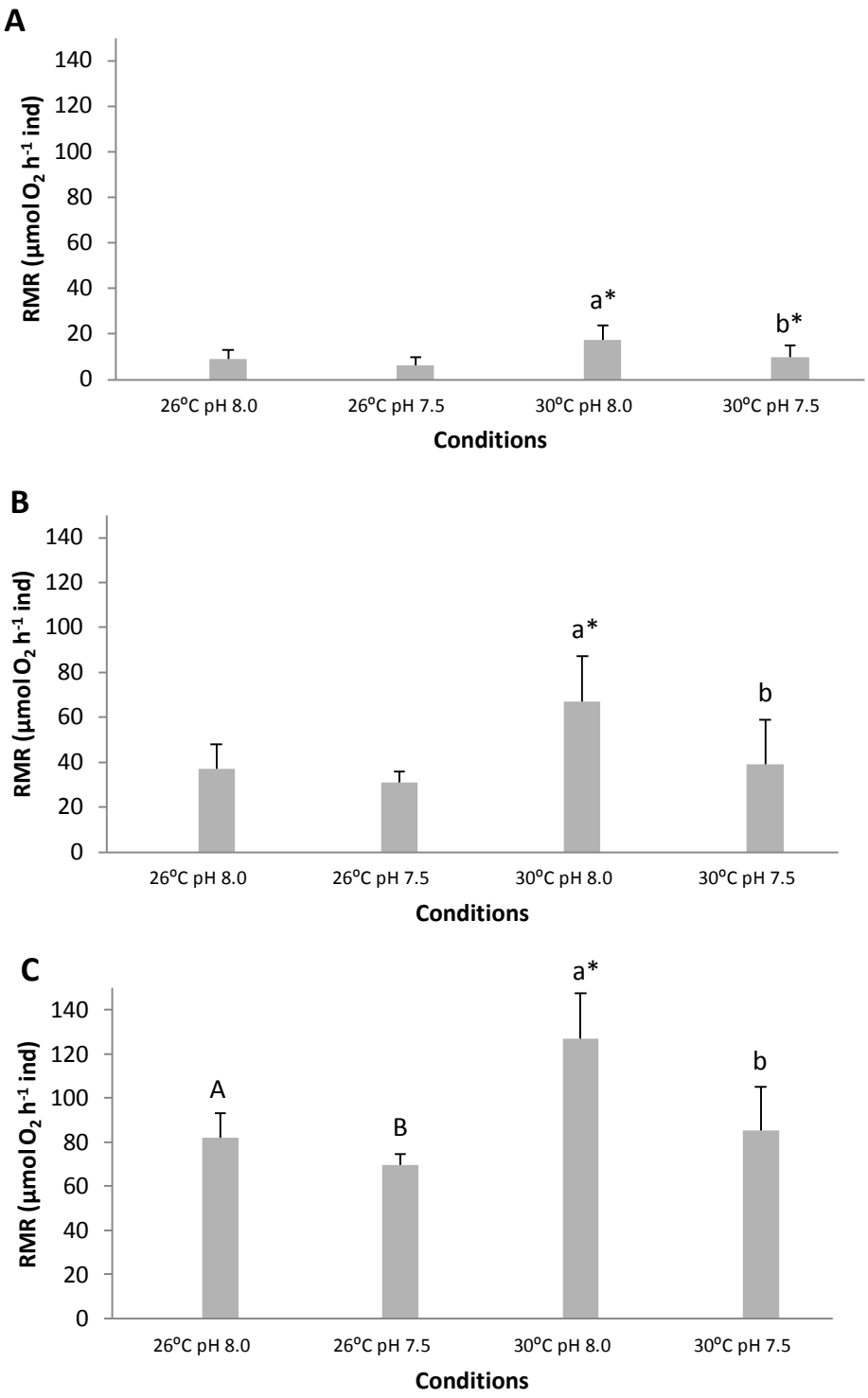

Figure 7. Impact of ocean acidification (ΔpH 0.5) and warming (+ 4 °C) on routine metabolic rate ($\mu mol^{-1}\ h^{-1}$ embryo) of A) early stage embryos; B) intermediate stage embryos; C) late/pre-hatching embryos of bamboo shark (*Chiloscyllium punctatum*). Values represent mean ± SD (n=5 per stage). Different upper- and lower-case letters represent statistical differences ($p<0.05$) between pH treatments at 26 °C and 30 °C, respectively ($p<0.05$). Asterisks represent significant differences between temperature treatments at the same pH condition. For more statistical details also see Table 3.

4. 6. Thermal sensitivity (Q_{10})

Temperature elicited a significant increase in RMR both at normocapnic (Q_{10} value of 5.37) and hypercapnic (Q_{10} value of 2.98) conditions ($p<0.05$). Embryonic Q_{10} values (i.e. thermal sensitivity of metabolism between 26 °C and 30 °C) ranged above 3 under normocapnia across all embryonic stages, and close to 1.5 in intermediate and late stages acclimated to low pH (grey line in Fig. 8).

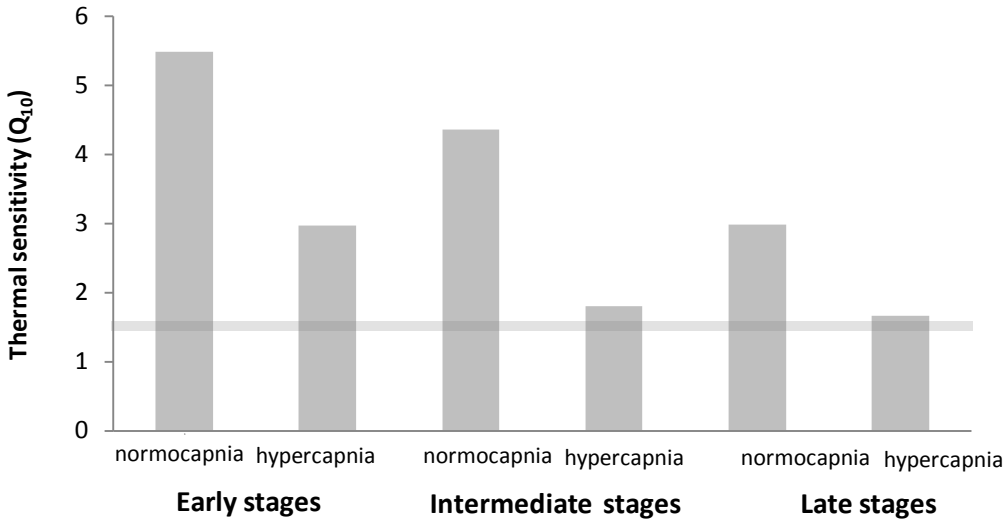

Figure 8. Impact of ocean acidification (ΔpH 0.5) and warming (+ 4 °C) on the thermal sensitivity (Q_{10}; between 26 °C and 30 °C) of early embryonic stage (left-hand side panels), intermediate embryonic stages (center panels) and late stage (right-hand side panels) of common bamboo shark (*Chiloscyllium punctatum*). Grey line represents a threshold below which the levels are indicative of active metabolic depression.

4.7. Juveniles' survival rates

Thirty days after hatching, survival rapidly declined in individuals experiencing both ocean warming and acidification (Fig. 9A, 9B, two-way ANOVA, $p<0.05$; Table 4). At present-day temperature, survival decreased significantly from 100% under normocapnia to 61% under hypercapnia. At future warming conditions, survival decreased from 71% under normocapnia to 44% under hypercapnia.

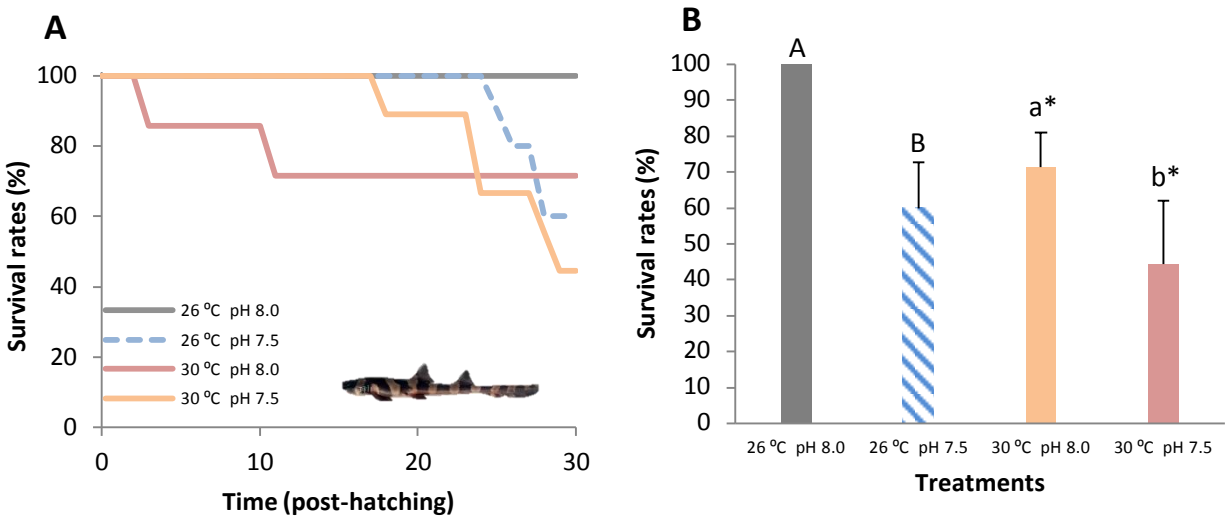

Figure 9. Impact of ocean acidification (ΔpH 0.5) and warming (+ 4 °C) on: A) survival rates during 30 days post-hatching (%) and B) juveniles' survival rates (%) of bamboo shark (*Chiloscyllium punctatum*). Asterisks represent significant differences between temperature treatments at the same pH condition. For more statistical details also see Table 4.

4.8. Fulton condition

Immediately after hatching (day 0), the Fulton condition (K) of juvenile bamboo sharks was significantly different in individuals which experienced future warming and hypercapnia (Fig. 10, two-way ANOVA, $p<0.05$; Table 4). However, while pH did not affect K under present-day thermal conditions (K between 0.37-0.39), K changed under the future warming condition (K = 0.34).

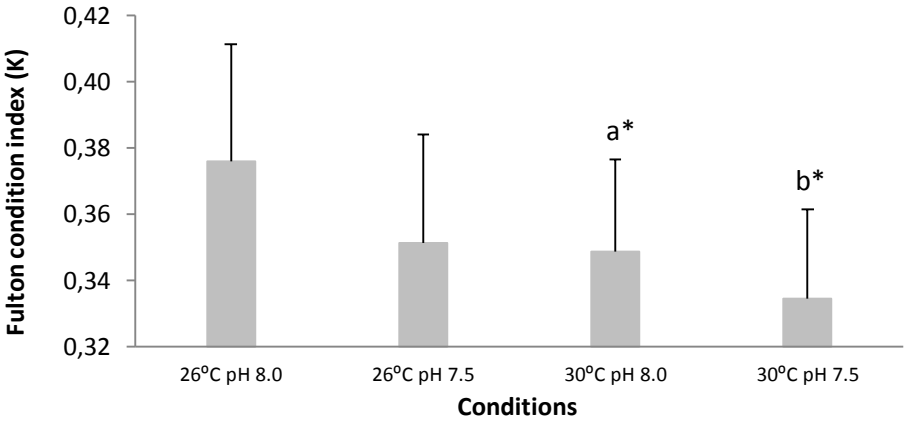

Figure 10. Impact of ocean acidification (ΔpH 0.5) and warming (+ 4°C) on Fulton condition on nearly-hatched juveniles (day 0) of bamboo sharks (*Chiloscyllium punctatum*). Different upper- and lower-case letters represent statistical differences between pH treatments at 26 °C and 30 °C, respectively ($p<0.05$). Asterisks represent significant differences between temperature treatments at the same pH condition. For more statistical details also see Table 4.

4.9. Juveniles' metabolic rates

The RMR of juvenile sharks (30 days after hatching) was significantly affected by temperature and pH (Fig. 11, two-way ANOVA, $p<0.05$). Moreover, there was a significant interaction between these two environmental variables ($p<0.05$; Table 4).

Although pH did not cause any relevant change at 26 °C (normocapnia: 82.6 µmol ± 12.3 O_2 h^{-1} ind^{-1}; hypercapnia: 74.6 µmol ± 3.9 O_2 h^{-1} ind^{-1}), it significantly decreased RMR under the future warming scenario (from 161 ± 2.6 O_2 h^{-1} ind^{-1} to 115 ± 17.2 O_2 h^{-1} ind^{-1}; $p<0.05$).

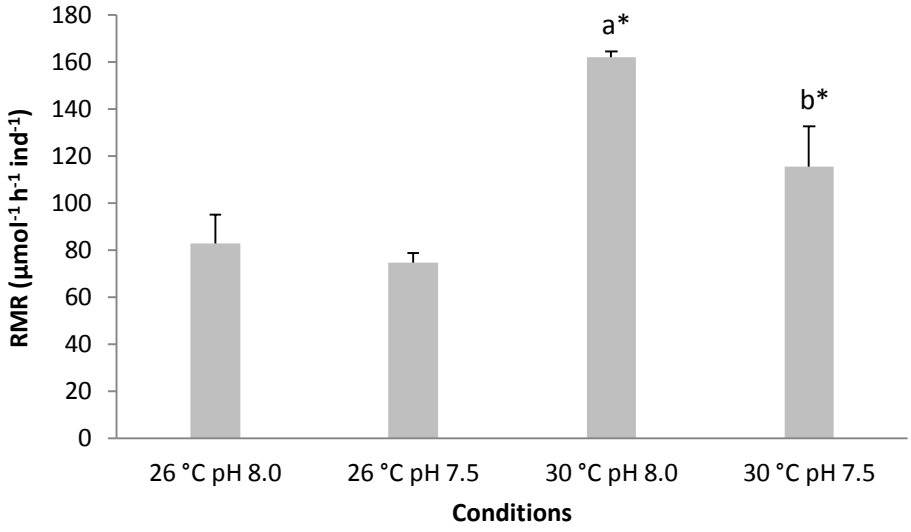

Figure 11. Impact of ocean acidification (ΔpH 0.5) and warming (+ 4 °C) on metabolic rates (µmolO_2 h^{-1} ind^{-1}) of 30 days post-hatching juveniles of bamboo shark (*Chiloscyllium punctatum*). Values represent mean ± SD. Different lower-case letters represent statistical differences between pH treatments at 30 °C ($p<0.05$). Asterisks represent significant differences between temperature treatments at the same pH condition. For more statistical details also see Table 4.

4.10. Ventilation rates

Ventilation rates of juvenile sharks showed similar trends, with the number of breaths per min increasing significantly with warming under normocapnia (Fig. 12, two-way ANOVA, $p<0.05$; Table 4). However, such increasing pattern with warming did not happen under hypercapnia. Additionally, the impact of ocean acidification on ventilation rates was only significant under the higher thermal scenario ($p<0.05$).

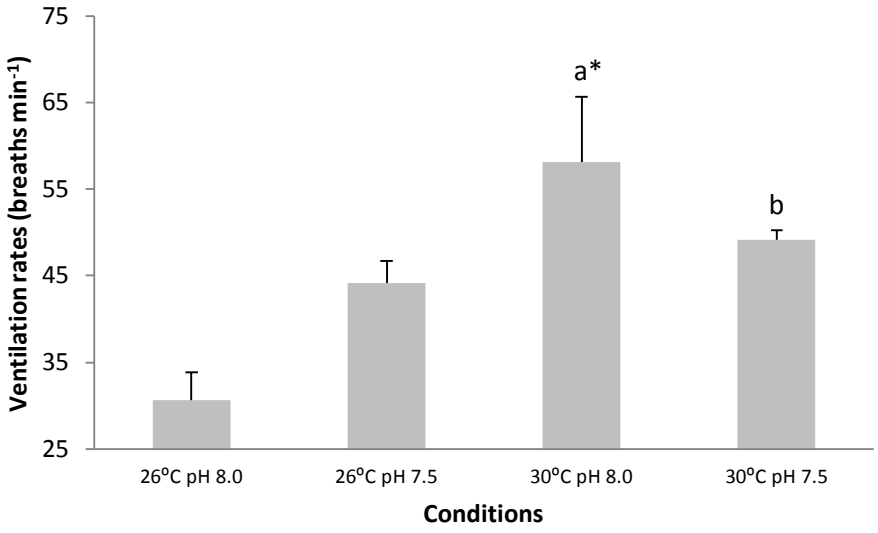

Figure 12. Impact of ocean acidification (ΔpH 0.5) and warming (+ 4^0C) on ventilation rates (number of breaths min^{-1}) of juveniles of bamboo shark (*Chiloscyllium punctatum*). Values represent mean ± SD. Different lower-case letters represent statistical differences between pH treatments at 30 °C ($p<0.05$). Asterisks represent significant differences between temperature treatments at the same pH condition. For more statistical details also see Table 4.

Table 2 - Results of two-way ANOVA evaluating the effects of temperature and pH on survival, development time, yolk consumption and specific growth rates (SGR) of bamboo shark embryos (* - p<0.05).

Embryos	df	MS	F	p
Survival				
Temperature (T)	1	675.0	1.6	0.001*
pH	1	75.0	2.4	0.159
T x pH	1	75.0	2.4	0.159
Error	8	31.2		
Development time				
Temperature (T)	1	3781.2	39.2	0.000*
pH	1	84.2	0.8	0.357
T x pH	1	3.0	0.0	0.861
Error	51	96.6		
Yolk consumption				
Temperature (T)	1	0.0	36.7	0.000*
pH	1	0.0	0.2	0.687
T x pH	1	0.0	0.0	0.928
Error	51	0.0		
SGR				
Temperature (T)	1	0.0	32.3	0.000*
pH	1	0.0	1.1	0.292
T x pH	1	0.0	0.7	0.394
Error	51	0.0		

Table 3 - Results of three-way ANOVA evaluating the effects of temperature, pH and stage of development on routine metabolic rates (RMR) of bamboo shark embryos (* - p<0.05).

	df	MS	F	p
RMR				
Temperature (T)	1	4937.1	28.7	0.000*
pH	1	1443.0	8.3	0.005*
Stage (S)	2	27998.6	162.7	0.000*
T x pH	1	145.1	0.8	0.363
T x S	2	750.7	4.3	0.018*
pH x S	2	171.1	0.9	0.378
T x pH x S	2	233.4	1.3	0.268
Error	43	172.1		

Table 4 - Results of two-way ANOVA evaluating the effects of temperature and pH on Fulton condition, survival, routine metabolic rates (RMR) and ventilation rates of bamboo shark juveniles. (* - $p<0.05$)

Juveniles	df	MS	F	p
Fulton condition				
Temperature (T)	1	0.0	5.7	0.048*
pH	1	0.0	3.9	0.026*
T x pH	1	0.0	0.0	0.886
Error	51	0.0		
Survival				
Temperature (T)	1	1564.1	33.7	0.000*
pH	1	3434.1	74.1	0.000*
T x pH	1	114.2	2.5	0.155
Error	8	46.3		
RMR				
Temperature (T)	1	10815.5	92.1	0.000*
pH	1	222.1	18.9	0.002*
T x pH	1	1106.6	9.4	0.015*
Error	16	117.5		
Ventilation rates				
Temperature (T)	1	594.3	21.8	0.000*
pH	1	324.4	8.3	0.003*
T x pH	1	211.3	7.8	0.015*
Error	32	27.2		

5. Discussion

One of the most important mechanisms that will allow marine organisms to cope with future environmental changes is acclimation (Donelson and Munday, 2012). Here we showed that, after a long-term acclimation process, which comprises an extensive embryonic period and 30 days post-hatching, the projected conditions of ocean acidification and warming are already outside the range of tolerance of this tropical shark. This finding was clearly supported by the high mortality (>50%; Fig. 3) and decreased survival in the juvenile phase (Fig. 9).

Early life stages are known to display narrow environmental tolerance windows due to developmental constraints and insufficient capacity of central organs (Pörtner and Farrell, 2008; Pörtner *et al.*, 2004b). Consequently, at high temperature borders, oxygen supply in shark embryos may have become limited via ventilatory and circulatory constraints and its combined effect with CO_2 should have decreased their thermal tolerance (Pörtner and Farrell, 2008). Based on this concept, the scope for aerobic performance in the bamboo shark should decline above the optimal temperature range, with consequences for locomotion, growth and reproduction.

In this study, ocean warming had more pronounced impacts on shark's early ontogeny than acidification. During embryogenesis, none of the measured parameters (survival, development time, yolk consumption and SGR) was significantly affected by hypercapnia (Table 2), with the exception of RMR at intermediate and pre-hatching stages (Fig. 7C). However, RMR under the warming scenario was significantly affected by pH (Fig. 7; Table 3). Energy expenditure rates were highly temperature-dependent under normocapnia, with Q_{10} values ranging between 3 and 6. However, under high CO_2 conditions, such rates became less temperature-dependent (Q_{10} values around 1.5), showing that hypercapnia was a crucial factor on embryo's RMR.

Reduced pH caused the embryos to become more hypometabolic (Fig. 8), which is expected to be accompanied by a reduction in protein synthesis and, consequently, decreased SGR (Fig. 6) (Hochachka and Somero, 2002; Storey and Storey, 2004).

Our findings corroborate the idea that subtle events in a species' early life stages can have "carry-over effects" to later periods of its life cycle. In other words, the variation in development rates (Fig. 4) and growth (Fig. 6) among embryos, due to initial differences in parental input (Burt *et al.*, 2011) and environmental conditions experienced throughout embryogenesis, seem to have been transmitted to the next developmental stages (juvenile sharks).

Summing up, although the vulnerability of tropical sharks to climate change is species-specific, factors such as temperature, sea level rise and freshwater input are assumed to elicit the greatest effects upon the estuarine, coastal inshore and reef species (Chin *et al.*, 2010). Such effects are likely to be expressed through changes in species abundance and distribution (e.g. poleward movements and migrations to deeper waters) (Perry *et al.*, 2005).

After hatching, under future ocean conditions, this relatively inactive bottom-dwelling shark displayed a more lethargic behavior (data not shown) and decreased metabolic and ventilatory capabilities, which might have cascading effects on its growth and subsequent reproduction. These effects may be even stronger in more active shark species displaying higher metabolic costs associated to energetically demanding life strategies (e.g. pelagic species with ram ventilation). Temperature-induced respiratory acidosis and hypercapnia cause short-term acid-base imbalance, but like in most fish, pH compensation in sharks is done rapidly and efficiently across the gills, through the direct transfer of acid–base equivalents between the animal and the external environment (Heisler *et al.*, 1988; Perry and Gilmour, 2006). As shark ventilation may be influenced by acid-base status (Heisler, 1988; Heisler *et al.*, 1988), the increased ventilation observed under the warming and acidification scenarios (Fig. 12) likely indicates acid-base imbalance (metabolic and respiratory acidosis). If compensation of acid–base imbalance was not achieved, the long-term exposure to reduced pH (and elevated pCO_2) would explain the reduction of shark metabolism (Fig. 11) (Guppy and Withers, 1999; Pörtner *et al.*, 2004a; Pörtner *et al.*, 1998). Greater degree of physiological impairment in shark embryos under hypercapnia had deleterious repercussions on juvenile fitness (Fig. 10) and survival (Fig. 9). One should keep in mind that metabolic depression,

or even suppression, is considered an adaptive strategy to cope with short-term hypercapnia (and hypoxia) but it is not advantageous under the chronically high CO_2 conditions predicted for the ocean of tomorrow (Rosa and Seibel, 2008; Rosa *et al.*, 2013).

Until now, ocean acidification was not considered as a primary climate-related threat to elasmobranchs; only indirectly through habitat and community changes (e.g. reef sharks) (Chin *et al.*, 2010; Field *et al.*, 2009). Here we showed that the physiological experiences and resulting phenotypes of developing shark embryos (under hypercapnia and warming) can be carried-over to later shark life stages with negative consequences (reduced survival). This phenomenon is, to our knowledge, described for the first time in sharks and should be kept in mind as a significant source of variation in fitness of these organisms. Experimental approaches are critical to directly assess risk and vulnerability of sharks to ocean acidification and warming, and can help managers and policy-makers to take proactive measures targeting most endangered species.

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