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Ciências
ULisboa

**Integrative taxonomy of arthropods as potential vectors of Viral
Haemorrhagic Rabbit Disease - genotype 2 (RHDV2) and as
potential new vectors of Myxomatosis**

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ACKNOWLEDGEMENTS

To both of my supervisors: Thank you! Thank you for this amazing opportunity of working under your supervision. To Prof. Maria Teresa Rebelo I want to thank for the amazing supervision, for being a great and dedicated professor, for helping me whenever I needed and for answering very quickly to all my requests and questions. To Prof. David Ramilo I want to thank for being a great supervisor, for giving advice, wisdom, and encouragement for this work and for life. I truly have a good friend in this man, such friendship, kindness, guidance and patience that I value. Thank you both for providing me with everything that I needed for this work.

I would like to offer my gratitude to the colleagues from Faculty of Veterinary Medicine: Prof. Isabel Pereira da Fonseca and Dr. Lidia Gomes. Their kindness and concern about my work, safety and happiness in the laboratory gave me the tools to be accepted and welcome in the workplace.

I would like to extend my gratitude to the colleague Msc Fábio Abade dos Santos, which gave me all the samples needed to this work and carried out the virological analyses, even as part of his PhD; to INIAV - Instituto Nacional de Investigação Agrária e Veterinária, I.P. and to the +Coelho 2 project which financed all the molecular biology reagents; and finally to the Research and Development (R&D) Center for Interdisciplinary Research in Animal Health (CIISA) which finances all Faculty of Veterinary Medicine' labs.

To my colleagues from the Laboratory of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine: You are the best! Ana Maria Filipe, Sara Rocha, Pedro Costa, Joana Sequeira, André Gomes and Claudia Lobo, thank you for all the support, funny times and help given during my stay there and for the opportunity to work with such brilliant minds. To Rita Diogo, I want to express all the gratitude for being my friend, for being there every time I needed and for answering very quickly to my messages whenever I needed. I really appreciate!

I wish to express my appreciation to all my colleagues and friends from Faculty of Sciences of the University of Lisbon; specially to Matthew Janeiro, Bárbara Monteiro, Camila Henriques and Luís Coelho for being great friends, for all the laughs together and the brilliant minds they have; and to Stephanie Andaluz, the amazing friend in which every day, every time, anywhere, the support that I needed arrived shortly! Thank you all for being by my side, I cannot be more grateful.

A special appreciation to Public Health Department of Cascais and all my co-workers for being amazing colleagues, for giving me all the necessary tools for my success as a professional and for accepting me as I am in the workplace. I am learning every day with you.

To my friends of all time, Teresa Carvalho, Paula Nunes and Sérgio Martins, thank you for all the time together, for being one call away and for being the support that I needed in my most difficult times. We know what we have been through and one "Thank you" is not enough. You all know what you mean to me.

I would also like to show my appreciation to my family, that without their unconditional support and love through all my life, I would not be in this position. Thank you for always giving me everything that I need in life. My Mother, Maria de Fátima, my father Manuel, my grandmother Josefa, my brother Ricardo, my sister-in-law Melissa, and my dog Ruben Octavio are truly the greatest family that anyone could ask for.

Finally, Agostinho da Silva, Amália Chapelas and José Biscaia Duarte, you are my shining stars that light my path every day.

Thank you all for showing me that everything is possible, I am sure none of this would be possible without you. I am eternally grateful to you!

Integrative taxonomy of arthropods as potential vectors of Viral Haemorrhagic Rabbit Disease - genotype 2 (RHDV2) and as potential new vectors of Myxomatosis

ABSTRACT

Rabbit Haemorrhagic Disease and Myxomatosis are highly infectious viral diseases that rapidly kill populations of European rabbit (*Oryctolagus cuniculus*), and, with some recent findings and studies, it quickly came to our understanding that those viruses have serious implications on the health of the European brown hare (*Lepus europaeus*).

The transmission of these viruses remains uncertain, with only some clearance in case of Myxomatosis, since some studies concluded that any biting or sucking arthropod could serve as a vector. However, viral transmission through mechanical vectors, such as insects, is of great epidemiological importance.

Therefore, the aim of this work is to perform an analysis of the morphological characteristics of several specimens of arthropods caught near a rabbit hutch in Alenquer, Portugal, between December 2018 and December 2019, in order to detect and quantify the viruses (through real-time PCR analysis) in those arthropods. Two more captures were carried out in this location, in the month of February 2020, after an outbreak of the two diseases.

A total of 30,522 specimens were identified, divided by 59 families/genus/species being represented mostly by Diptera (95.37%). The full screened month with most captured specimens in a 15-day sampling was February 2019 (9.36%). The specimen's abundance was greater in Spring than Winter, which was expected, due to higher temperatures.

Specimens infected with both viruses were found. Although, in small numbers they were all collected in Winter: Mycetophilidae (61 specimens) for Rabbit Haemorrhagic Disease Virus – Genotype 2 and Chironomidae (5), Ceratopogonidae (10), Lepidoptera (14), Muscidae (19), Scatopsidae (1), and *Culicoides obsoletus* (1) for Myxoma Virus.

Considering that vector-borne diseases are a major problem nowadays, causing economic losses of thousands of millions of euros on vector control each year to reduce vector-borne pathogens. More studies are important regarding new vectors of vector-borne pathogens with Public and Animal Health importance.

Keywords: *Oryctolagus cuniculus*; *Lepus europaeus*; Vector-born diseases; Portugal

Taxonomia integrativa de artrópodes como potenciais vetores da Doença Viral Hemorrágica do Coelho - genótipo 2 (RHDV2) e como potenciais novos vetores de Mixomatose

RESUMO ALARGADO

As famílias Leporidae e Ochotonidae pertencem à ordem dos mamíferos Lagomorpha, onde se incluem as lebres - do género *Lepus* - e os coelhos do género *Oryctolagus*. Lebres e coelhos podem ser vistos naturalmente em todos os continentes, exceto na Oceania e na Antártica, onde foram introduzidos. Coelhos e lebres, como presas que são, têm um comportamento comum: são crepusculares. O coelho europeu é único devido à sua grande diversidade de habitats: desertos, pântanos, campos, fazendas, bosques e florestas.

Uma das maiores forças por trás do declínio do coelho europeu (*Oryctolagus cuniculus*) e da lebre ibérica (*Lepus europaeus*) é a perda e fragmentação do habitat, muito conseguida através de agricultura intensiva moderna, produção massiva de gado, mudanças climáticas e aquecimento global, produtos químicos agrícolas, caça excessiva e caça furtiva.

Outro fator são duas doenças que surgiram no século XX: a doença hemorrágica do coelho e a mixomatose, tornando-se importantes e relevantes, pois o coelho europeu e a lebre ibérica são nativos desta região, importantes para a economia e o turismo, bem como para o ecossistema, pois constituem uma fonte alimentar fundamental para a Águia Imperial Ibérica e para o Lince Ibérico, que se encontram em vias de extinção. Para além disso, há medidas que terão de ser implementadas e estudos sobre doenças transmitidas por vetores terão de ser realizados, pois o estado de conservação do coelho-europeu está, desde 2012, classificado como “Espécie em perigo”.

A Doença Hemorrágica do Coelho é uma doença viral altamente infecciosa que rapidamente mata populações de coelho europeu e é caracterizada por um conjunto típico de características observadas *post-mortem* no hospedeiro infetado, nomeadamente: renomegalia, esplenomegalia, hepatomegalia, hemorragias no pulmão e no trato respiratório superior e icterícia.

Descrito pela primeira vez na China no início dos anos 80 do século XX, o vírus - *Vírus da doença hemorrágica do coelho*, da família *Caliciviridae* e género *Lagovirus* - rapidamente se espalhou por territórios onde *Oryctolagus cuniculus* estava presente, como a Península Ibérica, Itália, França, Reino Unido, Austrália, entre outros.

Em 2010, um novo surto surgiu em França, com um perfil genético e antigénico diferente, chegando no ano 2011 a Espanha e em 2012 a Portugal, onde dizimou populações de coelhos europeus de norte a sul, incluindo os arquipélagos e ilhas remotas da costa. Este novo vírus, designado por RHDV2, possui características diferentes do anterior, pois pode causar infeção em indivíduos jovens, é capaz de infetar hospedeiros de outras espécies e é bastante resistente no meio ambiente.

Tal como a Doença Hemorrágica do Coelho, a Mixomatose - doença de notificação obrigatória pela Organização Mundial de Saúde Animal - é outra das principais doenças virais do coelho europeu, podendo afetar a sua saúde e o seu bem-estar. Embora raros mundialmente,

alguns casos confirmados de mixomatose foram descritos na lebre ibérica. Normalmente ocorre na forma aguda ou hiperaguda, tem evolução rápida para septicemia, rinite produtiva, dispneia, lesões pulmonares, entre outras, até que ao fim de 10-14 dias ocorre a morte.

A Mixomatose foi reconhecida pela primeira vez no Uruguai no final do século XIX, tornando-se, em 1950, numa “arma biológica” quando uma estirpe da doença foi utilizada como agente biológico de controlo de coelhos na Austrália, chegando à Europa em 1952 pelo mesmo método, pois a redução de indivíduos foi um sucesso (>99%). Após esse sucesso, um declínio na letalidade foi observado como resultado da seleção natural e resistência ao vírus.

Até hoje, a Mixomatose só foi observada em coelhos europeus, mas alguns estudos mostram que ocorreu uma transmissão entre espécies. Em 2018 surgiu o primeiro surto de mixomatose em lebres na Península Ibérica, primeiro em Espanha e depois em Portugal.

Ao longo dos anos, algumas vacinas eficazes foram produzidas e estão disponíveis, embora a doença persista e possa coinfectar um indivíduo infetado com outros vírus.

Inicialmente pensava-se que o vírus da mixomatose se espalhava pelo contato direto de coelho para coelho; no entanto, alguns estudos comprovam que *Culex annulirostris* é um vetor mecânico deste vírus. Posteriormente, outros estudos concluíram que quase qualquer artrópode mastigador ou sugador poderia servir como vetor para mixomatose. Alguns exemplos disso são *Spilopsyllus cuniculi*, *Anopheles atroparvus*, *Aedes caspius*, *Aedes detritus*, entre outros.

Em Portugal, os mosquitos foram estudados pela primeira vez por Sarmiento & França em 1901, trabalho esse continuado por Cambournac (1938 e 1943), entre outros. Em 1999 é publicada pela primeira vez em Portugal uma chave de identificação para culicídeos de Portugal Continental, Açores e Madeira por Ribeiro e colaboradores.

Assim, o objetivo deste trabalho foi analisar das características morfológicas de vários artrópodes capturados numa quinta de produção cinegética de coelho bravo em Alenquer, Portugal, entre dezembro de 2018 e dezembro de 2019, com a finalidade de verificar a presença e a quantidade viral do genótipo 2 da Doença Hemorrágica do Coelho e/ou vírus da Mixomatose, por PCR em tempo real. Mais duas capturas foram realizadas neste local, no mês de fevereiro de 2020, após um surto das duas doenças.

As colheitas foram realizadas por um colaborador, no final dos primeiros 15 dias de cada mês, com auxílio de uma armadilha CDC light trap miniatura com uma lâmpada de luz negra, ligada continuamente, 24h/7 dias e preservadas a -20 °C. No laboratório, as amostras foram preservadas a -80 °C. A sua análise foi feita com a ajuda de um estereomicroscópio, em que as amostras foram colocadas numa placa de Petri em cima de uma base com gelo, separados com o auxílio de pinças, colocados em triplicado de 5 indivíduos (e posteriormente 10 indivíduos) por tubo e para uma identificação adequada foram utilizadas chaves de identificação dicotómicas. Para deteção e análise molecular do vírus, através de PCR em tempo real, as amostras foram enviadas para o Instituto Nacional de Investigação Agrária e Veterinária onde foram realizadas por um colaborador do projeto, enquanto as demais amostras foram mantidas a -80 °C.

O presente trabalho permitiu a colheita de dados relacionando famílias/géneros/espécies de artrópodes com mapas de precipitação total e médias de temperatura em Alenquer e o

conhecimento de alguns potenciais vetores da Doença Viral Hemorrágica do Coelho - genótipo 2 e de potenciais novos vetores de Mixomatose. Recolheram-se 30522 indivíduos divididos por 59 famílias/géneros/espécies, capturados durante 14 meses. A ordem com mais indivíduos capturados foi Diptera (95,37%) e dentro desta ordem, a família mais prevalente foi Psychodidae (57,06%); o mês totalmente analisado com mais indivíduos capturados foi fevereiro de 2019 (9,36%). No geral, 1 ordem, 4 famílias, 1 género e 1 espécie foram positivas para Doença Viral Hemorrágica do Coelho - genótipo 2 e Mixomatose (1 - RHDV2 e 6 - MYXV) e na maioria dos resultados positivos, o número de indivíduos capturados foi maior na primavera (abril de 2019) do que no inverno (dezembro de 2020, janeiro de 2019 e fevereiro de 2020), com exceção de dois resultados, onde houve redução do número de exemplares na primavera; e um resultado, onde os números se mantiveram iguais.

Milhões de euros são gastos anualmente no controlo de vetores com o objetivo de reduzir as doenças por eles transmitidas. Com as mudanças climáticas que enfrentamos no presente, este trabalho, pela interpretação dos mapas do IPMA e dos seus resultados, apoia a hipótese da possibilidade de aparecimento de novos vetores de doenças transmitidas por vetores e confirma a presença de vírus em novos artrópodes (corroborando as teorias da existência de possíveis novos vetores para Mixomatose e da existência de um possível vetor para a Doença Viral Hemorrágica do Coelho - genótipo 2). No entanto, são necessários mais estudos para confirmar novos vetores de doenças de importância para a Saúde Pública e Animal, bem como alguns dos resultados obtidos nesta dissertação.

Palavras-Chave: *Oryctolagus cuniculus*; *Lepus europaeus*; Vetores; Portugal

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

BTV - Bluetongue virus

CDC-LT - Centers for Disease Control and Prevention miniature light trap

EHDV - Epizootic hemorrhagic disease virus

FAO - Food and Agriculture Organization of the United Nations

INIAV - Instituto Nacional de Investigação Agrária e Veterinária, I.P.

IPMA - Instituto Português do Mar e da Atmosfera

MYXV – Myxoma Virus

rec-MYXV - Recombinant Myxoma Virus

OIE - World Organisation for Animal Health

RHD – Rabbit Haemorrhagic Disease

RHDV – Rabbit Haemorrhagic Disease Virus

RHDV2 - Rabbit Haemorrhagic Disease Virus – genotype 2

SDGs - Sustainable Development Goals

WHO - World Health Organization

1 INTRODUCTION

Leporidae Fischer von Waldheim, 1817 and Ochotonidae Thomas, 1897 are the only two existing families in the mammalian order Lagomorpha Brandt, 1855¹. Splitting roughly into two groups, the family Leporidae, contain the hares of the genus *Lepus* Linnaeus, 1758, containing 32 species, and the rabbits of the genus *Oryctolagus* Lilljeborg, 1873¹ of which derived over 100 domestic breeds from the European wild rabbit² (Figure 1.1).

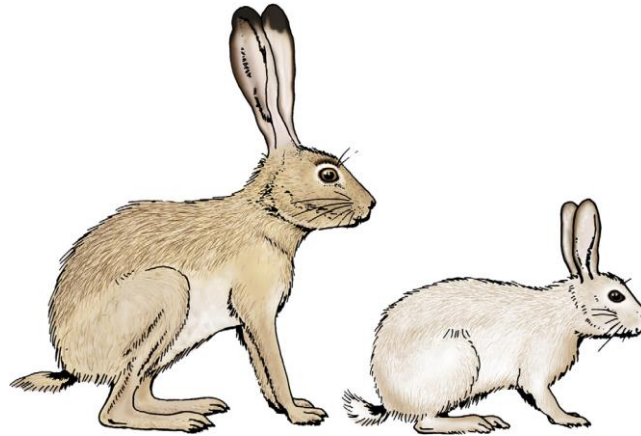


Figure 1.1 - Diagram comparing the bodies of a hare (left) and a rabbit (right). Adapted from¹

The name of European rabbit – from which all domestic breeds originate – is *Oryctolagus cuniculus* (Linnaeus, 1758). Rabbits are burrowing animals (in contrast to other species in Lagomorpha) and, therefore, the genus name is derived from the Greek words orukter (a tool for digging) and lagos (a hare)¹. There are many present-day European words for the English word “coney”, and the similarity are unique: Coelho [Portuguese], conejo [Spanish], coniglio [Italian], konijn [Dutch] and Kaninchen [German]. In the English dictionary, there are specific words to mean the young of a species (e.g., cat/kitten, hare/leveret, dog/puppy), but there is no word for the young of rabbits, therefore, often times, they are referred to as kits or bunny¹.

Hares and rabbits are found in both Old World and New World. They naturally occur on all continents, except Oceania and Antarctica, where they have been introduced, similarly to what happened in other large islands around the globe². Rabbits are herbivores, their body sizes varies from 300g to 5kg and their body form is unique: large eyes, big ears, round heads and extended, ricochet hind legs³. Rabbits and hares, as prey species, have a common behaviour: they are crepuscular, being most active during the early hours at sunrise and the late hours at sunset².

The European rabbit is unique because of its great diversity of habitat: deserts, swamps, fields, farms, woodlands, and forests. However, *Oryctolagus cuniculus* in North America, has not become feral and is only found as a domesticated animal¹.

Between V and X centuries A.D., monks kept rabbits in their monasteries as a food source in southern Europe. This is an example of domestication of *O. cuniculus*, long before agriculture altered the environment – as so as forest clearance. This domestication was sufficient to allow large numbers of rabbits to exist in the wild¹. In zoological institutions, domestic rabbits are frequently found in farms and in interactive exhibits as educational farms².

One of the greatest forces behind the decline of the European rabbit (*Oryctolagus cuniculus*) and Granada hare (*Lepus granatensis* Rosenhauer, 1856) is habitat loss and fragmentation. With modern intensive agriculture, the negative impact on these two species is superior than small scale farming⁴. Massive production of livestock and high ungulate numbers are responsible for resource competition and habitat degradation as well as climate change, agricultural chemicals, overhunting and poaching⁵.

Reforestation of old cultures in Spain and Portugal, especially in northern Iberian Peninsula, and densification of open scrubland areas have replaced the habitat for both rabbits and hares and their predators^{6,7}.

Another greatest force behind the decline of these two species, their health and consequences on ecosystems has been two diseases that appeared in the XX century: Rabbit Haemorrhagic Disease (RHD) and Myxomatosis (caused by Myxoma Virus - MYXV)⁸.

These diseases are important and relevant in the Iberian Peninsula since European rabbit and Granada hare are native to this region, being an important key for the economy and tourism⁹, as well as the ecosystem, since they are a fundamental food source for many critically endangered predators, such as the Iberian Imperial Eagle *Aquila adalberti* Christian Ludwig Brehm, 1861¹⁰ and the Iberian Lynx *Lynx pardinus* (Temminck, 1827)¹¹ and more than forty terrestrial and aerial predatory species¹². The incidence of these diseases may increase in the Iberian Peninsula due to global warming⁶.

Another concern shown in the study conducted by Carvalho, *et al.*¹³ is that some rabbits can reveal a coinfection of RHDV2 and MYXV. Other studies say that the etiological agents of both Myxomatosis and Rabbit Haemorrhagic diseases can be transmitted between wild and domestic rabbits through the action of biting/blood sucking insects¹⁴.

1.1 European Rabbit (*Oryctolagus cuniculus*) in the world

Individuals of the subspecies *Oryctolagus cuniculus cuniculus* Linnaeus, 1758 are thought to be descendants of primitive domestic rabbits that were deliberately introduced into the wild¹⁵. The distribution of the species *Oryctolagus cuniculus* can be seen through figure 1.2. This species is native in countries like Portugal, Spain and France; however, it is commonly found all around the globe, since it is thought that this species was introduced in western Europe as early as the Roman period^{15,16}.

Classified as “Nearly Threatened” back in 2008¹⁷, less than twelve years later, this species of Leporidae family, was classified as “Endangered”¹⁶. The same family has two recognized subspecies: *Oryctolagus cuniculus algirus* Loche, 1858 - which is located in Portugal, in the south of Spain, in North Africa and in several Atlantic and Mediterranean islands¹⁸ and *Oryctolagus cuniculus cuniculus* - occupying Central Europe, Australia, New Zealand and South America¹⁹.



Range
■ Extant (resident)

Compiled by:
IUCN (International Union for Conservation of Nature)

NE DD LC NT VU < EN > CR EW EX
Endangered



The boundaries and names shown and the designations used on this map do not imply any official endorsement, acceptance or opinion by IUCN.



Figure 1.2 - Distribution map of *Oryctolagus cuniculus*¹⁶.

1.2 Granada Hare (*Lepus granatensis*) in the world

The Granada hare or Iberian hare is endemic to Iberia Peninsula and its distribution range covers most of this site, making it one of the most important local game species²⁰, as can be seen in Figure 1.3. This species is native in countries like Portugal and Spain and was introduced in France²¹, however it is excluded from northern regions of Spain where the brown hare (*Lepus europaeus*, Pallas 1778) takes over²². It can be found on the island of Mallorca but it went extinct on Ibiza island²³.

Classified as “Least Concern” in 2019²¹, it is common to see Granada hares within its widespread geographic range as the current population is stable, with increasing numbers in northeastern Spain²¹. However, on Mallorca island and other points of Spain (western Galicia, western Asturias, north of the Ebro River) the population of this species is now considered rare²⁴.

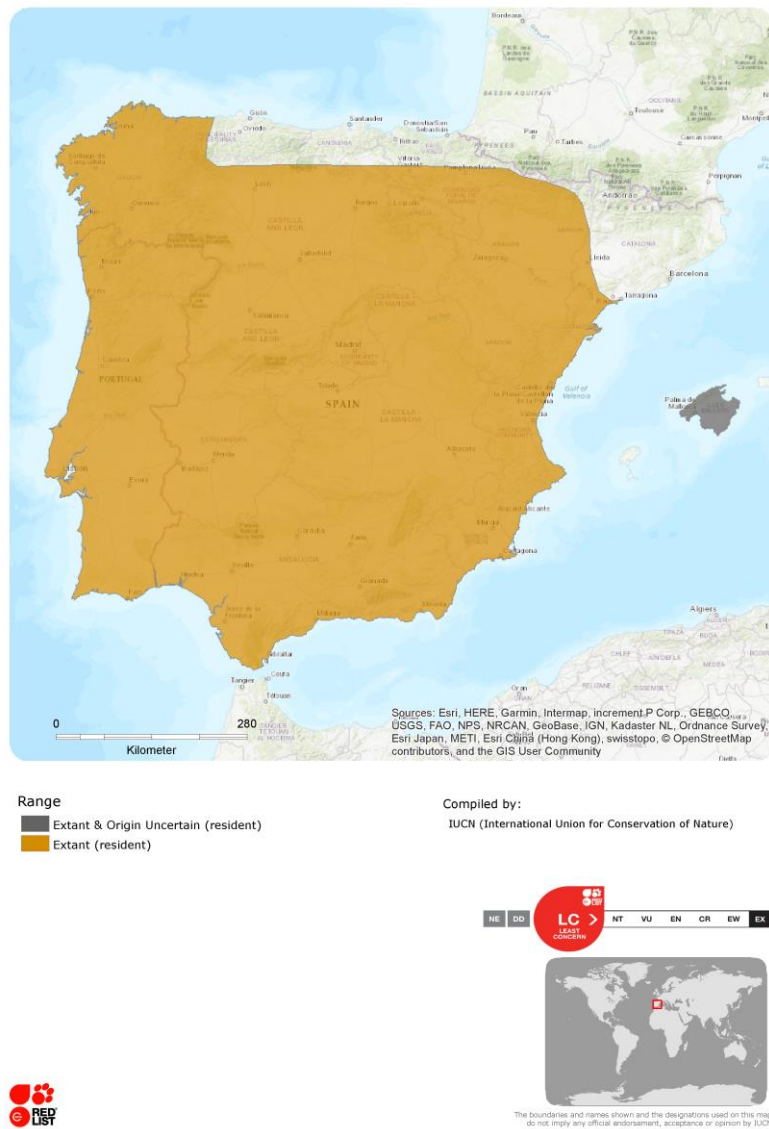


Figure 1.3 - Distribution map of *Lepus granatensis*²¹.

1.3 Haemorrhagic Rabbit Disease Virus - genotype 2 (RHDV2)

Rabbit Haemorrhagic Disease (RHD), known as a highly infectious disease and with a high mortality rate in populations of European Rabbit, is characterized due to the following *post-mortem* changes in affected individuals: splenomegaly, renomegaly, hepatomegaly, haemorrhages in the lungs and upper respiratory tract and, often, jaundice²⁵.

It was first described in the Asian continent, particularly in China, in the mid 80's – more accurately in 1984²⁶. A few years later, in 1986, the disease was reported in Italy²⁷, thus entering into the European continent. From here, it spread to the Iberian Peninsula, with the first cases of the disease occurring in 1988 in Spain²⁸ and 1989 in Portugal (revised in ⁵).

With molecular analysis for Haemorrhagic Rabbit Disease virus (RHDV) strains, it is possible to identify and classify six strains of virus that are related and well defined in the same phylogenetic group (G1 to G6), despite the low genetic level found^{30,31}. However, it appears that only adult individuals are not naturally resistant to lethal infection with classic RHDV strains³².

It is believed that due to the trade of contaminated meat, the number of intensive farming systems increased, both importation of live animals for slaughter and breeding purposes also grew and the disease has spread faster and has been the source of several outbreaks^{27,33}. Rabbit meat is one of the most important food sources in the European food and commercial chain, as it is a healthy source of protein and an essential part of the traditional Mediterranean diet²⁵. Just behind Italy, Spain is the second largest producer of rabbit meat in the European Union²⁵. The populations of European Rabbit, whether in nature or in rabbit-production farms, called rabbitries, for consumption or hunting, become a factor of great economic loss when they are affected by RHDV outbreaks.³⁴

More than two decades after the first cases of RHDV, in 2010, a new virus appeared with a different genetic and antigenic profile, designated as RHDV2. It was first identified on the European continent, in France³⁵. Once identified, it quickly spread to the rest of Europe, reaching countries such as Italy³⁶, Scotland³⁷, Great Britain³⁸, Sweden³⁹, Poland⁴⁰ and Iberian Peninsula – 2011 in Spain⁴¹, 2012 in Portugal⁴². Between late 2014 and early 2015, RHDV2 was detected in Azores archipelago⁴³ and in 2016 in Madeira archipelago⁴⁴.

This new variant of the virus has also been detected globally, having reached the Canary Islands⁴⁵, Morocco⁴⁶, Egypt⁴⁷ and Australia⁴⁸.

In 2017, a group of scientists proposed a new RHDV nomenclature for this strain - GI.2⁴⁹, since RHDV2 and RHDVb were used to identify it⁵⁰. This nomenclature is still used nowadays⁵¹.

Mainland Portugal has so far seen *Oryctolagus cuniculus* populations decimated by this disease in the Berlengas Islands⁵², in the North (Valpaços), Alentejo (Barrancos) and Algarve regions, causing high morbidity and mortality, affecting rabbits of any age group, both wild and domestic⁵³. Worldwide, a similar scenario was also observed⁵⁴.

As some of the main differences between the RHDV and the new variant RHDV2, it is found that: 1) the latter variant can cause infection in young individuals (with less than two months) who have not left burrows; 2) it is capable of affecting individuals vaccinated with

RHDV who, although protected against classical strains, are susceptible to infection by RHDV2^{35,36,41} and 3) it is capable of infecting hosts of other species. However, the results obtained from several studies conclude that they belong to the same Family (Leporidae). The RHDV2 was detected in Cape Hare, *Lepus capensis* Linnaeus, 1758⁵⁵ and also in Corsican Hare, *Lepus corsicanus* de Winton, 1898⁵⁶. Thus, we can say that this new virus, in its clinical characteristics, differs in terms of occurrence, duration and mortality rates³⁶.

Since the first cases of RHDV2 were described⁴², no more circulating RHDV strains have been seen in the clinical cases of Viral Haemorrhagic Rabbit Diseases, which suggests that the new variant has replaced the old⁵⁷. This discovery is possibly due to the selective advantages of this new virus that is able to break the existing immunity to the old virus⁵⁷.

As it has a very high mortality rate, reaching up to 80% in some cases, this disease is currently considered the main cause responsible for the large-scale reduction of the European Rabbit in the Iberian Peninsula, causing an imbalance in the existing ecosystem and promoting an cascade effect on Mediterranean species that feed on this herbivore⁵⁰.

One of the characteristics of the virus that stands out the most is the fact that it is quite resistant in the environment⁵³. RHDV2 can remain active in the decomposing organic matter for seven months, resisting to freezing temperatures (being able to stay for months in frozen rabbit meat), high temperatures (up to one hour at 50°C) and can resist environments with a pH between 4.5 and 10.5, making it resistant to both alkaline and acid environments⁵³.

However, the virus is sensitive to some environments and substances, such as 1-2% formaldehyde, 1% sodium hydroxide (caustic soda), and sodium hypochlorite (base component of bleach) at 0, 5%, which makes it inactive under these conditions⁵⁸.

More recent studies detected antibodies against RHDV or some segments of the RHDV genome in other animals⁵⁹ - Alpine musk deer *Moschus chrysogaster* Hodgson, 1839⁵⁹; Mediterranean pine vole *Microtus duodecimcostatus* (de Selys-Longchamps, 1839)⁶⁰ and Greater white-toothed shrew *Crocidura russula* (Hermann, 1780)⁶⁰. Although, it was not the cause of death of these animals⁵⁹, it shows the capacity of this virus to cross species barrier.

Some studies in England⁶¹, Scotland⁶², Australia⁶³, France⁶⁴, Spain and Italy⁶⁵ have shown and reported that this disease crossed the species barrier to hares and killed the host.

1.4 Myxoma Virus (MYXV)

Myxomatosis is one of the major viral diseases affecting the European domestic rabbit and have serious effects on rabbits' health, as well as on their welfare⁶⁶. It has rarely been reported in the European brown hare and a few and sporadic confirmed cases have been seen worldwide.⁶⁷ With this case, a central question is made: does an infectious agent becomes more or less virulent as it adapts to the new host after a successful cross species barrier?⁶⁸

This disease, listed as a notifiable diseases by the World Organisation for Animal Health (OIE), is a considerable problem in an economic perspective, as an entire rabbitry might be affected⁶⁹.

Frequently occurring in an acute or hyperacute form, myxomatosis can have a rapid course to septicaemia and death⁷⁰. The clinical signs of the disease can be productive rhinitis, dyspnea, pulmonary lesions, cutaneous myxomas, aural, and urogenital swelling and blepharitis and bacterial infections of the respiratory tract and conjunctiva with Gram-negative bacteria: *Pasteurella multocida* and *Bordetella bronchiseptica* (Ferry 1912) Moreno-López 1952 are frequently seen, which contribute to the lethality of the disease⁷⁰. Most rabbits die within 10-14 days of infection; however, highly virulent strains of the myxoma virus may cause death before the usual signs of infection have appeared⁶⁸.

First recognized in Uruguay in XIX century, this disease had little importance until 1950/51 when, with the dramatic spread of the introduced European rabbits in Australia, a MYXV strain was used as a biological agent, reducing and controlling the serious rabbit problem in that part of the world⁷⁰.

In 1952, and as result of the efficiency of the technique, mortality and morbidity of the strain, it was introduced into wild rabbits in France and, from this point, it spread over the majority part of Europe, including the United Kingdom (UK)⁷¹.

Although an initial massive reduction of the European rabbit population (>99%) was seen in both continents, a decline in case fatality rates was observed as result of natural selection, but also due to resistance to the virus⁷².

In the end of 1953, in Spain, myxomatosis was first diagnosed in domestic rabbits⁶⁶. Until 1978, there were outbreaks of classic or typical myxomatosis, in the form of pseudotumor – myxomas - depending on the susceptibility of the rabbit and viral strains involved⁷³. MYXV belongs to the *Poxviridae* family and the *Leporipoxvirus* genus⁶⁶. Since 1979, a form of atypical myxomatosis, described as “decreased cutaneous expression and continued respiratory problems” appeared, and, since then both forms - classic and atypical or “amyxomatous” – have occurred in this country⁷³. Different studies, based on 660 visited farms in Spain, reported a seasonal variation, with an increase during Autumn, from October to December⁷⁴.

Until this date, myxomatosis has been a disease restricted to the European rabbit with some sporadic cases in hares, as shown previously. However, some reports shown that a potential species jump has occurred, as a widespread mortality in the Iberian hare has been seen and reported.⁷⁵

During 2018, an outbreak of myxomatosis emerged on the Iberian Peninsula, first appearing in Spain during the Summer and leading to decimation of Iberian hare population - *Lepus granatensis*¹² - and was caused by a recombinant MYXV.^{76,77} Since the classical MYXV in *Oryctolagus cuniculus* was circulating together with the recombinant MYXV in *Lepus granatensis*, studies were initially suggesting that MYXV was in adaptation to efficiently multiply in hares. Although the recombinant MYXV was originally considered hare specific, it was being detected in *Oryctolagus cuniculus* and making great loses in the population; therefore, a more generalist, species and geographic independent designation may be preferable for the future: rec-MYXV (for recombinant myxoma virus).¹² In October of that year, the first case of MYXV in *Lepus granatensis* was found in Portugal.⁷⁸

A study, conducted by Abade dos Santos, *et al.*¹² detected myxomatosis in *Oryctolagus cuniculus* caused by the rec-MYXV, adding some concerns about the threat of extinction and the fragile conservation state of the wild rabbit.

Over the last years, some vaccines have been produced and available, showing effectiveness against the disease, although the disease persists⁶⁶ and, since the animal is vulnerable to other viral diseases, the virus can coinfect a host already infected with other viruses¹³.

1.5 Transmission routes of RHDV2

Knowledge about the RHDV has been growing, as well as the information concerning the disease' pathology. However, little is known about the transmission mechanisms of the virus by vectors⁷⁹. The same happens with RHDV2⁵³.

From the various studies conducted, it is known that RHDV2 is transmitted through direct contact with infected rabbits, by oral, conjunctival, or respiratory routes. It can be transmitted by exposure to the corpses of infected animals or indirectly through mechanical vectors, such as insects, birds or mammals.⁸⁰ It can also be transmitted through contaminated objects, beds, food and water. Repopulation with contaminated animals by humans, in places where the disease had not been detected, may play an important role in the spread of the disease⁵³. However, the transmission route in healthy rabbits, or how they get infected in first place, is unknown.⁸⁰

The transmission route of viruses by insects can take two forms: mechanical or biological⁸¹. In nature, mechanical vectors pick up an infectious agent on the outside of their bodies and transmits it in a passive manner; for example, dipterans from the family Muscidae Latreille, 1802 are capable of transmitting RHDV2, landing on dead rabbits and later resting on the eye of healthy rabbits⁸², although in the case of individuals of the family Calliphoridae Brauer & Bergenstamm, 1889, another less direct transmission method should be considered as they settle in dead animals, but are rarely seen around live rabbits⁷⁹.

A study conducted by a group of researchers, showed that flies belonging to the genus *Phormia* Robineau-Desvoidy, 1830 were able, in laboratory conditions, to transmit the disease seven hours after contamination⁸².

Also in laboratory conditions, fleas from the species *Spilopsyllus cuniculi* (Dale) and *Xenopsylla cunicularis* Smit, 1957 and *Culex annulirostris* Skuse, 1889 mosquitoes are also known to be able to transmit the virus to susceptible rabbits^{83,84}. Thus, transmission in a passive manner, as mechanical vector, has received special attention from researchers⁷⁹.

During 1998, in New Zealand, a field experiment was conducted, and the preliminary results showed that exposure of healthy rabbits to insects might have resulted in the transmission of the disease to the rabbits in open cages. Although the mode of transmission remains unclear and further research is required, the researchers indicates that, probably, it may have occurred by direct contact of the rabbits with *Oxysarcodexia varia* (Walker, 1836).⁸⁵

An Australian study supports the hypothesis, through laboratory data, that individuals of the species *Musca vetustissima* Walker, 1849 may have a role as a mechanical vector of RHDV⁸⁶,

since it is known that individuals of the family Muscidae feed naturally on live or dead animals and move between 7 and 15 km per day⁸⁷. It was also shown that the virus remained for more than 11 days in these individuals. However, the virus could only be detected for up to 7 hours on the insect legs⁷⁹.

A recent study affirms that detection of RHDV2 - and other lagovirus currently circulating in Australia - in carrion flies looks to be a good indicator to monitor the disease⁸⁸.

It is possible, in theory, to admit the possibility of transmitting the virus between rabbits, directly or indirectly, being the insects responsible for the spread of the infection, not only on the continent, but also on the Australian islands⁸⁶.

Another study concluded that the transmission of the virus by insects is of great epidemiological importance⁸⁹. It is known that the rabbit can be a source of food for various insects (by its flesh or blood, dead or alive), being part of their food chain. As an example, there are several species of insects of the genus *Culicoides* Latreille, 1809, which feed on the rabbit⁹⁰.

With all the existing doubts, the route of transmission remains uncertain, reinforcing that more research is needed⁸⁹.

1.6 Transmission routes of MYXV

MYXV shown its importance throughout the 20th century because of its use by the Australian government in the attempt to control the feral Australian population of *Oryctolagus cuniculus* and the subsequent illegal release of MYXV in Europe.⁹¹

With the originally thought that MYXV was spread by direct contact from rabbit-to-rabbit, some field-tests were conducted in Australia, mainly in the dry regions, during Autumn, Winter and Spring, but without considerable success in inducing widespread disease in the rabbit population⁹². However, shortly after these field tests and when the rainy season ended, several dead rabbits infected with MYXV were found alongside rivers, and the researchers explained this event with the seasonally expanded populations of *Culex annulirostris*, a mechanical vector for MYXV⁹³. Nevertheless, it rapidly became clear that nearly any biting or sucking arthropod could serve as a vector for the virus, which allowed MYXV to circulate over large areas^{92,93,94}. As an example, the European rabbit flea, *Spilopsyllus cuniculi*, a native stickfast flea that was imported into Australia, spread widely, and infected rabbits throughout the 1960s⁹². After their introduction and successful establishment in Australia as vectors of myxomatosis⁹⁵, studies about laboratory breeding, their physiology and successful establishment in the field, their role in changing myxomatosis epidemiology, and reductions in rabbit abundance as seen during Winter were published, as they caused outbreaks of myxomatosis, which killed a high proportion of young rabbits throughout south-eastern part of South Australia and adjacent western Victoria⁹⁶.

As previously said, Myxomatosis is normally transmitted rabbit-to-rabbit, when virus particles adhere to the piercing mouthparts of a biting insect vector⁹³. As seen in Australia and also in Great Britain, *Spilopsyllus cuniculi*, is the most important vector of the pathological agent⁹⁷. However for the majority of the scientists, other blood-sucking insects may play a minor role in some circumstances as vectors⁹⁷.

When infected by an arthropod carrying MYXV, a nonimmune animal develops a local plaque of thickened inflammatory tissue lesion of benign character. When a fresh arthropod feeds on the lesion its mouthparts become contaminated and it can transfer the pathogenic agent to the next rabbit it bites⁹⁸.

The entry of Myxomatosis into South American laboratory rabbit colonies happened due to infected *Aedes* Meigen, 1818 from wild local rabbits⁹⁸. At the same time, the death of wild rabbits was seen and almost confined to the immediate neighbourhood of streams, lakes, or temporary accumulations of water, and the circumstantial evidence pointed strongly to *Culex annulirostris* as the important vector, although there are other insects to this hypothesis to account: *Ochlerotatus theobaldi* (Taylor, 1914) or *Aedes aegypti* (Linnaeus, 1762)⁹⁸. Some studies shown that mechanical contamination of the mosquitos mouth-parts and transfer to other rabbits is purely by mechanical fashion, with the infection being initiated as a local lesion in the skin and not by injection of infected saliva into the blood^{93,98}.

As previously said, the virus is primarily spread by blood feeding arthropod vectors such as fleas or mosquitos, although transmission via fomites has also been described⁹⁹. Field studies verified the role of *Anopheles atroparvus* Van Thiel, 1927¹⁰⁰⁻¹⁰², *Aedes caspius* (Pallas, 1771), *Aedes detritus* (Haliday), *Culex modestus* Ficalbi, 1890¹⁰³, *Culiseta annulata* (Schrank 1776)¹⁰⁴, *Anopheles maculipennis* Meigen, 1818¹⁰⁵, the genus *Stomoxys* Geoffroy, 1762¹⁰⁶ more accurately *Stomoxys calcitrans* Linnaeus, 1758¹⁰⁷ in the transmission of myxomatosis.

Some studies say that the only way to prevent infection of pet rabbits, by not using biological or chemical products, is to protect animals from biting arthropods, by using mosquito nets around the rabbit hutch⁹⁷.

1.7 Taxonomy of arthropods

As already known for all biologists, taxonomy comes from the ancient Greek (taxis), meaning 'arrangement', and (-nomia), meaning 'method', and is the science of naming, defining and classifying groups of biological organisms on the basis of shared characteristics¹⁰⁸. Systematics, who comprises Classification, Taxonomy and Identification, is the scientific study of classes, diversity of organisms and their interrelations¹⁰⁸. Finally, identification is, according to a previously established Classification, the placing of an unidentified animal in the Class or group to which it corresponds¹⁰⁸.

1.7.1 Diptera

Diptera Linnaeus, 1758, are the most important group of arthropods as vectors of disease agents in human and veterinary medicine¹⁰⁹. They are known on all continents, with exception of Antarctica, from the average sea level¹¹⁰ to 4000 meters of altitude¹¹¹.

Adult mosquitoes or flies share the characteristics of most Diptera, presenting only one pair of wings, the body is covered by small scales that often form contrasting colour patterns, frequently used to identify species. Female mosquitoes have their mouthpiece adapted to suck blood from vertebrate animals. This particular fact makes mosquitoes the most important vectors of pathogenic agents¹¹², such as those represented in this document: RHDV2 and MYXV.

In Portugal, mosquitoes were first studied by Sarmiento & França in 1901¹¹³ but only by 1931 a monography with the description of 21 species were published¹¹⁴ and continued with the work of Cambournac (1938 and 1943) among others^{115,116}. In 1999, it was published for the first time in Portugal, an identification key for mosquitoes from mainland Portugal, Azores and Madeira, updated with 45 species and subspecies distributed in 15 subgenera and 7 genera¹¹⁷.

Males do not feed on blood, and their proboscis is adapted for ingestion mainly of nectars or products resulting from the fermentation of fruits¹¹⁸ being usually smaller than females of the same species, having feathery antenna^{110,112,118}.

The life cycle of mosquitoes and flies comprises an egg stage, four larvae stages, a pupal stage, and an adult form, as shown in the figure 1.4. The life cycle of mosquitoes comprises two phases: the first, necessarily aquatic, relating to immature forms (egg, larvae and pupa) - although they breathe atmospheric air and therefore have the need to return to the water surface; and a terrestrial/aerial phase corresponding to the adult mosquito¹¹⁹.

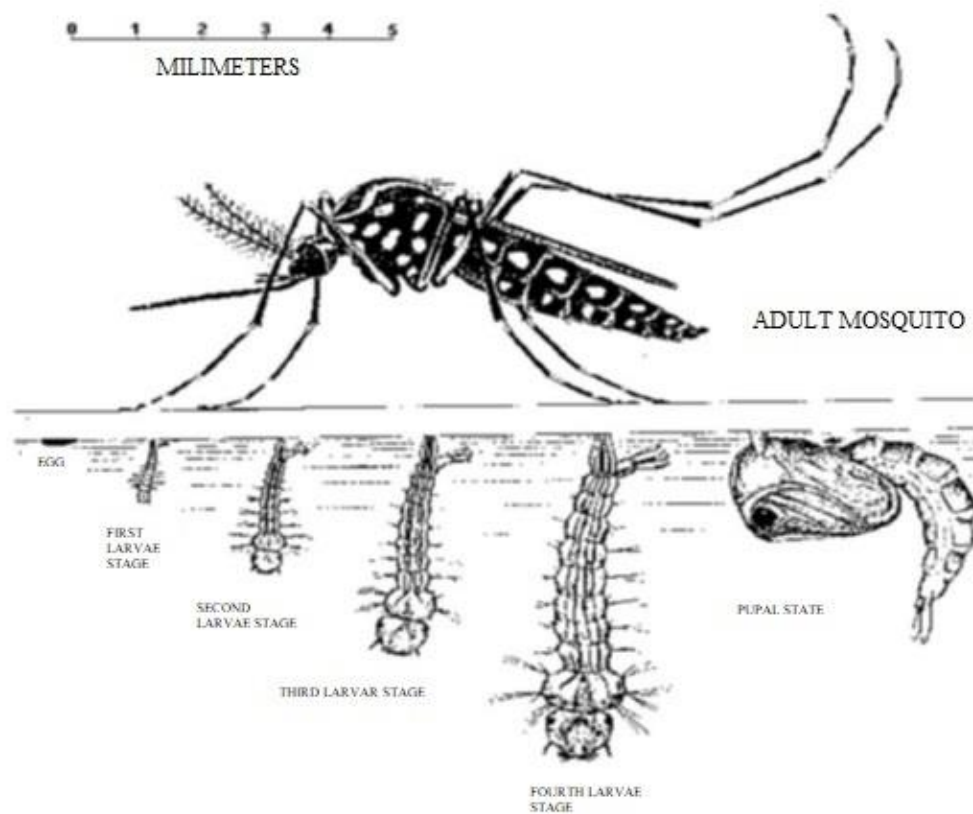


Figure 1.4 - Representation of the mosquito's life cycle. Adapted from^{110,119}

The oviposition takes place on the surface of water or moist soil. Although moist soil lacks on water, must have enough in order to permit the development of the larvae¹¹⁷. The habitats where the larvae develop are very wide-ranging: they can be wells, lakes, holes in rocks and trees

or abandoned containers. Some species are restricted to certain habitats and others can adapt to various ecological conditions. The oviposition depends on the species and the physiological condition of the female and it can reach from 100¹²⁰ up to 300 eggs¹¹⁷.

The location for oviposition is the decisive factor for the distribution of mosquito species, being freshwater environments the common desirable location, while some can tolerate highly polluted aquatic environments, nitrogen-rich waters and others are adapted to high salinity¹⁰⁴. With these specifications, few species develop in permanent aquatic systems, such as lakes and reservoirs. These habitats are usually deep, very open and do not provide protection against predators, such as those that belong to the aquatic fauna where the eggs are laid. Like other Diptera, mosquitoes are holometabolic^{117,120}.

The order Diptera is divided into two suborders: the Nematocera Latreille, 1825 and the Brachycera Zetterstedt, 1842¹²¹.

NEMATOCERA

Nematocera include mosquitoes with visibly long antennae with several articles. Commonly, specimens are slender and long-legged; however, some specimens have stout-bodied legs. The larvae of Nematocera typically have a well-developed head capsule, and the mandibles usually rotate at an oblique or horizontal angle¹²¹.

Culicidae

Culicidae Meigen, 1818, also known as the mosquito family, consist of 43 recognized genera¹¹⁷ incorporating about 3,600 species¹²².

Family Culicidae can appear in large numbers as larvae and adults and provide a major prey base for many vertebrates such as fish, birds, bats, or amphibians. Female adults do their meals by getting blood from animals – including humans – since they use their long and flexible proboscis¹¹⁷. Some of the male adults are important pollinators of flowers, who visit them to imbibe nectar¹²².

Many of them are well recognized for their importance as vectors of viruses, protozoa, bacteria and helminths, but there are several others of medical or veterinary importance, since many of the human pathogens are common with wild animal reservoirs¹²³. Considered one of the most annoying families of Insecta, because of the allergic reactions and the substantial blood loss they can cause due to their bites alone when they occur in large numbers, mosquitoes can be found in almost every imaginable environment where water exists¹²⁴.

The control methods to suppress mosquito populations and disrupt pathogen transmission, continue to be practiced in research and includes habitat modification, bed nets, insecticides, drug treatment, sterile release, and genetic manipulation¹²⁵.

Ceratopogonidae

Family Ceratopogonidae Newman, 1834, also known as “biting midges” or “no-see-ums”, contains 123 genera and 6,267 extant described species¹²⁶. The adults swarm around mammalian hosts, including humans, doing their meals from blood, since they are minute bloodsuckers. However, the larval feeding habits are most frequently of scavenging and predatory behaviour type¹²⁴.

Ceratopogonidae has four subfamilies: Dasyheleinae Lenz, 1934, Leptoconopinae Noe 1907, Forcipomyiinae Meigen, 1818 and Ceratopogoninae (sensu Wirth, 1965a), and are distributed worldwide, being found in different habitats^{126,127}, from sea level to up to 4000m in altitude - since they are resistant to cold¹¹¹ – and all over the world, with exception of Antarctica, Iceland, New Zealand, Patagonia and Hawaii islands^{127,128}.

One of the most important genus in Ceratopogonidae family for Veterinary Medicine and Public Health is *Culicoides* Latreille, 1809 since hematophagous females are known vectors of viruses, protozoans and filarial nematodes, like Schmallenberg virus (SBV), African Horse Sickness virus (AHSV), Bluetongue virus (BTV), among others⁹⁰.

Mycetophilidae

Mycetophilidae Newman, 1834, is a diverse and abundant family with insects known as Fungus-gnats, typically well known for their compact hump-backed appearance, long coxae and their well-developed tibial spurs, which generally has some mixture of black, brown and yellow patterned color¹²⁹. Their wings have a “Y”-shaped wing-vein¹³⁰. This family, very diversified with about 3000 species in 150 genera, is almost entirely cosmopolitan, although they can be found in a variety of ecosystems, like forested areas, normally in association with fungal habitats, around the globe, with the exception of Antarctica¹³¹.

The larvae of the insects of this family are translucent and wormlike, have a black head capsule and live in the growing medium of houseplants¹³⁰ – hence the cosmopolitan behaviour. Although they can cause plant damage, they are considered a minor pest of houseplants and the adults are a minor economically important insect¹²⁴. Adult Mycetophilidae do not bite and are harmless; however, if present in big numbers, they can be classified as pest¹³⁰.

In certain conditions Mycetophilidae can be considered beneficial to humans and their environment, since they play an important role in food chains in nature, as they are decomposers and recyclers of decaying organic matter of different types¹²⁴. Although, there are no records concerning pathogenic agents transmitted by insects of this family, some studies have been done by experimentally infecting them with iridescent virus (family *Iridoviridae*). However, almost nothing is known of such infection in the wild¹²⁹.

BRACHYCERA

The major part of the Diptera individuals belong to the suborder Brachycera, with about 80,000 described species and where the best known families of flies are: Muscidae and Drosophilidae Rondani, 1856¹²¹. This group is characterized by modifications in the larval head and mouthparts and by the short, three-segmented antennae¹³².

Muscidae

The family Muscidae includes, approximately 9,000 species in 190 genera. Fortunately, only a few of these contains medical or veterinary importance, due to its vectorial capacity, for being blood-feeding parasites, vectors of disease agents, parasitizing domesticated animals and wildlife¹³³, and because this family includes anthropophilic species - parasites that prefer or seek human as host rather than other animals – such as:

- i. The house fly (*Musca domestica*, Linnaeus, 1758), well known for its “filthy habits”, whose adults and immatures prefer a variety of filthy organic substrates, including latrines, household garbage and manure, which prudence orders that these flies should be minimized wherever human food is prepared and served¹³³;
- ii. The stable fly (*Stomoxys calcitrans* Linnaeus, 1758) well known for its “biting habits” which bites both humans and livestock¹²⁴;
- iii. The sweat flies, whose specimens prefer to feed persistently on perspiration¹³³.

Adults and larvae of this family can be identified by morphological, behavioural and ecological features, including habitats¹²⁴ and also by the nature of their mouthparts: the nonbiting Muscidae and the biting Muscidae¹³³. The nonbiting Muscidae uses their soft, fleshy, and sponging mouthparts to ingest liquids from substrates and animal tissues, since they are incapable of penetrating the skin. On contrary, biting Muscidae have piercing/sucking mouthparts that are able to penetrate skin in order to obtain blood from their meals¹³³.

Some Muscidae specimens form a cocoon prior to pupation, which is very uncommon among other families of order Diptera. They also can be predators on other insects, but mostly are scavengers or feed on pollen¹²⁴. Control of Muscidae in houses or stables often involves prevention using biological control of local breeding by elimination or modification of known larval source, application of repellents and screening to exclude adult flies from indoor areas¹³³.

Drosophilidae

The family Drosophilidae is commonly referred as “Vinegar flies”. They are generally small insects (1-6mm), usually with red eyes¹²⁴ and, the best known, is *Drosophila melanogaster* (Meigen, 1830) an abundant model organism for genetic research¹³².

Frequently found around overripe fruit, mushrooms, decaying vegetation and fungi, the larvae are maggot-like and obtain nutrients by consuming yeast and other microorganisms in decomposing dead plant or animal biomass¹³². Some are leaf miners, while others have a parasitic lifestyle and are predators of specimens of the suborder Homoptera Boisduval, 1829¹²¹.

Drosophilidae specimens are common in most households, flying around or crawling on overripe fruit. As said previously, *Drosophila melanogaster* is a common laboratory animal mostly used in genetic research¹³⁴. Though the flies are generally harmless, some species, especially *Drosophila repleta* Wollaston, 1858, are a potential vector by mechanical transmission of pathogens, since they can breed in animal faeces^{87,135}. Drosophilidae can also be lachryphagous like many other Diptera, feeding only on tears and perspiration and are known as vectors and intermediate hosts for *Thelazia callipaeda* Railliet & Henry, 1910, which parasitizes the eyes of wild and domestic animals, which include lagomorphs¹³⁴.

1.7.2 Lepidoptera

The order Lepidoptera Linnaeus, 1758 includes butterflies and moths and form the second largest diversity of insects, being around 180,000 species distributed in 34 superfamilies and 130 families and are found worldwide, especially in tropical locations and other wide variety of habitats¹³⁶. The name Lepidoptera refers to the presence of scales in their wings (from Greek *lepis* = scales, and *pteron* = wings), which forms the basis for the attractive colour patterns present in many species. The combination of these insects' features make them one of the most studied groups of organisms¹³⁶.

Lepidoptera adult insects feed at mud puddles, carrion and dung in a behaviour known as puddling, but they can also feed on nectar, pollen, liquids from fermented fruits, vegetable resins and some are even sudophagous, lachryphagous and hematophagous¹³⁷ - i.e. *Calyptra thalictri* (Borkhausen, 1790)¹³⁸. However, not all adults have this behaviour, since some have atrophied mouthparts, and, in this case, they consume the accumulated reserves obtained during the larval stage¹³⁹⁻¹⁴¹. Additionally, puddling intensity differs within species, among sex and age classes¹⁴².

Blood-feeding Lepidoptera have been observed piercing the skin of their hosts during feeding, looking for sodium¹⁴² or proteins¹⁴³, since these fluids provide them. These behaviours have negative implications on hosts' health and are a serious potential for pathogenic agents' transmission¹³⁷.

Some studies about Lepidoptera feeding on mammals refers some negative health effects: localized irritation and inflammation. This particular behaviour gives evidence on making hematophagous and lachryphagous Lepidoptera a potential vector for pathogen agents, though this has never been documented¹³⁷ and more studies are required.

1.8 Public Health, Animal Health, Plant Health, and the Environment – One Health

Some insects are invasive, can colonize new territories and can have, or are likely to have, environmental, economic, public, or animal health impact. These exotic species become invasive species because they establish and proliferate within an ecosystem, and they can adapt to both human and animal activities, since they are introduced mostly through globalization. This globalization and the occurrence of invasive species are associated with commercial transportation, human and animal travel and, climate change^{113,120}.

In many regions of the planet, climate change and intrinsic adaptations of species make the existence of the vector insect constant throughout the year, in any season or weather condition, leading to the presence of a larger number of specimens per year and a longer period of activity¹⁴⁴.

As said previously, vectors can transmit infectious disease agents and this transmission can occur biologically or mechanically (Figure 1.12). In biological transmission, the pathogenic agent replicates or matures in the vector prior of being transmitted to the next host – normally a vertebrate. In mechanical transmission, there is no maturation or replication of the pathogenic agent in the vector, transmitting physically from one vertebrate host to another. In those vectors who are hematophagous the transmission results, normally, from the contamination of oral parts¹¹¹.

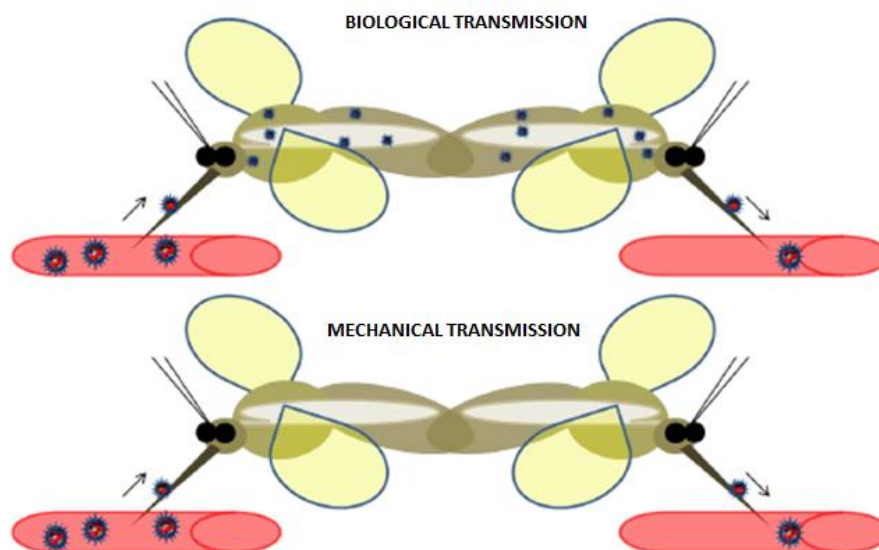


Figure 1.5 - Representation of the two possible known ways of transmitting pathogen agents by vectors, considering the maturation of the pathogen agent in the vector. Adapted from¹¹¹.

Vector-borne diseases are a dynamic – more or less specific, depending on the participants - interaction/relationship between four parts: the pathogen, the vertebrate host, the environment and the vector. The pathogen can be transmitted by multiple vectors and this one is specific to a particular group of hosts¹²¹.

For a successful transmission/infection, it is essential that the pathogen agent infect and mature (or replicate) in either the vector or the host. In many cases, this transmission/infection take place when the vector feeds on hosts' blood to encourage egg development or to fulfil other physiological needs¹²¹. The following blood meals can be a doorway to a new transmission/infection since the vector can transmit the pathogen to new potentially susceptible hosts. While for the vector the consequences of the interaction with the pathogen agent exerts little or no harmful effects, the pathogenic agent causes infection in the susceptible vertebrate host¹¹¹. The important species for vector control¹⁴⁵ in animal and/or human public health, belongs to the family Culicidae^{111,121}, Simuliidae Newman, 1834¹⁴⁶; the subfamily Phlebotominae¹⁴⁷; the genus *Culicoides*⁸⁹, among others.

The epidemiology of mosquito-borne diseases depends on three parameters: Vector competence – the ability of a vector to ingest, keep and transmit a pathogenic agent to a susceptible host¹⁴⁸; Vectorial efficiency - the efficiency of a given vector to transmit a pathogenic agent in a region, influenced by the interactions between exogenous factors (biotic and abiotic) and endogenous factors, related to the vector, which will result in the ability to transmit the agent; and Vectorial capacity – the number of new infections produced by the vector per case and per day¹⁴⁹.

With global warming and the climate changes, species can undergo on an evolutionary adaptation and migrate to areas with temperatures more satisfactory to their development, growth and expansion¹⁵⁰, not only making possible to (re)-introduction of exotic mosquito species, and therefore, new cases of diseases, but also other mosquito-borne diseases may be introduced, making necessary a constant surveillance in Animal and Public Health perspective¹⁰⁷.

'One Health' is an approach programmed to think and implement plans, legislation, policies and research by a group of multidisciplinary professionals, such as public health, animal health, plant health and the environment, of several sectors that work together in some areas (i.e. food safety, the control of zoonoses) to succeed better public health outcomes. One example of this strong partnership is the work that World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO) and World Organization for Animal Health (OIE) make to promote multi-sectoral responses, providing guidance on reducing risks of public health threats¹⁴⁵.

1.9 Sustainable Development Goals

The Sustainable Development Goals (SDGs) and the 2030 Agenda consist of 17 objectives, 169 goals, which were approved by the leaders of several countries, at a memorable summit at UN headquarters in 2015. These define the priorities and aspirations of sustainable development for 2030, where they seek to mobilize global efforts in areas that require global action by governments, companies and civil society to eradicate poverty and create a life with dignity and opportunities for all citizens of the world and those yet to come , within the limits of the planet¹⁵¹.

Therefore, this dissertation fits into two of the objectives¹⁵¹, highlighting the goals:

GOAL 3: GOOD HEALTH AND WELL-BEING:

- “Strengthen the capacity of all countries, in particular developing countries, for early warning, risk reduction and management of national and global health risks.”.

GOAL 15 – LIFE ON LAND:

- “Take urgent and significant action to reduce the degradation of natural habitats, halt the loss of biodiversity and, by 2020, protect and prevent the extinction of threatened species.”;

- “By 2020, introduce measures to prevent the introduction and significantly reduce the impact of invasive alien species on land and water ecosystems and control or eradicate the priority species.”;

- “By 2020, integrate ecosystem and biodiversity values into national and local planning, development processes, poverty reduction strategies and accounts.”.

2 Objectives

Knowledge of the feeding behaviour (blood, sweat, tears, puddling, etc.), preference of habitats and behaviour around animals and humans of Arthropods is vital in assessing their vectorial competence and determining host preferences. Therefore, this helps to understand the roles of these specimens in the epidemiology of different vector-borne diseases and will improve the knowledge about the arrival, development, and appearance of other vector-borne pathogens. A deeper study of the different Arthropods present in the rabbitries and their ecological preferences is required. The main aim of this study was to, by integrative taxonomy of arthropods, find potential vectors of Viral Haemorrhagic Rabbit Disease Virus - genotype 2 (RHDV2) and potential new vectors of Myxoma Virus (MYXV) with the help of real time PCR to detect and quantify those viruses. The specific goals included:

- i. Arthropod abundance, dominance, and frequency in a wild rabbit production farm located in Alenquer;
- ii. Viruses' detection and quantification in arthropods;
- iii. Relation between arthropod and climatic variables such as temperature and total precipitation.

3 Material and methods

The work was carried out in the Parasitology and Parasitic Diseases laboratory of the Faculty of Veterinary Medicine of the University of Lisbon, as well as in the Entomology Laboratory, in the Advanced Signal and Image Processing Laboratory of the Faculty of Sciences of the University of Lisbon and INIAV - National Institute for Agricultural and Veterinary Research.

3.1 Study area

Alenquer, a municipality of the district of Lisbon with 304,22km² of area, presents a unique and characteristic landscape, a transition between countryside and the plains, where a vast livestock, mostly aviaries and rabbits hutches, agricultural and wine region are predominant for more than eight centuries, making this the ancestral base of its economy¹⁵².

In a very schematic way, Alenquer can be divided into three very distinct zones: the mountainous zone (666m of altitude), the sub- mountain (280m of altitude) and the plain area (50m of altitude)¹⁵².



Figure 3.1 - Map of Portugal with Alenquer in red¹⁷⁴.

3.2 Collection of specimens

For this study, the specimens were collected, between December 2018 and December 2019, in a wild rabbit production farm, in Alenquer. The sampling was made between December 2018 and December 2019. This was done only once, at the end of the first 15 days of each month using a Center for Disease Control and Prevention miniature light trap (CDC-LT) baited with a blacklight (also referred to as a UV-A light), switched on continuously, 24h over 24h, to capture arthropods with day and/or night activity. The specimens were captured dry and preserved at -20 °C. Posteriorly, due to an outbreak of coinfection of RHDV2 and MYXV on the rabbit hutch, specimens were sampled in February 2020. This last sampling was divided in two samples: one sampled during the 10th and 11th February 2020 and another that lasted for the entire month; both captures were performed using the same methods described above. The sampling was carried out by a collaborator of the rabbit hutch, who had all the material available and the necessary knowledge practices to perform the task.

The specimens were brought to the laboratory in freezer containers (to maintain the temperature) and placed at -80 °C, because, in this way, the degradation of the viral RNA or DNA (RHDV2 and MYXV, respectively) would be slower (despite these being resistant viruses).

3.3 Morphological Identification of Specimens

The identification of the collected specimens was made through their morphological characteristics, after being separated by groups with common characteristics - ideally by family/genus/species. This procedure required the use of dichotomous identification keys, such as those by Thielman & Hunter¹⁵³, Barrientos¹⁵⁴ and Ramilo⁹⁰. The specimens were observed under a stereomicroscope (Olympus SZ51 - the magnification range of 8x to 40x), placed in a Petri dish on top of a base with ice, separated with the help of tweezers with super thin curved tips, 120mm, and watercolour brushes n°1. After separation, they were placed in *ependorfs*, duly identified, and preserved at -80 °C.

The separation of the specimens and their placement by family/genus/species in the tubes was initially done in triplicate of 5 specimens per tube and a fourth tube with the remaining specimens. After several molecular analysis performed with negative results, this method was revised and 10 specimens per tube were placed in triplicate to try to check if there was a greater viral load to be properly detected. Due to time constraints and with the purpose of doing a larger screening of the specimens, when the number of specimens identified reached around 1000 in a month, the screening stopped unless after molecular analysis a positive result was obtained. The counting of the specimens was done with the help of a handheld cell counter.

3.4 RNA and DNA detection and quantification

After specimen identification, 15 specimens of each family/genus/species identified were sent to INIAV for detection and quantification of the virus, by real time PCR, while the remaining specimens were kept at -80 °C. This analysis was carried out by MSc Fábio Abade dos Santos in the scope of his PhD work included in Project +Coelho 2 (INIAV)¹⁵⁵. After the detection of low viral load, 10 specimens were placed in tubes in triplicate, as mentioned above. If any of the groups analysed was found to be positive for the presence of the virus, the remaining individuals that have been preserved would be subject to a new morphological identification and / or

molecular analysis up to the species level (if they have not been previously and when possible) and sent again for detection and quantification of the virus.

3.5 Photographic records

Images used in this work were acquired on Zeiss STereo LUMAR stereoscope, equipped with a Hamamatsu Orca-ER CCD camera and GFP fluorescence filter set, controlled with the MicroManager v1.14 software. Those images were processed using the AxioVision SE64 software, giving a treated image.

4 Results

The data collected on this dissertation is found in annex 8.1.

4.1 Arthropod Abundance, Dominance and Frequency

A total of 30,522 specimens, divided by 59 families/genus/species, were identified. The most represented order was Diptera (95.37%), the family with the greatest representation in the samples was Psychodidae Newman, 1834 (57.06%) and the full screened month with most captured specimens in a 15-day sampling was February 2019 (9.36%) – Wintertime and April 2019 (8.35%) - Springtime. February 2020 was the highest in number of specimens (34.09%) but had a all-month sampling and, therefore, it cannot be compared with other months. The summary results can be shown at the table 4.1.

Table 4.1 - Collected specimens by family/genus/species.

Arachnida (0,17%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
Acari	3	0,01%
Arachnida	48	0,16%
Coleoptera (0,24%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
Coleoptera	74	0,24%
Collembola (0,01%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
Collembola	2	0,01%
Diptera (95,37%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
Anisopodidae	2	0,01%
Calliphoridae	3	0,01%
Carnidae	3	0,01%
Cecidomyiidae	2765	9,06%
Ceratopogonidae	1776	5,82%
<i>Culicoides</i> spp.	1	0,00%
<i>C. festivipennis</i>	2	0,01%
<i>C. imicola</i>	2	0,01%
<i>C. kurensis</i>	1	0,00%
<i>C. newsteadi</i>	2	0,01%
<i>C. obsoletus/ C. scoticus</i>	575	1,88%
<i>C. punctatus</i>	7	0,02%
<i>C. univittatus</i>	3	0,01%
Chironomidae	2214	7,25%
Chironomini	78	0,26%
<i>Ablabesmyia</i>	1	0,00%
Culicidae	339	1,11%
<i>Anopheles</i>	4	0,01%
<i>Culex</i> spp.	82	0,27%
<i>Psorophora</i>	1	0,00%
Ditomyiidae	6	0,02%

Drosophilidae	123	0,40%
Lauxaniidae	4	0,01%
Lonchopteridae	1	0,00%
Milichiidae	62	0,20%
Muscidae	194	0,64%
<i>Hydrotaea</i>	2	0,01%
Mycetophilidae	342	1,12%
Oдиниidae	63	0,21%
Opomyzidae	4	0,01%
<i>Palloptera ustulata</i>	5	0,02%
Phoridae	88	0,29%
Psychodidae	17417	57,06%
<i>Phlebotomus</i>	142	0,47%
Scatopsidae	583	1,91%
Sciaridae	592	1,94%
Simuliidae	3	0,01%
Sphaeroceridae	18	0,06%
Tipulidae	243	0,80%
Trichoceridae	1353	4,43%
Hemiptera (0,59%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
Cicadellidae	124	0,41%
<i>Empicoris vagabundus</i>	24	0,08%
Miridae	1	0,00%
<i>Blepharidopterus</i>	32	0,10%
Hymenoptera (0,52%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
<i>Anaphes nitens</i>	44	0,14%
Hymenoptera	116	0,38%
Lepidoptera (2,15%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
Lepidoptera	655	2,15%
Neuroptera (0,13%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
Chrysopidae	2	0,01%
<i>Chrysoperla carnea</i>	10	0,03%
<i>Semidalis</i>	28	0,09%
Orthoptera (0,29%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
Orthoptera	19	0,06%
Staphylinidae	69	0,23%
Psocoptera (0,51%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
Ectopsocidae	151	0,49%
Psocoptera	7	0,02%
Thysanoptera (0,02%)		
<u>Specimens</u>	<u>Total number of specimens</u>	<u>Total number of specimens (%)</u>
Thysanoptera	7	0,02%

The next pictures represent some of the identified specimens. Since the stereoscope used has a continuous zoom, it was not possible to calculate the magnification using the pixel size of the image and this feature will not be present in the pictures.



Figure 4.1 – Adult female of family Culicidae.
(Author's original)



Figure 4.2 - Adult male of family Culicidae.
(Author's original)



Figure 4.3 – *Culicoides* female specimen – *C. kurensis* (Left) *C. obsoletus sensu latu* (Right)
(Author's original)



Figure 4.4 – *Phlebotomus* specimen (Author's original)



Figure 4.5 – Specimen of family Mycetophilidae
(Author's original)



Figure 4.6 - Specimen of family Muscidae (Author's original)



Figure 4.7 - Specimen of family Drosophilidae, a known Brachycera (Author's original)



Figure 4.8 - Specimen of order Lepidoptera
(Author's original)

4.2 RNA and DNA detection and quantification

The results of detection and quantification of the viruses are presented in the Table 4.2.

The results show that, during February 2020 (Code JS0220), 61 specimens of Mycetophilidae family were positive for RHDV2, which represents 100% of the specimens collected and sent to the laboratory of that month.

Some order/families/genus/species were positive for MYXV, all captured in January 2019, with exception of specimens of the family Chironomidae Erichson, 1841, which were captured in December 2018. The results represent the number of specimens sent to the laboratory and the percentage of the specimens collected in that month: 10 specimens of Ceratopogonidae (4.17%); 14 specimens of Lepidoptera (100%); 19 specimens of Muscidae (100%); 1 specimen of *C. obsoletus* Meigen, 1818 (100%); 1 specimen of Scatopsidae Newman, 1834 (100%) and 5 specimens of Chironomidae (1.78%)

Table 4.2 - Positive results for RHDV2 and MYXV.

Specimens	Code	Number of specimens	Positive for
Mycetophilidae	JS0220	61	RHDV2
Chironomidae	JS1218	5	MYXV
Ceratopogonidae	JS0119	10	
Lepidoptera	JS0119	14	
Muscidae	JS0119	19	
<i>C. obsoletus</i>	JS0119	1	
Scatopsidae	JS0119	1	

4.3 Relation between arthropod and climatic variables

With the data of IPMA - Instituto Português do Mar e da Atmosfera, it was possible to associate climatic variables (the local temperature/precipitation - Alenquer) to collected samples. The summary results of precipitation and mean air temperature are represented in Tables 4.3 and 4.4, respectively. All the figures taken from IPMA's website can be found in Annex 8.1.2.

Table 4.3 - Summary results of precipitation by month

Months	Precipitation (mm)	Months	Precipitation (mm)
December 2018	25 – 50	July 2019	1 – 5
January 2019	25 - 50	August 2019	5 – 10
February 2019	10 - 25	September 2019	10 – 25
March 2019	25 - 50	October 2019	25 – 50
April 2019	50 - 100	November 2019	50 – 100
May 2019	10 - 25	December 2019	50 – 100
June 2019	10 - 25	February 2020	5 - 10

Table 4.4 - Summary results of mean air temperature by month

Months	Temperature (°C)	Months	Temperature (°C)
December 2018	10 – 12	July 2019	22 – 24
January 2019	10 – 12	August 2019	22 – 24
February 2019	10 – 12	September 2019	22 – 24
March 2019	14 - 16	October 2019	18 - 20
April 2019	14 – 16	November 2019	14 – 16
May 2019	18 – 20	December 2019	12 – 14
June 2019	18 - 20	February 2020	12 - 14

The total precipitation during the possible comparable months regarding the abundance of specimens (December 2018 and December 2019) was higher in December 2019 (25–50mm vs 50-100mm) as well regarding the mean temperature - about 2 °C. The total precipitation between the Winter months of positive samples for the virus (December 2018, January 2019 and February 2020) and Springtime (April 2019) was lower (25-50mm and 5-10mm vs 50-100mm) but the mean temperature was higher in April 2019 (about 2 °C to 4 °C).

Although weather data was not collected at the survey site, during the months of February 2019 and February 2020 the precipitation totals were different (10-25mm vs 5-10mm) and the mean temperature in February 2020 was higher - about 2 °C.

The relation between arthropod and climatic variables are shown next through the Figure 4.1, where is possible to see that, although the positive results for viruses were in Wintertime (December 2018, January 2019 and February 2020), the numbers of specimens collected was greater in the Springtime (April 2019): Ceratopogonidae (more 57%); Lepidoptera (more 179%); *C. obsoletus* (more 7.8%); and Scatopsidae (more 4.3%). The exceptions were those concerning the family Mycetophilidae (-90%) and family Chironomidae (-72%), where there was a reduction of the number of specimens in Springtime; and in the family Muscidae where the numbers remain

the same. It is also possible to see that the abundance of these groups maintained in the other months of the same season.

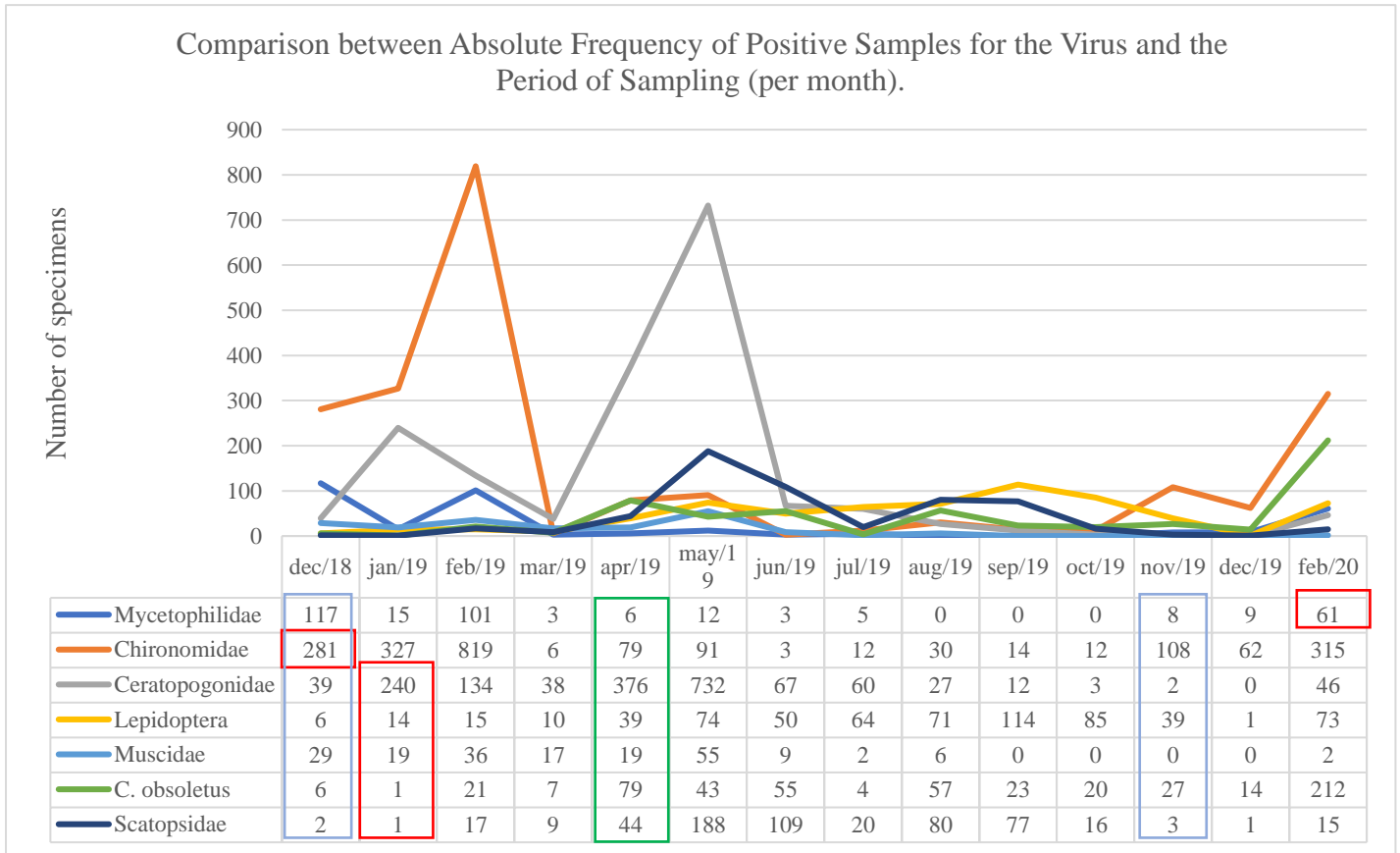


Figure 4.9 - Comparison between Absolute Frequency of Positive Samples for the Virus and the Period of Sampling (per month). The positive cases (in red) and the comparison with other month in other season - April 2019 – Springtime (in green); the abundance of the months in comparison - December 2018 and December 2019 (in blue).

5 Discussion

5.1 Arthropod Abundance, Dominance and Frequency

The order Diptera represented 95,37% of the total specimens collected. This observation was expected since, alongside with Coleoptera Linnaeus, 1758, this order ranks as one of the worlds' most numerous¹³² and the trap used in this work is manipulated to lure specially Diptera into the collection chamber.

The most abundant family was Psychodidae Newman, 1834 (57.06%) as expected, since they are widely distributed¹⁵⁶ and are most active at night, although they can also be seen during daylight and the females need a blood meal before reproduction¹⁵⁷. The results are concerning because the family Psychodidae includes species of public and veterinary importance: Psychodinae and Phlebotominae¹⁵⁶, known vectors for the transmission of leishmaniosis, Toscana virus and Vogt-Koyanagi-Harada disease¹⁵⁸, among others to humans and animals (including rabbits and hares)¹⁵⁹.

The full screened month with most captured specimens in a 15-day sampling was February 2019 (9.36%) with the family Chironomidae (29%) leading the most captured group just after the family Psychodidae (39%). The high numbers of the family Chironomidae are explained because they can be found in various environments, including temperate and tropical regions¹⁶⁰, in many freshwater habitats, have a wide range of tolerance to severe environmental conditions, for instance hypoxia, high salinity or low pH, making them one of the most abundant invertebrates¹⁶¹.

From all the collected families/genus/species, some are known vectors of pathogenic agents of public and veterinary importance, such as: those belonging to genus *Culicoides* - *Bluetongue virus* (BTV), *Epizootic haemorrhagic disease virus* (EHDV), *African horse sickness virus*, among others⁹⁰; family Drosophilidae - *Thelazia callipaeda*¹³⁴; family Culicidae - *Dirofilaria immitis*, *West Nile virus*, potentially MYXV, among others^{112,118,122,162}; and family Calliphoridae – Myiasis and potentially *Rabbit Haemorrhagic Disease virus*⁷⁹. With all these captures, is significant the scientific warning of this goal's work: find potential vectors of Viral Haemorrhagic Rabbit Disease - genotype 2 (RHDV2) but also find new vectors of Myxomatosis. Drosophilidae specimens are common in most households, flying around or crawling on overripe fruit. Though the flies are generally harmless, some species, especially *Drosophila repleta* Wollaston, 1858, are a potential vector by mechanical transmission of pathogens, since they can breed in animal faeces^{87,134}.

Although it was not possible to make a statistical analysis to compare the samplings from February 2019 and February 2020 – because in 2019 the sampling was done for 15 days and in 2020 for the full month – there was a massive increase in the abundance of specimens captured (more 72.54%), with 2,857 in February 2019 and 10,406 in February 2020, while the diversity of families/genus/species was comparatively similar (28 family/genus/species in February 2019 vs 30 family/genus/species in February 2020), corresponding a growth of 7.14%.

5.2 Viruses' detection and quantification

Concerning the positive finding of RHDV2 in Mycetophilidae captured in February 2020, despite the abundance has been rather inferior of the February 2019 captures (61 vs 101 specimens), the potential mechanical transmission of the virus by the specimens is equally

relevant¹¹⁷, as referred in other works^{10,52,66,79,85,86}. However, more work is needed to prove this hypothesis; for instance, it is essential to keep these insects in contact with the virus, permit their posterior contact with the rabbit at the laboratorial level and wait for those rabbits to show clinical signs, so we could say that these insects are mechanical vectors of this virus.

Still regarding these positive findings of RHDV2, family Mycetophilidae requires further studies on its ecological role and to assess its economic significance¹⁶³. Although fungus gnats have long been considered as pests, larvae of this family have also been responsible for infesting cultivated mushrooms causing extensive damage¹⁶³ or by serving as vectors of nematodes¹⁶⁴. The role of this family in decomposition may be more decisive than generally recognised. For instance, scientific community believes that they are responsible to carry putrefactive microorganisms into the decaying material¹⁶⁵ and may also stand as great pollinators of certain flowers, especially orchids¹⁶³. With this information is important to be aware of the results of this work, especially the potential vectorial competence of this family and importance of making more laboratory work regarding this matter, as previously mentioned.

Focusing on the positive findings of MYXV, the vectorial competence, efficiency and capacity of some specimens collected are different and known, and the results can be explained by the potential biological and/or mechanical transmission as presented by some previous studies^{78,81,94,97}.

Concerning the Chironomidae, only 5 specimens of the 15 analyzed were positive. Nevertheless, to confirm this result, because a contamination of the samples may have occurred, and, as said previously, giving the possibility of their vectorial competence, efficiency, and capacity, more laboratory experiments are needed.

As well as seen in family Chironomidae, the same happened during the screening of the Ceratopogonidae samples: of the total of 15 specimens analysed, 10 specimens were positive. On contrary of the family Chironomidae, these results are worrisome because rather different quantitative factors influence the possible transmission of myxomatosis⁹⁸ by Ceratopogonidae and *C. obsoletus* (referred in east region of Alentejo)⁹⁰.

The positive result found in a *C. obsoletus* specimen is particularly interesting, since the genus *Culicoides* is best known for being important for Veterinary Medicine and Public Health, as hematophagous females are known vectors of viruses, protozoans and filarial nematodes and *C. obsoletus* species is known to have a host preference for *Oryctolagus cuniculus*⁹⁰.

Little is known about the family Scatopsidae, and the individuals of this family are commonly known as "dung midges", being distributed throughout the world and its larval stages are present in decaying plant and animal material¹⁶⁶. Regarding family Muscidae, as said before, only a few of these contains medical or veterinary importance, due to its vectorial capacity. Some species of this family are blood-feeding parasites, vectors of disease agents, such as brucellosis, anaplasmosis and summer mastitis, parasitizing domesticated animals and wildlife¹³³. Thus, the need for more in-depth studies on the real vectorial capacity of these families is urgent to sustain the results presented.

Lepidoptera adults have a behaviour known as puddling and some are even hematophagous¹³⁷, since they have been observed piercing the skin of their hosts during

feeding^{142,143}. These behaviours have negative implications on hosts' health - localized irritation and inflammation¹³⁷- giving evidence to make hematophagous and lachryphagous Lepidoptera a potential vector for pathogen agents, though this has never been documented¹³⁷. Being this behaviour one of the possible explanations for the positive result given in this work, more investigation in this field is needed to support the theory that these insects are vectors of MYXV.

5.3 Relation between arthropod and climatic variables

For these two diseases (RHDV2 and MYXV), temperature and humidity appear to be the most important climate variables. Studies in Australia have shown that the mortality rates are high, occurring, for RHDV2, in early spring and being absent in the summer - only becoming active during the breeding season – and, for MYXV, during early summer or autumn¹⁶⁷. Many survivors of MYXV breed during autumn or early winter but many die due RHDV2 before raising their litters¹⁶⁷.

The ecology and reproduction of European hare is known and the specimens of this species are mostly nocturnal; however, they may start feeding in midafternoon or in summer can be seen during the day¹⁶⁸ during the breeding season which is continuous, producing eight litters a year, starting near the winter solstice¹⁵, but having its peaking in March and April¹⁶⁸.

The European rabbit is notoriously fertile; it is likely to breed opportunistically, at any season, from January to August, with peaks in spring - when pasture production is maximal - which contributes to its success as a colonist, like it is seen in many countries¹⁵.

Regarding the results presented in this study, climate variables may contribute to the geographic and seasonality stated for the RHDV2 and MYXV outbreaks by influencing the abundance and activity of the vectors involved in RHDV2 and MYXV transmission²⁹. It is well known and recognized by the scientific community that the climatic variations, especially temperature, disturb the life cycle history of arthropods. This phenomenon have particular importance when dealing with vector-borne diseases, since it has been found that nearly all biological processes (i.e the biting rate, the pathogen incubation rate and the mortality rate) happen at a quicker rate at higher temperatures, although not all processes change in equal manner¹⁴⁴.

A possible explanation for the increase of the number of positive specimens found during Springtime is that the presence of arthropods is influenced by the interactions between biotic and abiotic factors – as precipitation and mean temperature^{144,150}.

Knowledge of the thermal biology of species may help understand the population dynamics, especially their distribution and abundance, and may even provide insight into the epidemiology of the pathogens that they transmit¹⁶⁹.

The specimens of Mycetophilidae rest during daylight, but can be active during bright, moist conditions. Commonly found during autumn, they are extremely numerous when most other species are on decline. In winter they may be collected in heavy concentrations in patches of evergreen vegetation^{129,163}, which justifies the high numbers during the winter presented in this study.

Chironomidae are among the most abundant invertebrates in freshwater environments¹⁶¹, being distributed and ecologically adapted to many environments¹⁶⁰. During autumn is when this family has a highest density of larvae, while the lowest density occurs during winter time¹⁷⁰. The insects are known to tolerate harsh environmental conditions, such as low temperatures¹⁶¹. Thus, the results were expected for this family, since for most species the overwintering capability is not restricted, and, in some regions, development may continue throughout the winter¹⁶¹.

Culicoides – including *C. obsoletus* – are known to not tolerate low temperatures – although they can be found in negative temperatures - giving evidence that thermal physiology may be a crucial factor in their distribution and abundance¹⁶⁹. A variety of factors, including effects of temperature, can influence oviposition, survival and vectorial capacity¹⁷¹. Therefore, this behaviour can explain the results obtained, although not all *Culicoides* species can tolerate higher temperatures, like *C. sonorensis*, that decline as temperatures increase, as shown in a previous study¹⁷².

Despite the results obtained regarding total precipitation and mean temperature, is not scientific precise to make accurate conclusions, because studies that relate climate to the presence of certain insects must be done locally.

5.4 Sustainable development goals

The world is handling the worst public health and economic crisis in a century, making COVID-19 responsible for severe negative impacts on most SDGs. The crucial measures taken to respond to the immediate threat of COVID-19 led to a global economic crisis. This is a major obstacle for the world's ambition to achieve the SDGs. The only positive point in this dark picture is the reduction in environmental impacts resulting from industrial activity. However, all long-term effects of the pandemic remain very much unclear at this point¹⁷³.

5.5 Study limitations

Some limitations occurred during this dissertation, such as the time limitation to identify all the thousands of specimens collected. Thus, there was a need to subsample up to 1000 specimens per month, as explained in the methodology. Nevertheless, the diversity of specimens was identified, and the methodology was valid and accurate.

In order to avoid degradation of eventual viruses in the specimens, during the morphological identification, a Petri dish with ice was used. However, as it was not electric, it had to be replaced regularly and may have had an impact on the quality of the molecularly identified samples.

Due to safety reasons and not to cause stress to the rabbits, the author was not allowed to visit the facilities to take photographs nor to collect samples.

It is well known that COVID-19 was recognized by the WHO as a pandemic on March 11, 2020. In this context, several measures were taken to contain the spread of SARS-CoV-2/COVID-19 infection. One of these measures was the closing of many universities, including both Faculty of Veterinary Medicine and Faculty of Sciences of the University of Lisbon in which Parasitic Diseases laboratory, Entomology Laboratory and the Advanced Signal and Image Processing

Laboratory are. With the laboratories closed it was impossible to perform any work for a total of almost 6 months, although all the samples and materials were properly conserved.

Additionally, the detection and quantification of the viruses was affected by COVID-19 since most PCR machines were requested by health authorities to help during COVID-19 crisis when Portugal entered in emergency state. Once this point has been surpassed, the collaborator who performed these analyses found it impossible to carry out all the planned analyses, since the reagents used to perform the PCR tests were sold out worldwide.

6 Conclusion

The present work allowed the collection of data concerning different arthropods species captured in a rabbit hutch, showing their abundance, dominance, and frequency.

Since in the captures performed during February 2020, the family Mycetophilidae was positive for RHDV2; and in the captures made during January 2019 the order Lepidoptera, the families Ceratopogonidae, Muscidae, Scatopsidae and the species *C. obsoletus* were positive for MYXV, as well as for family Chironomidae captured in December 2018, is important to have more studies regarding the abundance, dominance, and frequency of arthropods.

Economic loss and vector-borne diseases are a major problem nowadays, as well as climate change and the emerging of new vector-borne diseases; for that, it is evident the necessity of more studies in order to collect, detect and prove by laboratory and field work, new vectors of vector-borne pathogens of Public and Animal health importance, in this case, RHDV2 and MYXV.

Through this methodology it was expected to show the species competence to carry the viruses, through mechanical or biological transmission. Further studies are needed to prove that these species can actually transmit the virus through mechanical or biological ways, but this point did not fit under this work.

Despite the results obtained regarding total precipitation and mean temperature more field work is needed and, to be properly analysed, thermometers and rain gauges must be placed in the sampling area to allow for more robust conclusions concerning the relation between the presence of some arthropods and these abiotic factors.

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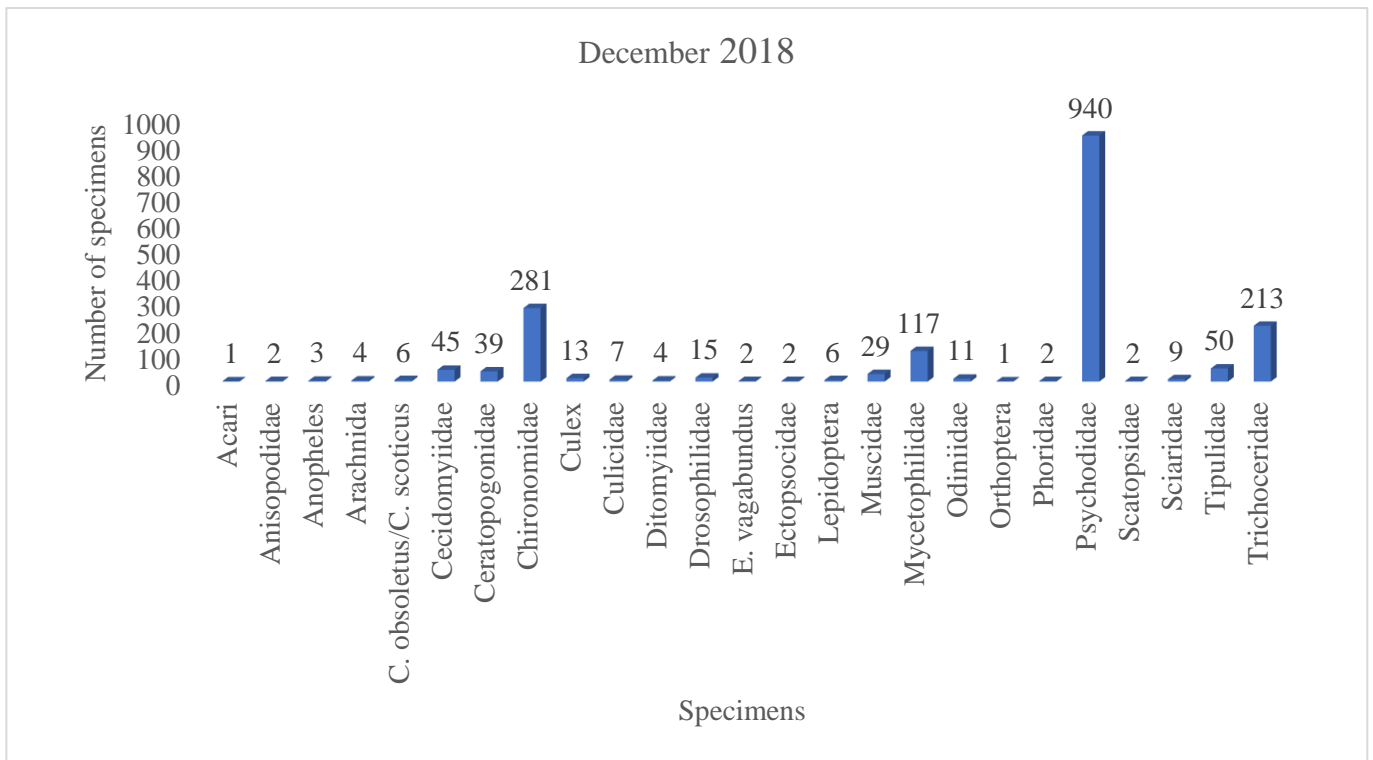
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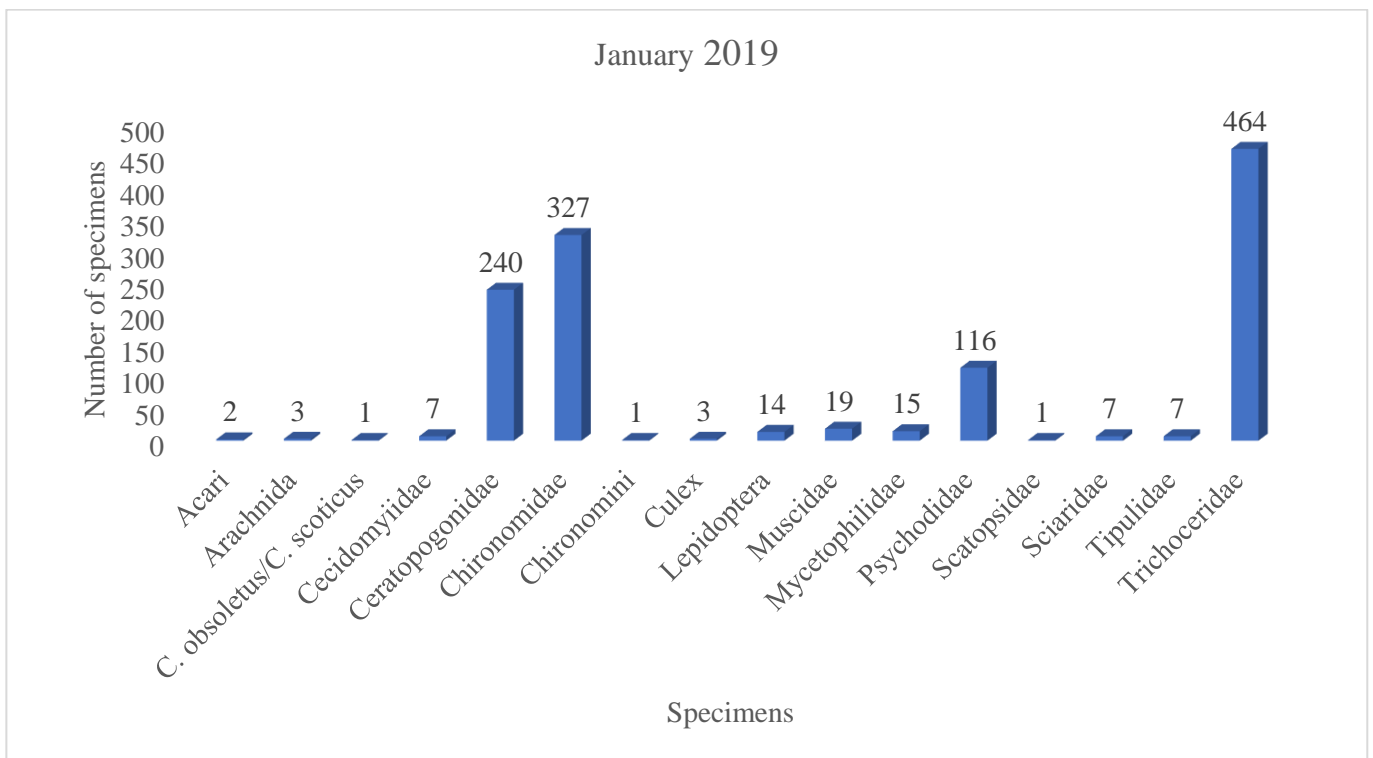
8 Annexes

8.1 Data analysis

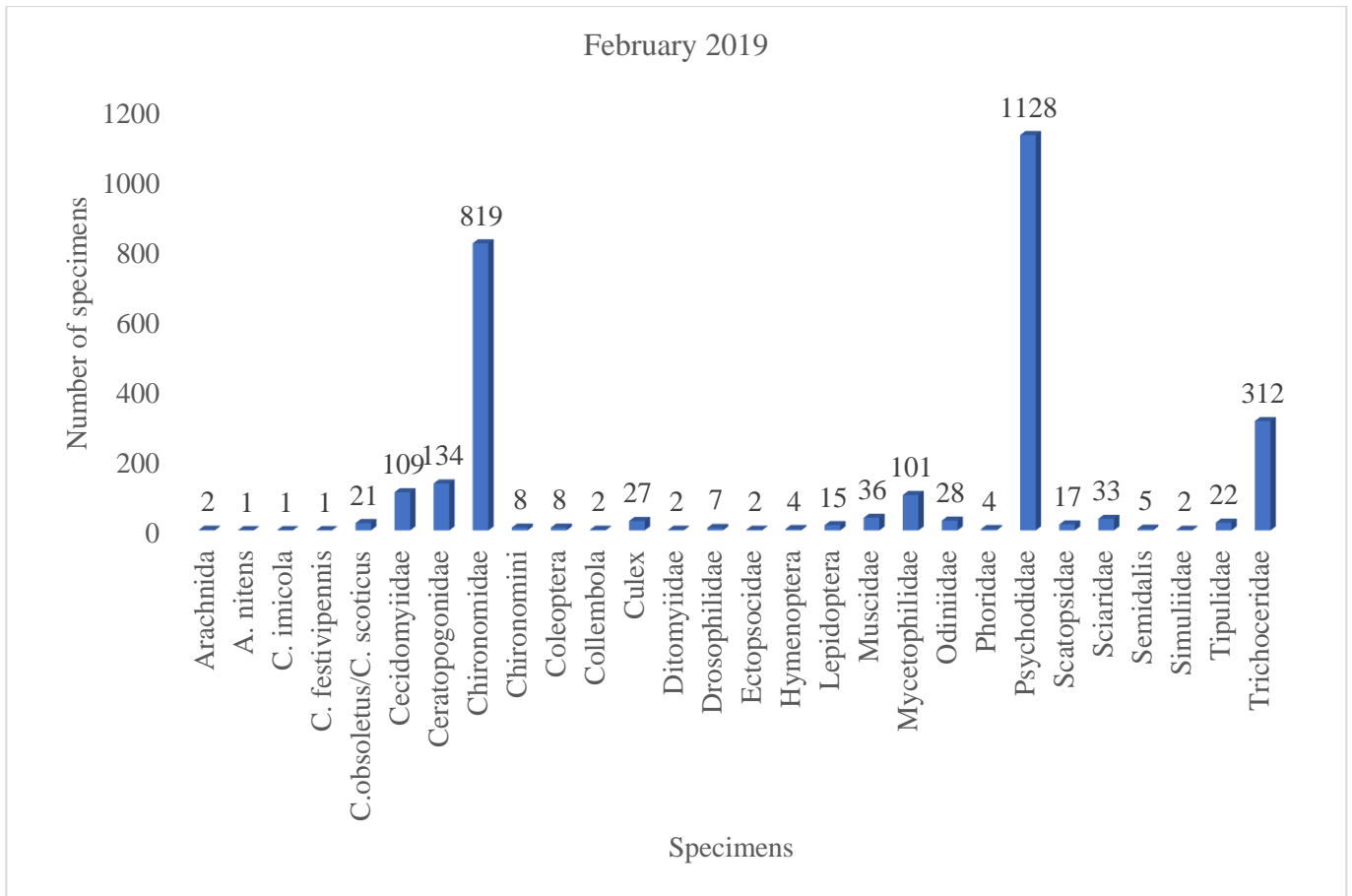
8.1.1 Collected specimens and morphological identification by family/genus/species.



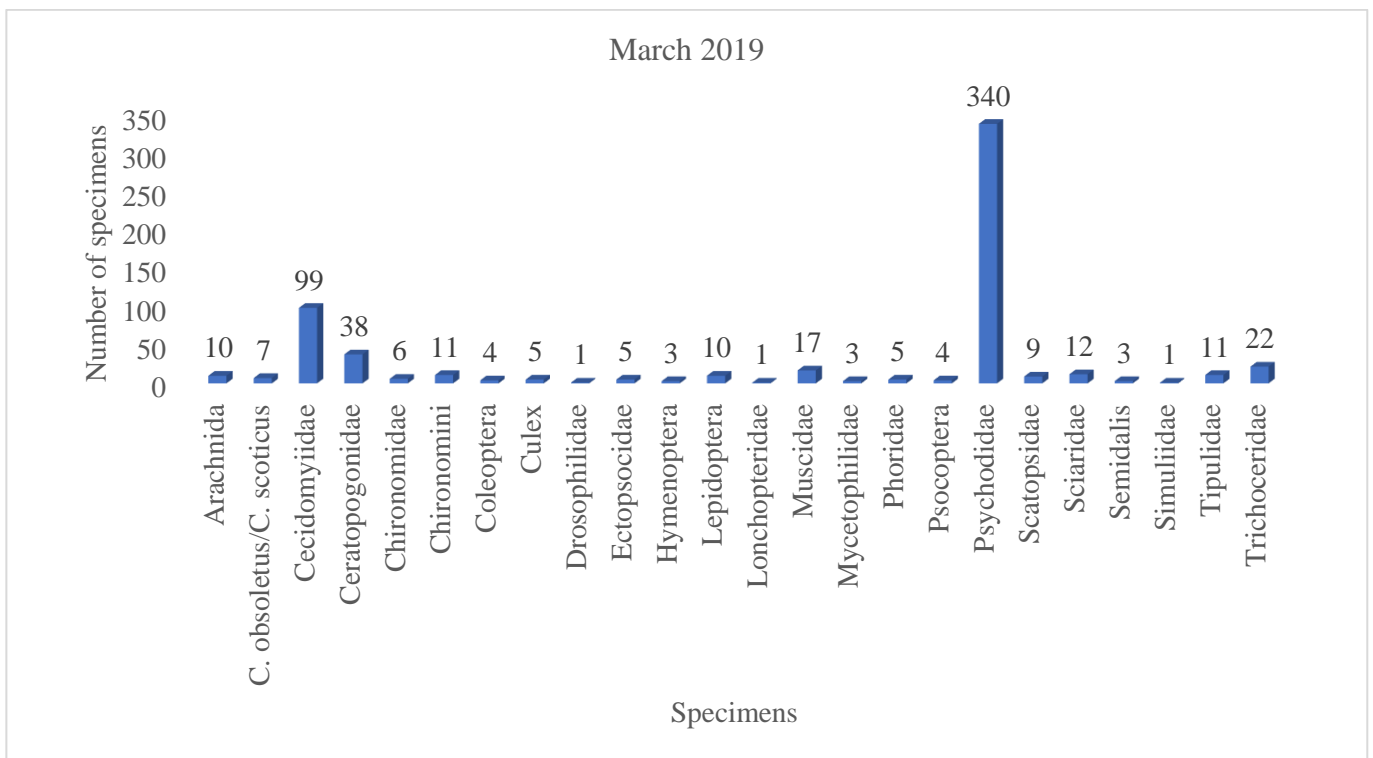
Annex 1a - Specimens collected in December 2018.



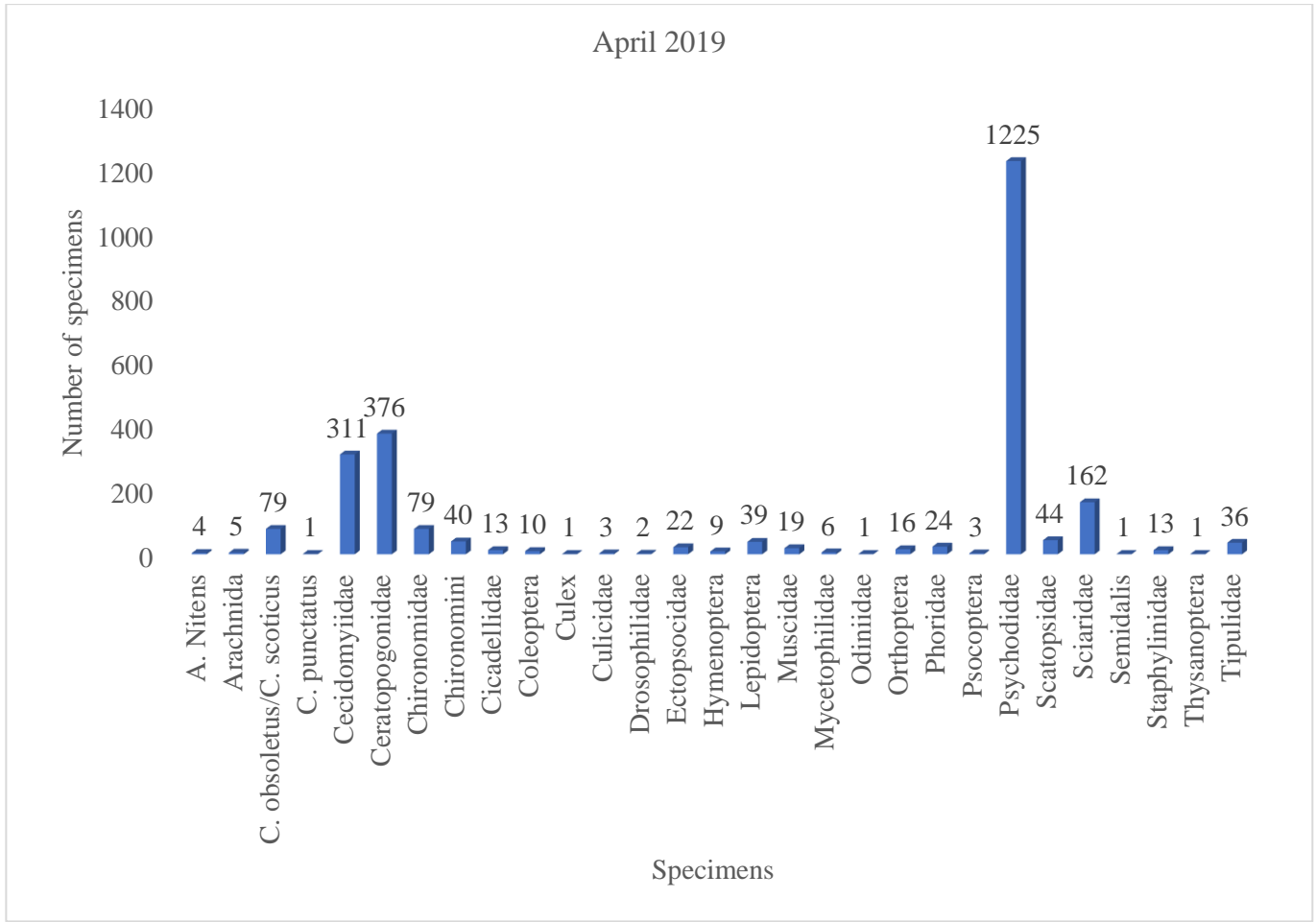
Annex 1b - Specimens collected in January 2019.



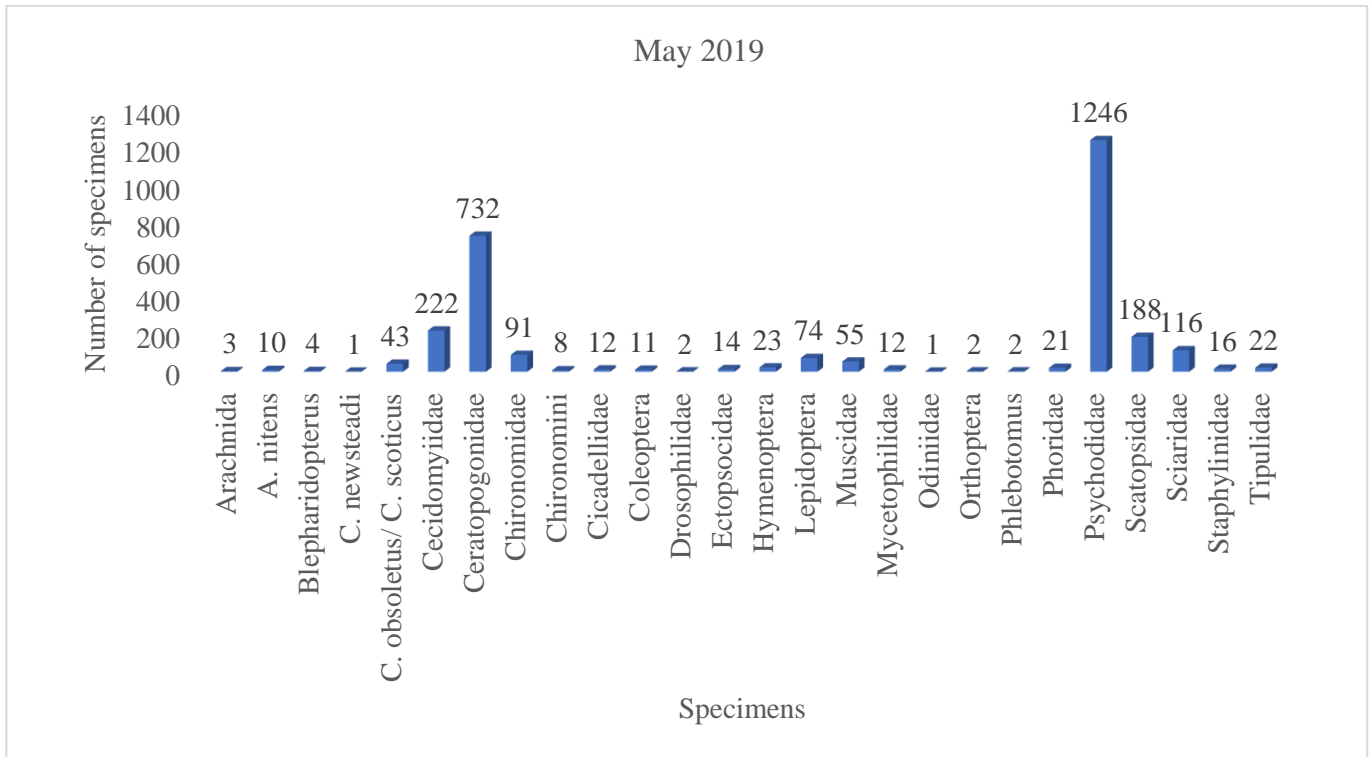
Annex 1c - Specimens collected in February 2019.



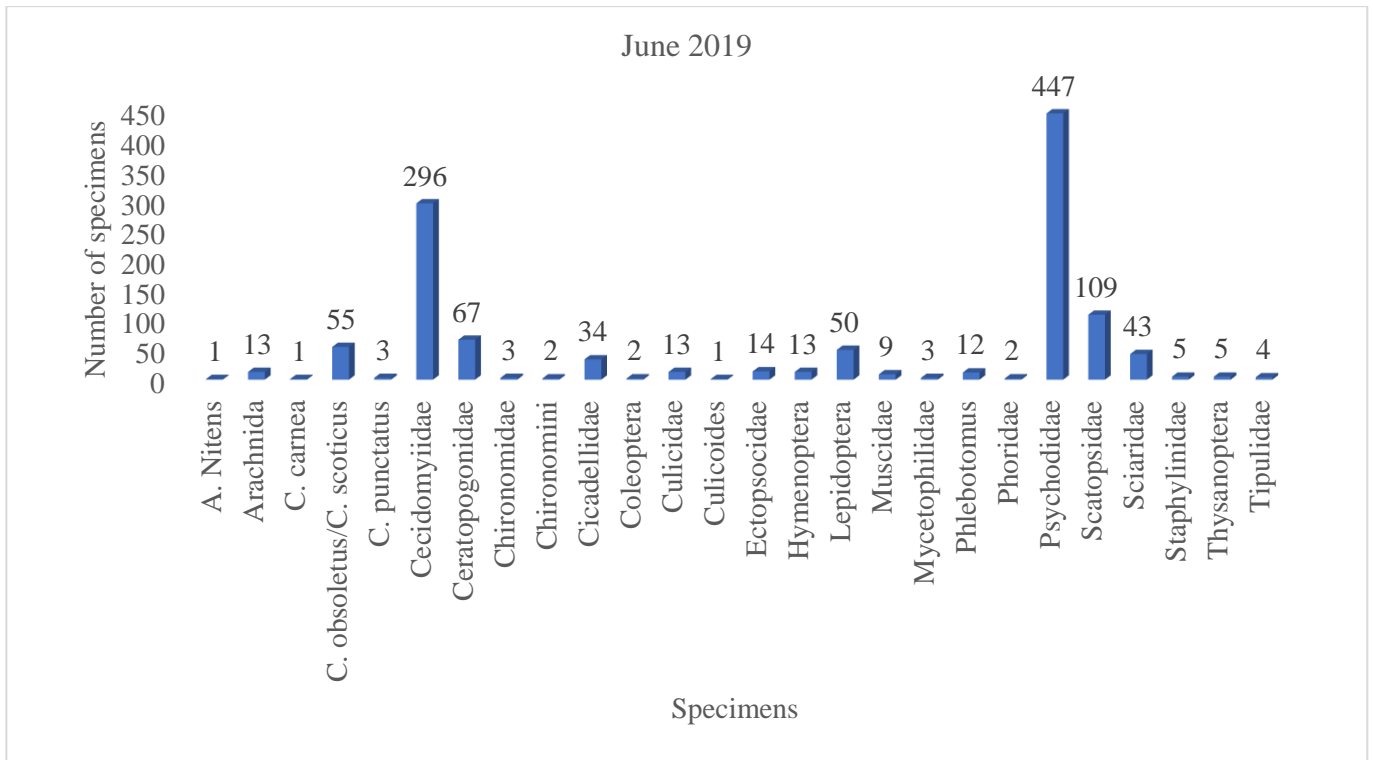
Annex 1d - Specimens collected in March 2019.



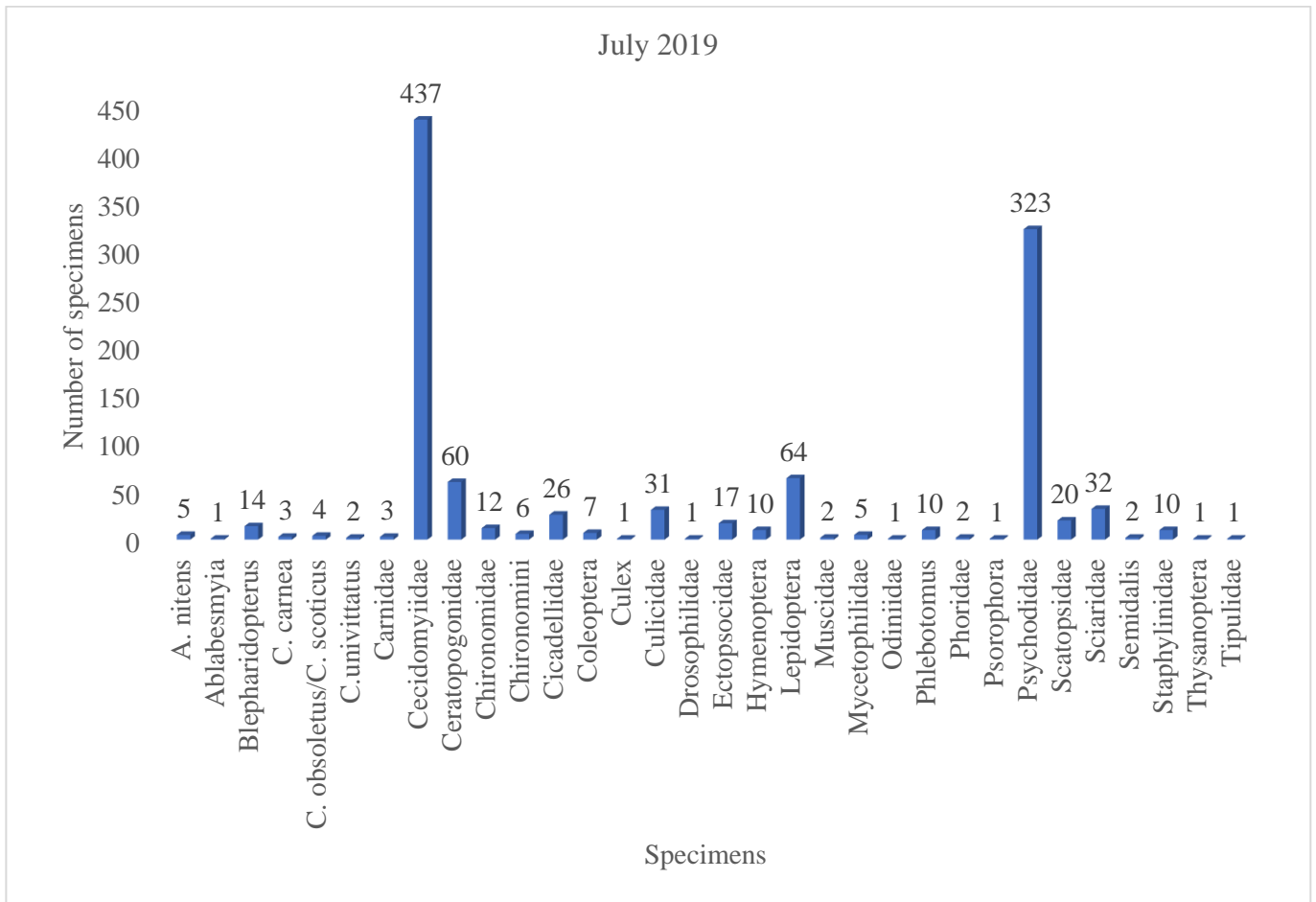
Annex 1e - Specimens collected in April 2019.



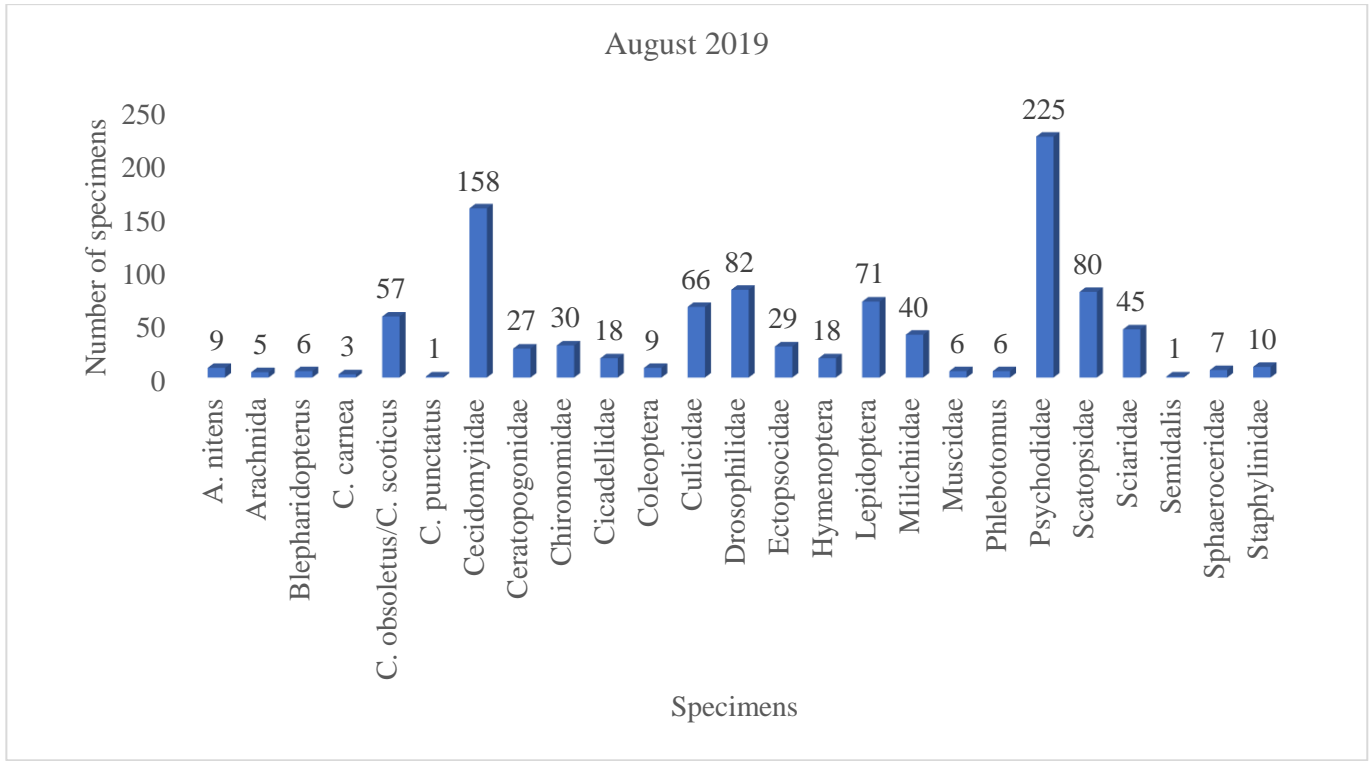
Annex 1f - Specimens collected in May 2019.



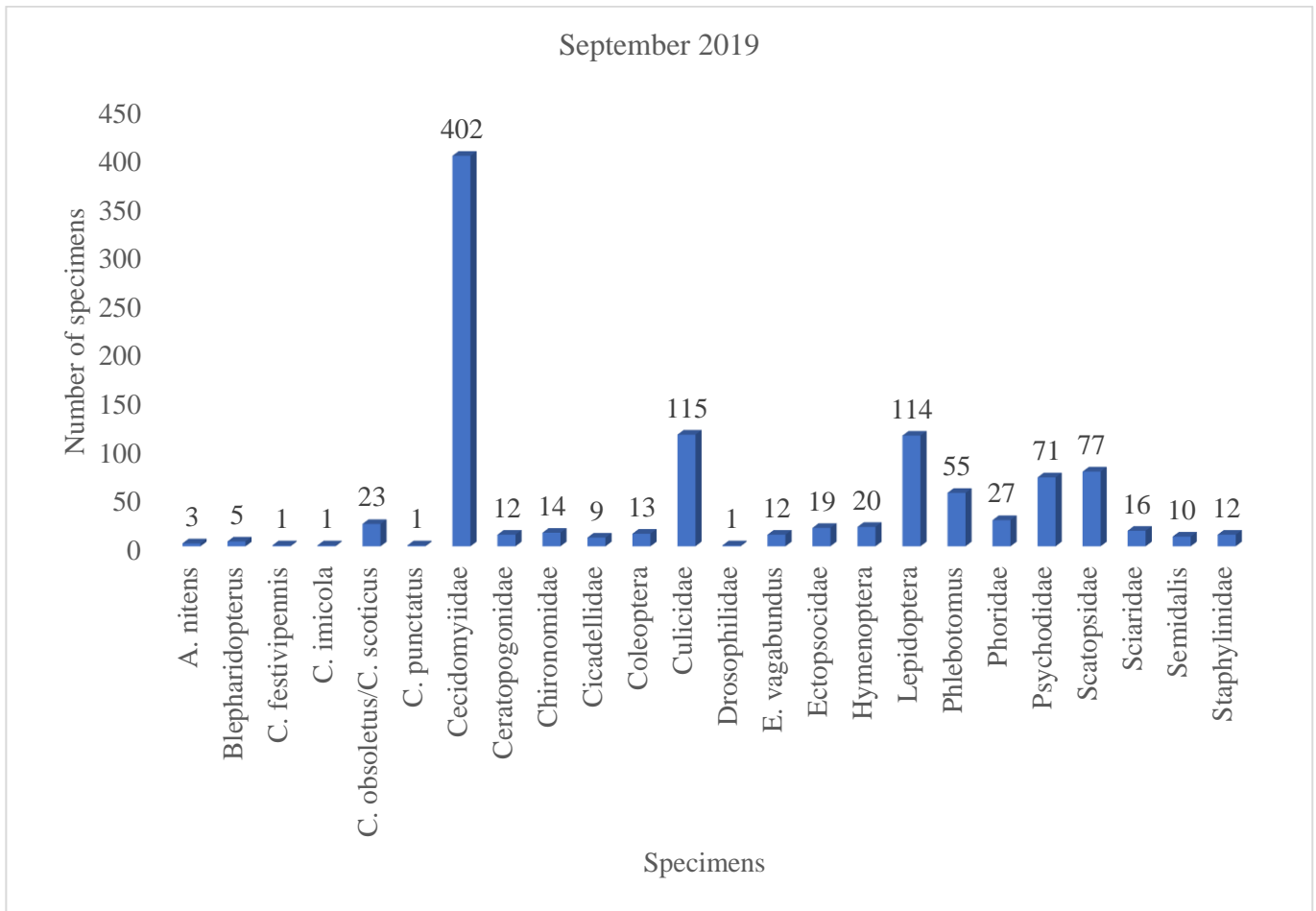
Annex 1g - Specimens collected in June 2019.



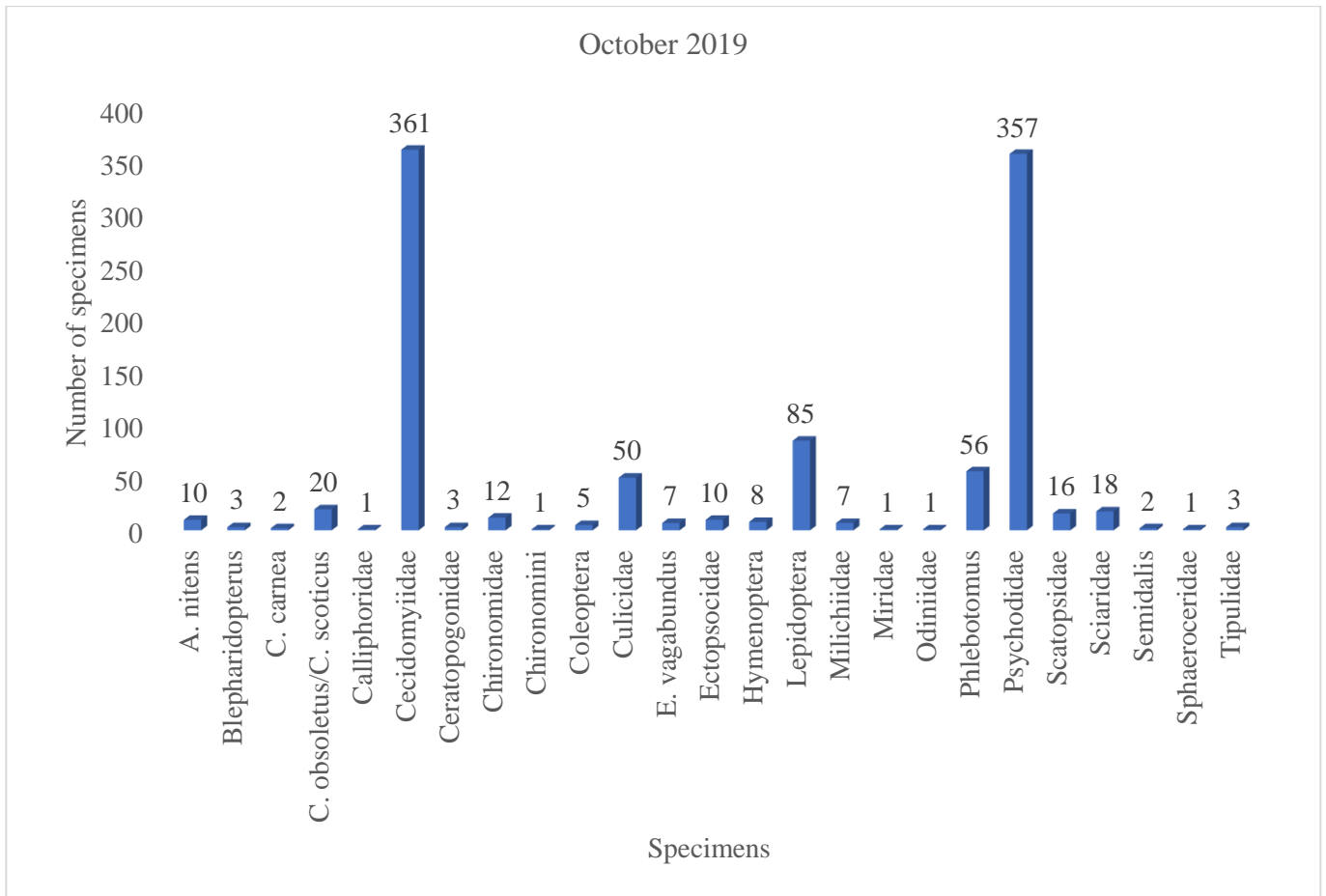
Annex 1h - Specimens collected in July 2019.



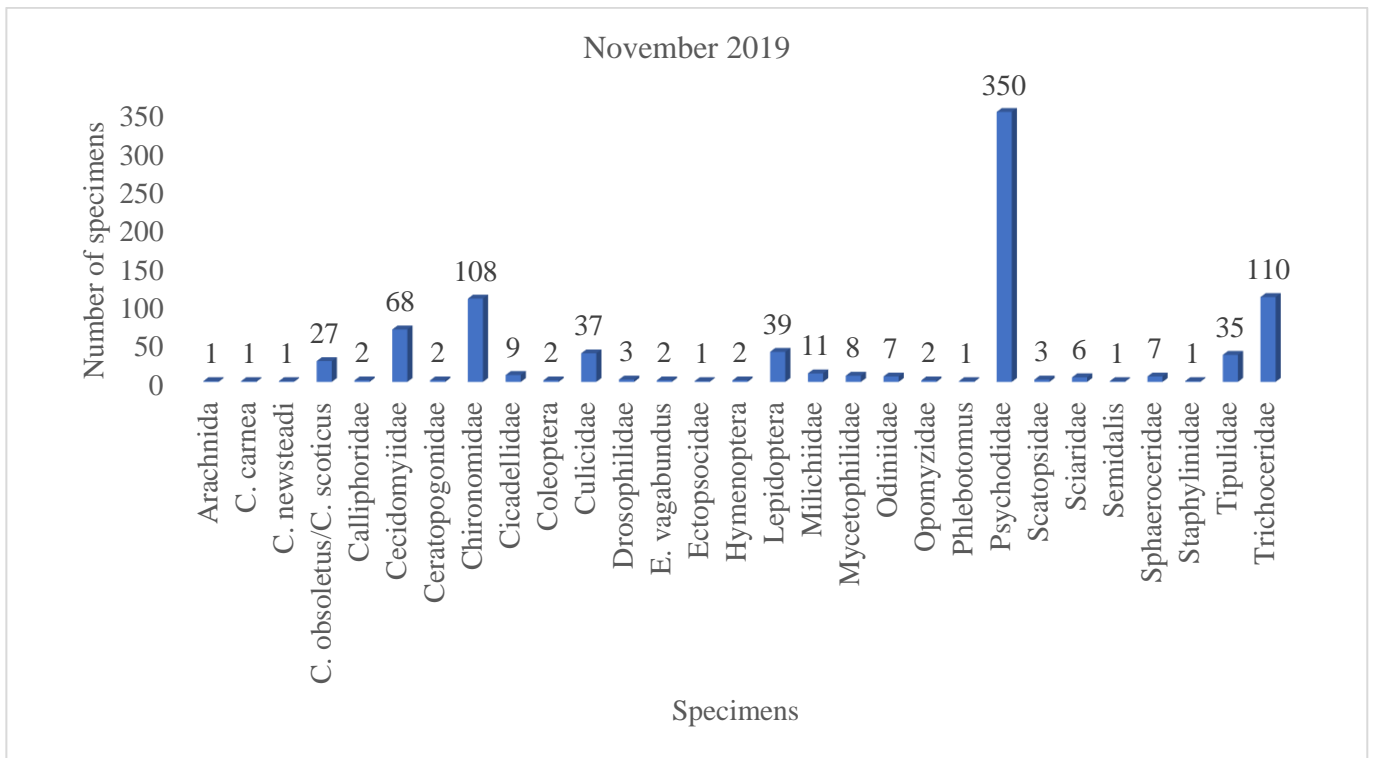
Annex Ii - Specimens collected in August 2019.



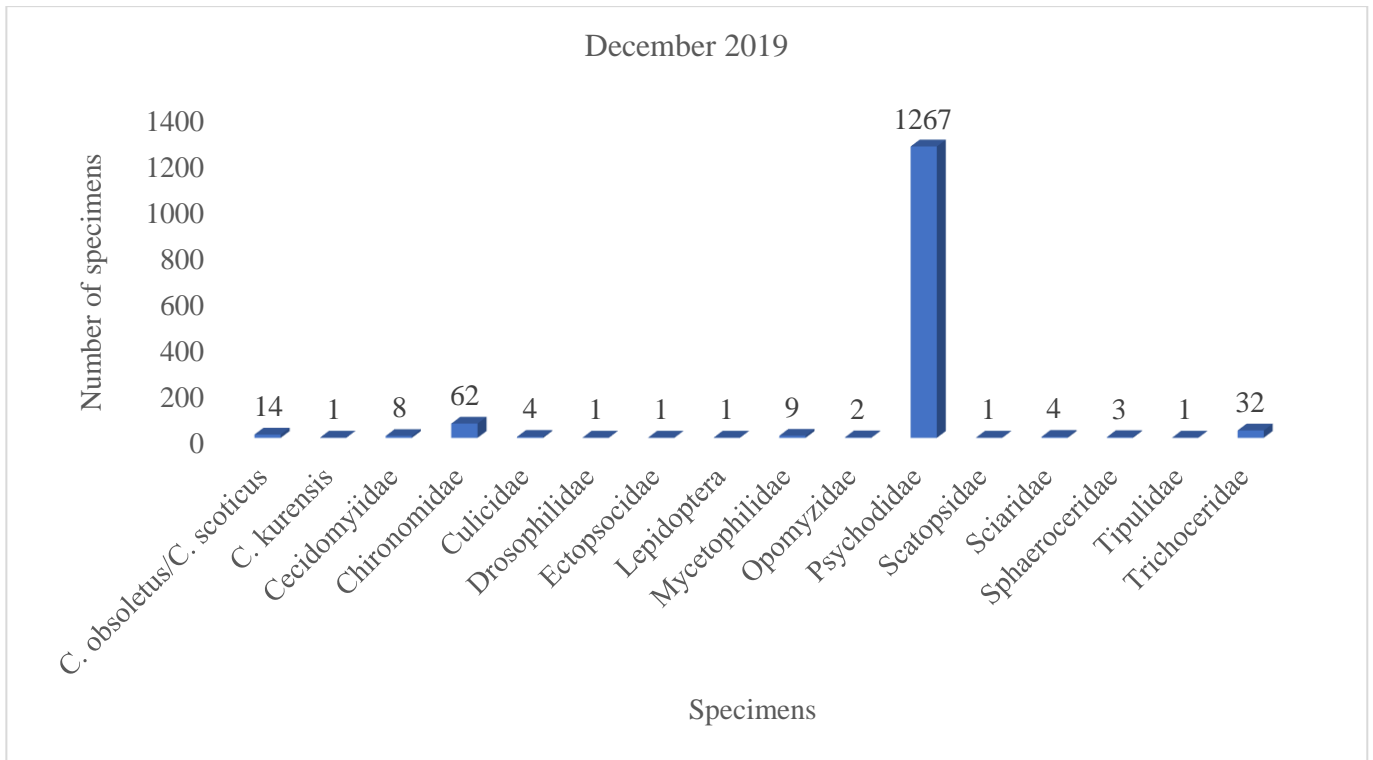
Annex Ij - Specimens collected in September 2019.



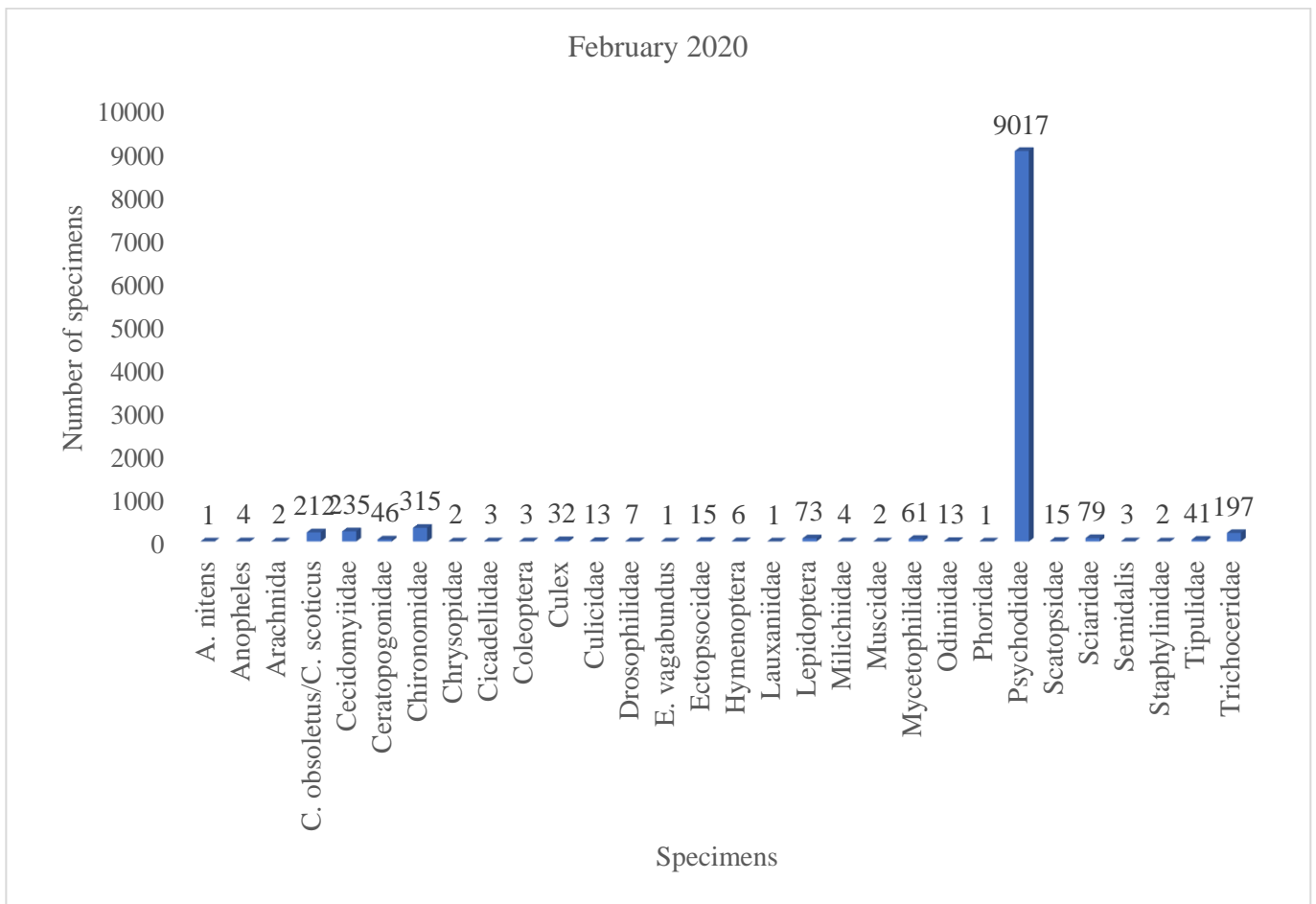
Annex 1k - Specimens collected in October 2019.



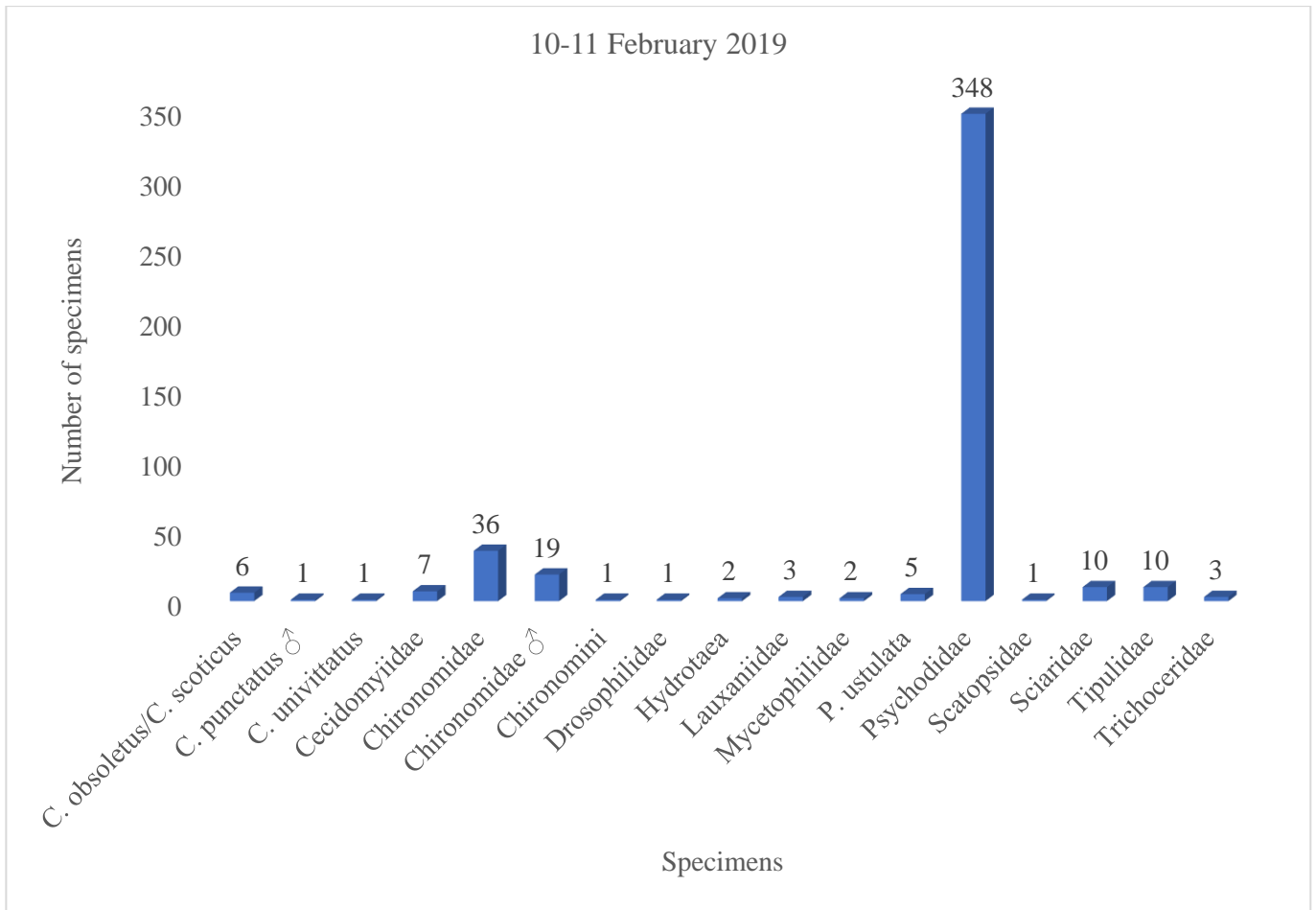
Annex II - Specimens collected in November 2019.



Annex 1m - Specimens collected in December 2019.



Annex 1n - Specimens collected in February 2020.

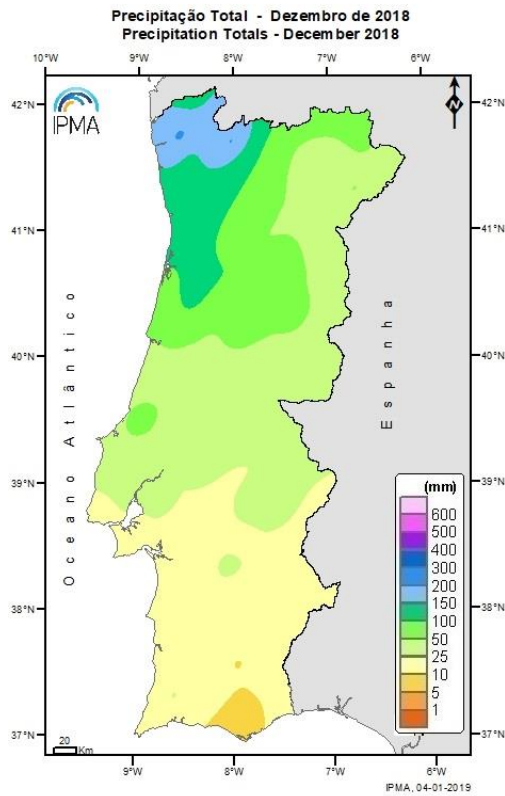


NOTE: All those individuals who had plumose antennae, were joined and identified as "Chironomidae ♂". In the case of "Chironomidae 2", there were 3 individuals that were identical to each other but different from those present in "Chironomidae" and "Chironomidae ♂".

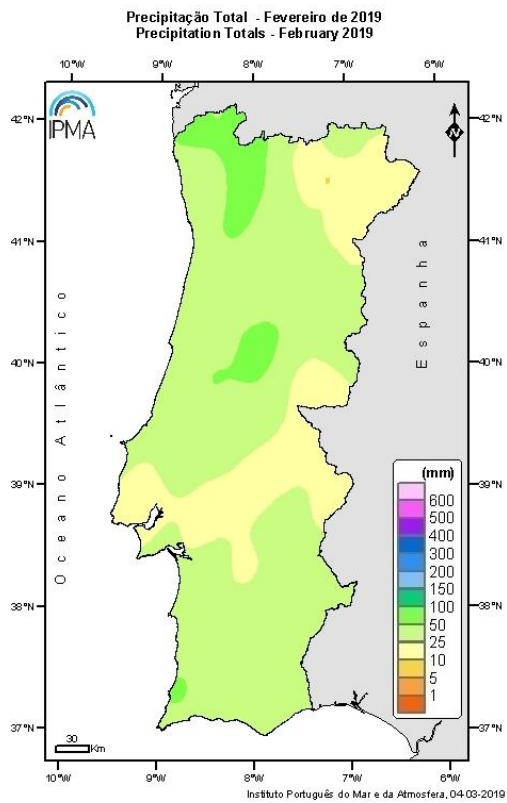
Annex 1o - Specimens collected in 10-11 February 2020.

8.1.2 Data collected from IPMA's website - Precipitation.

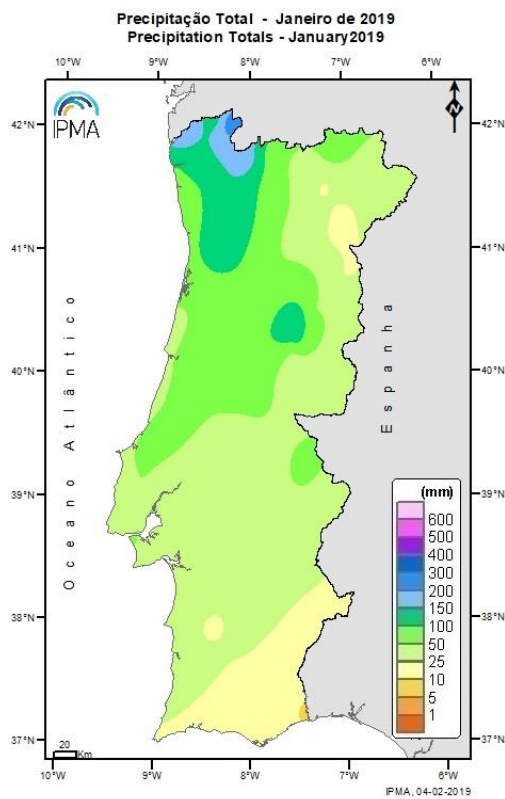
Annex 2a - Precipitation totals, December 2018 – IPMA, 2019



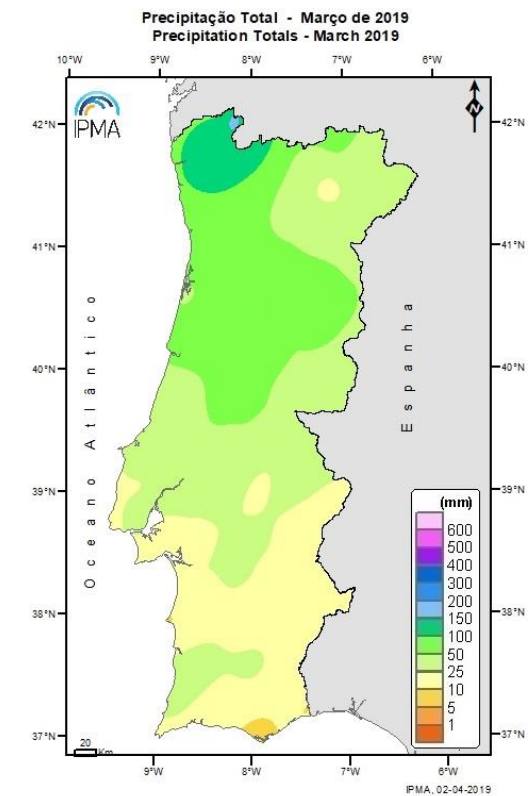
Annex 2c - Precipitation totals, February 2019 – IPMA, 2019



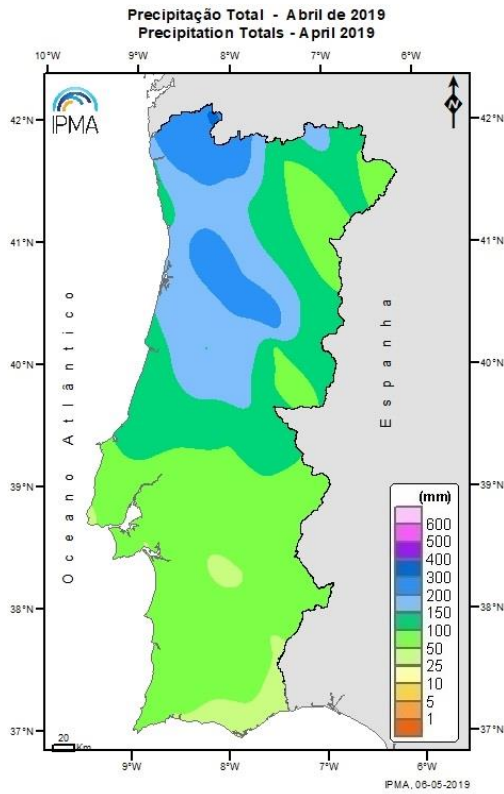
Annex 2b - Precipitation totals, January 2019 – IPMA, 2019



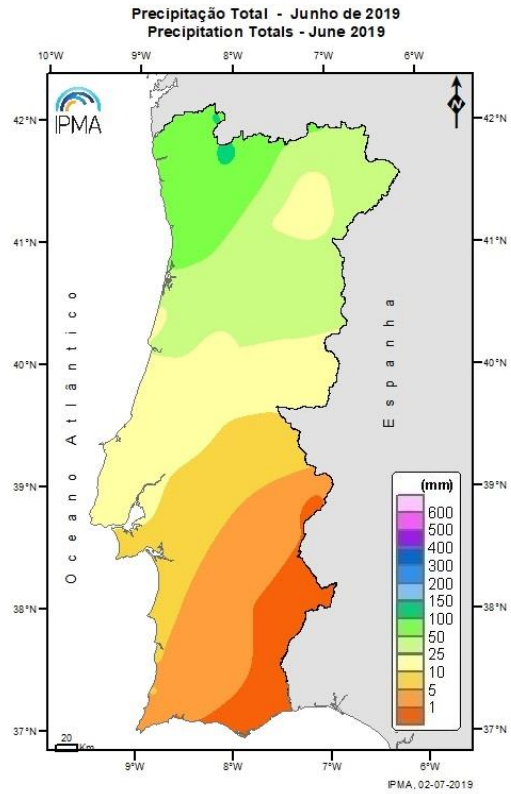
Annex 2d - Precipitation totals, March 2019 – IPMA, 2019



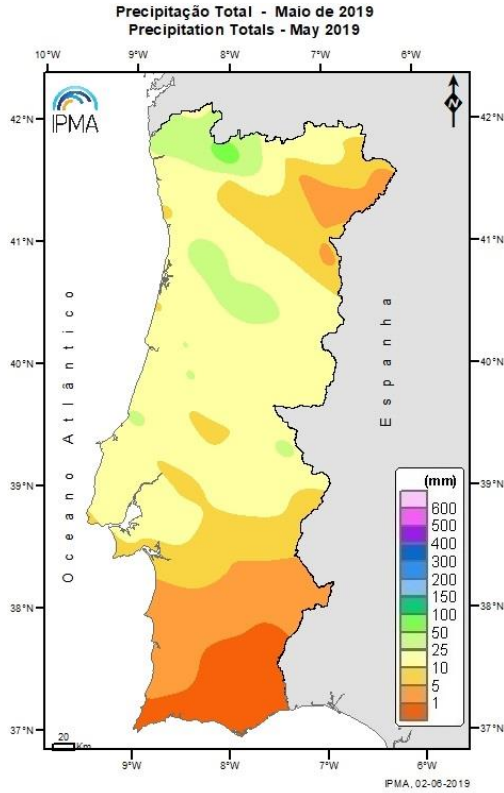
Annex 2e - Precipitation totals, April 2019 - IPMA, 2019



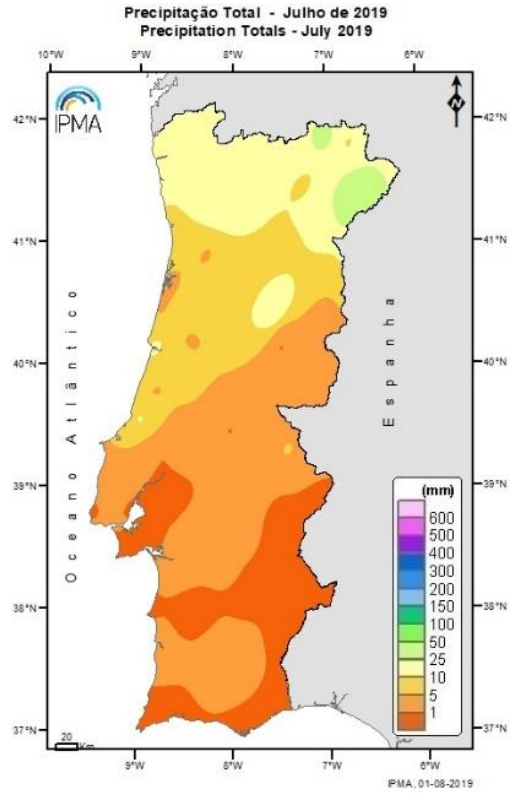
Annex 2g - Precipitation totals, June 2019 - IPMA, 2019



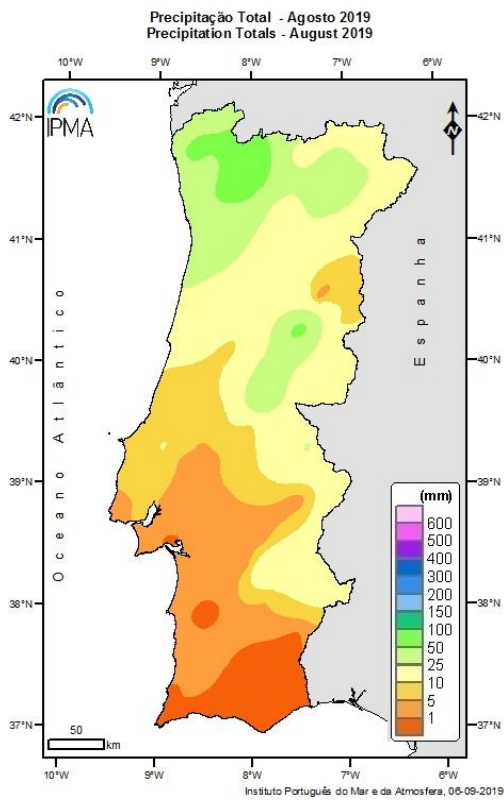
Annex 2f - Precipitation totals, May 2019 - IPMA, 2019



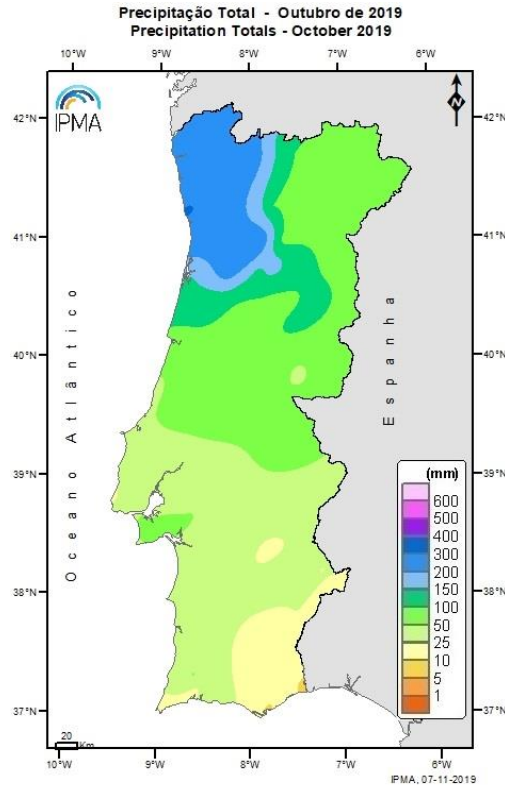
Annex 2h - Precipitation totals, July 2019 - IPMA, 2019



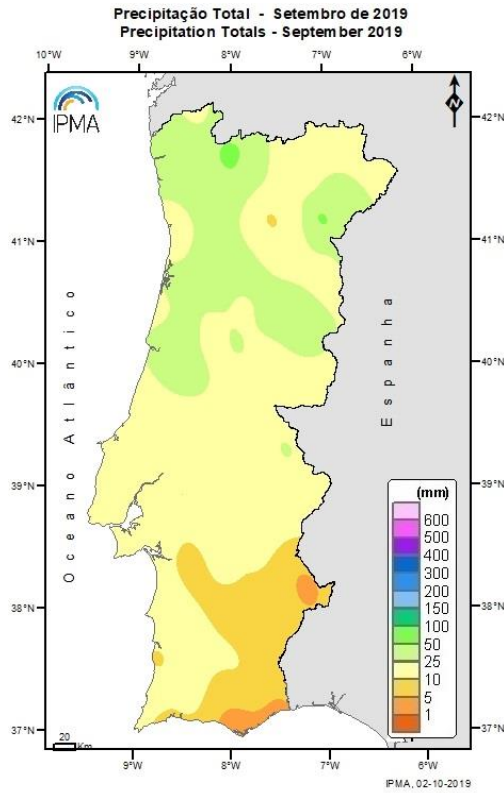
Annex 2i - Precipitation totals, August 2019 - IPMA, 2019



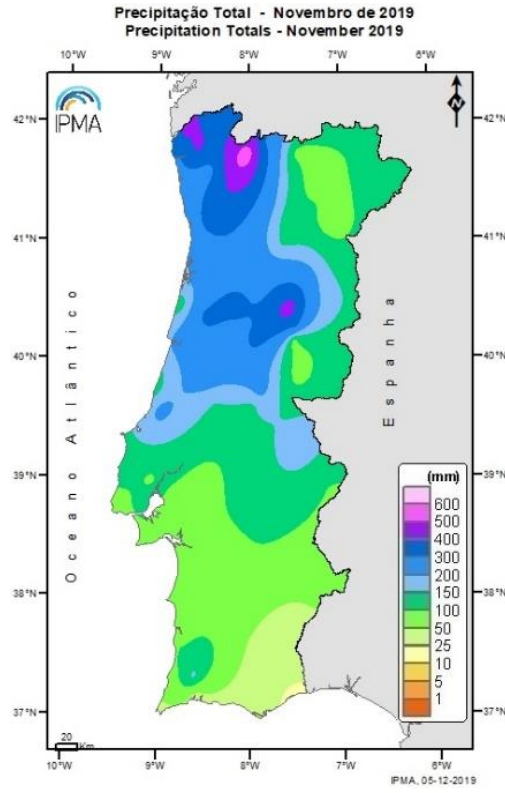
Annex 2k - Precipitation totals, October 2019 - IPMA, 2019



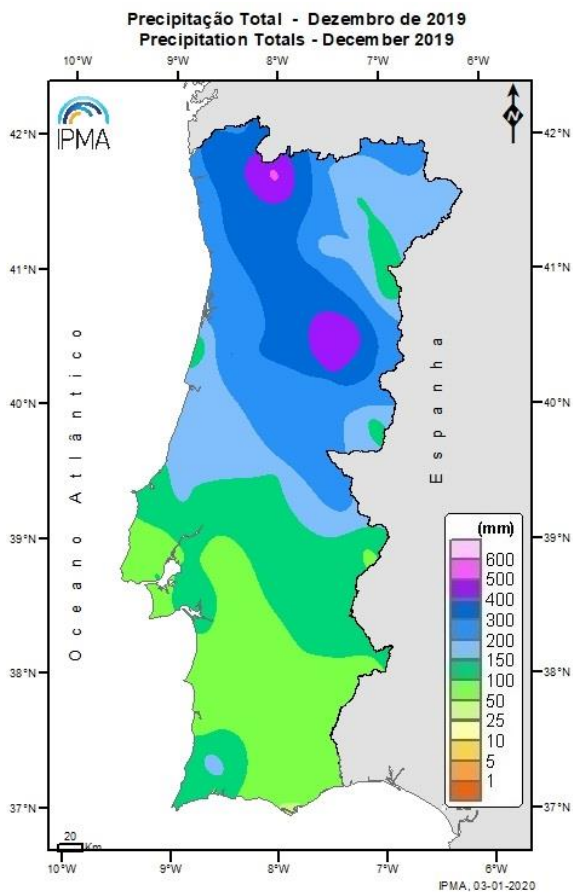
Annex 2j - Precipitation totals, September 2019 - IPMA, 2019



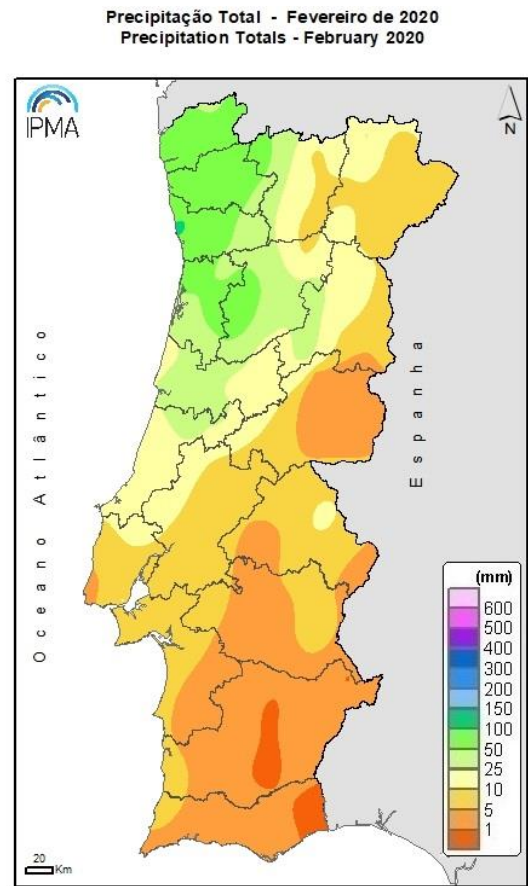
Annex 2l - Precipitation totals, November 2019 - IPMA, 2019



Annex 2m - Precipitation totals, December 2019 - IPMA, 2020

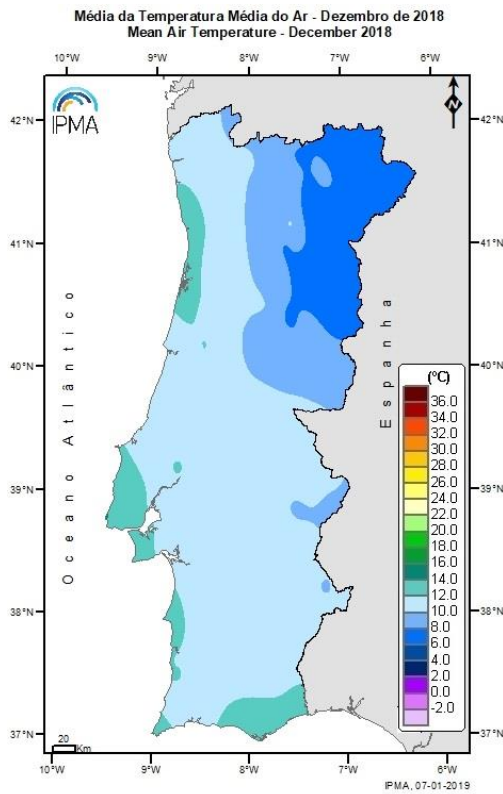


Annex 2n - Precipitation totals, February 2020 - IPMA, 2020

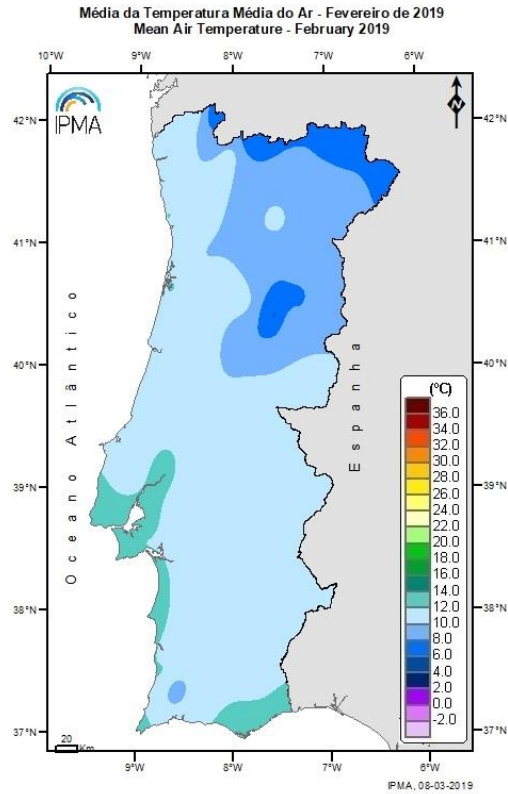


8.1.3 Data collected from IPMA's website – Mean air temperature.

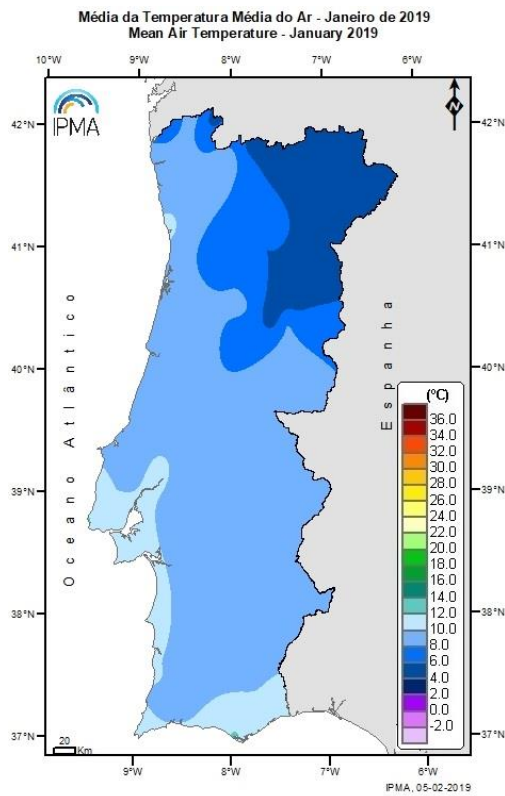
Annex 3a - Mean air temperature, December 2018 - IPMA, 2019



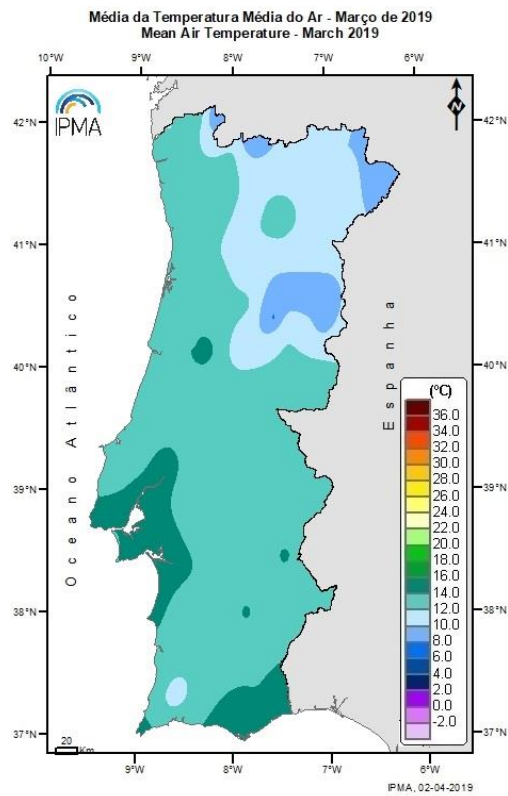
Annex 3c - Mean air temperature, February 2019 - IPMA, 2019



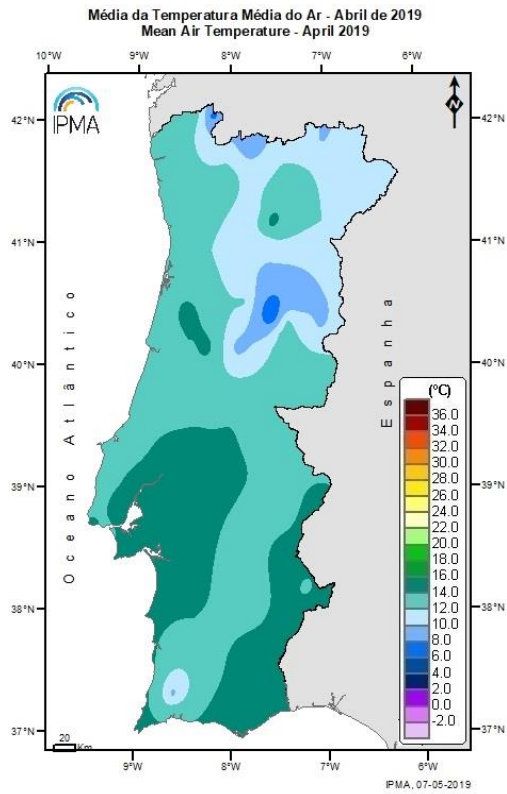
Annex 3b - Mean air temperature, January 2019 - IPMA, 2019



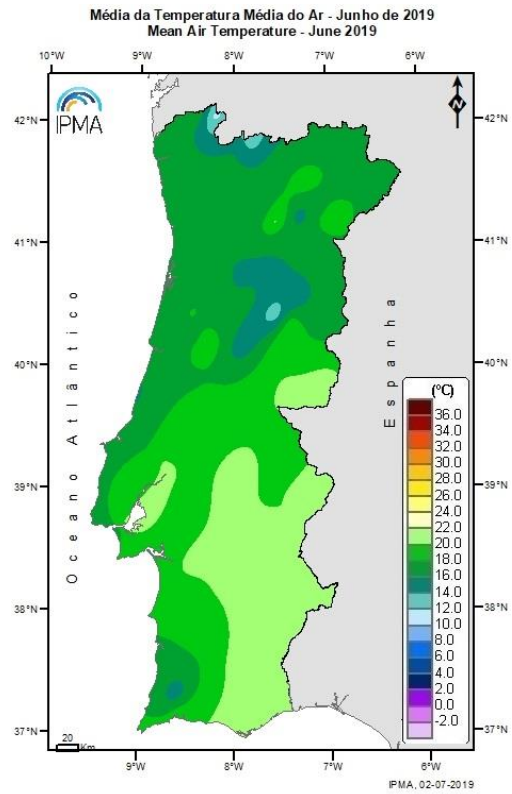
Annex 3d - Mean air temperature, March 2019 - IPMA, 2019



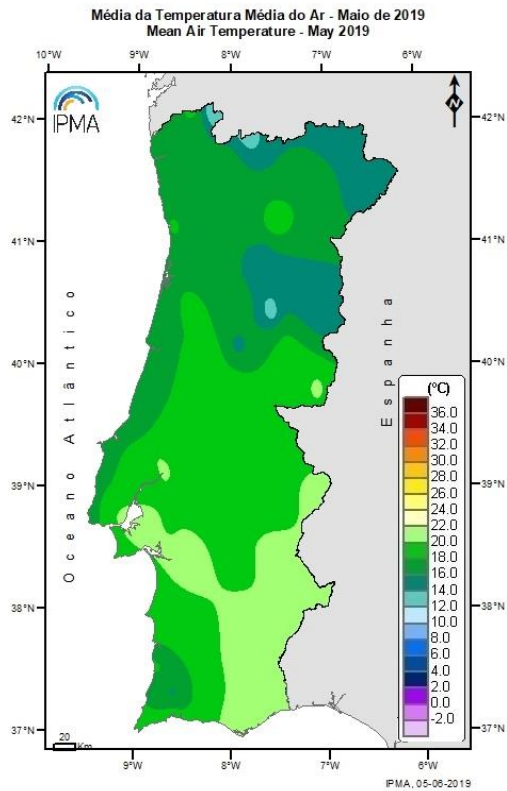
Annex 3e - Mean air temperature, April 2019 - IPMA, 2019



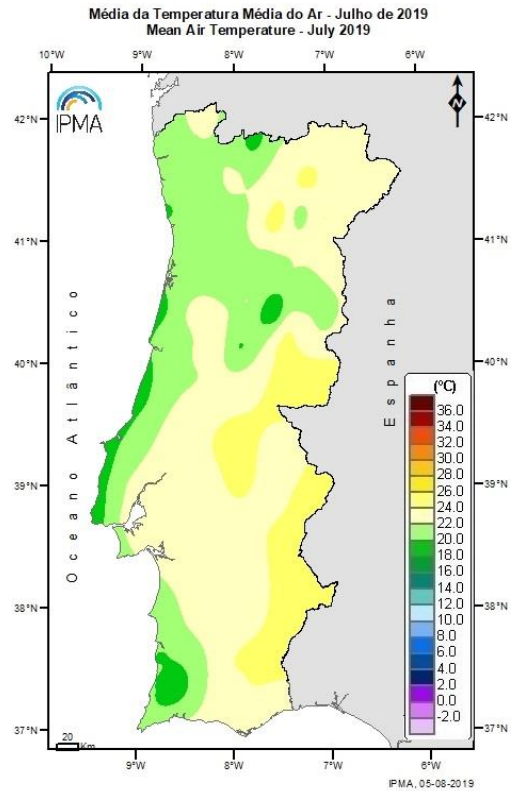
Annex 3g - Mean air temperature, June 2019 - IPMA, 2019



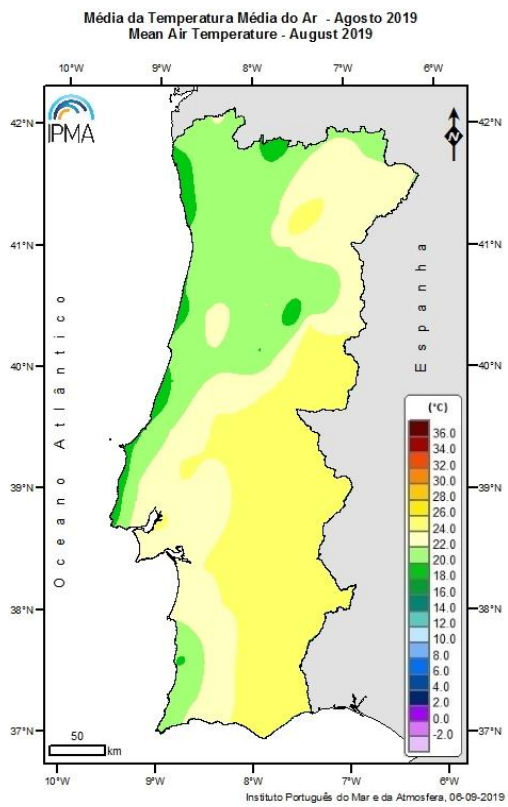
Annex 3f - Mean air temperature, May 2019 - IPMA, 2019



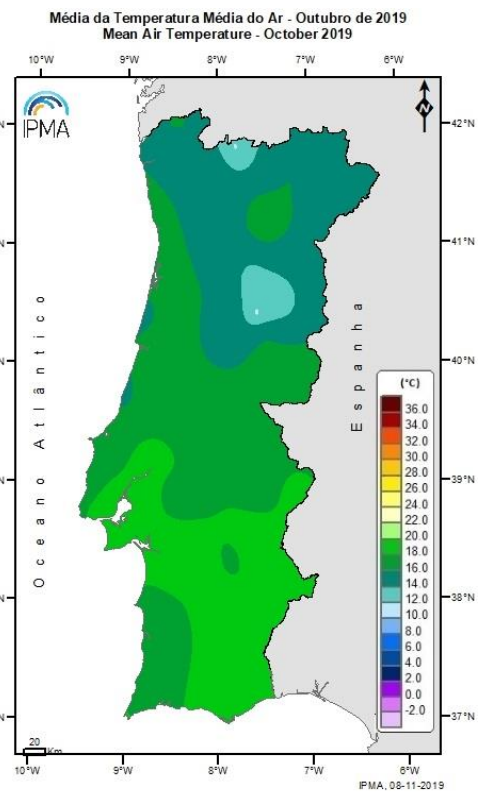
Annex 3h - Mean air temperature, July 2019 - IPMA, 2019



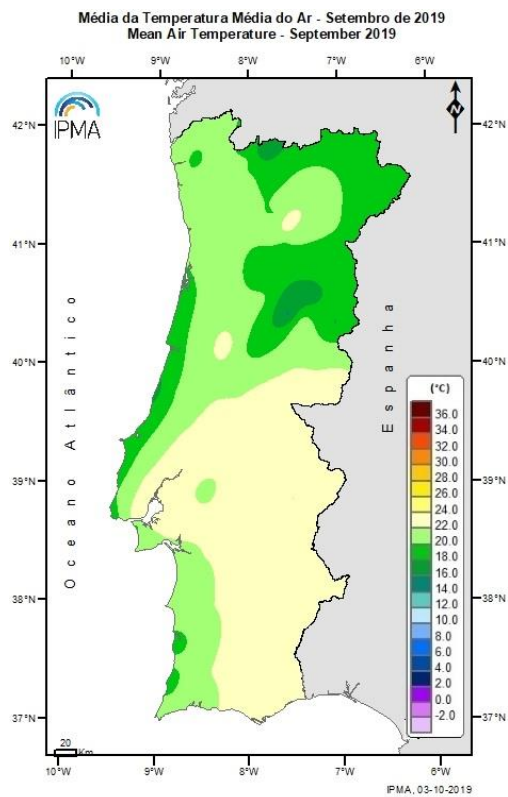
Annex 3i - Mean air temperature, August 2019 - IPMA, 2019



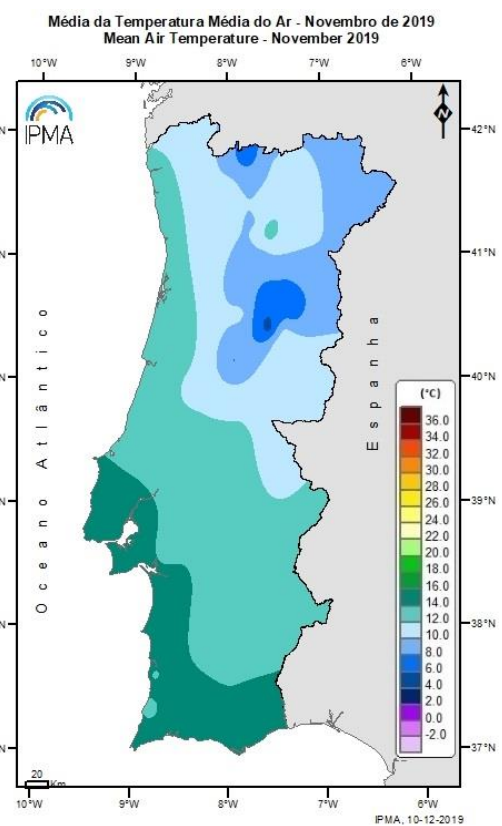
Annex 3k - Mean air temperature, October 2019 - IPMA, 2019



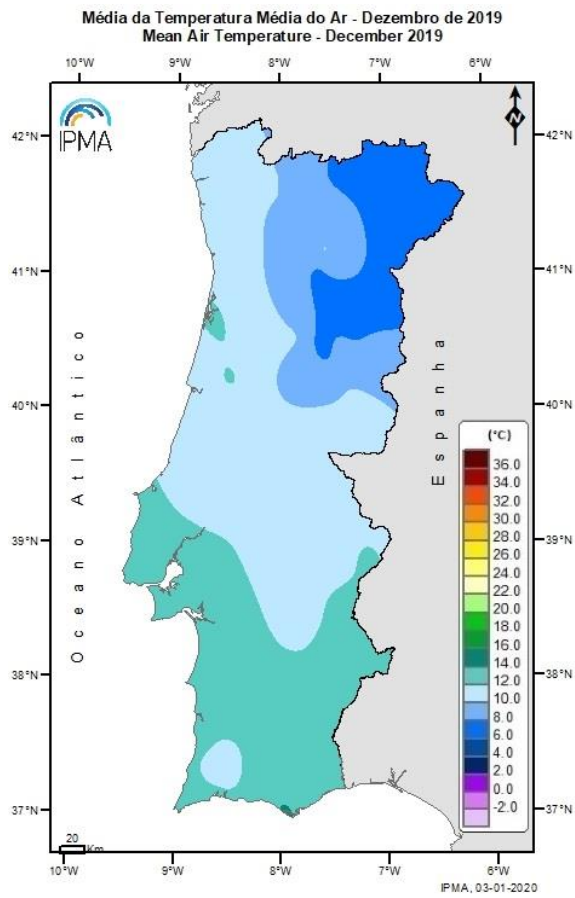
Annex 3j - Mean air temperature, September 2019 - IPMA, 2019



Annex 3l - Mean air temperature, November 2019 - IPMA, 2019



Annex 3m - Mean air temperature, December 2019 - IPMA, 2020



Annex 3n - Mean air temperature, February 2020 - IPMA, 2020

