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DEPARTAMENTO DE BIOLOGIA



**Unraveling the Mysteries of *Chrysomya albiceps* (Diptera, Calliphoridae):
Morphology, Potential Distribution and Forensic Applications**

“Documento Definitivo”

Doutoramento em Biologia e Ecologia das Alterações Globais
Especialidade em Biologia Ambiental e Saúde

Sérgio José Menezes Rodrigues Filho

Tese orientada por:
Professora Doutora Maria Teresa Rebelo (FCUL)

Documento especialmente elaborado para a obtenção do grau de doutor



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Nota prévia

A presente tese apresenta dois artigos científicos publicados (capítulo 2 e 3) e dois submetidos (capítulos 4 e 5), de acordo com o previsto no nº 2 do artigo 25º do Regulamento de Estudos de Pós-Graduação da Universidade de Lisboa, publicado no Diário da República, 2.^a série — N.º 57 — 23 de março de 2015. Uma vez que estes trabalhos foram realizados em colaboração, o candidato esclarece que participou integralmente na conceção dos trabalhos, obtenção dos dados, análise e discussão dos resultados, bem como na redação dos manuscritos.

Lisboa, 9 de Janeiro de 2024

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Resumo alargado

Existem cerca de 1000 espécies de moscas varejeiras (Diptera: Calliphoridae) no mundo, divididas em 150 gêneros que desempenham papéis importantes na natureza, atuando como polinizadores, parasitoides e bioindicadores ecológicos. Devido aos hábitos de sobrevoar materiais putrefatos, estas moscas estão ligadas a processos ecológicos centrais como a decomposição, sendo relevantes em ecologia, saúde humana, veterinária e ciências forenses. Por exemplo, os califorídeos podem ser carreadores mecânicos de patógenos, são causadores de miíases e podem ter a sua biologia associada a casos do foro criminal. Por outro lado, os seus corpos apresentam belas cores como verdes e azuis metálicos, razão do nome da família (Calliphoridae, em grego, significa “portadora da beleza”).

Entre essas espécies, *Chrysomya albiceps* se destaca por sua ampla distribuição, quase cosmopolita, resultante de eventos migratórios passados, o que a torna uma das espécies mais estudadas entre os califorídeos. Possui distribuição histórica centrada no Sul da Europa, no continente africano e no Oriente Médio. Em Portugal, ela já se destaca como uma das principais moscas pertencentes à fauna forense. Na Espanha e em outros países da Europa como Itália e Áustria, já foi relatada como evidência em casos criminais. Nos últimos 20 anos, *C. albiceps* começou a ser coletada no centro e leste da Europa, o que não era suposto acontecer por ser uma espécie que historicamente tolera ambientes tropicais/subtropicais. Na América, por exemplo, em poucos anos ela se expandiu por boa parte da porção sul do continente, por ser bem adaptada ao clima. Isso suscitou algumas questões! Quais os motivos que a fizeram conseguir se adaptar a estes locais mais frios no continente europeu? Com a variação da temperatura, pode haver alterações morfológicas na espécie? Em um mundo que está mudando constantemente e aceleradamente devido às alterações climáticas, como esta espécie se comporta perante estas mudanças? Estas são perguntas que a presente tese pretendeu responder. Apesar dos vários estudos realizados sobre *C. albiceps*, ainda existe uma lacuna de conhecimento sobre a sua relação com fatores ambientais, como a temperatura, a sua aplicação prática na entomologia forense e a anatomia de pequenas estruturas. Para que a entomologia forense seja eficaz, são necessários dois fatores fundamentais: conhecimento básico sobre a biologia das espécies forenses e identificação taxonômica acurada. Pelo papel fundamental que *C. albiceps* exerce neste cenário, a presente tese gerou resultados parcialmente novos sobre como a espécie pode ser usada na Entomologia Forense e como pode ser identificada de forma mais expedita nesse contexto.

Os objetivos desta pesquisa são derivados de duas hipóteses principais: a) a temperatura pode levar a variações morfológicas na espécie e b) a temperatura desempenha um papel significativo na formação da distribuição da espécie. Diante deste contexto, esta tese tem como objetivo principal investigar a influência da temperatura na morfologia da asa e na distribuição geográfica da espécie *C. albiceps*. Além disso, busca contribuir com informações valiosas para o campo da entomologia forense, fornecendo aplicações práticas da relação biologia-temperatura e descrições detalhadas de ultraestruturas anatômicas. Para atingir esses objetivos, empregamos técnicas de morfometria geométrica, algoritmos de *machine learning*, cálculos envolvendo a biologia da espécie e sua associação com a temperatura (como graus-dia acumulados - GDA) e microscopia eletrônica de varredura (MEV). Os principais resultados podem ser resumidos da seguinte forma: existem diferenças significativas no formato das asas entre machos e fêmeas, o que ajuda na identificação expedita do sexo, e o tamanho das asas apresenta uma correlação negativa com a temperatura. De acordo com o modelo e as variáveis testadas nesta tese, uma parte substancial da Europa é climaticamente adequada para *C. albiceps*. Entre as variáveis examinadas, a temperatura média anual e a faixa de temperatura anual foram consideradas os melhores indicadores de adequação climática. As projeções futuras indicam um aumento na adequação climática devido às variações de temperatura. Os cálculos envolvendo o GDA validam o uso potencial da *C. albiceps* como um indicador forense em investigações criminais. A análise MEV revelou sensilas anteriormente não documentadas associadas ao aparelho olfativo e um possível dimorfismo sexual no aparelho locomotor da *C. albiceps*. Esses resultados ressaltam a abundância de possibilidades inexploradas para o estudo do *C. albiceps*. Por ser uma espécie com alto poder competitivo, potencial agente de miíases, polinizador, indicador forense e potencial vetor mecânico de patógenos, entendemos que *C. albiceps* deve ser uma espécie não negligenciada na Europa e no resto do mundo. Esperamos que esses resultados possam fornecer suporte teórico para estudos futuros e ajudar a entender melhor a morfologia dessa espécie, como ela reage em determinadas condições e como ela pode ser usada nas ciências aplicadas, em particular na entomologia forense.

Palavras-chave: temperatura; *Chrysomya albiceps*; morfologia; distribuição geográfica potencial; Entomologia Forense.

Abstract

Blowflies, specifically *Chrysomya albiceps*, hold significant importance in several fields, including forensics, sanitary, veterinary sciences, and ecology. Despite being a well-studied species, there are still knowledge gaps regarding the interplay between *C. albiceps* and temperature, its practical application in forensic entomology, and the analysis of anatomical ultrastructures. This thesis aims to bridge these gaps by investigating the relationship between temperature and morphology, temperature and distribution, and providing practical insights into the temperature-biology relationship and the description of two anatomical ultrastructures of *C. albiceps* for forensic purposes. To achieve these objectives, we employed geometric morphometry techniques, machine learning algorithms, estimates involving species biology and its association with temperature (such as Accumulated Degree Days - ADD), and scanning electron microscopy (SEM). The key findings can be summarized as follows: significant differences in wing shape exist between males and females, aiding in sex identification, and wing size exhibits a negative correlation with temperature. According to the model and the variables tested in this thesis, a substantial area of Europe is climatically suitable for *C. albiceps*. Among the variables examined, mean annual temperature and annual temperature range were found to be the best predictors of climatic suitability. Future projections indicate an increase in climate suitability due to temperature variations. The calculations involving ADD validate the potential use of *C. albiceps* as a forensic indicator in criminal investigations. SEM analysis revealed previously undocumented sensillae associated with the olfactory apparatus and a possible sexual dimorphism in the locomotor apparatus of *C. albiceps*. These findings underscore the abundance of unexplored possibilities for the study of *C. albiceps*. It is anticipated that these results would provide a theoretical foundation for future research endeavors, contributing to an enhanced understanding of the species for practical applications across various scientific domains.

Key-words: temperature; *Chrysomya albiceps*; morphology; potential distribution; Forensic Entomology.

Table of contents

Acknowledgements.....	VII
Resumo.....	IX
Abstract.....	XI
Chapter 1.....	1
General Introduction.	1
1.1 The world is changed	3
1.2 Measuring structures	4
1.3 Where are the species?	5
1.4 The Diptera.....	5
1.5 Calliphoridae	6
1.6 History, classification and distribution of the family Calliphoridae	6
1.7 <i>Chrysomya albiceps</i> species.....	9
1.8 When insects become evidence?	12
1.9 Looking too closely	13
1.10 Putting the pieces together	14
1.11 Thesis objectives and structure.....	14
1.12 References	15
Chapter 2.....	31
“Size does matter: intraspecific geometric morphometric analysis of wings of the blowfly <i>Chrysomya albiceps</i> (Diptera: Calliphoridae)”	31
2.1 Abstract	34
2.2 Introduction	35
2.3 Methods.....	37
2.3.1 Specimen Collection and Identification of Species	37
2.3.2 Slide Preparation and Capture of Images	38
2.3.3 Wing Morphometrics landmarks	39
2.3.4 Variables used in Statistical Analysis.....	40
2.3.5 Allometry.....	41
2.3.6 Wing Shape Analysis	41
2.3.7 Wing Size analysis	42

2.3.8 Error measurement	42
2.4 Results	43
2.4.1 Error measurement results	43
2.4.2 Allometry results	43
2.4.3 Wing Shape results	44
2.4.4 Wing Size results	48
2.5 Discussion	51
2.5.1 A brief discussion about sample size issues	51
2.5.2 Allometry discussion	52
2.5.3 Wing Shape Discussion	53
2.5.4 Wing Size discussion	54
2.6 Conclusion	56
2.7 Credit Statements	56
2.8 Acknowledgements	56
2.9 Ethics approval and consent to participate	57
2.10 References	57
Chapter 3.....	65
"Where in Europe is <i>Chrysomya albiceps</i>? Modelling present and future distributions".	
.....	65
3.1 Abstract	68
3.2 Introduction	69
3.3 Material and Methods.....	71
3.3.1 <i>Chrysomya albiceps</i> records.....	71
3.3.2 Climatic Data.....	72
3.3.3 Modelling and Model Evaluation.....	73
3.3.4 Plotting	73
3.4 Results	74
3.5 Discussion	80
3.6 Conclusion.....	83
3.7 References	83
3.8 Supplementary Material 1	90
3.9 Supplementary Material 2	112
3.10 Supplementary Material 3	121
Chapter 4.....	125

"First experimental entomological evidence of Post Mortem Interval calculation from Portugal".....	125
4.1 Abstract	128
4.2 Introduction	129
4.3 Methods	131
4.3.1 Study Area	131
4.3.2 Sampling design – casework simulation	132
4.3.3 Insect rearing	132
4.3.4 Data analysis.....	132
4.4 Results	133
4.4.1 Collecting adults and rearing insects	133
4.4.2 Casework simulation	134
4.5 Discussion	138
4.5.1 Collecting adults and rearing insects	138
4.5.2 Casework Simulation	140
4.6 Conclusion.....	142
4.7 References	142
Chapter 5.....	155
“Characterization of <i>Chrysomya albiceps</i> (Diptera: Calliphoridae) tarsi and head by Scanning Electron Microscopy.”	155
5.1 Abstract	158
5.2 Introduction	159
5.3 Methods.....	160
5.3.1 <i>Chrysomya albiceps</i> specimens	160
5.3.2 Laboratory Procedures	160
5.3.3 Image Analysis	161
5.4 Results	161
5.5 Discussion	166
5.6 Conclusion.....	169
5.7 Acknowledgements	169
5.8 References	170
Chapter 6.....	175
Overall discussion.....	175
6.1 General Discussion.....	177
6.1.1. Wing size and wing shape	177

6.1.2. Climate suitability of <i>Chrysomya albiceps</i>	178
6.1.3. <i>Chrysomya albiceps</i> and Forensic Entomology	180
6.1.4. The ultrastructures of <i>C. albiceps</i>	181
6.2 Conclusion.....	182
6.3 References	184
7 Annex.....	189

In our hands Themis placed
the Scales of Law and Order.
The power to protect,
and a prophecy for destiny.
Would the scales be empty
or filled with life?
Silent faces starewaiting,
pleading, asking
only for the right to be.
And when you look up my child,
what will you see?
In our hands was placed earth's eternal life.
On silent pages, the sun ticks away time.
The Sands of the ages
running through the
hourglass of our existence . . .
When you ask me my child
'What, Daddy, will you leave behind for me?'
For you 'I will open the gate . . .'

Michael J. Samways, in his 2005 book *Insect Diversity Conservation*

Chapter 1

General Introduction.

Introduction

1.1 The world is changed

The world is currently experiencing various climate changes (IPCC, 2021). In part, this is caused by anthropogenic activities that increase the emission of pollutant gases in the atmosphere, such as the burning of fossil fuels, cement production, flaring, and inadequate management of natural resources (Jia et al., 2019). This can alter natural cycles of rainfall, wind and other gases present in the atmosphere, which has consequences at the level of forest fires and even human health (Pecl et al., 2017). Europe, for example, is warming faster than the global average, especially in Eastern Europe, Scandinavia and the Iberian Peninsula (IPCC, 2021). Not only that, but this increase is also predicted to continue in the coming years (ESOTC, 2022). This requires humanity to respond quickly in understanding how these processes work and how they affect living beings.

Climate change is seen as one of the main threats to the planet's biodiversity (Harvey et al., 2023), which act at all possible ecological scales, from the genotype of a species to the level of ecosystems (Garrett et al., 2006; Seidl et al., 2017), and from organisms above (Hill et al., 2021) to under (Singh et al., 2019) the ground. Changes in temperature mainly affect creatures/species whose development directly depends on this factor, such as insects (Gullan & Cranston, 2014).

Insects are the largest group of organisms in the world. The number of described species has exceeded one million, or about 66% of all animal species (Zhang, 2011; Eggleton, 2020). The great species richness of the group brings with it numerous varieties of ecological habits, forms, and potential ecological niches (Basset & Lamarre, 2019). They are ectothermic organisms with significant roles in maintaining crucial ecological services like pollination, decomposition, and are natural pest control agents (Schowalter et al., 2018). In recent years, a wealth of evidence has emerged regarding the impact of climate change on these creatures (Samy et al., 2016; Pureswaran et al., 2018; Hill et al., 2021; Harvey et al., 2023). These studies have explored various aspects, ranging from prey-predator interactions (Laws, 2017) to phenology (Forrest, 2016), geographic expansion (Kiritani, 2013), and even potential extinction (Wagner, 2020). The effects can be remarkably specific, to the extent that they can influence specialized scientific fields like Forensic Entomology (Turchetto & Vanin, 2004). Due to the

often unpredictable nature of these responses (Jactel et al., 2019), it is crucial to employ both broad-scale and targeted investigations to unravel the mechanisms behind such responses.

Temperature is the main variable altered by climate change. Studies involving insect responses to temperature change can raise several questions. One way to study this problem is to know whether under different temperatures, the organism has its morphology or physiology modified (Garzón & Schweigmann, 2018), a capacity known as phenotypic plasticity. In recent years, studies have been dedicated to measuring biological structures changes, many of them using a tool known as geometric morphometry (Bookstein, 1991; Rodrigues-Filho et al., 2022).

1.2 Measuring structures

Geometric morphometry (GM) is a recent tool that has helped in several areas of biology, including genetics, ecology and entomology (Dujardin, 2008). GM techniques measure size, shape and the relationship between size and shape, known as allometry (Cooke and Terhune, 2015; Tatsuta et al., 2018). Measurements are made using x,y points in orthogonal planes and analysed using multivariate statistical techniques (Dujardin, 2008). In insects, it has been extensively used in recent years, measuring structures such as membranous wings and elytra (eg, Sontigun et al., 2017; Benítez et al., 2020).

Wing shape in insects can be used to identify sexual dimorphism (Sontigun et al., 2019). However, it is mainly used for identification between species (Sauer et al., 2020), as it is less influenced than wing size by environmental factors (Gómez et al. 2014; Gómez & Correa, 2017). Sontigun et al. (2017) classify shape as a more stable trait that is very informative about evolutionary and phylogenetic relationships. Wing size is already more used to extract information on how the organism responds to environmental factors (Battán-Horenstein & Peretti, 2011; Gallesi et al., 2016; Garzón & Schweigmann, 2018), but can also be used to characterise sexual dimorphism (Jiménez-Martín et al. 2020). In addition to the aforementioned factors, geometric morphometry has already been used to understand competition between species (Carmo et al. 2018; Ivorra et al., 2022), biological invasion processes (Laparie et al., 2016), microhabitat effects on wing shape evolution (Chazot et al., 2016), response to seasonal effects (Gallesi et al., 2016) and varied environmental factors (Zhou et al., 2020), and even taxonomy (Gomes & Correa, 2017; Sontigun et al., 2019; Szpila et al., 2019; Limsopatham et al., 2021). Some of these works are focused on insects of the order Diptera (Sontigun et al.,

2017, 2019; Szpila et al., 2019, 2022, Rodrigues-Filho et al., 2022), given the ease of finding them in nature and the diverse importance of the members of the order.

1.3 Where are the species?

Temperature effects can be investigated in another way by assessing the climatic suitability for the potential distribution of organisms (Phillips et al., 2006). This approach helps to understand whether bioclimatic factors play a role in shaping species distribution patterns. A notable example is the case of *Aedes albopictus* (Skuse, 1894) in Europe during recent years, as studied by Cunze et al. (2016), along with other Diptera species (Miličić et al., 2018; Milić et al., 2019; Rodrigues-Filho et al., 2023). Such studies utilize methods like species distribution models (SDMs), which involve analyzing occurrence data to generate models that depict species' responses to bioclimatic variables (Lobo et al., 2016).

In recent years, species distribution models (SDMs) have gained significant popularity as valuable tools for comprehending the potential distribution of organisms based on climate suitability (Liu et al., 2005, 2016). Moreover, these models offer the capability to project future distribution scenarios using global climate models (Hosni et al., 2020, 2022). This potential holds great promise for supporting enhanced natural resource management (Souza & De Marco, 2014) and predicting the spread of pests and vectors (Cunze et al., 2016; Urvois et al., 2021). The availability of extensive web-based databases like GBIF, in addition to the existing species literature, enables the generation of reliable species distribution models, thereby facilitating informed decision-making processes.

To be able to generate SDMs with confidence, several software and statistical packages have been developed in recent years (Elith et al., 2006). It is possible to develop these models using approaches integrated with GIS techniques, for example (Souza & DeMarco, 2014). An algorithm known as Maximum Entropy, run by the Maxent program (Phillips et al., 2006 Phillips & Dudik, 2008) has been widely used (Cunze et al., 2016; Samy et al., 2016; Mulieri and Patitucci 2019; Hosni et al., 2020, 2022), with evidence that it performs better than other methods such as GARP and BIOCLIM (Elith et al. 2006). Insects are a good model to perform this type of method, including multidiverse orders such as Coleoptera (Urvois et al., 2020) and Diptera (Samy et al., 2016; Rodrigues-Filho et al., 2023).

1.4 The Diptera

Diptera (Di = two; ptera = wings) is an order of insects mainly characterized by having one pair of functional wings, while the second is reduced to a structure responsible for flight balance called haltere (Oliveira-Costa, 2011). Flies (Diptera: Brachycera) and mosquitoes (Diptera: Nematocera) fit this order, which has approximately 160000 species (Pape et al., 2011). The difference between the two suborders is basically the number of antenna segments formed in the adult stage, while in the larvae, the difference is identified by the cephalic structures (Oliveira-Costa, 2011). In continental Portugal, there are about 1475 species of Diptera, whose surveys can be consulted in Carles-Tolrá & Hjorth-Andersen (2002) and Ferreira et al. (2020, 2021). Among these organisms, there are the family Calliphoridae, whose members are known as blowflies.

1.5 Calliphoridae

The calliphorids constitute about 1000 species distributed in 150 genera (Rognes, 1997). Members of the family have a vast number of shapes, habitats, feeding habits, colours and behaviours (Shewell, 1987). Sometimes they are metallic and green, sometimes blue, cupric or violet, or even without the metallic luster noticeable at first glance (Wolff & Kosmann, 2016). This shine is even responsible for the name Calliphoridae, which in Greek means: "bearer of beauty" (Greenberg & Kunich, 2002). They can be small or large, ranging in size from 4 to 16 mm (Shewell, 1987) and are present in all biogeographic regions, from the north of the globe to subantarctic islands (Laparie et al. 2016). Calliphorids are organisms that occupy forest (Vasconcelos et al. 2013), rural (Arias-Robledo et al. 2019) and urban (Greenberg et al. 2003) habitats, so they are easily recognised and related to the human species. Another factor that makes them famous is feeding on decaying matter and faeces, especially females that need protein to produce eggs. They also feed on sugar solutions such as nectar (Zumt, 1965, Vargas & Wood, 2010), acting as pollinators of some species like *Mangifera indica* L. (Ramírez & Davenport, 2016).

1.6 History, classification and distribution of the family Calliphoridae

The oldest record of Calliphoridae in geological history dates back 70 million years, to a species called *Cretaphormia fowleri* (McAlpine, 1970), described through emerged pupae.

However, this record is notably dubious (Grimaldi, 2005) and regarded as a probable Cyclorrhapha, a classification denoting the way some flies exit the pupa, by means of a circular hole at the extreme tip of the casing. In addition, there are records of puparia of the species *Cochliomyia macellaria* (Fabricius, 1775) associated with bones of *Teratornis merriami* (Miller, 1909), a Pleistocene bird of prey (Pierce, 1945), puparia of the species *Phormia regina* (Meigen, 1826), *Protophormia terraenovae* (Robineau-Desvoidy, 1830) and *Cynomya cadaverina* (Robineau-Desvoidy, 1830) in prehistoric excavation sites (Teskey & Turnbull, 1979) and mummified bodies (Giordani et al., 2018). Interestingly, it is from blowflies that the earliest record of insects recognised in human writing 3600 years ago (Greenberg & Kunich, 2002).

Until the recent past, the classification of Calliphoridae still lacked a proposal that would evidence the group as monophyletic (Rognes, 1997, Kutty et al. 2010, Singh & Wells, 2013, Cerretti et al. 2017, Kutty et al. 2019). This has changed as per the work of Yan et al (2021). The proposal subdivides Calliphoridae into 8 subfamilies: Ameniinae, Bengaliinae, Calliphorinae, Chrysominae, Luciliinae, Phumosiinae, Rhiniinae and Rhinophorinae and highlights monophyleticism in Calliphoridae. For didactic purposes, in this thesis, the eight subfamilies proposed above will be adopted. Of these, both Ameniinae and Phumosiinae have very restricted distributions. Together with Bengaliinae, these are the three subfamilies that do not have a recorded distribution in Portugal.

The subfamily Ameniinae is distributed in the Eastern and Australasian regions, except for New Zealand and Tasmania (Crosskey, 1965). The little evidence available on the biology of the group suggests that, like the family Mesembrinellidae in the Neotropical region, the Ameninae are macrolarviparous, i.e., larvae are retained in the uterus until near maturity (Ferrari, 1976). In addition, there is evidence that the group is parasitic on terrestrial molluscs (Colless, 1998).

Phumosiinae is distributed over the same regions as Ameniinae. It is a subfamily that is known to parasitize nests of anurans of the family Rhacophoridae (Rognes, 2015, Banerjee et al. 2018).

The Bengaliinae are distributed across the same regions as the two aforementioned families including the Afrotropical region. They are species associated with termite nests, ectoparasites of elephants and deer, and are also causers of myiasis (Rognes, 2011).

Rhiniinae is distributed across Afrotropical, Palearctic, Eastern and Australasian regions, and the biology of members of the species is also poorly known (Thomas-Cabianca et

al., 2023). In Portugal, for example, little is known about the most common species occurring in the country, *Stomorphina lunata* (Fabricius, 1805). Some adult species are floral visitors and others are associated with ant and termite nests (Thomas-Cabianca et al., 2023).

Rhinophorinae are distributed almost entirely throughout the Old World, with few species outside the Palearctic Region, specifically in Australia and Ecuador. They are known to be the only parasitoids of garden armadillos among all insects (Cerretti et al., 2020).

Calliphorinae are distributed all over the planet (Whitworth & Rognes, 2012). They include, among other genera, the species of the genus *Calliphora*. All species of this genus develop in decomposing organic matter (Zumpt, 1965). Among these species is the most common blowfly in Portugal, *Calliphora vicina* Robineau-Desvoidy, 1830, which occurs in the adult stage in all seasons (Prado e Castro et al., 2012). Besides it, still occur in Portugal *Calliphora vomitoria* (Linnaeus, 1758), a species restricted to the colder season of the year and *Calliphora loewi* (Enderlein, 1903), a fly recently found in Insular Portugal (Prado e Castro et al., 2016). *Calliphora vicina* attracts itself to much of the decomposing materials and has veterinary (Zumpt, 1965), forensic and medical (Smith, 1986) importance. It is such an important species that by 1990 it had about 900 scientific publications devoted to it (Rognes & Blackith, 1990).

The Luciliinae are also a subfamily widely distributed throughout the world (Smith, 1986). They are important species from forensic, medical and veterinary aspects. *Lucilia sericata* (Meigen, 1826), for instance, is an important forensic indicator, besides being frequently cited as a myiasis-causing species (Zumpt, 1965). The same author says that this species was used in past centuries as an aid in the wound healing process, before being replaced by antibiotics and other modern pharmaceutical solutions. There is experimental evidence that this species competes with *Chrysomya albiceps* (Wiedemann, 1819) in the Iberian Peninsula and that under certain conditions, its body and population size can be reduced (Ivorra et al., 2022). Prado and Castro et al (2012) demonstrated that *L. sericata* is the second most abundant species of *Lucilia* spp. in cadaveric baits (piglet) in autumn in Portugal. Besides them, other species of the genus occurring in Portugal are: *Lucilia ampullacea* (Villeneuve, 1922), *Lucilia caesar* (Linnaeus, 1758), *Lucilia illustris* (Meigen, 1826) and *Lucilia silvarum* (Meigen, 1826) (Prado e Castro et al., 2011, 2012; Rebelo et al., 2014).

Chrysominae constitute another subfamily whose species are widely distributed throughout the world (Smith et al., 1986). They have representatives with very varied behaviours, from primary and secondary myiasis causers, to pollinators, pathogen carriers and

forensic indicators (Sivell, 2021). In recent decades, four species of this subfamily have made it to the American continent (Baumgartner, 1988). One of these species is *C. albiceps*, the target species of this thesis. It occurs in Portugal together with another species of the genus called *Chrysomya megacephala* (Fabricius, 1794) (Prado e Castro & Garcia, 2009).

1.7 *Chrysomya albiceps* species

Chrysomya albiceps is characterized by metallic green body, dark bands on the abdomen, gena in different shades of brown, 5 to 7 proepisternal (propleural) setae, whitish anterior spiracle (Grella et al., 2015) and white polyinosity on the last segment of the abdomen (Dores, 2016). Some of these features can be visualised below (Fig. 1).



Figure 1: *Chrysomya albiceps* specimen observed in a forensic experiment in Lisbon, Portugal.

The species is one of the best studied forensic indicators in the world (Grassberger et al. 2003, Corrêa et al. 2019; Williams & Villet 2019; Al-Qahtni et al. 2021). Originally, its geographical distribution encompassed Africa, the Iberian Peninsula, and the Middle East (Seguy 1930-1932; Holdaway 1933). However, in the 1970s, this species was dispersed by ships to South America (Guimarães 1979; Gagné 1981; Baumgartner & Greenberg 1984). Since

then, it has spread in a few years over much of the territory (Baumgartner 1988) and is considered to be a population displacer of the species *Cochliomyia macellaria* (Fabricius, 1775), as it is a voracious predator in its larval phase, feeding on other species or even on itself (Faria et al. 1999). In the early 2000s, *C. albiceps* started to be identified in new areas of Europe (Povolný 2002; Grassberger et al. 2003). Since then, the distribution of the species has increased in the old continent towards other Central and Eastern European countries (Table 1). Due to these factors, it is listed in the Invasive Species Compendium as a species threatening livelihoods and the environment worldwide (CABI, 2019). Furthermore, *C. albiceps* causes secondary myiasis, acting as enhancer of attacks on livestock and domestic animals in synergy with other species of blowflies (Zumpt 1965; Schnur et al. 2009).

In studies from Portugal, *C. albiceps* has already been recorded in studies of genetics (Cainé et al. 2009; Ferreira et al. 2020), forensic entomology (Prado e Castro et al. 2012), forensic faunistics (Farinha et al. 2014), geographical distribution (Prado e Castro et al. 2016). The species in the adult stage occurs from late spring to late autumn (Prado e Castro et al., 2012). Recently, it was recorded that the species, besides being collected in cadaveric baits such as piglets, dogs and cats, was also collected in a cadaver of *Balaenoptera acutorostrata* (Lacépède, 1804), the minke whale (Rodrigues-Filho et al., 2022). Furthermore, no potential for rabbit haemorrhagic disease virus carriage has been found in *C. albiceps*, although it has been found in species of the genus *Calliphora* (Lopes et al., 2023). Although it is a well-studied species, it still lacks many studies to elucidate important aspects of its biology, which includes the practical work of Forensic Entomology.

Table 1: List of countries/regions in Europe where *Chrysomya albiceps* is recorded.

Country	Studies
Albania	Verves 2003
Austria	Povolný 2002; Grassberger and Frank 2004
Azores	Verves 2003
Belarus	Makovetskaya and Verves 2018
Belgium	Gosselin and Braet 2008
Bosnia & Herzegovina	Verves 2003
Bulgaria	Verves 2003
Croatia	Verves 2003
Cyprus	Verves 2003
Czechia	Verves 2003
France	Grassberger et al. 2003
Germany	Povolný 2002, Kotrba et al. 2012
Georgia	Verves 2003
Greece	Verves 2003
Hungary	Ujvári and Bozó 2014
Italy	Introna et al. 1998; Vanin et al. 2009; Lambiase and Camerini 2012
Lithuania	Lutovinovas and Markevičiūtė 2017
Macedonia	Klekovska et al. 2017
Malta	Verves 2003
Insular Portugal	Martínez-Sánchez et al. 2002; Prado e Castro et al. 2016
Continental Portugal	Cainé et al. 2009; Prado e Castro et al. 2009, 2011, 2012; Farinha et al. 2014; Rebelo et al. 2014
Moldavia	Verves 2003
Poland	Szpila et al. 2008; Michalski and Szpila 2016, Szpila et al. 2019
Romania	Iancu and Parvu 2013; Iancu et al. 2016
Slovakia	Verves 2003
Slovenia	Verves 2003
Spain	Martínez-Sánchez et al. 2000, 2001, 2002, 2015; Martín-Vega and Baz, 2013; Martín-Vega et al. 2017
Switzerland	Amendt et al. 2015
Turkey	Verves 2003
Ukraine	Verves 2004; Verves and Khrokalo 2018

1.8 When insects become evidence?

Forensic Entomology is a tool that supports judicial forum processes with basic and applied knowledge of insect biology and ecology (Dias-Filho et al., 2022). Although its use in forensic institutions is not so widespread, it is an area in science that is growing steadily (Oliveira-Costa et al., 2011). The first record of its informal use dates to the 13th century, from a case reported by a Chinese lawyer named Song Ci, in a book called *Xi Yuan Ji Lu*, whose rough translation would be "The Washing Away of Wrongs" (Benecke, 2001). The case concerned a murder in which the victim was killed by a sickle whose user was unknown. On the day after the murder, all villagers had to deposit their scythes in a public space in front of the local residents. One of the scythes attracted saprophytic flies and the owner was coerced and eventually admitted the murder.

After centuries of this report, the first time that Forensic Entomology was formally used, with estimation of post mortem interval (PMI) dates back to the 19th century, made by a French physician, named Louis François Étienne Bergeret, in 1855 (Benecke, 2001). Jean Pierre Mégnin, in his seminal work called *La faune des cadavres application de l'entomologie à la médecine légale* (1894), launched a work on the insects that occurred in corpses. In the book, he calls them workers of death and comments that they occur in the so-called waves, that is, different insects occurring in different phases of the cadaveric phases. This characterizes the first record of what would be an entomological succession in a corpse. In the book, Mégnin cites two species of blowflies that are part of the workers of death, namely: *C. vomitoria* and *L. caesar*.

Forensic Entomology has four major fields of application: a) urban: deals with cases involving synanthropic insects that may somehow be targeted within a civil/criminal lawsuit, such as pests in real estate (e.g. termites, cockroaches and ants) (Dias-Filho et al., 2022); b) stored products: deals with cases in which there may be insect infestation in food products (Dias-Filho et al., 2022); c) environmental: deals with cases in which insects can serve as environmental bio-indicators or as legal good to be preserved and/or protected by the State (Dias-Filho et al., 2022); and, finally, the most common d) medico-legal: deals with cases in which the biology and ecology of insects can be applied to solve crimes of violent deaths situations of neglect, abuse, and in toxicology contexts (Catts & Goff, 1992; Byrd & Castner, 2009).

One of the ways in which medico-legal Forensic Entomology can be used is to calculate the Post Mortem Interval (PMI) (Amendt et al., 2007). The most widely used method for this is the Accumulated Degree Days (ADD) calculation which uses temperature data and basic knowledge about the temperature range of forensic species (Oliveira-Costa et al., 2011). In the literature, no record of this calculation has been found in the forensic history of Portugal, so in this thesis it is believed to be the first time that evidence is provided from the ADD. Unfortunately, even with the volume of work done on this species, there are no records in many countries of actual cases in which it is involved (see details in chapter 4).

Forensic methods require reliability not only in the biology and ecology of species, but also in their correct identification (Byrd & Castner, 2009). In recent years, different ways to identify forensic species have emerged, including genetics, biochemistry, geometric morphometry and anatomical ultrastructure images of insects.

1.9 Looking too closely

Recent work has demonstrated a great diversity of sensilla, bristles and differentiated structures in insects from images made in scanning electron microscopy (Sukontason et al., 2006; Setzu et al., 2011; Friedemann et al., 2014; Carriço et al., 2020). Besides being impressive images, they bring to light many insights into how basic insect systems, such as the locomotion (Gorb, 1998) and olfactory (Fernandes et al., 2004) systems work. In blowflies, this kind of work becomes even more important because of their forensic and medical importance (Sukontason et al., 2006).

Among the Calliphoridae species that have had their ultrastructures studied, there are *Calliphora erythrocephala* (Gorb, 1998), *Triceratopyga calliphoroides* (Rohdendorf, 1931) (Zhang et al. 2014), *Hemilucilia segmentaria* (Fabricius, 1805) (Alvarez-Garcia et al. 2022), *Cochliomyia hominivorax* (Coquerel, 1858) and *C. megacephala*, *Chrysomya rufifacies*, *C. nigripes* and *Lucilia cuprina* (Sukontason et al, 2004). *Chrysomya albiceps* has never had its ultrastructures of the locomotion apparatus studied. The olfactory system, on the other hand, had never been studied in European populations, the only record being made by Darilmaz et al. (2019). As we talked about earlier, due to its recent spread in central and eastern European countries, along with its role in forensic entomology, the correct species identification is of utmost necessity.

1.10 Putting the pieces together

Climate change has reshaped ecosystems worldwide, enabling the migration of species into previously inhospitable areas beyond their presumed thermal limits (Turchetto & Vanin, 2004). In recent years, the expansion of *C. albiceps* into colder European regions (Rodrigues-Filho et al., 2023) suggests potential alterations in its characteristics. Given its acknowledged forensic significance, countries newly inhabited by this species should reconsider investigative approaches for criminal cases, acknowledging its potential impact on local fly communities. Additionally, considering its uncommon prevalence in many parts of Europe, it becomes crucial to update and enhance the taxonomic recognition of *C. albiceps* due to the shifts in its distribution.

1.11 Thesis objectives and structure

This thesis aims to investigate the influence of temperature on the wing morphology and geographical distribution of the species *C. albiceps*. Additionally, it seeks to contribute valuable insights to the field of Forensic Entomology by providing practical applications of the biology-temperature relationship and detailed descriptions of anatomical ultrastructures. The objectives of this research are derived from two primary hypotheses: a) temperature leads to morphological variations in the species, and b) temperature plays a significant role in shaping the species' distribution.

This thesis consists of six chapters, structured as follows:

Chapter 1: General Introduction. In this chapter, we discuss the family Calliphoridae and the species *C. albiceps*.

Chapter 2: **Rodrigues-Filho, S. J. M.**, Prado E Castro, C., Lopes, L. F., da Fonseca, I. P., & Rebelo, M. T. (2022). Size does matter: intraspecific geometric morphometric analysis of wings of the blowfly *Chrysomya albiceps* (Diptera: Calliphoridae). *Acta Tropica*, 235, 106662.

<https://doi.org/10.1016/j.actatropica.2022.106662>

Geometric morphometry was used to ascertain whether wing shape and wing size vary by sex, over time, temperature and type of carcass sampled.

Chapter 3: **Rodrigues-Filho S. J. M**, Lobato F. S., Abreu C. H. M, Rebelo, M. T. (2023). Where in Europe is *Chrysomya albiceps*? Modelling present and future potential distributions. *NeoBiota* 85, 81-99. <https://doi.org/10.3897/neobiota.85.96687>

A machine learning algorithm was used to estimate potential distributions of *C. albiceps*, focusing our discussion on the recent dispersal of the species across Europe.

Chapter 4: **Rodrigues-Filho, S.J.M.**, Fialho, A. C., Reis, C., Fonseca, I. P. & Rebelo, M; T. (2023). First experimental entomological evidence of Post Mortem Interval calculation from Portugal. *Forensic Science International*. (Submitted).

This chapter intends to provide the first evidence from Portugal about Post Mortem Interval, using ADD calculations.

Chapter 5: **Rodrigues-Filho, S.J.M.**, Prado e Castro, C., Reis, Fonseca, I. P. & Rebelo, M; T. (2023). Characterization of *Chrysomya albiceps* (Diptera: Calliphoridae) tarsi and head by Scanning Electron Microscopy. *Current Research in Insect Science*. (Submitted).

Scanning electron microscopy was used to access anatomical ultrastructures of the olfactory and locomotor apparatus of *C. albiceps*.

Chapter 6: The last chapter is an overall discussion, where the most important findings are integrated and debated.

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Chapter 2

“Size does matter: intraspecific geometric morphometric analysis of wings of the blowfly *Chrysomya albiceps* (Diptera: Calliphoridae)”

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Size does matter: intraspecific geometric morphometric analysis of wings of the blowfly

***Chrysomya albiceps* (Diptera: Calliphoridae)**

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2.1 Abstract

Blowflies have forensic, sanitary and veterinary importance, as well as being pollinators, parasitoids and ecological bioindicators. There is still little work with real data and from experiments assessing the relationship between blowflies' morphologic features and environmental and demographic factors. The present work tests whether the variation, in the shape and size, of *Chrysomya albiceps* (Wiedemann, 1819) wings is influenced by the following factors: 1) time; 2) temperature; 3) sex and; 4) different types of carcasses (pig, dog/cat and whale). Male and female wings from four different sites collected in six different years were used to obtain wing size and shape of *C. albiceps*. Analyses between wing shape and the variables tested had low explanatory power, even though they had statistical support. However, it was possible to identify differences in wing shape between males and females, with good returns in sex identification. The comparison between wing size and the variables tested showed that wing size has a negative relationship with temperature, significant differences between sexes, slight variation over time and no influence by carcass types. Furthermore, wing size influenced wing shape. Understanding population-specific characteristics of *C. albiceps* provide important insights about how the species reacts under specific conditions.

Key-words. Calliphoridae; Sexual dimorphism; Temperature; Wing shape; Wing size.

2.2 Introduction

Insect wings are membranous structures with functions ranging from flight to protection from external shocks (Parchem et al. 2007; Salcedo and Socha, 2020). Furthermore, they are important structures in the morphological distinction of groups (Gullan and Cranston, 2014). One of the ways to study morphology of these structures and that has grown in recent years is Geometric Morphometry - GM - (Cooke and Terhune 2015; Tatsuta et al. 2018). GM consists of a set of multivariate statistical techniques to visualize and analyze the morphology of insects through landmarks in an orthogonal space (Dujardin 2008). The landmarks generate 'x,y' coordinates, used to generate shape and size variables to be associated with covariates of interest (Bookstein 1991). Geometric Morphometry applied to wings is used to elucidate evolutionary effects, influence of environmental factors and ecological relationships (Chazot et al. 2016; Ivorra et al. 2019; Lemic et al. 2020), in addition to serving as a complement to molecular analyses (Sauer et al. 2020). Wing shape has been widely used to identify differences between species (Sontigun et al. 2019; Limsopatham et al. 2021) and between populations (Limsopatham et al. 2018). The GM of wing size is studied along with shape (Garzón and Schweigmann 2018), as it can influence wing shape, an association known as allometry (Benítez et al. 2013). Recently, wing size has been used to answer how competition can be a limiting factor between two blowflies under experimental conditions (Carmo et al. 2018; Ivorra et al. 2022).

Insect wing shape is less influenced than size by environmental factors (Gómez et al. 2014; Gómez and Correa, 2017). This demonstrates that shape is a more useful trait than size in species identification, so there are works that focus only on variations in shape (e.g., Grzywacz et al. 2017; Sontigun et al. 2019). Research that focus on wing size seek to provide answers about how the development of the studied model may respond to environmental factors

such as temperature and diet (Klingenberg, 2010). Temperature, for instance, may favor the population rise of some insects, increasing the likelihood of developing more generations in their seasons of occurrence (Lehmann et al. 2020; Abarca and Spahn, 2021). However, with population increase, intraspecific competition for resources increases, which can generate a negative relationship between wing size and temperature, as seen in the blowfly *Chrysomya megacephala* (Fabricius, 1794) (Reigada and Godoy, 2005). Furthermore, seasonality is a factor that does little to explain the body size of calliforids (Gião and Godoy, 2006).

Blowflies have forensic, sanitary and veterinary importance, as well as being pollinators, parasitoids and ecological bioindicators (Zumpt, 1965; Ramírez and Davenport, 2016; Wolff and Kosmann, 2016). Wing shape in blowflies has already been used to study adaptation of invasive populations (Laparie et al. 2016), develop tools for taxonomic identification (Sontigun et al. 2017; Macleod et al. 2018; Szpila et al, 2019), analyze population differences (Hall et al. 2014), evaluate responses to interspecific competition (Macedo et al. 2020) and characterize sexual dimorphism (Brown 1979; Lyra et al. 2009; Vásquez and Liria 2012; Hall et al. 2014; Laparie et al. 2016; Sontigun et al. 2017; Macedo et al. 2018; Szpila et al. 2019; Jiménez-Martín et al. 2020). In *Chrysomya albiceps* (Wiedemann, 1819), wing size does not differ between sexes (Jiménez-Martín et al. 2020). However, in similar species such as *Chrysomya rufifacies* (Macquart, 1842) and other species of the same genus, differences were detected (Sontigun et al. 2017).

Chrysomya albiceps is one of the best studied forensic indicators in the world (Grassberger et al. 2003, Corrêa et al. 2019; Williams and Villet 2019; Al-Qahtni et al. 2021). It is a species distributed in Africa, the Iberian Peninsula, the Mediterranean and the Middle East (Séguy 1930-1932; Holdaway 1933). In recent decades, *C. albiceps* is invasive in South America and responsible for the displacement of species such as *Cochliomyia macellaria* (Fabricius, 1775) (Faria et al. 1999), due to its predatory behavior in the larval stage

(Grassberger et al. 2003; Rosa et al. 2006; Faria et al. 2007). Food resources of *C. albiceps* in nature are rich, but temporary and unevenly distributed, so it is expected to find variations in some morphological characters and body size of the species (Battán-Horenstein and Peretti, 2011). The species has already been collected using pig, dog/cat, rabbit and rat carcasses, (Early and Goff, 1986; Moura et al. 1997; Carvalho et al. 2000; Grassberger et al. 2003; Velásquez, 2008; Prado e Castro et al. 2011; Mashaly et al. 2020). However, it is still unknown how the difference in carcass types may affect wing size and wing shape in blowflies. In Portugal, the species generally occurs throughout the mainland and Madeira Island, appearing from late spring to autumn (Prado e Castro et al. 2009a; Farinha et al. 2014). Continuous monitoring of the occurrence of species such as *C. albiceps* is of fundamental importance to prevent health emergencies (Vanin et al. 2009). This includes work on how morphological traits may vary according to different conditions.

The present work tests whether there is variation in the shape and size of *C. albiceps* wings: 1) over time; 2) by temperature influence; 3) by sex and; 4) by types of carcasses (pig, dog/cat and whale), using Portuguese populations.

2.3 Methods

2.3.1 Specimen Collection and Identification of Species

Chrysomya albiceps specimens were obtained from six different years and come from four sites in Portugal (Table 2.1). The specimens were kindly provided by the second (2004, 2006 and 2007), the fourth (2014) and the third (2017) authors. The 2021 specimens were collected by the first author. The collections in 2004, 2006, 2007, 2014 and 2021 were made through Forensic Entomology experiments. In the 2004, 2006 and 2007 trials, an adapted Schoenly trap (Prado e Castro et al. 2009b) was used to collect the flies. The trap surrounded

the carcass, while the flies were captured in collecting cups attached to the trap. In 2014, flies were also collected in cups attached to a small cage constructed from metal and wood that enclosed the carcass. In 2021, flies were collected via deadly jar, using metal tweezers. The carcasses were surrounded by a cage built from wood and cloth that allowed the entrance of the collector, to facilitate the collection of the insects. In all these experiments, collection was done daily, at least during the period when *C. albiceps* was present in the carcass. The 2017 flies were collected manually, via metal forceps, and deposited directly into a flask with 70% alcohol. The specimens were given for this work already identified and had the identification confirmed by the first author using the works of Rognes (1990) and Grella et al (2015).

2.3.2 Slide Preparation and Capture of Images

The right wing of each specimen was detached by using a combination of fine-tipped forceps. Each wing was positioned in ventral face on a slide and a drop of mounting medium was added before the apposition of a coverslip. The slides were left at room temperature for 7 to 10 days for drying and avoiding bubbles. Images of the slides were taken using a digital camera (Zeiss Stereo Lumar v.12) attached to a stereomicroscope with magnification at 14.8x and objective at 1.2x.

Table 2.1. Source data and sample size of the specimens of *Chrysomya albiceps* used in this study.

City	Location	Year	Method	n Male/Female
Coimbra	40°12'42.64"N; 8°27'10.56"W	2004	Pig carcass	95/95
Lisbon	38°42'27.46"N; 9°10'56.30"W	2006	Pig carcass	97/97
Lisbon	38°42'27.46"N; 9°10'56.30"W	2007	Pig carcass	100/100
Lisbon	38°42'56.10"N; 9°11'37.23"W	2014	Dog/Cat carcass	26/73
Lisbon	38°43'7.31"N; 9° 9'1.36"W	2017	Whale carcass	37/7
Lisbon	38°42'56.10"N; 9°11'37.23"W	2021	Dog/Cat carcass	98/100

Note: Although not related to the objectives of the study, the presence of *C. albiceps* in whale carcasses, *Balaenoptera acutorostrata* (Lacépède, 1804), in Portugal has been recorded for the first time.

2.3.3 Wing Morphometrics landmarks

The set of images was transformed into a TPS file using the tps Utility program software (tpsUtil32, v. 1.78, Rohlf 2019) to avoid biases in the landmarks digitization process. To obtain the wing coordinates, the software tpsDig2 v. 2.31 (Rohlf 2017) was used. In total, 16 landmarks were digitized on the wings of the specimens (Figure 2.1), which were used to obtain the 'x,y' coordinates and subsequent size and shape metrics.

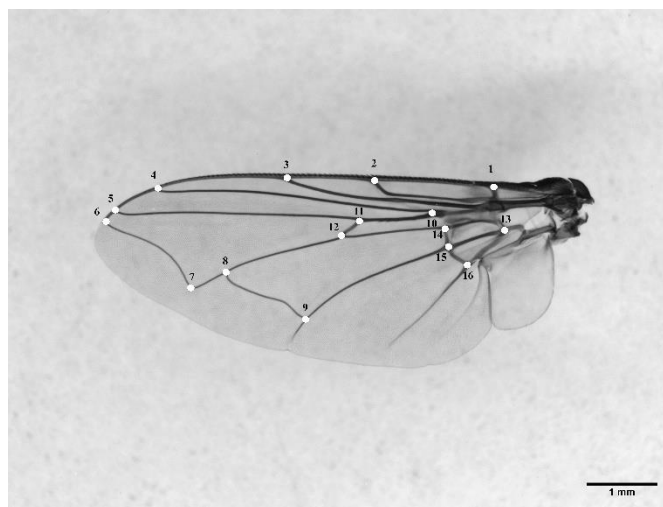


Figure 2.1. Landmarks on the wing of *Chrysomya albiceps*.

2.3.4 Variables used in Statistical Analysis

In the statistical analyses, two variables were mainly used: the Centroid Size (CS), used to perform the analyses with the wing size; and the Procrustes Coordinates (PC), used for the analyses of the wing shape. The procedure is done by importing the TPS file in MorphoJ (Klingenberg, 2011) and running in the software a Procrustes Fit following the path "Preliminaries → New Procrustes Fit → Align by Principal Axes → Perform Procrustes Fit". This step is necessary not only to generate the variables used in the analyses, but also to remove variations due to differences in scale, position, and orientation of the coordinates (Sontigun et al, 2017). The output of the procedure is the maintenance of the raw coordinates, a data matrix with the PC and CS. In shape analysis from MorphoJ, the variables "Sex" (Male or Female), "Year" (Year of Collection), Type of Carcass = "Pig" + "DogCat" + "Whale" were used as classifiers; and as covariates, the maximum (TMAX), minimum (TMIN) and mean temperature (TAVG) of the month in which the specimens were collected. All temporal variables were obtained at <https://www.ipma.pt/pt/oclima/series.longas/> (IPMA, 2022). Moreover, "TMAX",

“TMIN”, “TVG”, “Sex”, “Year”, “Pig”, “DogCat” and “Whale” were also used as independent variables in the wing size analyses (more details in later sections).

2.3.5 Allometry

The effect of allometry, when wing size influences shape variation, was evaluated by simple linear regression in MorphoJ. For this, the CS was used as independent variable and the PC as dependent variable. Complementarily, it was tested if this relationship could be influenced by sexual dimorphism, by grouping the sex variable in a multiple regression with 10,000 rounds of randomization. The residuals of the PC *vs* CS regression were subjected to the Discriminant Function Analysis (DFA) to find out whether sexual dimorphism is altered when excluding the allometric effect. The DFA underwent cross-validation test with Mahalanobis Distance (MD) associated with permutation test with 10,000 rounds. Furthermore, it was also tested the relationship of the residuals with TMAX, TMIN and TAVG.

2.3.6 Wing Shape Analysis

The variation in wing shape was analyzed in the MorphoJ. To test the difference between wing shapes by sex, DFA was used with permutation test with 1,000 permutation runs and MD. The DFA further underwent a cross-validation test to assess whether the difference was adequately evaluated. To test whether there was a difference between wing shapes by sex and year, Canonical Variable Analysis (CVA) was used. To test for variation in wing shape by type of carcass (Pig, Dog/Cat and Whale), a CVA was performed too. In both CVA, the significance level of permutation tests with MD was obtained from 10,000 permutation rounds. To test whether the variation in *C. albiceps* wing shape can be explained by temperature

variation, a simple linear regression with 10,000 permutation rounds was performed in MorphoJ: PC *vs* TMAX, PC *vs* TMIN and PC *vs* TAVG.

2.3.7 Wing Size analysis

The difference between wing size was tested by Sex, Year of Collection and Type of Carcass (CS *vs* Sex, CS *vs* Year and CS *vs* Pig, Dog/Cat, Whale). In this step, the RStudio Program (RStudio Team 2021) was used. The data did not reach normality (Shapiro-Wilk Test) nor homogenized variance (Levene Test). Therefore, the Wilcoxon rank sum test with continuity correction was used to assess the size difference by Sex. The difference in wing size per Year was assessed using the Kruskal-Wallis rank sum test. As a post hoc test, a pairwise evaluation was performed using Wilcoxon rank sum test with continuity correction with Bonferroni correction. The wing size difference by Type of Carcass was tested in the same way (CS *vs* Pig, Dog/Cat and Whale). Kernel regressions were used to find out whether the wing size of *C. albiceps* can be explained by temperature variation (CS *vs* TMAX, CS *vs* TMIN and CS *vs* TAVG). This method is used to identify non-linear relationships between two random variables. The estimator used in Kernel Regression was Local-Constant, the type of Regression was Second-Order Gaussian and the Bandwidth Selection Method was Least Squares Cross-Validation.

2.3.8 Error measurement

A sub-sample of 30 wings digitized twice was separated to find out if there was measurement error of the landmarks. A procrustes ANOVA was then performed combining the two coordinate datasets. This procedure is described by Benítez et al. (2020).

2.4 Results

2.4.1 Error measurement results

When the square mean value exceeds the error value, it means that there was no measurement error during landmarking. Consequently, it is assumed that the analyses were not biased by any kind of procedural error in the measurements. (Table 2.2).

Table 2.2. The Procrustes Anova result comparing two subsamples of *Chrysomya albiceps* wing images to assess measurement error. If $MS > Error I$, it is assumed that the analyses were not biased by any kind of procedural error in the measurements.

Centroid size		Wings			
Effect	SS	MS	df	F	p
Individual	11,002012	0,379380	29	65811,8	<0.0001
Error 1	0,00017	0,000006	30		
Shape					
Effect	SS	MS	df	F	p
Individual	0,02034992	0,0000250615	812	274,3	<0.0001
Error 1	0,00007674	0,0000000914	840		

2.4.2 Allometry results

Wing size explained the changes in wing shape, PC vs $CS = 10,6591\%$ ($p < 0.0001$). Considering this influence, all further analyses were performed when removing the allometric effect. Even so, some results are shown to highlight the practical differences of removing the allometric effect (Table 2.3). It was evidenced that allometry influences sexual dimorphism in *C. albiceps* (predicted = 5,6919%, $p < 0.0001$). Furthermore, the explanatory predictions between PC and temperatures decreased (values not shown). Allometry also caused changes in

sex discrimination (Table 3, MD= 5.4159, $p < 0.0001$). Cross-validation confirmed the values found in the DFA - T-Square $p < 0.0001$.

Table 2.3. Allocation of groups by Sex using Discrimination Function Analysis in MorphoJ (DFA, $p < 0.0001$). The values in bold show how the correct identification of sexes is improved after removing the influence of wing size on wing shape.

DFA	Allocated to		DFA	Allocated to	
Group	Female	Male	Group	Female	Male
Female	467	5	Female	470	2
Male	2	451	Male	1	452
Cross-Validation	Allocated to		DFA	Allocated to	
Group	Female	Male	Group	Female	Male
Female	467	5	Female	469	3
Male	2	451	Male	2	451

Note: Bolded values are the DFA result after removing allometric effect.

2.4.3 Wing Shape results

DFA by Sex confirmed variation in shape - MD= 4.9262, $p < 0.0001$ - (Figure 2.2) and allocated the two groups (Table 2.3). Cross-validation confirmed the values found in the DFA -T-Square $p < 0.0001$. The success in group identification reached 99.36% for females and 99.55% for males. Variation was also found between sexes in the same year as evidenced by CVA. Despite the high overlap between points on the graph, the first two canonical variables were responsible for explaining 80.70% (Figure 2.3), which denotes that the analysis identified dimorphism not only by Sex among all specimens, but also dimorphism among specimens by Year, which is confirmed in Table 2.4. The MD distance between the groups analysed by CVA

also showed that the wing shapes of flies collected at the same site are less distant than flies collected between different sites. There was also a lot of overlap of points in the CVA of the wing shape by type of carcass (Figure 2.4). The MD distance was low between each of the methods (Pig vs DogCat= 1.4681, Pig vs Whale= 2.6016, DogCat vs Whale= 1.9885), but the result had statistical support at $p < 0.0001$. Little variation in shape as a function of temperatures was found by regression analysis, even the results had statistical support (Table 2.5) ($p < 0.0001$).

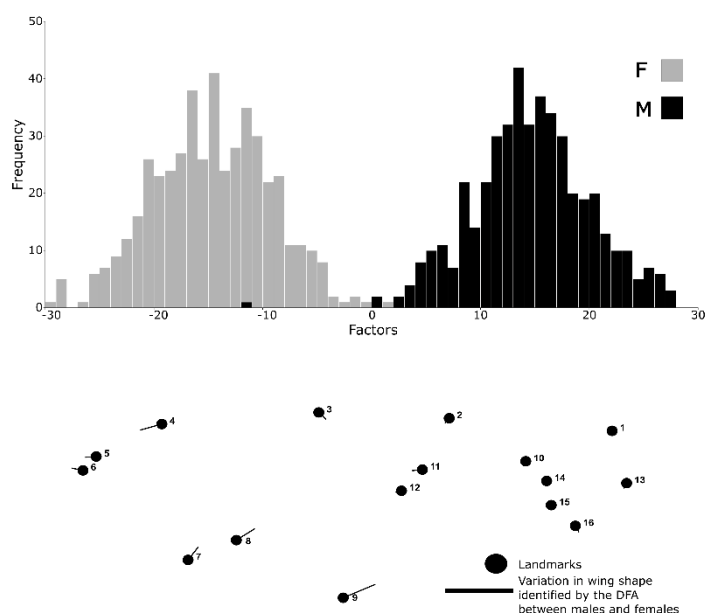


Figure 2.2. Confirmed variation in wing shape between males and females of *Chrysomya albiceps* (analysis made in MorphoJ). At the top: histogram representing values of the discriminant scores for the original data variation. At the bottom: diagram indicating shape differences between the two group means (the most variable landmarks are at more apical points). F= Female; M= Male; DFA = Discriminant Function Analysis.

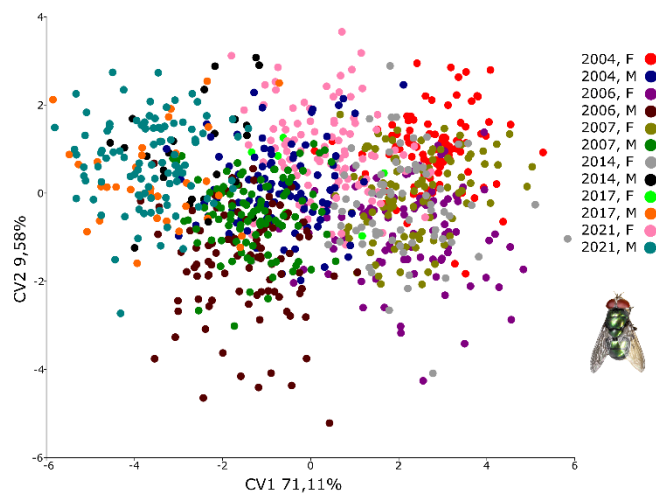


Figure 2.3. Scatterplot representing Canonical Variate Analysis in MorphoJ discriminating the wing shape of *Chrysomya albiceps*, considering the variables Sex and Year together. Each color represents a different combination of the variables Year and Sex in which *C. albiceps* was collected. F= Female; M= Male, CV = Canonical Variate.

Table 2.4. Mahalonobis Distances found by Sex and Year analyzed together by Canonical Variate Analysis and permutation test with significance obtained at 10,000 rounds ($p < 0.05$). M= Male, F= Female.

Mahalonobis Distance Values												
Years & Sex	2004, F	2004, M	2006, F	2006, M	2007, F	2007, M	2014, F	2014, M	2017, F	2017, M	2021, F	
2004, M	3,8391											
2006, F	2,277	3,4579										
2006, M	5,0628	2,2536	3,8399									
2007, F	2,2809	3,5137	2,0858	4,5293								
2007, M	4,6211	2,0814	3,9482	2,2698	3,9204							
2014, F	2,4595	3,4222	1,8028	4,0584	2,1249	4,1156						
2014, M	5,5501	2,8115	5,1181	3,062	5,2641	2,7816	4,9655					
2017, F	4,5794	3,7606	3,9488	4,2036	4,0093	3,7295	4,0813	4,5023				
2017, M	6,6413	3,5545	6,047	3,2792	6,152	3,2992	5,9257	2,6319	4,4375			
2021, F	2,8806	2,0949	2,7511	3,2719	2,8182	2,8781	2,6723	3,4747	3,0308	4,3114		
2021, M	6,5506	3,3241	6,0523	3,2686	6,2562	3,2498	5,8767	1,7265	4,9814	2,0113	4,2051	

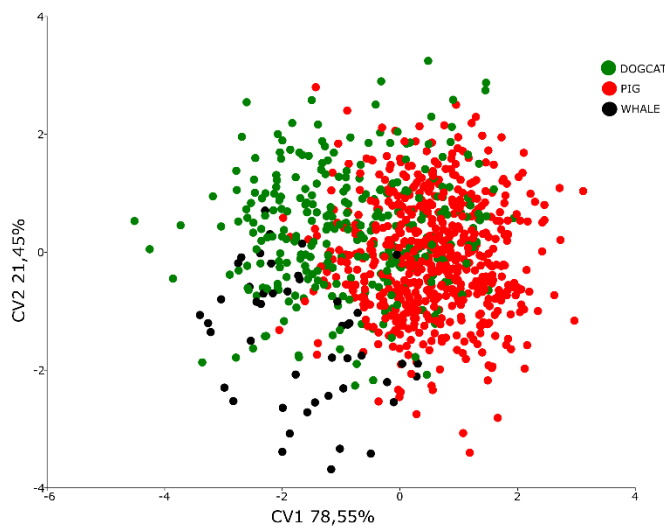


Figure 2.4. Scatterplot representing Canonical Variate Analysis in MorphoJ discriminating the wing shape of *Chrysomya albiceps*, considering the type of carcass. Each animal and its respective colour represents the type of carcass in which the *C. albiceps* individuals were collected. CV = Canonical Variate.

Table 2.5. Values from the regression analysis in MorphoJ between Procrust coordinates (PC) and temperatures (TMAX, TMIN and TAVG).

Regression Test	Total SS	Predicted SS	Residual SS	% predicted
PC vs TMAX	0,41349661	0,00666912	0,40682749	1,6129
PC vs TMIN	0,41349661	0,00900937	0,40448724	2,1788
PC vs TAVG	0,41349661	0,00765520	0,40584142	1,8513

Note: bolded values are the percentage of explanatory prediction of one variable as a function of the other.

2.4.4 Wing Size results

Variation in wing size by Sex was found by the difference in medians detected by the Wilcoxon rank sum test (Figure 2.5). The W value is not an estimate of how different the medians are, but rather the number of times the median of one group is smaller than that of the other. Variations was also found in wing size by Year (Figure 2.6) and between the types of carcasses (Figure 2.7). When testing whether wing size can be influenced by temperature, a non-linear relationship by Kernel

regression (Figure 2.8) was obtained. All relationships had statistical support $p < 2.22 \times 10^{-16}$ and similar values in R^2 . However, in other outcomes of the analysis, the relationship between wing size and TMAX ($R^2 = 0.2800388$, Residual Standard Error = 0.605526, $h = 0.4862875$), TMIN ($R^2 = 0.3369224$, Residual Standard Error = 0.5811118, $h = 0.03400968$) and TAVG ($R^2 = 0.3367556$, Residual Standard Error = 0.5811856, $h = 0.4910393$) showed different results. The h value is a smoothing parameter known as Bandwidths. Very high or small values indicate that the model did not work very well to describe the relationship between two variables. Apparently, this is what happened in CS vs TMIN. On the other hand, CS vs TAVG proved to be a good description of the relationship between wing size and temperature.

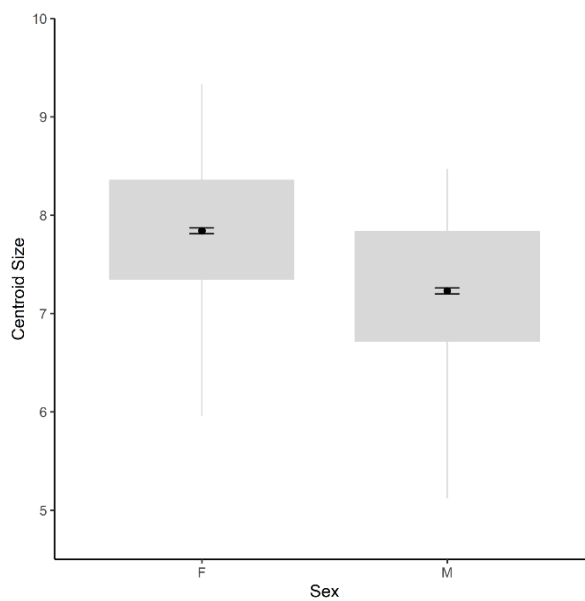


Figure 2.5. Boxplot representing CS (Centroid Size) vs Sex: Difference in wing size between F= females and M= males of *Chrysomya albiceps*. $W = 160563$, $p < 2.2 \times 10^{-16}$. CS female = 7.84; CS male = 7.22.

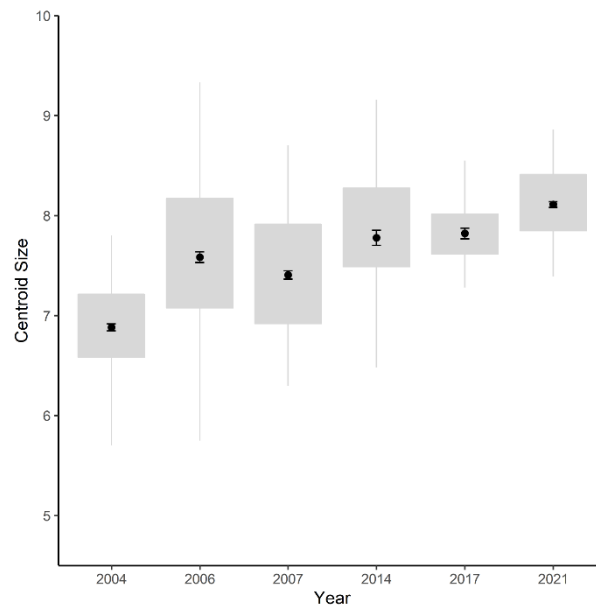


Figure 2.6. Boxplot representing CS (Centroid Size) vs Year: Difference between wing size of *C. albiceps* over time in the years sampled for this study. $H = 331.76$, $df = 5$, $p < 2.2e-16$. The values of Pairwise comparisons using Wilcoxon rank sum test with continuity correction was: 2004 vs 2006= $p < 2.2e-16$; 2004 vs 2007= $p < 6.0e-15$; 2004 vs 2014= $p < 2.2e-16$; 2004 vs 2017= $p < 2.2e-16$; 2004 vs 2021= $p < 2.2e-16$; 2006 vs 2007= $p < 0.05739$; 2006 vs 2014= $p < 0.24193$; 2006 vs 2017= $p < 0.95188$; 2006 vs 2021= $p < 8.3e-13$; 2007 vs 2014= $p < 5.4e-06$; 2007 vs 2017= $p < 0.00034$; 2007 vs 2021= $p < 2.2e-16$; 2014 vs 2017= $p < 0.99999$; 2014 vs 2021= $p < 0.00558$; 2017 vs 2021= $p < 5.3e-05$.

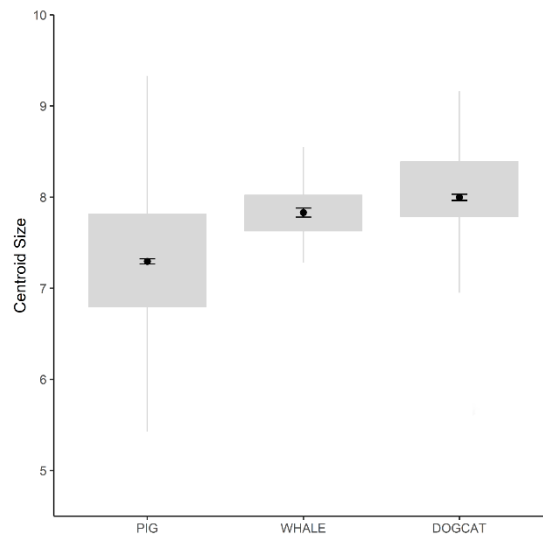


Figure 2.7. Boxplot representing CS (Centroid Size) vs Type of Carcass (Pig, Whale and Dog/Cat). Each animal represents the type of carcass in which the *Chrysomya albiceps* individuals were collected. $H = 215.89$, $df = 2$, $p < 2.2e-16$. The values of Pairwise comparisons using Wilcoxon rank sum test with continuity correction was: Pig vs Whale= $p < 2e-08$; Pig vs Dog/Cat= $p < 2e-16$; Whale vs Dog/Cat= 0.0012

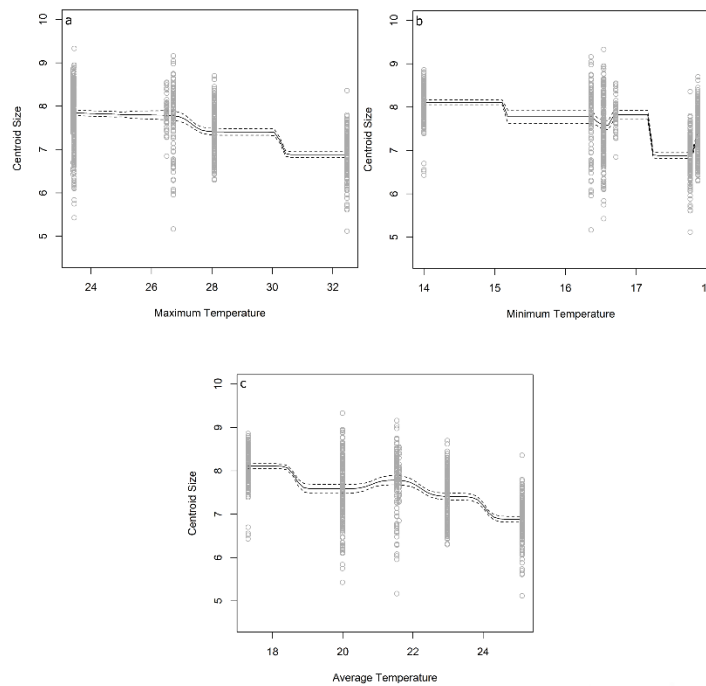


Figure 2.8. Scatterplot representing CS vs TMAX (top, left), CS vs TMIN (top, right) and CS vs TAVG (bottom, mid): Relationship between wing size (Centroid Size = CS) of *Chrysomya albiceps* and maximum (TMAX), average (TAVG) and minimum temperature (TMIN) of the insect sampling periods, estimated by Kernel regressions.

2.5 Discussion

2.5.1 A brief discussion about sample size issues

Some studies in the literature that evaluated sexual dimorphism in Calliphoridae did not detect intraspecific sexual dimorphism for some species, either in wing shape, wing size, or both (Sontigun et al. 2017; Szpila et al. 2019; Jiménez-Martín et al. 2020). In all these studies, a common point is the low sample size for some species, an experimental limitation that can generate ambiguous interpretations and hide existing relationships in nature (Bissonette, 1999). For instance, there is no way of knowing whether the failure to detect sexual dimorphism in wing size or shape in *C. albiceps* in previous works is the result of low sample size, regional differences between populations, or the method of obtaining the landmarks. However, the sample size in the present work was greater than

in previous works. Therefore, sexual dimorphism may have been detected in the present work by this factor. Modern geometric morphometry is indeed a cheap and very useful tool for integrative taxonomy (Schlick-Steiner et al. 2010). A low sample size can return high assertiveness values in species identification (Sontigun et al. 2017; Szpila et al. 2019). Thus, specific investigations on how sample size may interfere with the detection of patterns in wing shape and wing size are needed.

The Figures 2.4 and 2.7 show the differences between wing shape and wing size as to the Type of carcass in which the flies were collected. These results should be interpreted with caution because of the disparity in sample size between the groups tested. This was an effort to start a discussion on the subject, but the low sample size did not allow a robust analysis.

2.5.2 Allometry discussion

The present work found allometric effect on wing shape variation of *C. albiceps* with good statistical support (see section 3.2). However, this effect did not influence so much the sex discrimination of the species (see Table 2.3), but it influenced the results of the temperature effect on the wing shape (results not shown). Possibly, this happened because the size of insect structures is directly influenced by temperature, which potentiates the effect of size on shape. In other Diptera species, allometry was also identified (Sontigun et al. 2019, Oliveira-Christe et al. 2020, Limsopatham et al. 2021). Sontigun et al. (2017) found a low allometric effect that did not impact the identification of blowflies. However, allometry can play an important role in detecting sexual dimorphism. For practical purposes, it is suggested that all work involving phenotypic variation should remove the allometric effect before any analysis, as recommended by Sontigun et al. (2017).

2.5.3 Wing Shape Discussion

Changes in the wing shapes of blowflies have already been identified due to seasonality and biological invasion (Laparie et al. 2016), competition (Macedo et al. 2020) and allometry (Szpila et al. 2019). In the present study, effects with good statistical support were identified, however, with low explanatory power. Furthermore, a strong effect of wing size on wing shape (allometry) was observed (see section 3.2), a similar conclusion to that of Szpila et al. (2019). This is the reason for presenting the DFA results before and after removing the allometric effect (see Figure 2.2).

Discrimination Function Analysis identified sexual dimorphism in *C. albiceps* with a high success rate (Figure 2.2), which is encouraging for scholars less experienced with Calliphoridae taxonomy. Dimorphism in wing shape is identified for several species of blowflies (Szpila et al. 2019), including other species of the genus *Chrysomya* (Sontigun et al. 2017). However, some studies have not found sexual dimorphism in *C. albiceps* (Vásquez and Liria, 2012; Szpila et al. 2019; Jiménez-Martín et al. 2020). In the present work, this may have occurred because it reflects regional variation in flies (Hall et al. 2014), an allometric effect sufficient to cause differences in wing shape (Szpila et al. 2019), or divergent niche between sexes (Shine, 1989; Fryxell et al. 2019). Females have a competitive advantage for finding animal carrion (Campobasso et al. 2001). Examining Figure 2.2, the wing shape between the two sexes is more stable at the base, while the most variable points are at more apical points, a detail also noted by Jiménez-Martín et al. (2020) in the species of blowflies from the Iberian Peninsula. The possibility of using the wing shape of *C. albiceps* as a tool for sex identification in the species was confirmed, provided that allometry is identified. This does not exclude the use of traditional taxonomic criteria, nor the importance of considering geographic variation to complement identification. These results are valuable for Forensic Entomology, for instance, when dealing with damaged specimens that are found at crime scenes.

The variations in wing shape by Year/Sex (see Figure 2.3) and by Type of Carcass (see Figure 2.4) are not clear. In Figure 2.3, the sum of the two main covariates was 80.69%. Considering the 12

groups tested, this figure shows that wing shape does not vary by Year and Sex, when analyzed together. The non-detection of variation in wing shape over the six years sampled indicates that there is a strong genetic component driving these changes in *C. albiceps*, which makes wing shape a stable criterion for taxonomic identification. In Figure 2.4, the two main covariates explained the variation in shape by 100%, which denotes that the analysis did not separate the three groups tested in the CVA. As seen in section 4.1, this may have happened because of the sampling disparity between the groups tested, mainly because of the low sample size of specimens collected on the whale carcass.

2.5.4 Wing Size discussion

In this work, there is a clear difference in wing size by sex in *C. albiceps* (see Figure 2.5). The identification of sexual dimorphism by size is contrary to another work with the same species in the Iberian Peninsula (Jiménez-Martín et al. 2020). In that work, the differences the wing size is not as variable and do not have statistical support. This difference between the two results may have occurred because of the difference in sample size (see section 4.1), or natural differences between local populations. For instance, the females of blowflies need protein nutritional sources for the development of their eggs (Shewell, 1987). It is expected that due to this factor, females need to move over a larger distances in their habitats, since guilds of carrion-feeding species are donor-controlled, i.e., oviposition of these species depends on the random supply of resources in nature (Polis and Strong 1996). Consequently, female flies accessing animal carcasses are expected to be larger and better competitors.

Size variations in insects are often associated and correlated to environmental factors (Battán-Horenstein and Peretti 2011; Gallesi et al. 2016). A negative relationship between wing size and temperature has been found elsewhere (Garzón and Schweigmann 2018), including *C. megacephala* (Reigada and Godoy, 2005). In the present work, the same conclusion was reached: the higher the temperature, the further the wing size decreases, in a non-linear relationship (Figure 2.8). Although

C. albiceps has a competitive advantage in carcasses environments, in temperate environments it is a seasonal species. The species range is restricted in Portugal to the warmer months of the year (Prado e Castro et al. 2009a, 2012, 2016), so population size increases with temperature. This generates greater competition for resources and consequent trade-offs in population parameters such as size.

Chrysomya albiceps is a species that competes voraciously for resources in the larval stage (Faria et al. 2007). It is recorded feeding on other species and on itself, especially in environments where larval density is high, such as in animal carcasses (Ullyett 1950). In Forensic Entomology experiments using animal carcasses, *C. albiceps* usually appears in the adult stage in two moments. The first, when eggs are laid, in the first days of colonization, and the second at the birth of new individuals from the initial egg laying (since this species does not migrate from cadavers to pupate). As adult size in these flies basically depends on the life history of their larval stage, it was expected that different Carcass Types could influence the wing size of *C. albiceps*. However, this is not what the present work found, as seen in Figure 2.7. On the other hand, population parameters such as weight and body size are density dependent characteristics and influenced by factors as temperature (Reis et al. 1994; Tarone et al. 2011). Moreover, it is not excluded that, as with the wing shape, no difference was detected because of the lack of sample size.

Over the years, a slight difference in wing size was noted. However, it is notable that the greatest difference expressed is between the years 2004 and 2021 (Figure 2.6). This indicates that other factor may have caused the increase in wing size in *C. albiceps*, as the relationship with temperature was negative. Riback and Godoy (2008) found no relationship between seasonality and wing size and tibia size of *C. albiceps* over two years, indicating no influence of temperature change on some morphological traits of the species. Under experimental conditions, the wing size of *C. albiceps* is larger in the presence of *Lucilia sericata* (Meigen, 1826) than when the species interacts with itself (Ivorra et al. 2022), which confirms the results obtained by Ullyett (1950). Therefore, it is

plausible that the most important factor for the body size and structures of *C. albiceps* is its peculiar larval behavior, which not only feeds on animal carcasses, but also preys on other species.

2.6 Conclusion

Unlike what was previously known, *C. albiceps* has sexual dimorphism in both wing shape and wing size. Both were influenced by temperature, although this influence was low in the case of the wing shape. One of the reasons for this is the relationship between wing size and wing shape, a phenomenon already well known. Studies with information on species populations in different locations demonstrate how the same species can have different developments, so it is important to assess regional differences in species such as *C. albiceps*. The results of this work can be used as a standard for future studies and provide further information on the natural history of this species, as well as being useful for fields such as Forensic Entomology.

2.7 Credit Statements

Sérgio J. M. Rodrigues-Filho: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft preparation, Project Administration. Catarina Prado e Castro: Resources, Writing – Review & Editing. Luís Filipe Lopes: Resources, Writing – Review & Editing. Isabel Pereira da Fonseca: Resources, Writing – Review & Editing. Maria Teresa Rebelo: Conceptualization, Methodology. Resources, Writing – Review & Editing, Supervision, Funding Acquisition.

2.8 Acknowledgements

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2.9 Ethics approval and consent to participate

Ethical approval for this research project was given by CEIE (Ethics Commission for Research and Teaching from Faculty of Veterinary Medicine, University of Lisbon). Written and oral consent for cat/dog/pig were obtained for every case from owners or responsible shelter centre personnel.

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Chapter 3

"Where in Europe is *Chrysomya albiceps*? Modelling present and future distributions".

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Where in Europe is *Chrysomya albiceps*? Modelling present and future potential distributions.

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3.1 Abstract

Chrysomya albiceps (Wiedemann, 1819), a species of blowfly (Diptera: Calliphoridae), historically distributed throughout Southern Europe, has recently dispersed to cooler regions in Europe, which is an intriguing phenomenon. In this work, we used Maxent software to formulate climate suitability using a machine learning technique to investigate this fact. The bioclimatic variables that best explained the climate suitability were Annual Mean Temperature (67.7%) and Temperature Annual Range (21.4%). We found that *C. albiceps* is climatically suitable for several parts of Europe, except for high altitude areas like the Swiss Alps. In warmer countries such as Portugal, Spain and Italy, the entire coastal territory was the most suitable for the species. Future scenario models show that in these eastern countries and some northern areas, climate suitability has increased. This increase is reinforced when comparing the gains and losses in climate suitability between the present-day model and the future scenario models. These changes are most likely caused by changes in temperature, which is the main explanatory factor among the tested variables, for the climate suitability. As one of the most important species in forensic contexts and a potential myiasis agent, the expansion of *C. albiceps* to new locations cannot be neglected, and its expansion must be carefully monitored.

Keywords: Species distribution modelling; Climate suitability; Maxent; European continent; blowflies; Calliphoridae.

3.2 Introduction

As seen in recent years, the world is warmer and this phenomenon is influenced by anthropic activities such as fossil fuels burning, cement production, flaring, forest management and other land uses (Jia et al. 2019). According to the Intergovernmental Panel on Climate Change - IPCC (2021), an increase in the global average temperature is forecast for the coming years, considering the predicted scenarios. Those changes can modify global dynamics of the ecosystems by facilitating the invasion of exotic species, the dispersion of disease vector species and the emergence of agricultural pests (Wagner 2020), mainly insects (Samy et al. 2016; Iwamura et al. 2020; Wang et al. 2020). Predicting the distribution of insects in the context of climate change has become one of the great challenges of the 21st century.

Blowflies (Diptera: Calliphoridae) are a common group of insects, widespread throughout the world (Rognes 1997; Wolff and Kosmann 2016), including *Chrysomya albiceps* (Wiedemann, 1819). The biology and ecology of this species can be used to estimate the post-mortem interval of a corpse, as well as giving clues if the corpse has been changed location (Martín-Vega et al. 2017). Beyond the forensic importance, understanding the potential distribution of this species is essential for the following reasons: 1) *C. albiceps* is a mechanical vector of pathogens. The species can also cause severe primary and secondary myiasis in livestock, domestic animals and humans (Zumpt 1965; Schnur et al. 2009; Sotiraki and Hall 2012); 2) sympatry with similar species such as *Chrysomya rufifacies* (Macquart, 1842) and *Chrysomya putoria* (Wiedemann, 1830) may induce taxonomic misidentification (Erzinclioglu 1987; Grella et al. 2015); 3) the species is a facultative predator of other blowflies in larval stage in Neotropical Region (Faria et al. 1999, 2007) and Palearctic Region (Ivorra et al. 2022); and 4) more studies are needed on their driving forces in specific geographical areas, especially at smaller scales (Hosni et al. 2022). Recently, research groups have used a maximum entropy algorithm with Maxent software (Phillips et al. 2006) to model current and future

niche distributions of blowfly species (Mulieri and Patitucci 2019; Hosni et al. 2020), including *C. albiceps* (Hosni et al. 2022).

Maxent (Phillips et al. 2006; Phillips and Dudik 2008) has been used in recent years to estimate and predict scenarios of potential distribution of the species according to ecological niches favorable to the target species. This tool allows generating niche estimator models through bioclimatic data and the present occurrence of the species (Phillips et al. 2017). The tool is extremely popular and has been used systematically in recent years, as it has been shown to perform much better than other methods such as GARP and BIOCLIM (Elith et al. 2006). In this work, it was used to understand what the climate suitability of *C. albiceps* looks like, providing clues about its potential distribution under different climate scenarios.

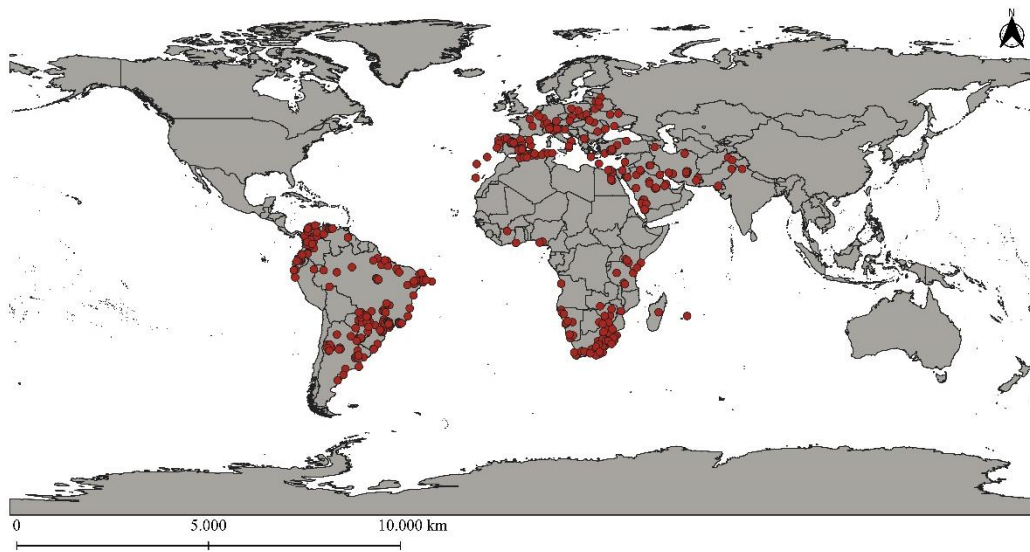
The historical distribution of this species encompasses Africa, the Middle East, and Southern Europe (Séguy 1930-1932; Holdaway 1933). In the early 2000s, *C. albiceps* began to be identified in new areas of Europe (Povolný 2002; Grassberger et al. 2003). Since then, the distribution of the species has increased on the old continent towards other central and eastern European countries (Makovovetskaya and Verves 2018). The recent and rapid dispersion of this species has generated two hypotheses: a) that the species' distribution is changing due to a more suitable climate (Povolný 2002; Gosselin and Braet 2008); b) that populations of *C. albiceps* are adapting to European winters (Makovetskaya and Verves 2018). Furthermore, the diapause mechanisms of the species are still not well understood (Michalski and Szpila 2016) and can also be a decisive factor in dispersion.

This study aimed to enhance our understanding of the climate suitability of *C. albiceps* and the climatic factors that influence its potential distribution. To achieve this, the study utilized geographic coordinates and bioclimatic variables to model the current and future distribution of *C. albiceps*. For that purpose, a maximum entropy machine learning technique was used. The discussion focused on the European region, given the recent expansion of the species in this continent.

3.3 Material and Methods

3.3.1 *Chrysomya albiceps* records

A total of 671 occurrence records were obtained from scientific papers, monographs, and dissertations present in the following databases: <https://www.biodiversitylibrary.org/>, <https://pubmed.ncbi.nlm.nih.gov/>, <https://scholar.google.com/>, <https://www.scielo.br/>, www.elsevier.com and <http://periodicos.capes.gov.br/>. The keyword searched was "*Chrysomya albiceps*" (see references in supplementary material 1). The Global Biodiversity Information Facility (www.gbif.org) was also used as a source of coordinates, with "species only" criterion and with the following filters: "material sample and preserved specimen" and "including coordinates". Some records from Brazil, using attractant traps by the first author, are included. Doubtful and repeated records were excluded. Records not coupled with collected specimens, records with photo-based identification and single records in remote areas (China, for example) were not used. Thereafter, the coordinates were refined with the filter of 20 km distance from each other using the package "spThin" in the RStudio program (Aiello-Lammens et al. 2015; RStudio Team 2021). The 20 km filter was used considering that species of the Calliphoridae family can reach distances of 3.5 km per day (Tsuda et al. 2009) and their adult life cycle can exceed several days (Norris 1965). After the coordinate refinement, 413 coordinates remained which were used to run the model (Figure 3.1, see also supplementary material 2). Although several coordinate points are lost after refinement, these steps are important to improve model fit.



3.3.2 Figure 3.1. Occurrence points of *Chrysomya albiceps* recorded from the scientific literature and in the GBIF database.

Nineteen bioclimatic variables from the Worldclim database with a spatial resolution of 2.5 arc-min (Fick and Hijmans 2017) were used to generate the present day model. For the predictions of the future, the Global Climate Model IPSL-CM6A-LR of Coupled Model Intercomparison Project (CMIP6) (Boucher et al. 2020) was used, for two shared socioeconomic pathways (SSPs: SSP1-2.6 and SSP5-8.5) for two future periods (2041-2060 and 2061-2080). IPSL-CM6A-LR was used because it has high climatic sensitivity (Qin et al., 2021). Next, a dimensional reduction procedure and exclusion of highly correlated variables was initiated. This process is necessary to avoid contributions of variables that generate interpretation problems to the models (Hosni et al. 2022). A correlation between the variables was then performed in the R Program (RStudio Team 2021), using the raster package (Hijmans 2022) (supplementary material 3, Table 1), to exclude highly correlated variables ($r > 0.7$). The best explanatory variable was chosen among the variables that correlated. The resulting variables used to fit the model were Bio1 (Annual Mean Temperature), Bio2 (Mean Diurnal Range, mean of monthly max temp – min temp), Bio7 (Temperature Annual Range), Bio12 (Annual Precipitation) and Bio15 (Precipitation Seasonality, Coefficient of Variation).

3.3.3 Modelling and Model Evaluation

The maximum entropy technique was used for modelling. The model input configuration (for present-day and future models) was: 100 replicates (70% calibration and 30% test), convergence threshold = 0, 0001, multiple regularizer = 1, maximum interactions = 500, and output in cloglog format with default prevalence = 0.6, for all potential models generated. The replicates were controlled using the Subsample replacement resampling method (Mulieri and Patitucci 2019), where data selected for testing cannot be selected for training. The performance of the generated models was evaluated using the Area Under the Roc Curve (AUC), a tool present in Maxent's output. Models with $AUC > 0.75$ are considered useful (Elith 2002). Model validation was assessed using True Skill Statistics (TSS). Finally, the Jackknife test was used to assess the importance of each variable for the construction of the present and future models.

3.3.4 Plotting

The suitability maps were plotted using the "Maximum training sensitivity plus specificity Cloglog threshold" (Liu and Shi 2020) obtained from the Maxent output (Threshold > 0.4). Climate suitability maps are reliably generated using this threshold (Liu et al. 2005). ArcGIS software was used to produce the maps (ESRI 2018). In Liu and Shi (2020), 4 suitability classes are used to visualize the maps. An additional class was created in the present study, namely: Unsuitable, Low, Medium, High, and Very High. To create these classes, we used the Reclassify function from ArcGis. To facilitate visualization of the European areas on maps, the region was divided into 4 sub-regions: Southern, Western, Eastern, and Northern. In addition, a comparison of climate suitability areas gains and losses between the different scenarios tested is provided (Hosni et al. 2022).

3.4 Results

The model generated from the potential distribution on present days had good performance (AUC= 0.886; sd= 0.007; TSS = 0.67). In this model, the variables that contributed the most to its construction were bio1 (67.7%) and bio7 (21.4%) (Figure 3.2). In Figure 3.3, it is shown how the predicted probability of presence changes according to the variation of bioclimatic variables. The variables with the highest gain were also bio1 and bio7 (see supplementary material 3, Figure 1).

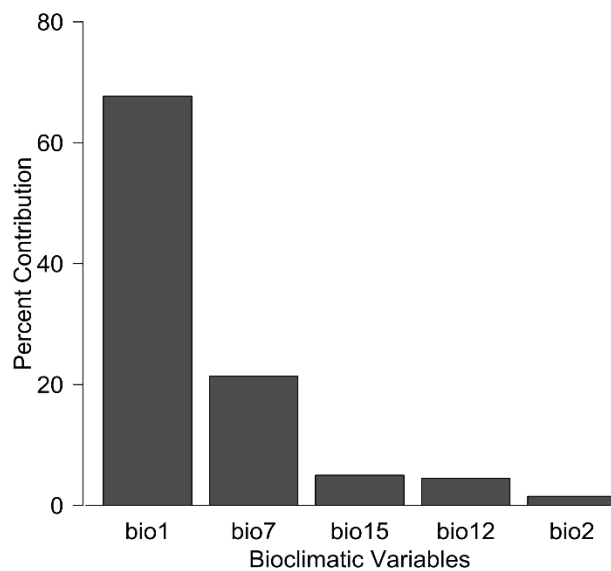


Figure 3.2. Relative contribution of bioclimatic variables to the construction of the current climate suitability model of the species *Chrysomya albiceps*. bio1 = Annual Mean Temperature, bio2 = Mean Diurnal Range, mean of monthly max temp – min temp), bio7 = Temperature Annual Range, bio12 = Annual Precipitation and bio15 = Precipitation Seasonality, Coefficient of Variation.

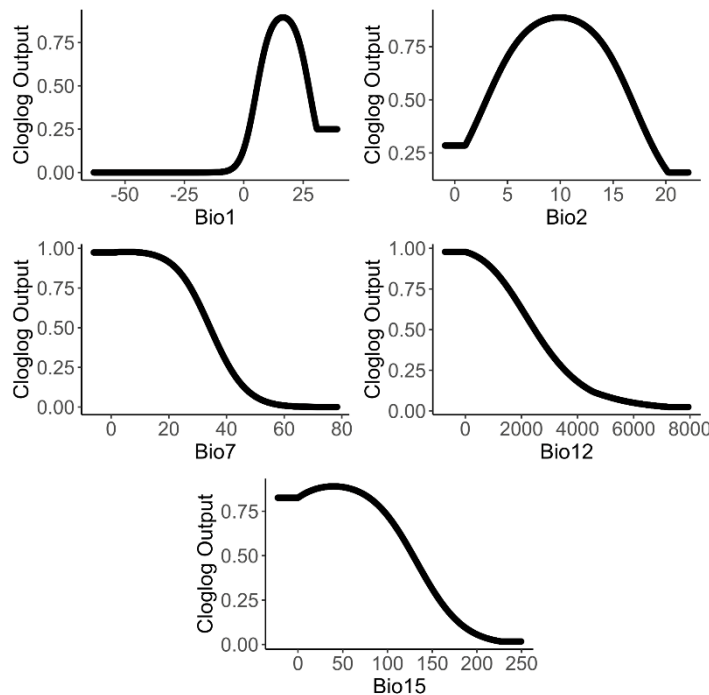


Figure 3.3. Response curves of the main bioclimatic variables in the construction of descriptive models of the climate suitability of *Chrysomya albiceps*. Bio1 = Annual Mean Temperature, Bio2 = Mean Diurnal Range, mean of monthly max temp – min temp), Bio7 = Temperature Annual Range, Bio12 = Annual Precipitation and Bio15 = Precipitation Seasonality, Coefficient of Variation.

Climate suitability for the species *C. albiceps* has been shown for the entire territory of Europe (Figure 3.4), except for high altitude sites such as the Swiss Alps and Northern Europe. In warmer countries, such as Portugal and Spain, the entire coastal territory was shown to have a highly suitable climate for the occurrence of the species. Furthermore, nearby countries with higher latitudes, such as France and Belgium, also showed a highly suitable climate in their coastal areas. To the east, still in the Mediterranean area, Italy, Malta, Albania and Greece followed the same pattern. Colder countries like Poland have medium climate suitability in almost all their territory. Neighboring countries like Belarus and Lithuania have lower climate suitability, however, the occurrence of the species is already confirmed on their territories, (Lutovinovas and Markevičiūtė 2017; Makovetskaya and Verves 2018), which may mean that *C. albiceps* can establish itself even in countries with low climatic suitability.

The predictive future models of this work indicate that more areas in Eastern Europe will have increased climate suitability (Figures 3.5, 3.6, 3.7 and 3.8). Portugal, considering the 4 predictive scenarios had little variation in the amount of climate suitability. Spain showed variation in all 4 scenarios. In many areas in the north-western and central parts of the country, the climate suitability decreased. However, territories in the east had the climate suitability increased from Medium to High. A part of the territories of France and Belgium lose much of their climate suitability, from very high to high. In the United Kingdom, considering the 4 scenarios, there is a tendency towards a decrease in the climate suitability (mainly in Ireland and Scotland). Interestingly, in Scotland, more areas fall into the Low/Medium category when the least optimistic scenarios are considered (Figures 3.6 and 3.8).

Variation in climate suitability was observed across the predictive scenarios, with greater improvements in the least optimistic scenarios compared to the optimistic scenarios (Figure 3.9). The most substantial increases in climate suitability were detected in Eastern and Northern Europe. Conversely, the SSP1-2.6 and SSP5-8.5 scenarios of 2070 resulted in the most significant declines in climate suitability, with the loss distributed across all European sub-regions.

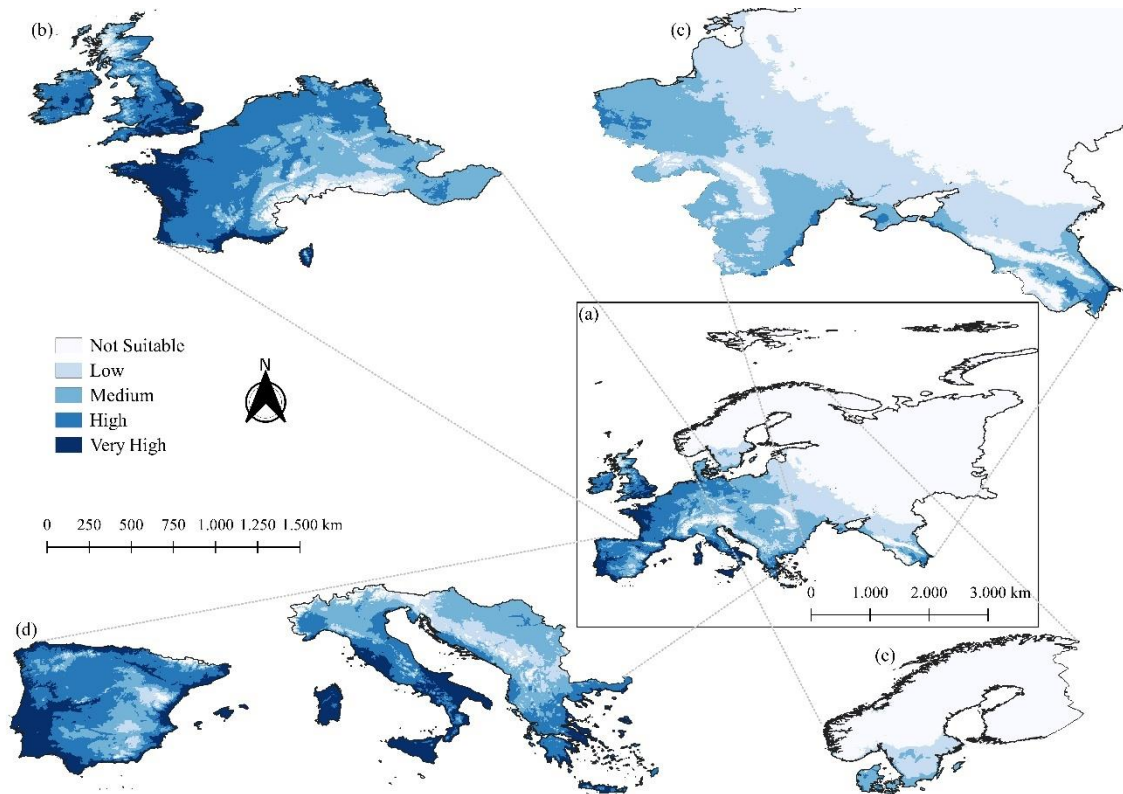


Figure 3.4. Climate suitability model of *Chrysomya albiceps* for present-day in the Europe (a) and sub-regions Western (b), Eastern (c), Southern (d) and Northern (e). Model ran in Maxent and figure redrawn in ArcGIS software.

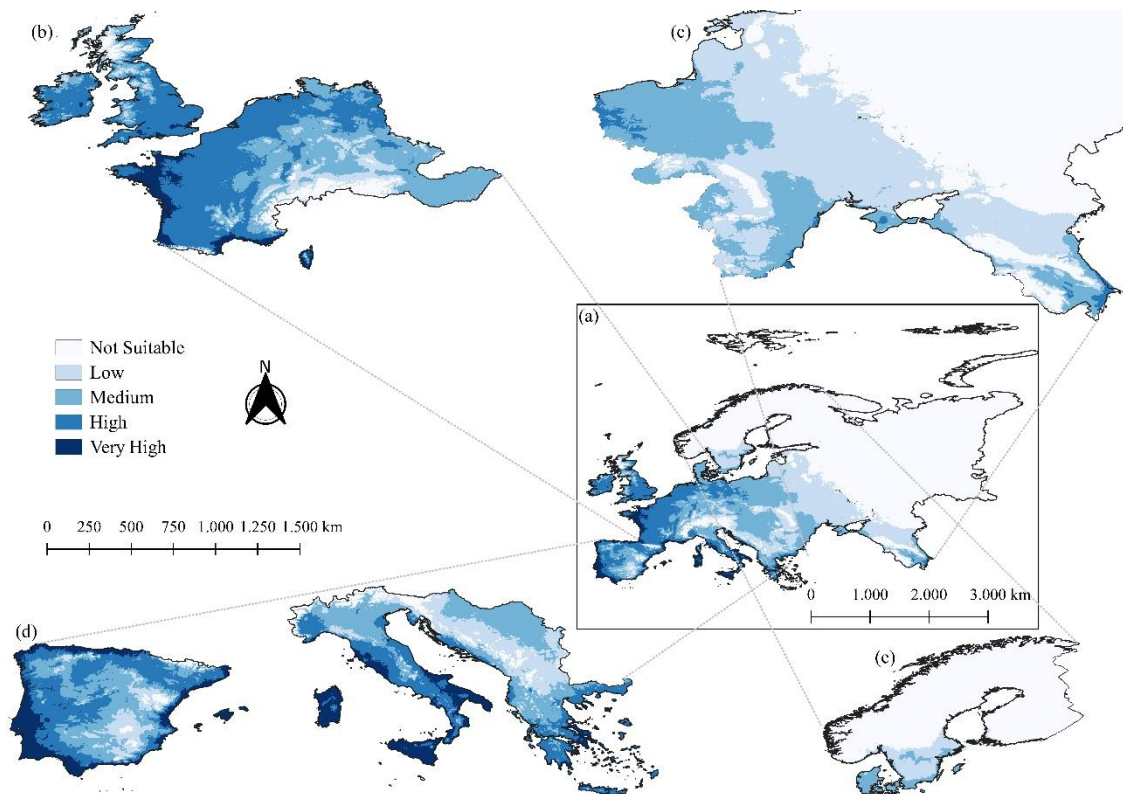


Figure 3.5. Climate suitability model of *Chrysomya albiceps* for the year 2050 in the most optimistic scenario (SSP1-2.6) in the Europe (a) and sub-regions Western (b), Eastern (c), Southern (d) and Northern (e). Model ran in Maxent and figure redrawn in ArcGIS software.

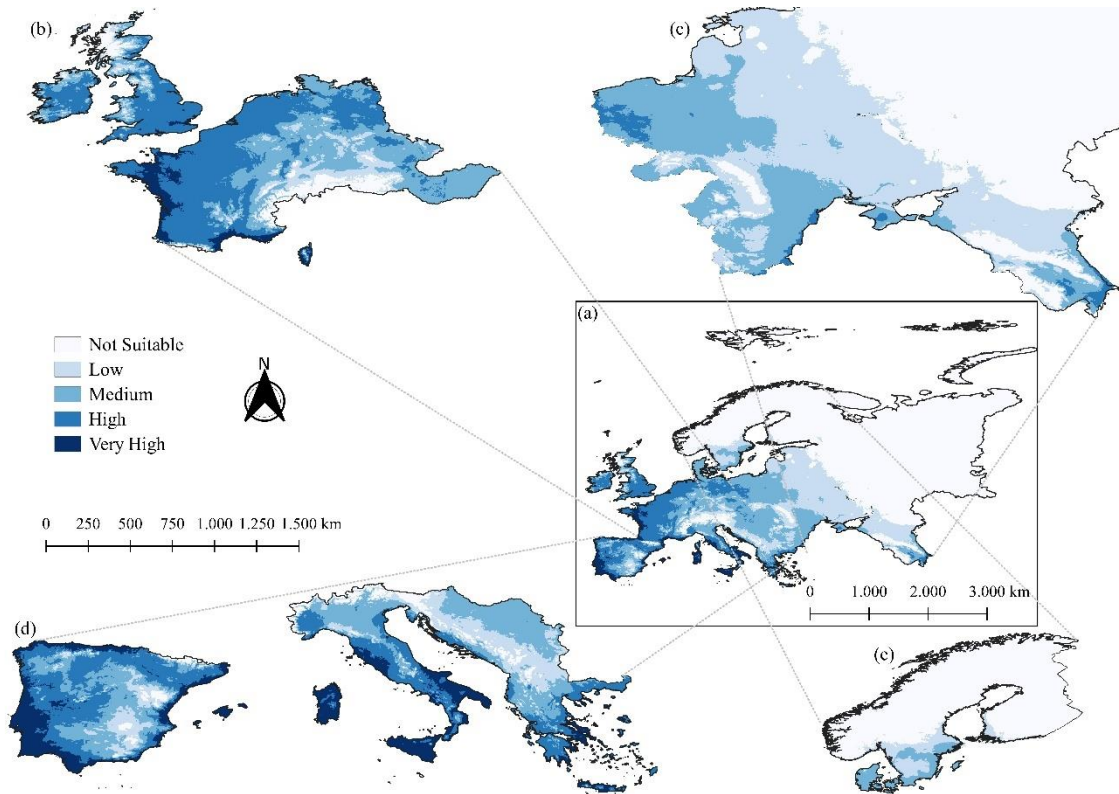


Figure 3.6. Climate suitability model of *Chrysomya albiceps* for the year 2050 in the least optimistic scenario (SSP5-8.5) in the Europe (a) and sub-regions Western (b), Eastern (c), Southern (d) and Northern (e). Model ran in Maxent and figure redrawn in ArcGIS software.

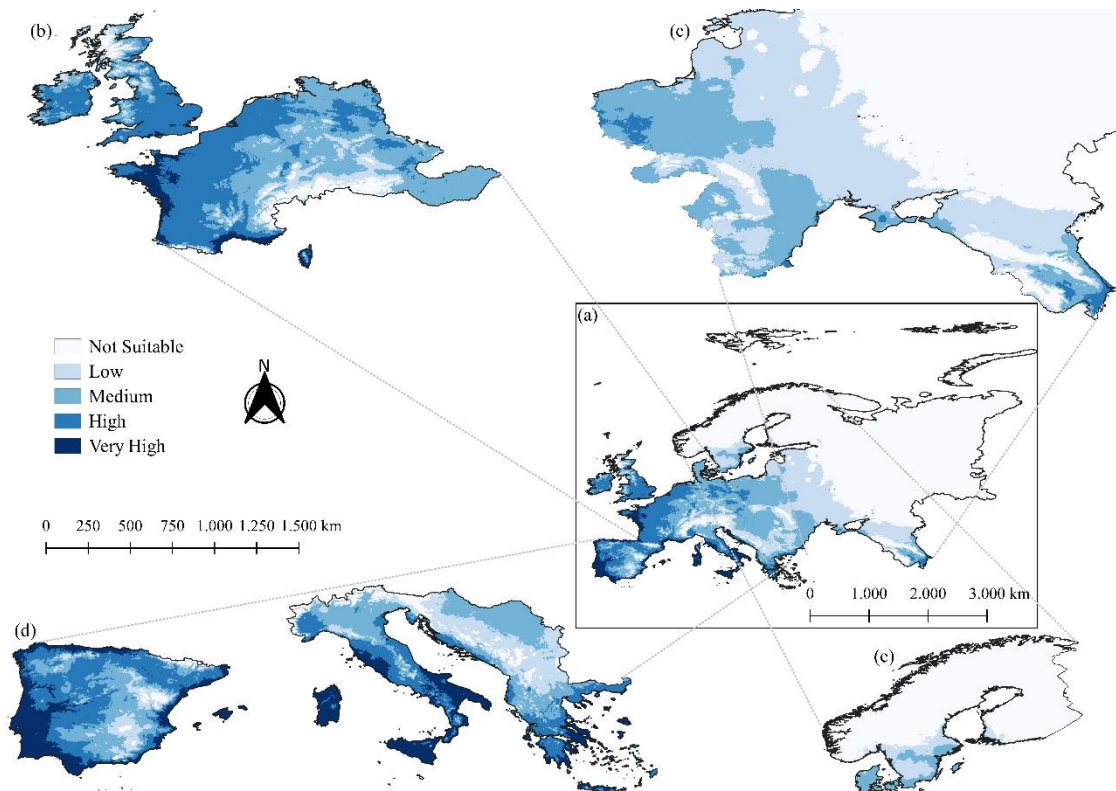


Figure 3.7. Climate suitability model of *Chrysomya albiceps* for the year 2070 in the most optimistic scenario (SSP1-2.6) in the Europe (a) and sub-regions Western (b), Eastern (c), Southern (d) and Northern (e). Model ran in Maxent and figure redrawn in ArcGIS software.

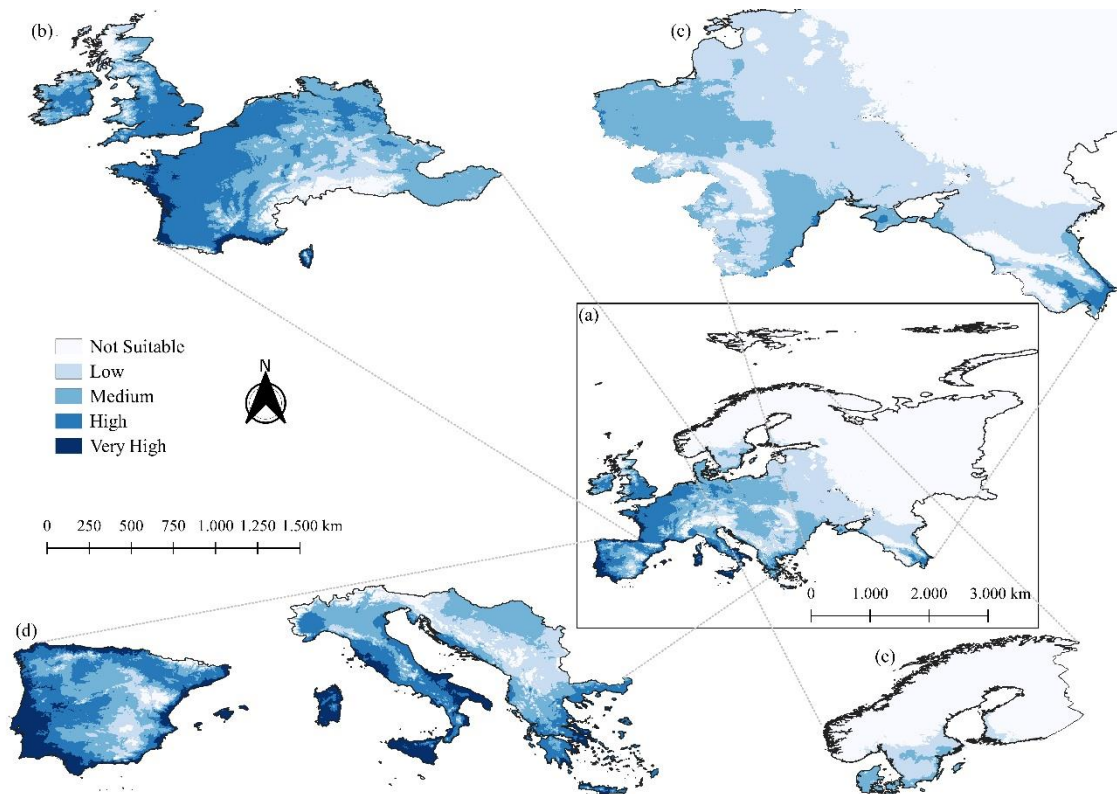


Figure 3.8. Climate suitability model of *Chrysomya albiceps* for the year 2070 under least optimistic scenario (SSP5-8.5) in the Europe (a) and sub-regions Western (b), Eastern (c), Southern (d) and Northern (e). Model ran in Maxent and figure redrawn in ArcGIS software.

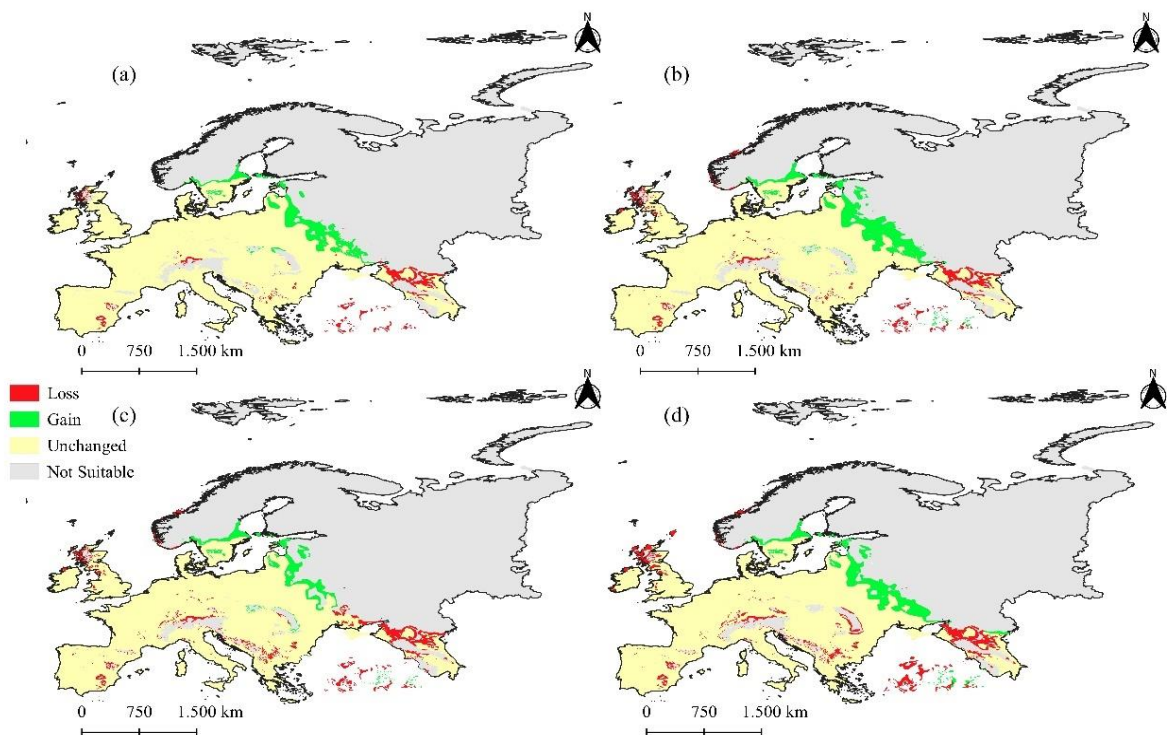


Figure 3.9: Variations in climate suitability in the 2050-SSP1-2.6 (a), 2050-SSP5-8.5 (b), 2070-SSP1-2.6 (c) and 2070-SSP5-8.5 (d) predictive scenarios. Gains (in km²) from climate suitability were at a=286.77, b=451.67, c=142.82 and d=334.61. Losses (in km²) from climate suitability were at a= 196.2, b=257.19, c=297.99, d=383.78. Threshold > 0.4.

3.5 Discussion

Changes in the climate suitability for the occurrence of *C. albiceps* between present and future scenarios have been observed based on the tested variables in the models. These differences are particularly noticeable in Eastern Europe, towards the recent geographic expansion of the species. It appears that climate change is partly responsible for this dispersal, making cooler areas more prone to *C. albiceps* occurrence. The variables bio1 and bio7, which are related to temperature, contributed to almost 90% of the variance in the models. Therefore, changes in temperature (Figures 3.4, 3.5, 3.6, 3.7, and 3.8) are highly likely to explain the observed differences between present and future scenarios, such as the evenly distributed losses in climate suitability and the gains in suitability concentrated in Eastern Europe (Figure 3.9). Consequently, *C. albiceps* may expand to new areas with climate suitability. For instance, according to Sivell (2021), the species is already considered a potential occurrence in the UK.

In the present work, it is demonstrated from a maximum entropy modelling that the most enlightening explanatory variables tested to understand the potential distribution of *C. albiceps* are the bio1 (Annual Mean Temperature) and the bio7 (Temperature Annual Range) (Figures 3.2 and 3.3). Not only that, but the generated model also demonstrates that if the bio1 is removed, the model loses much of its explanatory power (see supplementary material 3, Figure 1). Bio1 and was found to be the most useful variable in explaining the potential distribution of *C. albiceps* worldwide in the work of Hosni et al. (2022). Bio11 (Mean Temperature of Coldest Quarter) was also identified as an important variable in this study. Similarly, these two variables were found to be the most important in explaining the potential future distribution of *Chrysomya bezziana* (Villeneuve, 1914) (Hosni et al. 2020). For other subtropical/tropical insects like *Aedes albopictus* (Skuse, 1894), which has recently colonized Europe, bio11 is considered to be the limiting variable for its potential distribution (Cunze

et al. 2016). In contrast, bio15 (Precipitation Seasonality - Coefficient of Variation) has been identified as the best explanatory variable for the potential distribution of species in the family Syrphidae in Europe (Miličić et al. 2018; Milić et al. 2019).

Climate suitability in the tested models is also explained by bio7 (Temperature Annual Range), a variable related to seasonality (Figure 3.2, supplementary material 3). In subtropical regions, such as Portugal and Spain, the abundance of *C. albiceps* is seasonally dependent, increasing during the hottest periods of the year. The colder seasons limit the species, as its abundance decreases under such conditions (Prado e Castro et al. 2012).

In Figure 3.9, the gains and losses in climate suitability can be seen, with most gains concentrated in eastern and northern Europe, while suitability losses are distributed more evenly across the continent. These results are consistent with the predictions of the IPCC (2021), which anticipate the highest levels of global warming in northern and eastern Europe, as well as in northern Scandinavia and the interior areas of Mediterranean countries. The various future scenarios demonstrate that much of the areas remained unaltered (Figure 9), including southern Europe, in contrast to the results of Hosni et al. (2022), who, when evaluating the potential distribution of *C. albiceps* worldwide, stated that the species would practically disappear from the same region. Southern Europe is one of the oldest regions where *C. albiceps* historically occurred (Holdaway 1933). Even though notable climate changes may occur in the region (IPCC 2021), it is unlikely that the species would stop occurring in these regions.

Chrysomya albiceps, being poikilothermic, has its development, physiology, and distribution greatly influenced by temperature (Marchenko 2001; Hosni et al. 2022). Therefore, it was expected that temperature would be the variable that would best explain its climate suitability in Europe. The mean annual temperature range for the species is between 9°C and 27°C, as noted by Hosni et al. (2022). The life cycle of the species has been studied under experimental conditions between 11°C and 40°C in various locations around the world (Queiroz and Milward-de-Azevedo 1991; Aguiar-

Coelho and Milward-de-Azevedo 1995; Queiroz 1996; Marchenko 2001; Al-Misned et al. 2002; Kheirallah et al. 2007; Richards et al. 2009; Beuter and Mendes 2013; Al-Shareef and Al-Qurashi 2016; Salimi et al. 2018; Kordshouli et al. 2021), including Europe (Grassberger et al. 2003). In this context, the development of the species is interrupted at the upper temperature threshold of 37°C (Kordshouli et al. 2021), and at the lower temperature thresholds of 15°C (Grassberger et al. 2003) and 13°C (Marchenko 2001). Makovetskaya and Verves (2018) hypothesized that survival at these temperatures is sufficient for the species to spread to more sites in Europe, in addition to the Asian portions of southern Russia. Climate predictions suggest that the temperature in the old continent may increase from 1.2-3.4 °C to 4.1-8.5 °C in the coming years, in more and less optimistic scenarios (IPCC 2021). In this climate scenario, the hypothesis of Makovetskaya and Verves (2018) may be confirmed.

The models generated in this work can be used to help predict potential future distributions of *C. albiceps*. To better understand this species distribution around the world is an important contribution to Forensic Entomology. For instance, Turchetto and Vanin (2004) comment that the tropical species of forensic interest *Hermetia illucens* (Linnaeus, 1758) arrived in Italy in 1956, but only recently reached the colder areas of the country. This species is reported by the same authors as a superior competitor to the indigenous species. If conditions are suitable, *C. albiceps* can rapidly spread into new areas, changing the composition and dynamics of native blowfly communities, and consequently, the micro-ecosystems shaped by corpse decomposition (Baumgarten and Greenberg 1984; Braack and Retief 1986). This fly may be responsible for resetting the Post Mortem Interval due to its action on animal carcasses by preying on other species that may have arrived first (Grassberger et al. 2003). Since the beginning of the 21st century, *C. albiceps* is reported as a potential forensic species in Central Europe (Povolný 2002; Grassberger et al. 2003). Nonetheless, there are actual cases since 1995 where the species was recorded in Switzerland on the corpse of a man (Amendt et al. 2015). This indicates that the species already occurred sporadically in colder areas,

but only really started to definitively colonize new areas a few years later. This periodic colonization is exemplified in Poland by Michalski and Szpila (2016). In addition, another reason not to neglect *C. albiceps* dispersal throughout Europe and neighboring countries is the report in Bulgaria of sheep myiasis, as well as in northern Morocco (Sotiraki and Hall 2012).

3.6 Conclusion

Annual Mean Temperature and Temperature Annual Range were the variables that contributed the most to the climate suitability model in the present work. From the model generated, it is concluded that much of Europe is climatically suitable for *C. albiceps*. In future scenarios, the suitability increases in northern and eastern Europe, with areas of gains concentrated in these locations, which appears to align with the recent geographical dispersion of the species across the continent. Meanwhile, losses of areas appear to be more evenly distributed. These changes in climate suitability may have implications for the potential future distribution of the species, which could colonize new areas in Europe depending on the climatic dynamics in the coming years. Being one of the most important species in the forensic field, besides being a potential myiasis agent, the dispersion of *C. albiceps* to new locations should not be neglected.

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3.8 Supplementary Material 1

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3.9 Supplementary Material 2

species,longitude,latitude

Chrysomya albiceps,6.767,5.883

Chrysomya albiceps,31.3,30.317

Chrysomya albiceps,30.84,29.32

Chrysomya albiceps,-5.567,9.55

Chrysomya albiceps,5.738,5.543

Chrysomya albiceps,31.183,27.233

Chrysomya albiceps,28.003,-26.171

Chrysomya albiceps,26.167,-29.083

Chrysomya albiceps,14.883,-22.983

Chrysomya albiceps,26.433,-33.05

Chrysomya albiceps,4.733,36.067

Chrysomya albiceps,31.167,30

Chrysomya albiceps,30.55,30.983

Chrysomya albiceps,30.817,27.567

Chrysomya albiceps,13.35,-8.9

Chrysomya albiceps,17.3,-22.367

Chrysomya albiceps,2.234,36.261

Chrysomya albiceps,3.241,36.777

Chrysomya albiceps,-1.229,35.074

Chrysomya albiceps,2.901,36.759

Chrysomya albiceps,3.465,36.751

Chrysomya albiceps,7.373,37.004

Chrysomya albiceps,1.308,36.147

Chrysomya albiceps,6.642,36.36

Chrysomya albiceps,1.922,36.565

Chrysomya albiceps,0.575,36.254

Chrysomya albiceps,0.149,35.39

Chrysomya albiceps,2.872,36.511

Chrysomya albiceps,2.918,35.989

Chrysomya albiceps,1.322,35.367

Chrysomya albiceps,4.059,36.698

Chrysomya albiceps,15.028,-22.667

Chrysomya albiceps,46.167,39.05

Chrysomya albiceps,28.9,-28.7

Chrysomya albiceps,42.5,18.3

Chrysomya albiceps,-8.6,40.7

Chrysomya albiceps,-6.9,41.6

Chrysomya albiceps,-79.8,-4

Chrysomya albiceps,-47.7,-0.9

Chrysomya albiceps,-49.6,-2.3

Chrysomya albiceps,18.7,-34

Chrysomya albiceps,-77.8,-0.7

Chrysomya albiceps,-77.8,-1

Chrysomya albiceps,11.6,48.1

Chrysomya albiceps,-77.9,-0.5

Chrysomya albiceps,-78.1,-0.4

Chrysomya albiceps, -73.5, 5.7
Chrysomya albiceps, -80.4, -4.2
Chrysomya albiceps, -78.6, -0.5
Chrysomya albiceps, -67.5, -30.7
Chrysomya albiceps, 16.6, 49.2
Chrysomya albiceps, -74.1, 4.6
Chrysomya albiceps, 17.1, 49.7
Chrysomya albiceps, 42.4, 17.8
Chrysomya albiceps, 46.3, 24.4
Chrysomya albiceps, 50.1, 26.1
Chrysomya albiceps, 41.2, 30.6
Chrysomya albiceps, 44.2, 26.9
Chrysomya albiceps, 36.4, 28.3
Chrysomya albiceps, -58.8, -34.6
Chrysomya albiceps, -74.1, 11.2
Chrysomya albiceps, -73.4, 3.5
Chrysomya albiceps, 29.8, 30.9
Chrysomya albiceps, 11.1, 44.7
Chrysomya albiceps, -79, -0.4
Chrysomya albiceps, -69.9, -4.2
Chrysomya albiceps, 76.8, 31.3
Chrysomya albiceps, 73.1, 31.3
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Chrysomya albiceps, -74.9, 11
Chrysomya albiceps, -75.8, 5.9
Chrysomya albiceps, -74.4, 4.8
Chrysomya albiceps, -75.1, 6.4
Chrysomya albiceps, 26.5, -33.3
Chrysomya albiceps, -74.6, 6.4
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Chrysomya albiceps, -75.6, 1.9
Chrysomya albiceps, -73.4, 7.5
Chrysomya albiceps, -74.4, 6.5
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Chrysomya albiceps, 31.5, -29.3
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Chrysomya albiceps, 30.4, -29

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Chrysomya albiceps, -35.633, -5.117
Chrysomya albiceps, -54.615, -20.407
Chrysomya albiceps, -75.6, 5.733
Chrysomya albiceps, -61.408, 7.295
Chrysomya albiceps, -47.173, -22.573
Chrysomya albiceps, -46.884, -23.186
Chrysomya albiceps, -46.942, -22.372
Chrysomya albiceps, -47.154, -22.761
Chrysomya albiceps, -68.03, 10.473
Chrysomya albiceps, -65.265, -26.71
Chrysomya albiceps, -35.721, -7.741
Chrysomya albiceps, -35.291, -8.118
Chrysomya albiceps, -35.224, -8.359
Chrysomya albiceps, -36.205, -7.957
Chrysomya albiceps, -35.327, -7.657
Chrysomya albiceps, -35.255, -7.851
Chrysomya albiceps, -38.201, -8.956
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Chrysomya albiceps, -75.585, 1.603
Chrysomya albiceps, -42.566, -22.741
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Chrysomya albiceps, -67.621, 10.275
Chrysomya albiceps, -77.467, 1.267
Chrysomya albiceps, -73.945, 5.06
Chrysomya albiceps, -72.93, 11.54
Chrysomya albiceps, -75.475, 10.398
Chrysomya albiceps, -54.86, -20.442
Chrysomya albiceps, -57.833, -34.867
Chrysomya albiceps, -57.7, -34
Chrysomya albiceps, -43.436, -4.899
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Chrysomya albiceps, -47.117, -23
Chrysomya albiceps, -54.177, -24.009
Chrysomya albiceps, -32.015, -8.113
Chrysomya albiceps, -64.574, -31.909
Chrysomya albiceps, -52.357, -31.867
Chrysomya albiceps, -67.033, 10.333
Chrysomya albiceps, -66.81, 10.462
Chrysomya albiceps, -38.296, -7.986
Chrysomya albiceps, -34.953, -8.346
Chrysomya albiceps, -35.017, -7.601
Chrysomya albiceps, -34.036, -7.578
Chrysomya albiceps, -51.081, -0.035
Chrysomya albiceps, -52.487, -0.764

3.10 Supplementary Material 3

Bioclimatic variables are derived from the monthly temperature and rainfall values in order to generate more biologically meaningful variables. These are often used in species distribution modeling and related ecological modeling techniques. The bioclimatic variables represent annual trends (e.g., mean annual temperature, annual precipitation) seasonality (e.g., annual range in temperature and precipitation) and extreme or limiting environmental factors (e.g., temperature of the coldest and warmest month, and precipitation of the wet and dry quarters). A quarter is a period of three months (1/4 of the year).

They are coded as follows:

BIO1 = Annual Mean Temperature

BIO2 = Mean Diurnal Range (Mean of monthly (max temp - min temp))

BIO3 = Isothermality (BIO2/BIO7) ($\times 100$)

BIO4 = Temperature Seasonality (standard deviation $\times 100$)

BIO5 = Max Temperature of Warmest Month

BIO6 = Min Temperature of Coldest Month

BIO7 = Temperature Annual Range (BIO5-BIO6)

BIO8 = Mean Temperature of Wettest Quarter

BIO9 = Mean Temperature of Driest Quarter

BIO10 = Mean Temperature of Warmest Quarter

BIO11 = Mean Temperature of Coldest Quarter

BIO12 = Annual Precipitation

BIO13 = Precipitation of Wettest Month

BIO14 = Precipitation of Driest Month

BIO15 = Precipitation Seasonality (Coefficient of Variation)

BIO16 = Precipitation of Wettest Quarter

BIO17 = Precipitation of Driest Quarter

BIO18 = Precipitation of Warmest Quarter

BIO19 = Precipitation of Coldest Quarter”.

Text taken from <https://www.worldclim.org/data/bioclim.html>.

Supplementary Material 3 (cont.)

Table 1: Results of the correlation between bioclimatic variables. Variables that correlated more than $r > 0.7$ were excluded. The variables chosen were 01, (Bio1, Annual Mean Temperature), 02 (Bio2, Mean Diurnal Range, mean of monthly max temp – min temp), 07 (Bio7, Annual Temperature Range), 12 (Bio12, Annual Precipitation) and 15 (Bio15, Precipitation Seasonality, Coefficient of Variation).

	bio1	bio2	bio3	bio4	bio5	bio6	bio7	bio8	bio9	bio10	bio11	bio12	bio13	bio14	bio15	bio16	bio17	bio18	bio19
bio1	1	0.5719931	0.6737438	-0.506819	0.9698852	0.9756394	-0.329241	0.9411637	0.8981416	0.9781624	0.9811504	0.5297063	0.5310819	0.3061108	-0.390711	0.5532099	0.3220337	0.5091690	0.3078828
bio2	0.5719931	1	0.4816126	-0.166669	0.6320895	0.4886692	0.1166024	0.5150983	0.5274025	0.5876881	0.5354805	0.0199353	0.1110564	-0.110100	0.0794160	0.1082065	-0.112174	0.0782774	-0.0763739
bio3	0.6737438	0.4816126	1	-0.843666	0.5320096	0.7712939	-0.711951	0.4890012	0.7633434	0.5392398	0.7750362	0.6349907	0.6320783	0.3414478	-0.029126	0.6414096	0.3708368	0.4561934	0.4818679
bio4	-0.506819	-0.166669	-0.843666	1	-0.291930	-0.673795	0.9545043	-0.261682	-0.704754	-0.317477	-0.662585	-0.557523	-0.565801	-0.275884	0.0123942	-0.567903	-0.308212	-0.336015	-0.434050
bio5	0.9698852	0.6320895	0.5320096	-0.291930	1	0.8968049	-0.093896	0.9640252	0.8119696	0.9972829	0.9080332	0.4218468	0.4272985	0.2467439	-0.407301	0.4502346	0.2549674	0.4503045	0.216201
bio6	0.9756394	0.4886692	0.7712939	-0.673795	0.8968049	1	-0.524678	0.8659820	0.9386849	0.9119451	0.9977989	0.6017174	0.5945545	0.3520379	-0.359757	0.6153033	0.3736043	0.5221868	0.3899465
bio7	-0.329241	0.1166024	-0.711951	0.9545043	-0.093896	-0.524678	1	-0.093757	-0.549939	-0.133193	-0.498119	-0.542329	-0.515720	-0.317407	0.0258399	-0.518278	-0.350114	-0.308605	-0.461481
bio8	0.9411637	0.5150983	0.4890012	-0.261682	0.9640252	0.8659820	-0.093757	1	0.7192087	0.9709604	0.8733860	0.4592953	0.4534631	0.2847711	-0.464594	0.4791644	0.2947116	0.5280222	0.2195668
bio9	0.8981416	0.5274025	0.7633434	-0.704754	0.8119696	0.9386849	-0.549939	0.7192087	1	0.8206508	0.9390608	0.5091588	0.5108055	0.2743824	-0.285103	0.5253629	0.2973613	0.3708593	0.3686774
bio10	0.9781624	0.5876881	0.5392398	-0.317477	0.9972829	0.9119451	-0.133193	0.9709604	0.8206508	1	0.9204630	0.4495346	0.4490518	0.2709852	-0.426866	0.4728226	0.2806526	0.4763732	0.2372337
bio11	0.9811504	0.5354805	0.7750362	-0.662585	0.9080332	0.9977989	-0.498119	0.8733860	0.9390608	0.9204630	1	0.5836018	0.5872210	0.3267456	-0.337692	0.6067175	0.3474086	0.5137911	0.3652132
bio12	0.5297063	0.0199353	0.6349907	-0.557523	0.4218468	0.6017174	-0.542329	0.4592953	0.5091588	0.4495346	0.5836018	1	0.9026615	0.7446565	-0.324000	0.9333597	0.7780758	0.8323654	0.7599111
bio13	0.5310819	0.1110564	0.6320783	-0.565801	0.4272985	0.5945545	-0.515720	0.4534631	0.5108055	0.4490518	0.5872210	0.9026615	1	0.4580209	-0.090497	0.9905486	0.4919029	0.7626161	0.6184907
bio14	0.3061108	-0.110100	0.3414478	-0.275884	0.2467439	0.3520379	-0.317407	0.2847711	0.2743824	0.2709852	0.3267456	0.7446565	0.4580209	1	-0.474135	0.5007800	0.9925366	0.6350684	0.6576646
bio15	-0.390711	0.0794160	-0.029126	0.0123942	-0.407301	-0.359757	0.0258399	-0.464594	-0.285103	-0.426866	-0.337692	-0.324000	-0.090497	-0.474135	1	-0.147821	-0.486599	-0.313457	-0.287920
bio16	0.5532099	0.1082065	0.6414096	-0.567903	0.4502346	0.6153033	-0.518278	0.4791644	0.5253629	0.4728226	0.6067175	0.9333597	0.9905486	0.5007800	-0.147821	1	0.5350195	0.7918719	0.6465268
bio17	0.3220337	-0.112174	0.3708368	-0.308212	0.2549674	0.3736043	-0.350114	0.2947116	0.2973613	0.2806526	0.3474086	0.7780758	0.4919029	0.9925366	-0.486599	0.5350195	1	0.6538815	0.6871357
bio18	0.5091690	0.0782774	0.4561934	-0.336015	0.4503045	0.5221868	-0.308605	0.5280222	0.3708593	0.4763732	0.5137911	0.8323654	0.7626161	0.6350684	-0.313457	0.7918719	0.6538815	1	0.4226274
bio19	0.3078828	-0.076373	0.4818679	-0.434050	0.2162010	0.3899465	-0.461481	0.2195668	0.3686774	0.2372337	0.3652132	0.7599111	0.6184907	0.6576646	-0.287920	0.6465268	0.6871357	0.4226274	1

Supplementary Material 3 (cont.)

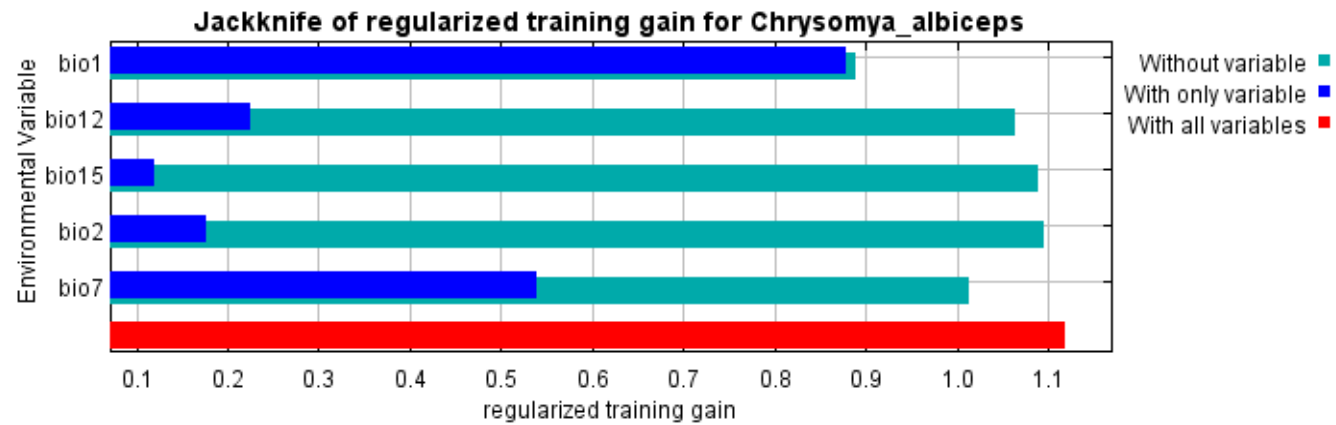


Figure 1: Results of the jackknife test of variable importance. This test is part of the output of the Maxent program.

Chapter 4

"First experimental entomological evidence of Post Mortem Interval calculation from Portugal".

The current chapter will be published as a research paper:

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This chapter followed the Guide for Authors of the journal in which it was submitted. The only changes made were in the size of the margins, the spacing of the bibliography and the reference number of the tables/figures. The link to the Guide for Authors of the Journal is in the Annex of this thesis.

First experimental entomological evidence of *Post mortem* Interval calculation from Portugal

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4.1 Abstract

Insects play a vital role in forensic investigations, especially in violent deaths. In Portugal, there is a wealth of knowledge regarding cadaveric fauna, but practical information on how insects can aid in forensics is limited. To address this gap, a study was conducted to assess the feasibility of using Accumulative Degree Days (ADD) - a technique that links temperature and insect biology to the Period of Insect Activity (PIA) in corpses. The study simulated a natural death casework and collected 4,984 insects associated with the decomposition of two cadaveric baits, including immatures that had undergone development in the laboratory. The results indicated that *Chrysomya albiceps* Wiedemann 1819, *Calliphora vicina* Robineau-Desvoidy, 1830, and *Lucilia sericata* Meigen, 1826 were the three species of blowflies present on the baits, with *C. albiceps* being the most prevalent (n=2,575) and significant forensic indicator in Portugal. The ADD calculation was successfully applied to all three species, with estimates of 13 days for *C. albiceps*, 10 days for *C. vicina*, and 7 days for *L. sericata* for the PIA. The PIA estimate for *C. vicina* was the most accurate in indicating the date of death, while the PIA for *L. sericata* was far from the actual date of death, indicating that it was a secondary species. Overall, this study demonstrated how environmental temperature data and insect biology can be effectively utilized in forensic investigations during the autumn season in Lisbon.

Key-Words: Calliphoridae, Accumulative Degree Days, Forensic Entomology.

4.2 Introduction

Insects are useful to forensics by providing clues about buildings in poor condition, pest attacks on stored products, environmental crimes, and, in particular, cases of violent death (Catts & Goff, 1992; Dias-Filho et al., 2022). Data on geographical distribution, biology and ecology of insects are recurrent in the construction of forensic entomology reports (Keh, 1985; Byrd & Tomberlin, 2009). These provide inputs into whether a corpse was removed from the scene of death, time of decapitation, specific locations of trauma on the body, drug use, or how long ago a corpse died (Amendt et al., 2007; Rivers & Dahlen, 2014).

The time that elapses between the death and the discovery of the corpse is known as the *Post mortem* Interval (PMI) (Catts & Haskell, 1990). However, some authors consider that this term is not adequate to describe the period when insects start their activities in the body, because their access is not always immediate. Therefore, this period is defined as Period of Insect Activity (PIA) (Tomberlin et al., 2011). During this time, strong odours are released by the corpse, attracting local fauna. Generally, the first insects to appear are blowflies (Diptera: Calliphoridae), as they occur in many locations around the world and have a specialised olfactory system for detecting putrefactive materials (Cammack et al., 2016).

Different methods are used to estimate the time of death. A widely used method of making this estimate is the accumulated degree-day (ADD) calculation (Byrd & Tomberlin, 2009; Oliveira-Costa, 2011). The technique originates from Agricultural Entomology (Arnold, 1960) and works by using species development according to temperature to find out how many thermal units are needed for the insect to develop (Amendt et al., 2011). It is widely used in recent years for estimation of PMI in real cases of wildlife forensic entomology, forensic veterinary, real deaths and academic studies (Bourguignon et al., 2006; Pujol-Luz et al., 2008; Myburgh et al., 2013; Defilippo et al., 2015; Amat et al., 2021).

The ADD data can only be used if information are available on the bionomic development of the forensic species (Oliveira-Costa, 2011). This type of data is not yet available for the entire forensic fauna, however, it was possible to apply the calculation in the present work for the species *Chrysomya albiceps* Wiedemann 1819, *Calliphora vicina* Robineau-Desvoidy, 1830 and *Lucilia sericata* Meigen, 1826, due to the presence and regularity of these species in forensic experiments from Portugal (Prado e Castro et al, 2009; Prado e Castro et al., 2011; Prado e Castro et al., 2012; Farinha et al., 2014; Prado e Castro et al., 2016; Fuentes-Lopez et al., 2020).

Chrysomya albiceps has been identified as a reliable forensic indicator in numerous real cases across the world. Studies in Brazil (Andrade et al., 2005; Oliveira & Vasconcelos, 2010; Vairo et al., 2015; Thyssen et al., 2018; Vasconcelos et al., 2019), Colômbia (Ramos-Pastrana & Wolff, 2017), Spain (Arnaldos et al., 2005; Ivorra et al., 2019; Arnaldos & García, 2021), Italy (Introna et al., 1998; Vanin et al., 2009; Bonacci et al., 2021), Germany (Lutz et al., 2021), Kuwait (Al-Mesbah et al., 2011), Saudi Arabia (Alajmi et al., 2016), Iran (Moemenbellah-Fard et al., 2018), Egypt (Tantawi et al., 2018), India (Sharma et al., 2018) and Poland (Grzywacz et al., 2021; Michalski et al., 2021) have all found this species to be useful in determining *post mortem* intervals. *Calliphora vicina* has also been classified as an important forensic indicator in real cases. A study in Spain by Arnaldos et al. (2005) found this species to be present in all cases reported by the authors. In last years, *C. vicina* has been reported in real cases in Iran (Moemenbellah-Fard et al., 2015), Turkey (Açıkgöz & Açıkgöz, 2018), Finland (Pohjoismäki et al., 2010), Poland (Kadej et al., 2020), Italy (Introna et al, 1998; Turchetto et al., 2001; Introna et al., 2011; Magni et al., 2013; Bonacci et al., 2021), Germany (Fremdt et al., 2012; Lutz et al., 2021), Canada (Anderson, 1995; (VanLaerhoven & Merritt, 2019) and the United States (Adair, 2008; Hildebrand et al., 2022). *Lucilia sericata* has also been recorded in real cases in numerous countries. These include Brazil (Vairo et al., 2015), Canada (Anderson, 1995), Mexico (Flores-Pérez et al., 2017), Spain (Arnaldos et al., 2005; Arnaldos & Garcia, 2021), Italy (Campobasso et al., 2009; Marchetti et al., 2013; Introna et al., 2021), France (Dourel et al., 2010),

Belgium (Bourguignon et al., 2006), Finland (Pohjoismäki et al., 2010), Sweden (Grzywacz et al., 2014) and Turkey (Açikgöz & Açikgöz, 2018). These records indicate that *L. sericata* can also be a useful tool in determining *post mortem* intervals in criminal investigations.

Although *C. albiceps*, *L. sericata*, and *C. vicina* are widely distributed (Prado e Castro et al., 2016), the literature search for real cases revealed few geographical records of these species. This suggests that they are underused as forensic indicators, despite their global recognition. Lack of specialized experts in Forensic Entomology, resulting in the failure to collect entomological evidences, and the non-publication of basic data in scientific literature may explain this phenomenon. Consequently, this study is expected to address this gap in Portugal and complement previously published research in other countries.

This work simulated a casework to evaluate the feasibility of using PMI calculations in the city of Lisbon, estimating how long three species of blowflies developed their activities on two cadaveric baits (dog and cat). Moreover, it is expected to provide knowledge to local authorities to use insects in the elucidation of criminal cases.

4.3 Methods

4.3.1 Study Area

Lisbon is a coastal city whose climate has Mediterranean and Atlantic influence. The city's climate classification according to the Köppen system is Csa - temperate climate with hot and dry summer (IPMA, 2022). The experiment was conducted at the Faculty of Veterinary Medicine of the University of Lisbon (38°42'55.85 "N, 9°11'37.45 "W), in September/October 2021. The area had predominant vegetation of grasses, shrubs, fruit trees and access by people was restricted.

4.3.2 Sampling design – casework simulation

Two Shannon-style adapted traps (Barbosa et al., 2017) were installed, placed 5m away from each other and were designed to facilitate internal access. A dog, *Canis lupus familiaris* (Linnaeus, 1758) and a cat, *Felis catus* (Linnaeus, 1758) were placed as cadaveric baits in each one. The animals were frozen and died at an earlier date. However, the date of death was considered as the day of the beginning of the experiment, when the corpses were thawed and placed in the field. The study was approved by the Ethics and Welfare Committee of FMV/ULisboa.

Daily surveys were made in the traps, in the morning, to register and collect the existing eggs, larvae, and adults of blowflies. Adult insects were collected with a killing jar and tweezers, while eggs were collected and transported in moistened filter paper. Around the two baits, pitfalls were placed in order to collect creeping species. Each pitfall was filled with 50 ml of alcohol 70°.

4.3.3 Insect rearing

Diptera immatures were transported daily to the Entomology Laboratory of the Faculty of Sciences of the University of Lisbon. After being separated by morphotypes, they were placed in plastic flasks containing 100g of substrate (vermiculite) and commercial cat food (pate style). Each jar was protected by veil style fabric to prevent the occurrence of parasites. The insects were left at room temperature and each jar was daily checked. Temperatures were recorded by a thermo-hygrometer left permanently in the rearing room.

4.3.4 Data analysis

The calculation of ADD was performed according to the following formula: $ADD = (value\ T - value\ LDT) \times Development\ Time$, where ADD = accumulated degree day, T = available

temperature; and LDT = lower development threshold. The formula is calculated according to the table provided in results section, where the expected ADD value was subtracted by the ADD value obtained, using the initial part of the formula above: value T - value LDT. Then, PIA was calculated by the following formula: $PIA = \text{expected ADD} - \text{obtained ADD}$, and subtracted the values until the expected ADD value was less than or equal to the obtained ADD value. The values of LDT and expected ADD have been explored from different works (Queiroz, 1996; Grassberger & Reiter, 2001; Marchenko, 2001; Grassberger et al., 2003; Donovan et al., 2006; Rueda et al., 2010; Grisendi et al., 2015; Roe & Higley, 2015; Salimi et al., 2018). However, temperature data from Marchenko (2001) were chosen for the species *C. albiceps* and *C. vicina* because it is a more detailed study. In Marchenko (2001), data for the species *L. sericata* were not used because $n=1$, besides being secondary data. Therefore, for this species, the data was obtained from Grisendi et al. (2015). Air temperatures used in calculation were obtained from the website www.accuweather.pt. The adaptations made in the simulation calculations for the species are described in the results section.

In addition, the results of laboratory rearing of insects that were obtained from the carcasses are provided as well as the developmental timeline of the three species according to the ADD calculations, and the estimated date of death. The timeline was made in Rstudio (Rstudio Team, 2022).

4.4 Results

4.4.1 Collecting adults and rearing insects

A total of 4,984 insects were collected from the carcasses from 24 september to 30 october. The three most abundant orders were Diptera ($n= 4,568$), Hymenoptera ($n=338$) and Coleoptera ($n=73$). The remainder is comprised by orders such as Hemiptera and Lepidoptera. The Diptera included the families Calliphoridae ($n=2,587$), Sarcophagidae ($n=136$), Muscidae (935), Polleniidae

(n=1, genus *Pollenia*) and Stratiomyidae (n=1, *Hermetia illucens* Linnaeus, 1758). Three species composed the cadaveric fauna of blowflies: *C. albiceps* (n=2,575), *L. sericata* (n=12), and *C. vicina* (only identified at laboratory when hatched from rearing). Among the Hymenoptera, the main groups collected were ants, and the exotic species *Vespa velutina* Lepeletier, 1836, recorded for the first time on European animal carcasses. This wasp was observed preying on flies of the Sarcophagidae family and the *Lucilia* genus. Among the Coleoptera, the families Dermestidae and Staphylinidae were recorded.

In insect rearing, 3,071 califorids emerged as adults, distributed among 3 species: *C. albiceps* (n male=1,588; n female=1,376), *C. vicina* (n male=6; n female=3) and *L. sericata* (n male=53; n female=45). Larvae of *H. illucens* were collected but did not thrive in rearing. Adults emerged during the period 12 October to 03 November. Notably, some specimens of *C. albiceps* took 24-28 days to initiate the pupal stage. During their time in rearing, all larvae were reared at an average temperature of 24.01°C.

4.4.2 Casework simulation

The results are based on the date of death of two carcasses, which occurred on September 24th, 2021, within the vicinity of the Faculty of Veterinary Medicine at the University of Lisbon. Throughout this study, the average temperature for rearing the three species remained at 24.01°C, whereas the average environmental temperature during this period was 20.62°C. In Lisboa, during the period of experiment, temperatures ranged from 11°C to 30°C in 2021 (Time and Date, 2023).

When calculating the ADD for *C. albiceps* and *C. vicina*, similar results were obtained (Tables 4.1 and 4.2) in determining the time of death. However, the results for *L. sericata* were not satisfactory (Table 4.3), since the estimated PIA did not come close to the actual date of death of the cadaveric baits used in the experiment. Among the three species, *C. vicina* provided the most precise estimation (as illustrated in Figure 4.1).

Table 4.1: ADD calculation using the development of *Chrysomya albiceps* species collected from animal carcasses. T= temperature in °C. The PIA indicated by the calculation is 13 days (development egg to adult).

<i>C. albiceps</i> (stage)	Date (day/month)	T (air)	ADD obtained	ADD expected
	08/10	23	10	96
	07/10	23.5	10.5	86
	06/10	22.5	9.5	75.5
	05/10	19.5	6.5	66
	04/10	18	5	59.5
	03/10	18	5	54.5
Empty pupa	02/10	21	8	49.5
	01/10	21.5	8.5	41.5
	30/09	18	5	33
	29/09	21	8	28
	28/09	19.5	6.5	20
	27/09	21.5	8.5	13.5
	26/09	20.5	7.5	5

Note: the ADD calculation was based on empty pupae collected from the carcass on 08/10. On the following day, a new wave of *C. albiceps* insects appeared on the carcass, which indicated the birth of the first adults on 08/10, when the first empty pupae were found below the dog carcass. The average temperature among the temperatures used in this calculation was 20.57°C.

Table 4.2: ADD calculation using the development of the species *Calliphora vicina* collected from animal carcasses. T= temperature in °C. The PIA indicated by the calculation is 10 days (development egg to pupa).

<i>C. vicina</i> (stage)	Date (day/month)	T (air)	T (rearing)	ADD obtained	ADD expected
Pupa	04/10		23.75	21,75	189.23
	03/10		24	22	167.48
	02/10		23.95	21.95	145.48
	01/10		24	22	123.53
	30/09	18		16	101.53
	29/09	21		19	85.53
	28/09	19.5		17.5	66.53
	27/09	21.5		19.5	49.03
	26/09	20.5		18.5	29.53
	25/09	21		19	11.03

Note: The ADD calculation was based on pupae emerging as adults on 04/10 and collected from the canine carcass on 30/09. Therefore, it was chosen to use the air temperature from day 30/09 to day 25/09 and the rearing temperature from day 04/10 to day 01/10. The average temperature among the temperatures used in this calculation was 21.72°C.

Table 4.3: ADD calculation using the development of the species *Lucilia sericata* collected from animal carcasses. T= temperature in °C. The PIA indicated by the calculation is 7 days (development egg to pupa).

<i>L. sericata</i>	Date	T	T	ADD	ADD
(stage)	(day/month)	(air)	(rearing)	obtained	expected
Pupa	06/10		23.9	13.3	79.8
	05/10		23.75	13.15	66.5
	04/10		23.75	13.15	53.35
	03/10		24	13.4	40.2
	02/10		23.95	13.35	26.8
	01/10	21.5		10.9	13.45
	30/09	18		7.4	2.55

Note: The ADD calculation was based on pupae emerging as adults on 06/10 and collected from the canine carcass on 01/10. Therefore, it was decided to use the air temperature on 30/09 and 01/10 and the rearing temperature from day 06/10 to day 02/10. The average temperature among the temperatures used in this calculation was 22.69°C.

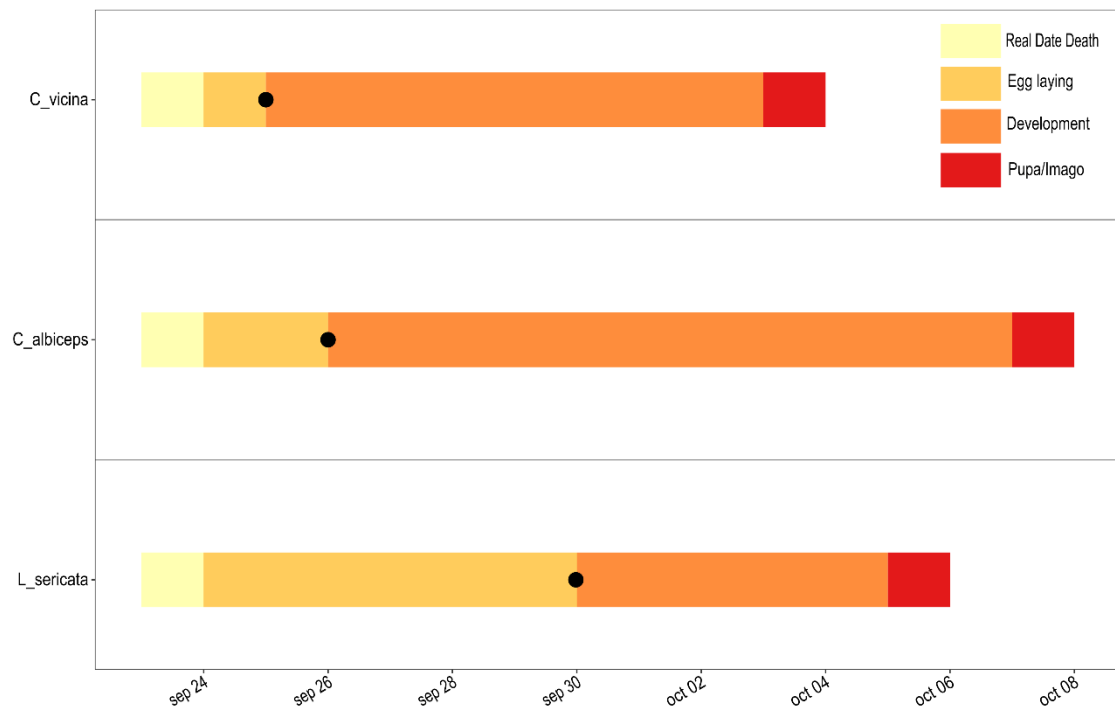


Figure 4.1: Estimated Period of Insect Activity – PIA – based on ADD calculation of three forensic species: *Calliphora vicina*, *Chrysomya albiceps* and *Lucilia sericata*. The black dot indicates the exact date of death indicated by the calculation.

4.5 Discussion

4.5.1 Collecting adults and rearing insects

Prado e Castro et al. (2012) collected 11 species of Calliphoridae from swine animal carcasses in previous experiments, in contrast to our experiment that only collected three species. This difference may be due to variations in the collection effort and fly capture methods employed. The present study aims to investigate the feasibility of using ADD calculation based on Calliphoridae species found in Lisbon. Efforts were not focused on the faunal succession of insects, but on the immatures that could be used as entomological evidence in real cases. As we will see below, the three

species collected in this study have been previously used in forensic cases, including in the Iberian Peninsula (Arnaldos et al., 2005; 2021). This indicates that the data collection was adequate.

Chrysomya albiceps is one of the most widely used forensic indicators in the world (Grassberger et al., 2003, Corrêa et al., 2019; Williams & Villet, 2019; Al-Qahtni et al., 2021). This species is a dominant insect on animal carcasses as it is highly competitive in the larval stage. In addition to preying on larvae of other species, it can cannibalize co-specific individuals (Faria et al., 1999; Grassberger et al. 2003; Faria et al., 2007). Originally, it was a species that occurred in Europe, Africa and Asia (Middle East), but in the mid-1970s it was dispersed to South America (Séguy 1930-1932; Holdaway 1933; Laurence, 1981; Laurence, 1986). *Calliphora vicina* is a blowfly associated with urban and temperate environments. Being common in Europe, it is a species of forensic importance (Greenberg, 1991; Limsopatham et al., 2018). In Portugal, it is a species that occurs throughout the year, mainly in autumn (Fuentes-Lopes et al., 2020) although abundance decreases in summer (Prado e Castro et al., 2012). It has dispersed to South America, occurring in part of the American continent (Salanitro et al., 2022). *Lucilia sericata* is a widely distributed species around the world, inhabiting urban and rural sites. Besides being a species also commonly found on corpses and carcasses (Byrd & Tomberlin, 2009; Tarone et al., 2011), the species is a cause of myiasis in sheep (Hira et al., 2004), which is why it is known as sheep blowfly.

Besides Calliphoridae adults, some notable remarks were the presence of *H. illucens*, a species also already used forensically (Turchetto et al., 2001; Pujol-Luz et al., 2008; Martínez-Sánchez et al., 2011). Another significant observation was the common occurrence of *V. velutina*, likely due to a nearby nest that facilitated its presence as a predator of the flies. *Vespa velutina* has a wide diet, easy to disperse and can cause economic losses due to predation of honey bees (Laurino et al., 2020). Moreover, it can be an ecological threat to other native species such as flies. Although the number of individuals predated by the wasp was not quantified in this experiment, it is an important factor that should be studied and considered in forensic contexts within European countries.

4.5.2 Casework Simulation

Temperature and development thresholds

The first species used was *C. albiceps*. The life cycle of this species in controlled temperatures has been studied several times (Queiroz & Milward-de-Azevedo, 1991; Aguiar-Coelho & Milward-de-Azevedo, 1995; Queiroz, 1996; Marchenko, 2001; Al-Misned et al, 2002; Grassberger et al., 2003; Kheirallah et al., 2007; Richards et al., 2009; Beuter & Mendes, 2013; Al-Shareef & Al-Qurashi, 2016; Salimi et al., 2018; Kordshouli et al., 2021). These studies investigated developmental temperatures ranging from 11°C to 40°C. The lower development threshold for survival and development of this species varies among the populations studied, but the lowest value was 10.2°C, estimated by Marchenko (2001). However, the species was only observed to develop from 13°C onwards in the same study. The species also does not develop well at very extreme temperatures, with its development rendered impossible from 37°C (Kordshouli et al., 2021) and/or 38°C (Dias, 2016).

Calliphora vicina is a species more adapted to temperate/subtropical climates (Salanitro et al., 2022), and its abundance tends to be limited in higher temperatures (Prado e Castro et al., 2012). The life cycle of this species has been studied at temperatures ranging from 3.5°C to 35°C, (Reiter, 1984; Davies & Ratcliffe, 1994, Donovan et al., 2006; Díaz Martín et al., 2014, Salimi et al., 2018). The lower developmental threshold for *C. vicina* is noted as 1°C by Donovan et al. (2006) and 1.8°C by Defilippo et al., (2013), based on linear regression. Marchenko (2001) points this threshold at 2°C. Furthermore, the development of *C. vicina* was interrupted from 29°C (and 30°C according to the works by Marchenko (2001) and Reiter (1984), respectively. Díaz-Martín et al. (2014) inferred that the maximum temperature threshold for development is between 31°C and 34°C.

The final species utilized was *L. sericata*. Numerous studies have examined its lifecycle development under varying temperature conditions, including Ash & Greenberg (1975), Greenberg

(1991), Wall et al. (1992), Davies & Ratcliffe (1994), Grassberger & Reiter (2001), Marchenko (2001), Kheirallah et al. (2007), Kim et al. (2007), Gallagher et al. (2010), Tarone et al. (2011), Karabey & Sert (2014, pupal stage only), Grisendi et al. (2015), Cervantès et al. (2018), Pruna et al. (2019), Wang et al. (2020) and Okpara & VanLaerhoven (2023), with temperature ranges from 7.5°C to 37°C. Marchenko (2001) suggested that the minimum developmental threshold for *L. sericata* survival is 9°C, while others estimated it to be 8°C (Reibe et al., 2010), 8.6°C (Cervantès et al., 2018), 10.6°C (Grisendi et al., 2015), between 7.5°C and 10°C (Roe & Higley, 2015), 10°C (Pruna et al., 2019), 9.6°C (Shiravi et al., 2011), and 9.19°C (Wang et al., 2020). These findings indicate that *L. sericata* has poor tolerance to colder temperatures. For instance, a study in Portugal by Prado e Castro et al. (2012) revealed that this species was only found once in the winter in a pig carcass environment.

The ADD calculation shows that *C. albiceps* developed in 13 days (egg to adult), averaging $T \cong 21^{\circ}\text{C}$, whereas *C. vicina* and *L. sericata* developed from egg to pupa in 10 to 7 days, at $T \cong 22^{\circ}\text{C}$, and $T \cong 23^{\circ}\text{C}$, respectively. At these temperatures, Marchenko (2001) established that the development of *C. albiceps* in the laboratory from egg to adult is 17.2 days, whereas that of *C. vicina* from egg to pupa is 9.6 days. As data from Grisendi et al (2015) were used for *L. sericata* and the authors did not rearing the species at 22°C and 24°C, the average number of rearing days of the egg-to-pupa period from the work of these authors was calculated, which theoretically amounts to 7 days of development. Analyzing the period of *C. albiceps*, this amount would exceed the period of decomposition of cadaveric baits. However, this calculation in Table 1 disregards the impact of other temperature factors, such as larval mass temperature, soil temperature, and local temperature, on species development in cadaver environments (Oliveira-Costa et al., 2011). *Calliphora vicina* had the egg-pupa period similar to the same period detailed by Marchenko (2001). Besides the species did not form larval masses, the specimens did not pupate in the soil in the field, but only at the laboratory, which may have influenced the development time similar to the work of Marchenko (2001). *Lucilia sericata* showed the same development time to the theoretical calculation derived from the work of

Grisendi et al. (2015), indicating that the specimens used in the ADD calculation may have colonised the carcass after *C. albiceps* and *C. vicina*. This is partially odd with information from Miralbes (2002), who mentions *L. sericata* as the primary species over *C. albiceps*.

4.6 Conclusion

Three species were identified as potential indicators of PIA for criminal investigations: *C. vicina*, *C. albiceps* and *L. sericata*. However, a satisfactory result was achieved only with the first two. This study intends to be a practical evidence of how environmental temperature data and insect biology can be related to aid criminal investigations during the autumn season in Lisbon. ADD data from non-native populations of Portugal were used, which does not exclude the need for works investigating the bionomics of local populations. Basic research with native populations makes the data more reliable and can be of great value to criminal investigations.

4.7 References

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Chapter 5

“Characterization of *Chrysomya albiceps* (Diptera: Calliphoridae) tarsi and head by Scanning Electron Microscopy.”

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Characterization of *Chrysomya albiceps* (Diptera: Calliphoridae) tarsi and head by Scanning Electron Microscopy.

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5.1 Abstract

This study focuses on the ultrastructures associated with olfaction and locomotion in *Chrysomya albiceps*, a blowfly species of sanitary, medical, veterinary and forensic importance. The tarsi and heads, collected from specimens used in Forensic Entomology research, were characterized through scanning electron microscopy analysis. The findings revealed a diverse array of sensillae not previously documented, both in the head and attachment pulvilli. Additionally, potential evidence of sexual dimorphism in *C. albiceps* tarsal claws was observed. These studies on the ultrastructures of medically and forensically relevant species are crucial for enhancing identification, expanding the knowledge of corpses detection by these insects, and even contributing to the recognition of insect body parts at crime scenes. These results would provide valuable resources for future research involving *C. albiceps*, as well as contribute to forensic reports worldwide.

5.2 Introduction

In Forensic Entomology, it is important to understand how and how fast are the responses of sarcosaprophagous flies in the detection of corpses (Oliveira-Costa, 2011). Consequently, studies that classify and describe these species are essential. A way to achieve this is by producing species identification keys and DNA databases (Byrd & Castner, 2010). Furthermore, contemporary techniques utilized in forensic scenarios include geometric morphometry, cuticular hydrocarbon patterns, and scanning electron microscopy (SEM) images (Dias-Filho et al., 2022).

In recent years, SEM has been extensively studied to enhance our understanding of typical insect structures (Sukontason et al., 2005; Braga et al., 2016). They also serve to understand pathogen carriage, bristle patterns, feeding habits, as well as practical applications in forensic scenarios (Sukontason et al., 2006; Zhang et al., 2014; Carriço et al., 2020). As an example, Carriço et al. (2020) remarked that SEM can be employed to identify insect body parts discovered at crime scenes.

Homicide scenes constitute propitious oviposition environments for insects (Keh, 1985). When a corpse is present, various chemical signals attract organisms that specialize in consuming ecological resources derived from decomposing materials (Catts and Goff, 1992). Blowflies (Diptera: Calliphoridae) are particularly drawn to these environments, and they have a well-known attraction to them (Smith, 1986). This characteristic has established blowflies as significant organisms in medical, forensic, veterinary, and ecological contexts (Wolff and Kosmann, 2016). To maintain such behaviours, species in the family rely on biological advantages that include specialised systems to detect these materials and to stick to surfaces near the materials (Sukontason et al., 2006; Cammack et al., 2016).

Chrysomya albiceps (Wiedemann, 1819), a blowfly, is known for being a skilled competitor, owing to its ability to prey on other insect species during the larval phase (Ivorra et al., 2022), including other individuals of the same species (Faria et al., 2007). Additionally, evidence suggests that *C. albiceps* can spread quickly in propitious environments (Baumgartner, 1988), leading to issues

such as displacement of other species and alteration of local forensic dynamics (Faria et al., 1999; Spindola et al., 2017). This species was initially distributed throughout southern Europe (Holdaway, 1933); however, it has recently expanded its range to central and eastern Europe, even in colder regions where it was formerly thought to be unable to survive (Grassberger et al., 2003, Rodrigues-Filho et al., 2023). As a result, greater attention is required to identify *C. albiceps* in its early stages. Currently, only one description of adult ultrastructures of this species exists (Darılmaz et al., 2019). The other are of immature stages (Greenberg and Singh, 1995; Shaheen and Fathy, 2008, Mendonça et al., 2010, Elshehaby et al., 2019), none of which were performed in Europe, making this the first time that ultrastructures of adults in European populations have been described.

In order to increase the database of anatomical characterizations of insect species, this paper describes ultrastructures of the head and attachment pulvilli of the blowfly *C. albiceps*, discussing possible applications for Forensic Entomology.

5.3 Methods

5.3.1 *Chrysomya albiceps* specimens

Twenty males and 20 females of *C. albiceps* were selected to photograph the structures of the head and tarsus. These specimens were obtained through Forensic Entomology experiments, employing either manual collection or the use of deadly jars. Ten males and 10 females were collected in 2004, kindly provided by the second author. These individuals were collected in Coimbra, using piglets as cadaveric baits. Another 10 males and 10 females came from Lisbon, from an experiment carried out in 2021, using dog and cat as cadaveric baits.

5.3.2 Laboratory Procedures

Flies had their head and right anterior tarsus detached. Each pair of structures was attached to a double-sided adhesive and glued on a drawing pin. All specimens were dehydrated at room temperature for one week. Following the dehydration process, each pair underwent gold dusting for a minimum of one hour. Then, each pair was photographed in a Scanning Electron Microscope at Faculty of Sciences of University of Lisbon, for further analysis.

5.3.3 Image Analysis

The images of the fly heads were carefully examined to determine the positioning and types of sensillae present on the antennae. In the images of the tarsals, the empodium, pulvilli and tarsal claws were observed. Details of the sensillae of the pulvilli are explored. The works of Hunter and Adserballe (1996), Keil (1999) and Johannes-Kelling (2001) were used for the classification of the sensillae. Sensillae of the same type were classified as different types following the size criterion

5.4 Results

The female head of *C. albiceps* has a largest space between the eyes than males, which configures sexual dimorphism in the species (Fig. 1).

The antennae of *C. albiceps* have three segments: scape (Sc), which is the base of the antenna, followed by the pedicel (Pd) and then the post-pedicel (Pp), where a plumose arista is located (Fig. 5.2). The scape has three types of sensilla: microtrichia, chaetic and trichoidea (Fig. 5.3). The pedicel is also covered by the same types of sensillae (Fig. 5.3 and Fig. 5.4). However, the chaetic sensillae are of different sizes, being classified into I, II and III. On the surface of the post-pedicel, microtrichia, trichoidea and three sizes of basiconic sensillae were found (Fig. 5.5), in addition to the arista (Fig. 5.6).

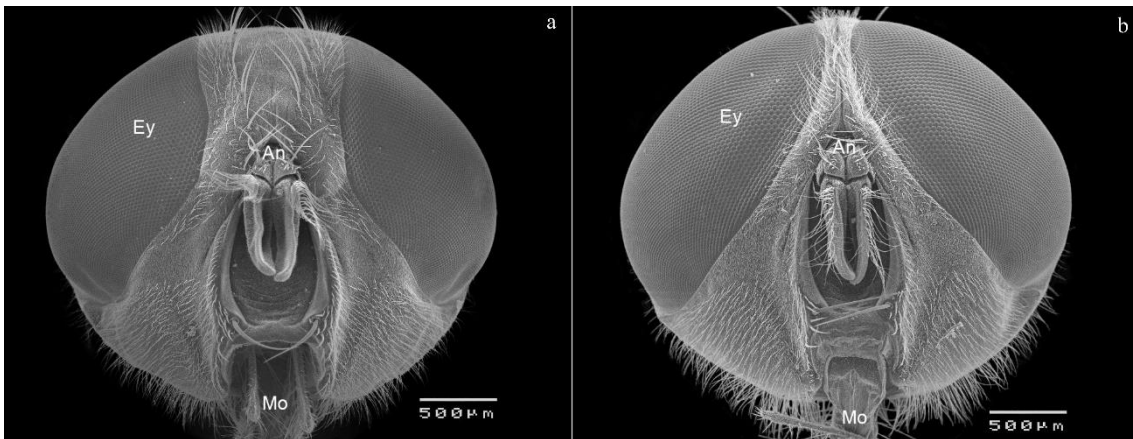


Figure 5.1: SEM images of the heads of the species *Chrysomya albiceps*: a) female; b) male. Ey= Eyes; An= Antennae; Mo= Mouth apparatus.

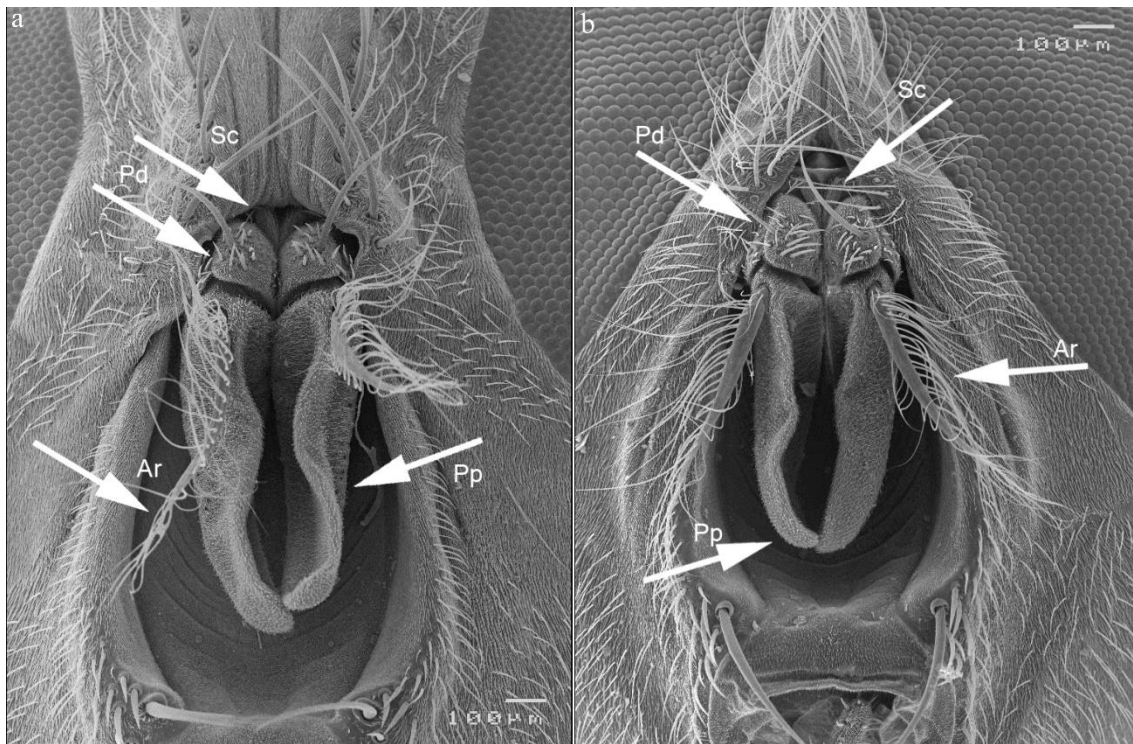


Figure 5.2: SEM images of the ultrastructures of the antennae of *Chrysomya albiceps*. a) female; b) male. Sc= Scape; Pd= Pedicel; Pp= Post-pedicel; Ar= Arista.

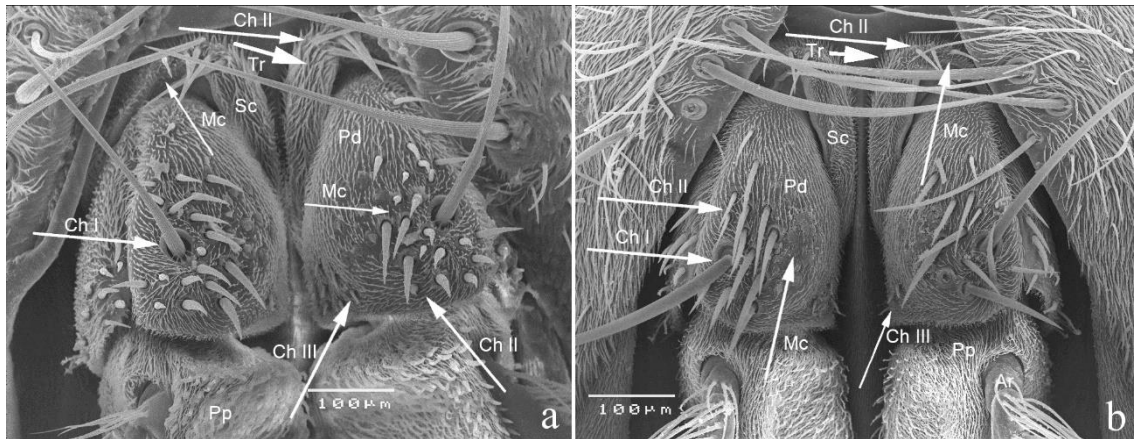


Figure 5.3: SEM images of the ultrastructures of the Scape and Pedicel of *Chrysomya albiceps*. a) female; b) male. Sc= Scape; Pd= Pedicel; Pp= Post-pedicel; Ar= Arista; Mc= Microtrichia; Ch= Chaetic; Tr= Trichoidea

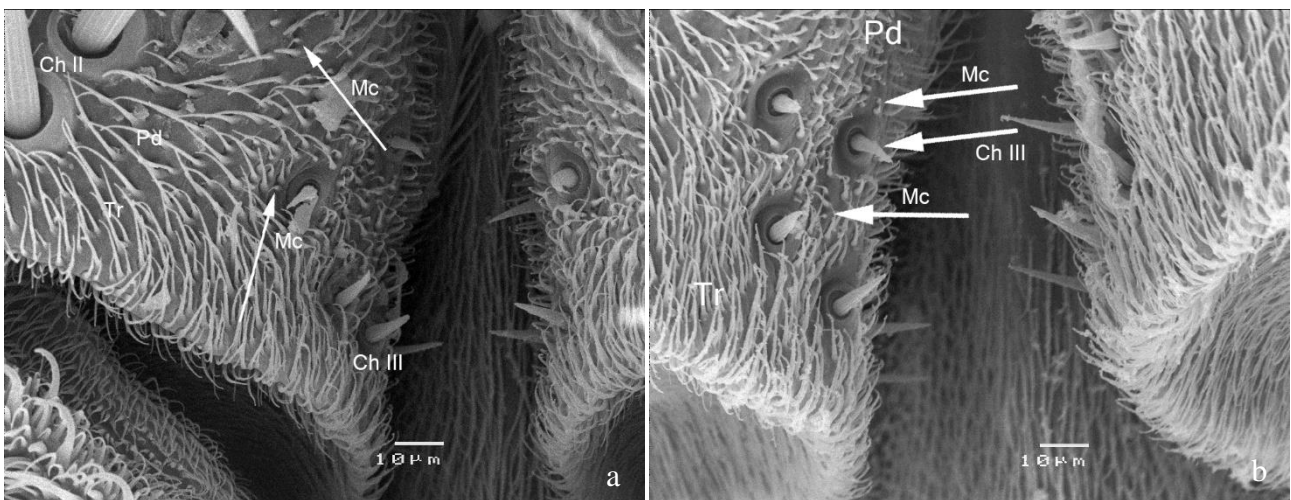


Figure 5.4: SEM images of the ultrastructures of the Pedicel of *Chrysomya albiceps*. a) female; b) male. Pd= Pedicel; Mc= Microtrichia; Ch= Chaetic; Tr= Trichoidea.

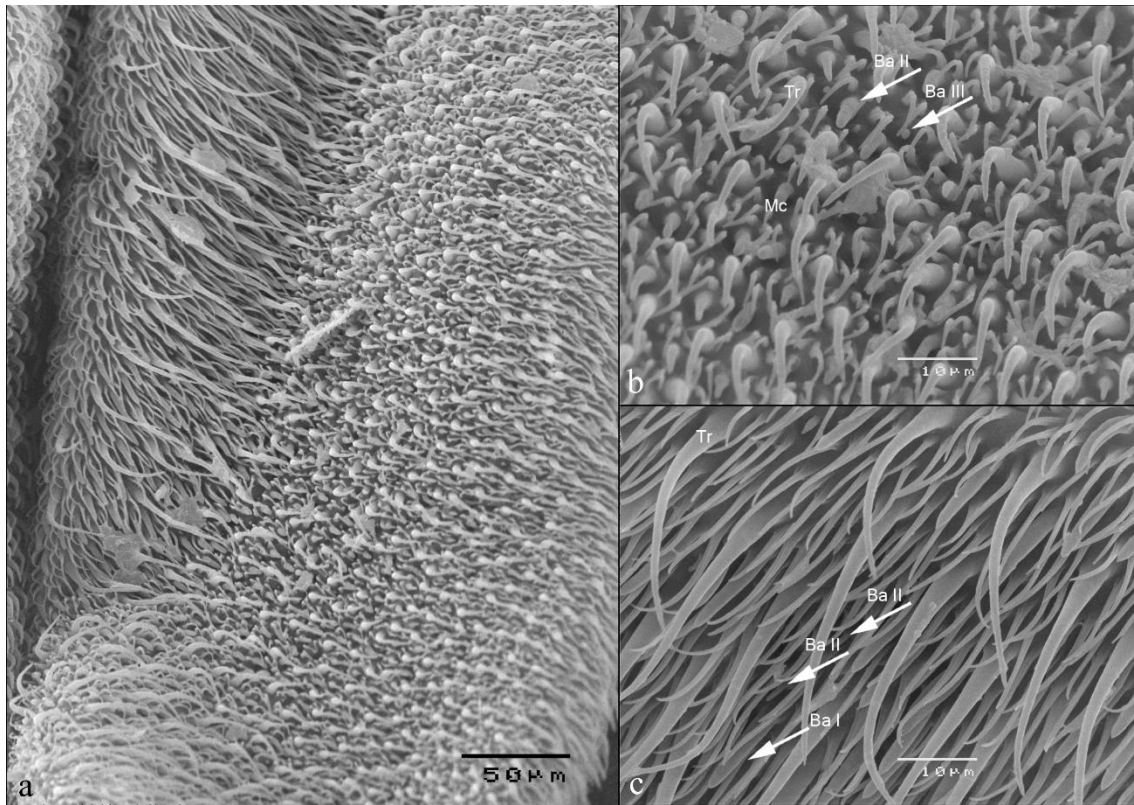


Figure 5.5: SEM images of the ultrastructures of the Post-pedicel of *Chrysomya albiceps*. a) Post-pedicel's details; b e c) Zoom on details of the post-pedicel. Pd= Pedicel; Pp= Post-pedicel; Mc= Microtrichia; Tr= Trichoidea; Ba= Basiconic.

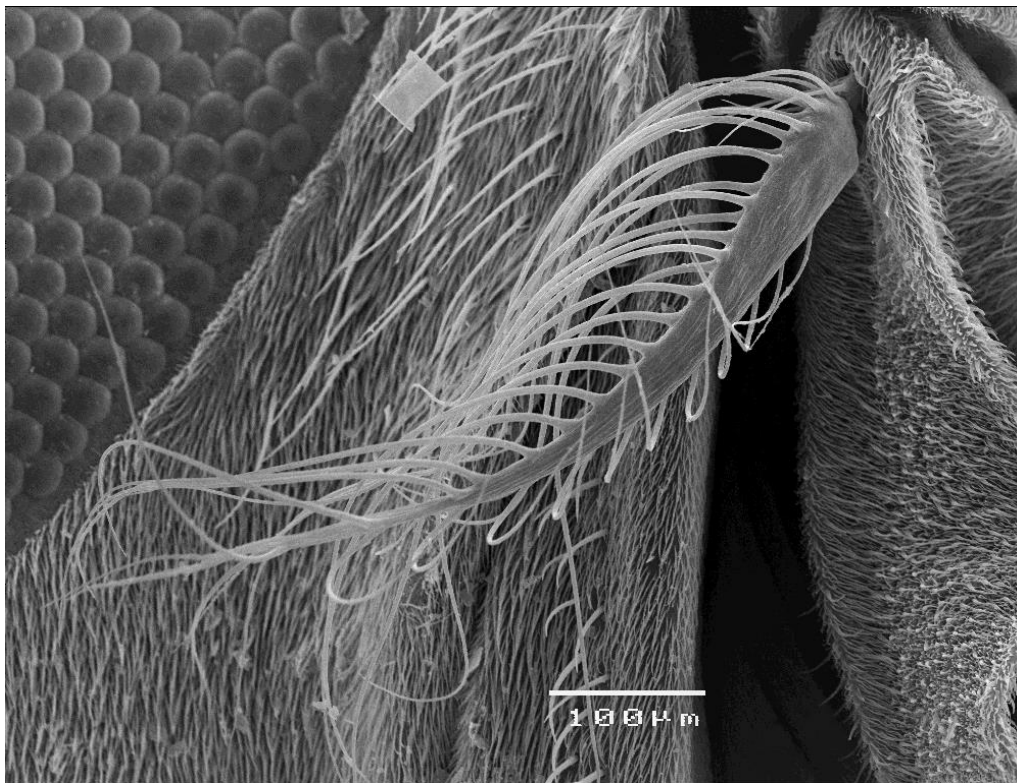


Figure 5.6: SEM images of the ultrastructures of the Arista of *Chrysomya albiceps*.

The tarsal claws are partially covered by microtrichia (Fig. 5.7). The empodium is arrow-shaped, located between the two pulvilli of the tarsus, and is a structure surrounded by microtrichia (Fig. 5.8). In the pulvilli of *C. albiceps*, only one type of setae tenente setae was detected, with the tip in the spoon style (Fig. 5.9). In these latter images, it is possible to see lipoid substances adhered to the surface of the tenente setae of the pulvilli.

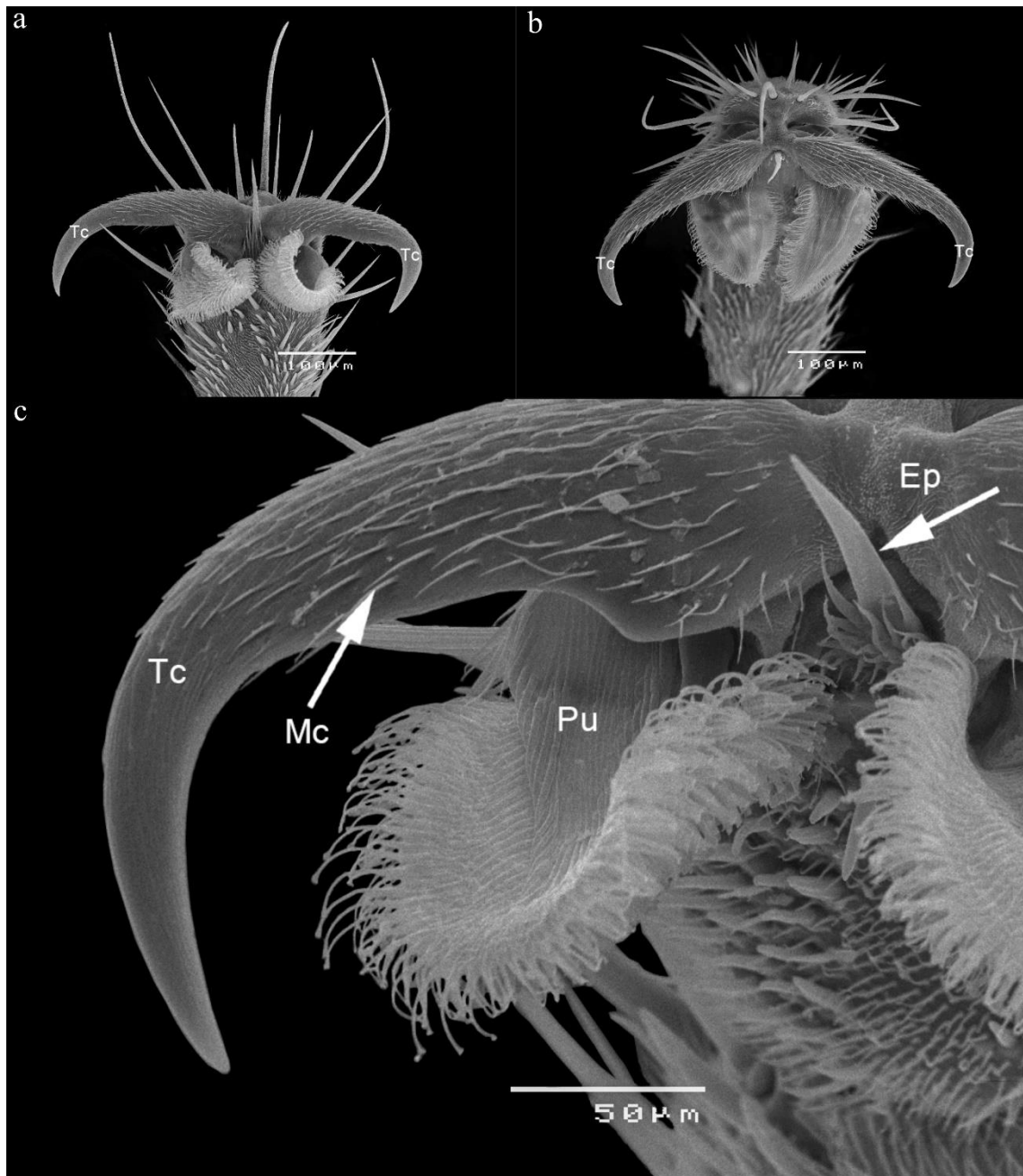


Figure 5.7: SEM images of the ultrastructures of the attachment pulvilli of *Chrysomya albiceps*. a) female; b) male. Tc= Tarsal claw; Mc= Microtrichia; Pu= Pulvilli; Ep= Empodium.

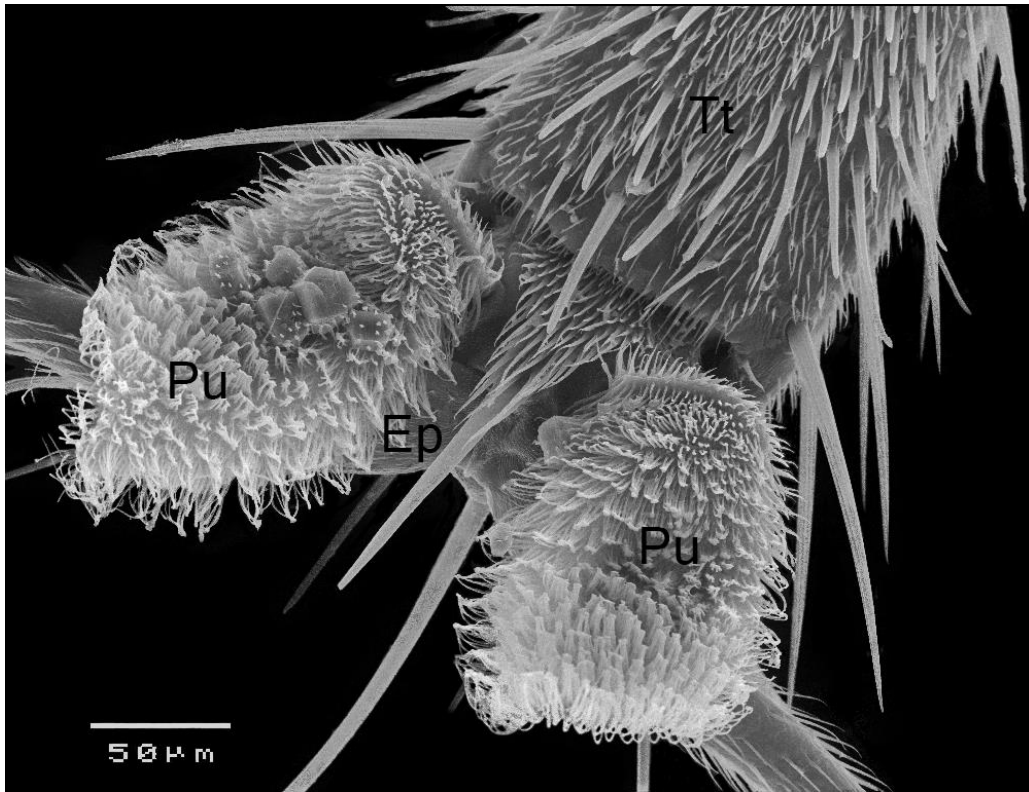


Figure 5.8: Detailed SEM images of the empodium *Chrysomya albiceps*. Tt= Terminal tarsomere; Pu= Pulvilli; Ep= Empodium.

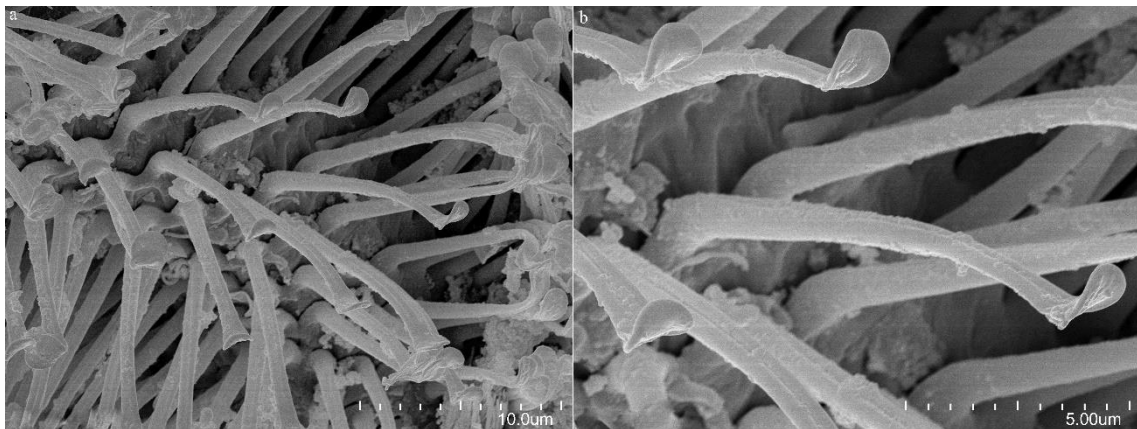


Figure 5.9: Detailed SEM images of the spoon-like tenent setae of the pulvilli of *Chrysomya albiceps*.

5.5 Discussion

The pattern of sensillae observed in the antennae of *C. albiceps* in this study was found to be similar to the pattern described by Darilmaz et al. (2019). The exception is that in the present work,

three sizes of basiconic sensillae were visualized, present in the post-pedicel, a pattern also found in *Drosophila melanogaster* (Meigen, 1830) (Shanbhag et al., 1999) and in calliphorids such as *Triceratopyga calliphoroides* (Rohdendorf, 1931) (Zhang et al., 2014) and *Hemilucilia segmentaria* (Fabricius, 1805) (Alvarez-Garcia et al., 2022). Furthermore, the pattern found of chaetic sensillae in *C. albiceps* is found in flies of the genus *Ophyra* (Carriço et al., 2015), families Sarcophagidae (Pezzi et al., 2016) and Mesembrinellidae (Caetano et al., 2018). All antenna segments showed much of their surface covered by trichoid sensillae and microtrichia, which is a common in Oestroidea (Hunter and Adserballe, 1996; Johannes-Kelling, 2001, Sukontason et al., 2004, 2007; Setzu et al., 2011; Zhang et al., 2013a, 2013b, 2014; Carriço et al., 2015, 2020; Pezzi et al., 2016; Caetano et al., 2018).

In the case of *Hypoderma bovis* (Latreille, 1818), it has been hypothesized that microtrichia, trichoid sensillae, and basiconic sensillae may serve an olfactory function (Hunter and Adserballe, 1996). Similar findings have been reported in houseflies, where trichoid sensillae are also associated with olfaction (Johannes-Kelling, 2001). Indeed, in Oestroidea flies, this hypothesis regarding the sensory function of these sensillae is frequently cited (Setzu et al., 2011; Carriço et al., 2020; Alvarez-Garcia et al., 2022). However, there is also a possibility that trichoid sensillae may have a mechanoreceptor function (Keil, 1999; Sukontason et al., 2004) or even possess thermosensitivity (Zacharuk, 1985). In a study by Gao et al. (2020), it was proposed that basiconic sensillae in species of the genus *Drosophila* are involved in host localization and the selection of different *Drosophila* species. In other insects, these sensillae are commonly associated with olfactory function (Zacharuk, 1985; Hunter and Adserballe, 1996; Fernandes et al., 2004; Sukontason et al., 2004; Setzu et al., 2011; Pezzi et al., 2016). Chaetic sensillae have been linked to mechanoreception in flies (Wang et al., 2012; Pezzi et al., 2016; Carriço et al., 2020; Alvarez-Garcia et al., 2022). As for microtrichia, they are not innervated (Hunter and Adserballe, 1996; Johannes-Kelling, 2001), and their exact functions remain unknown. However, Gorb (1998) suggests that the microtrichia in the head support system of dragonflies may function as friction systems.

Although the characterization of sexual dimorphism was not one of the study's objectives, an exploration was conducted among the sampled populations to identify any potential differences. Sexual dimorphism in Calliphoridae species is typically determined by assessing the distance between the eyes and the genitalia. However, when the head and the last abdominal segment are absent, limited alternatives remain for identifying the sex of the specimen. For instance, previous studies by Jimenez-Martin et al. (2020) and Szpila et al. (2019) did not observe sexual dimorphism in wing size or shape of *C. albiceps*. Conversely, Rodrigues-Filho et al. (2022) discovered sexual dimorphism in both wing shape and size, noting that female wings tend to be larger. This finding suggests the possibility of additional undiscovered differences or variations dependent on local population characteristics.

In this study, no distinct structures on the antennae of males and females were identified, similar to other flies in the genus *Chrysomya* (Sukontason et al., 2004) and the family Calliphoridae (Fernandes et al., 2004). This suggests that males and females may be sensitive to the same types of odors (Chapman, 1982). However, dimorphism was observed in the number of certain antennal sensillae in *Protophormia terraenovae* (Robineau-Desvoidy, 1830) (Setzu et al., 2011), indicating the need for further investigations into the ultrastructures of blowfly antennae.

In regards to the tarsal claws depicted in figure 5.7, it appears that the claws of males are coated with larger microtrichia. This pattern was consistent among the other specimens photographed. Caution should be exercised when interpreting these results, and further work is required to confirm them through statistically supported analyses. Niederegger et al. (2002) identified sexual dimorphism in the density of tenente setae in the pulvilli of *Calliphora vicina*. Although females of this species are generally larger than males, the pulvilli of males do not follow this body pattern. The authors hypothesized that this may indicate a possible grasping function during copulation, distinct from the already known locomotion function. It is not ruled out that a similar type of dimorphism may occur in *C. albiceps*, considering that microtrichia may serve as a friction system (Gorb, 1998).

In *C. albiceps*, the empodium exhibits multiple microtrichia at its base, similar to *Calliphora calliphoroides* (= *Triceratopyga calliphoroides*) and *Lucilia sericata* (Meigen, 1826) (Wang et al., 2016). The spoon-shaped tenente style setae on the pulvilli are similar to those found in another species of the genus, *Chrysomya villeneuvei* (Patton, 1922), but differ from *Chrysomya chani* (Kurahashi, 1979), *Chrysomya nigripes* (Aubertin, 1932), and *Chrysomya pinguis* (Walker, 1858) (Sukontason et al., 2006). This type of setae can also be found in other calliphorids such as *C. calliphoroides* and *L. sericata* (Wang et al., 2016), as well as in other Oestroidea species like *Portschinskia magnifica* (Pleske, 1926) (Yang and Zhang, 2014), *Sarcophaga portschinskyi* (Rohdendorf, 1937), and *Muscina stabulans* (Fallen, 1817) (Wang et al., 2016), and in other Diptera such as *Episyrphus balteatus* (De Geer, 1776) (Gorb, 1998). The presence of tenent setae with a spoon-shaped tip allows flies to increase their contact points on surfaces, enhancing adhesion and facilitating the locomotion of these insects (Sukontason et al., 2006).

5.6 Conclusion

The findings of this study offer valuable insights into the ultrastructures of *C. albiceps*. Descriptions of ultrastructures serve as valuable tools for improved species detection and identification. This study represents the first characterization of *C. albiceps*' antenna ultrastructures in Europe and the first worldwide description of the attachment pulvilli' ultrastructures. These data hold the potential to support future technical and scientific advancements in Forensic Entomology, facilitating the discovery of new evidence and enhancing forensic reports.

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Chapter 6

Overall discussion

6.1 General Discussion

The general discussion of the thesis has been structured according to the selection of the main findings in each chapter and aims to give a broad view of their significance.

6.1.1. Wing size and wing shape

In recent years, there has been an observed increase in wing size among *C. albiceps*. Initially, the hypothesis put forward to explain this phenomenon was the rise in temperature across Europe (IPCC, 2021). However, a non-linear regression analysis revealed a negative relationship between temperature increase and wing size. Similar patterns have been observed in *C. megacephala* (Reigada & Godoy, 2005) and *Aedes albopictus* (Garzón & Schweigmann, 2018). Notably, the most significant difference in wing size was observed between the first year analyzed (2004) and the last (2021), - suggesting that another factor likely accounts for this increase. Based on the existing knowledge within the genus *Chrysomya*, a hypothesis that relates this phenomenon to the competitive ability of *C. albiceps* during the larval stage can be suggested. As discussed in previous chapters, the species can act as a predator of other Calliphoridae species, and even cannibalize itself under conditions of local food scarcity (Faria et al., 1999). Holometabolous insects primarily undergo growth during this larval stage by consuming sufficient food to support metamorphosis (Gullan & Cranston, 2014). While the development of *C. albiceps* is influenced by temperature (Al-Shareef & Al-Qurashi, 2016), the expected relationship between body size and temperature was not found in this analysis.

The sexual dimorphism in wing size of *C. albiceps* was a surprising result. Generally, local populations may develop different traits and this may be common depending on the geographical distribution of a given species. For example, *C. albiceps* can be mistakenly with *C. rufifacies* by developing a similar taxonomic trait (Grella et al., 2015). In Argentina, the body size of *C. albiceps* differs between females and males, as well as experimental populations are larger than wild ones (Battán-Horenstein & Peretti, 2011). However, a study performed in Spain found no dimorphism in the wing size of the species (Jiménez-Martín et al., 2020), which led to the hypothesis that, due to the proximity between Spain and Portugal, besides the climatic similarity, the same result would be found in the present work. As seen in Chapter 2, dimorphism was identified, with the result following the work of Battán-Horenstein & Peretti (2011), in which females are larger. Three possible explanations were raised: 1) sample size: the present thesis worked with over 900 wings, divided almost equally, between the two sexes, a much more representative sample than that of Jiménez-Martín et al. (2020);

2) natural differences between the populations: even with the small distance between the countries, the populations of the two countries are different due to local conditions and resources; 3) method of obtaining the landmarks. The females of blowflies need protein nutritional sources for the development of their eggs (Shewell, 1987). It is expected that due to this factor, females need to move over a larger distances in their habitats, since guilds of carrion-feeding species are donor-controlled, i.e., oviposition of these species depends on the random supply of resources in nature (Polis & Strong, 1996). Consequently, female flies accessing animal carcasses are expected to be larger and better competitors.

Studies on wing shape in the genus *Chrysomya* differ on whether there is sexual dimorphism or not. Sontigun et al. (2017) analysed 6 species of the genus: *C. megacephala*, *C. chani*, *C. pinguis*, *C. nigripes*, *C. rufifacies* and *C. villeneuvei*. No dimorphism was identified in *C. pinguis* nor in *C. nigripes*. The same result was found by Szpila et al. (2019) in the case of *C. megacephala*. They further analysed the blowflies *C. albiceps*, *C. vicina*, *C. megacephala*, *L. ampullacea*, *L. caesar*, *L. sericata*, *L. silvarum* and *P. terraenovae*, but found no dimorphism in the wing shape of these species, but only in *C. vomitoria*. In other works, no dimorphism was found in *C. albiceps*, (Vásquez & Liria, 2012; Szpila et al., 2019; Jiménez-Martín et al., 2020). As seen in Chapter 2, dimorphism in the wing shape of *C. albiceps* was identified. Moreover, the success rate in discriminating males and females was almost 100%, which indicates that wings of males and females of the species, in the case of the populations sampled in this thesis, are quite different. In the present work, this may have occurred because it reflects regional variation in flies (Hall et al. 2014), an allometric effect sufficient to trigger differences in wing shape (Szpila et al. 2019), or divergent niche between sexes (Shine, 1989; Fryxell et al. 2019). Females have a competitive advantage for finding animal carrion (Campobasso et al. 2001).

An important detail found is that landmarks closer to the wing base are more stable in variation than landmarks further away, a detail also noted by (Jiménez-Martín et al. 2020). As seen in Battán-Horenstein & Peretti (2011), the thorax size of *C. albiceps* females is larger than that of males, which may require the wings to be larger and more variable in shape than those of males (Jiménez-Martín et al. 2020). The same authors hypothesize that it is possible that the base of the wings may be less variable because of proximity to the muscles involved in flight activity.

6.1.2. Climate suitability of *Chrysomya albiceps*

In 1933, Holdaway suggested that *C. rufifacies* occurred over a wider temperature range than *C. albiceps* and *C. putoria*. He made a map that became one of the first historical distributions created for *C. albiceps*. Exactly 90 years later, *C. albiceps* not only occurs on another continent, but has spread easily through both tropical and sub-tropical environments, as well as temperate environments in eastern Europe (Baumgartner & Greenberg, 1984; Michalski & Szpila, 2016). The question that permeated chapter 3 of this thesis was to understand why *C. albiceps* has managed to spread so easily in recent years across Europe, increasing its geographical distribution to points where it was not supposed to appear due to unsuitable climate (Grassberger et al., 2003). A species can spread because it has competitive advantages in tolerating environmental conditions and using resources (Begon & Townsend, 2021). In an ever-changing world, there are striking examples of species spreading across multiple locations (Cunze et al., 2016; Urvois et al., 2016). The case of the genus *Chrysomya* in the Americas is an example of how humans can be major carriers of exotic species (Baumgartner, 1988; Vitousek et al., 1997). However, its spread into Europe raised other questions, such as phenotypic plasticity and evolutionary adaptations to colder climates. Here, the first insights are provided into which environmental predictor variables can be used to study this.

Two hypotheses have been born in recent times about how *C. albiceps* has spread in Europe: a) that the species' distribution is changing due to a more suitable climate (Povolný, 2002; Gosselin & Braet 2008); b) that populations of *C. albiceps* are adapting to European winters (Makovetskaya & Verves 2018). Povolny (2002) found *C. albiceps* in Central and Eastern Europe a few years ago. However, the species never gave any clues that it was definitively establishing itself in these areas, which led him to hypothesize that the species was migratory and took advantage of less severe winters to reach more distant places of its historical distribution. To this movement, the same author named pulsation. In the present thesis, this hypothesis was re-signified, because it is believed that these temporary changes in distribution are nothing more than manifestations of the climatic changes, and that this was the reason why the species was able to establish in these colder areas. Regarding the hypothesis, postulated by Makovetskaya & Verves (2018), they comment on the possible expansion of *C. albiceps* to other European territories, in addition to the Asian portion of southern Russia.

In this thesis, it was shown that the main bioclimatic variables responsible for making the climate suitable for *C. albiceps* are related to temperature: Annual Mean Temperature (bio1) and Temperature Annual Range (bio7). Together, they contributed almost 90% to the climate suitability model. That is, among the variables tested, those that best explain the climatic suitability for *C. albiceps* are related to annual temperature trends (bio1) and seasonality (bio7). *Chrysomya albiceps* is a species whose adults are abundant throughout the year in tropical environments (Barros-Souza &

Ferreira-Keppler, 2013). However, in subtropical and temperate environments, this abundance increases only in the hottest periods of the year (Prado e Castro et al., 2012). It is also a ectothermic animal (Gullan & Cranston, 2014). These characteristics align with the result obtained in this thesis since to be able to spread more easily, the species needs to be in adult stage and requires multiple generations per year during the warmer periods. It is widely known that the species takes too long to develop in cooler temperatures (Marchenko, 2001), so it is natural for the species to have a lateness to establish (but not impossible) in a cooler location. Another result of this study that aligns with this assumption is the fact that *C. albiceps* already occurs in places where there is low climatic suitability. This is the case in countries like Belarus and Lithuania.

When analysing the losses and gains of areas where there is a change in climatic suitability, a large part of the gains is concentrated in eastern and northern Europe. There is relevant potential for the species to occur in these locations, as these gains are aligned with the direction in which the recent increase in the species' distribution across Europe has occurred. These results are also very consistent with the IPCC (2021) climate models, as it is predicted by the report that the temperature increase in these locations has not only been high but tends to continue in the coming years. Hosni et al (2022) analysed the potential distributions of *C. albiceps* globally. In their paper, they comment that there is a possibility in the coming years that the species will disappear from locations such as southern Europe, where it has been known to occur for a long time (Holdaway, 1933). In the present thesis, losses of climatic suitability are evenly distributed across the continent. In much of southern Europe, suitability remains unchanged, which seems to make more sense, as it is very unlikely that the species would cease to occur in these locations where its distribution is well established. Furthermore, the species survives over a wide temperature range, even near deserts (Akbarzadeh et al., 2018).

6.1.3. *Chrysomya albiceps* and Forensic Entomology

Real cases of forensic entomology in the Iberian Peninsula have already been described, mainly in Spain (Arnaldos et al., 2001; Arnaldos & García, 2021). So far, Portugal has a database of insects colonising human carcasses that has been assembled through genetic identification (Cainé et al., 2009), taxonomic identification (Prado e Castro et al., 2012) and review work (Rebelo et al., 2014). None of these works from Portugal, however, estimated the PMI as done in Chapter 4 of this thesis.

The present thesis confirmed what is already well-known in other countries: *C. albiceps* is not only part of the Portuguese cadaveric fauna but can also serve as evidence in ADD calculations. This

is a new step towards understanding which species in Portugal, the Iberian Peninsula and other countries can be used for this calculation and become evidence in legal cases. There are several species in the region that are part of the cadaveric fauna (Prado e Castro et al., 2012; Rebelo et al., 2014), not only feeding on the carcass, but ovipositing as well. Depending on the conditions to which the corpses are exposed, it is possible that species other than those found in this study serve as models for the ADD calculation.

The ADD calculation shows that *C. albiceps* developed in 13 days (egg to adult), averaging $T \cong 21^{\circ}\text{C}$. At these temperatures, Marchenko (2001) established that the development of *C. albiceps* in the laboratory from egg to adult is 17.2 days. This period overlaps the time when there was development of *C. albiceps* on the baits on which it was collected. This result may seem surprising, but it is not. Marchenko (2001) worked with specimens from colder locations in Europe and in a controlled environment. The development of flies in cadaveric environments can accelerate depending on which flies are collected and on which part of the cadaver. There are differences in heat accumulation, for example, in flies collected at the periphery of the cadaver and those that are collected in the larval masses formed by the accumulation of specimens, where the temperature can increase by several degrees (Byrd & Castner, 2009). In addition, microtemperature and soil temperature must also be considered (Dias-Filho et al., 2022).

6.1.4. The ultrastructures of *C. albiceps*

In this thesis, the diversity of sensillae present in two anatomical apparatus of *C. albiceps* was presented: olfactory and locomotion. The sensillae of the olfactory apparatus had already been studied in Turkish populations of the species (Darilmaz et al., 2009). In their work, these authors found that there were 4 types of sensillae in the pedicel (erroneously called scape in Figure 6 of their paper) of the species. In the same work, however, they did not explore the other antennal segments, which under-sampled the possible additional types of sensillae with some sensory function. In this thesis, 5 types of sensillae were described in the pedicel of *C. albiceps*, as microtrichia was added to the types characterized by Darilmaz and collaborators. Moreover, the other antennal segments were explored, which increased the diversity of sensillae described in the literature.

No differences were found in the patterns of sensilla and structures on the antennae of *C. albiceps*. This confirms similar patterns found in the genus *Chrysomya* (Sukontason et al., 2004). However, it is recommended that further studies be carried out on the antennae of this species in order to verify the number of sensilla in the ultrastructures.

This work also explored the locomotion apparatus of *C. albiceps* that had never been formally described. The analysis, focused on the pulvilli of the species, was able to show the spoon-shaped arrows in the pulvilli of *C. albiceps*, as well as describing a possible sexual dimorphism (to be confirmed in further work) in the microtrichia present in the tarsal claws of the species. Niederegger et al. (2002) identified sexual dimorphism in the density of tenente setae in the pulvilli of *C. vicina*. Although females of this species are generally larger than males, the pulvilli of males do not follow this body pattern. The authors hypothesized that this may indicate a possible grasping function during copulation, distinct from the already known locomotion function. It is not ruled out that a similar type of dimorphism may occur in *C. albiceps*, considering that microtrichia may serve as a friction system (Gorb, 1998).

6.2 Conclusion

The main aim of this thesis was to study the impact of temperature on the morphology and distribution of *C. albiceps* and unexpected and new results were found, with the following stand out:

- a) Unlike what was previously known, *C. albiceps* has sexual dimorphism in both wing shape and wing size. Both were influenced by temperature, although this influence was low in the case of the wing shape;
- b) In fact, a negative relationship between wing size and temperature has been found: the higher the temperature, the further the wing size decreases, in a non-linear relationship;
- c) Over the years, a slight difference in wing size was noted. However, it is notable that the greatest difference expressed is between the years 2004 and 2021;
- d) In wing shape, the success in group identification reached 99.36% for females and 99.55% for males;
- e) Annual Mean Temperature and Temperature Annual Range were the variables that contributed the most to the climate suitability model presented in the thesis;
- f) From the model generated, it is concluded that much of Europe is climatically suitable for *C. albiceps*;
- g) In future scenarios, the suitability increases in northern and eastern Europe, with areas of gains concentrated in these locations, which appears to align with the recent geographical dispersion of the species across the continent;

- h) Both *C. albiceps* and *C. vicina* demonstrated viability in determining the time of death based on the ADD (Accumulated Degree Days) calculation, being these data new for Portugal and of great relevance in forensic entomology;
- i) The sensillae of *C. albiceps* olfactory apparatus are more diverse than previously seen by other authors, which opens new routes for future investigations on corps location;
- j) Among the sensillae found in the locomotor apparatus of *C. albiceps*, described by the first time in the species, a possible sexual dimorphism was perceived in the microtrichia of the species.

Being a species with high competitive power, potential myiasis agent, pollinator, forensic indicator and potential mechanical vector of pathogens, *C. albiceps* should not to be neglected in Europe and the rest of the world. We hope that this thesis results can provide theoretical support for future studies and help to better understand what this species looks like, how it reacts under certain conditions, and how it can be used in a theoretical-practical context in applied sciences.

6.3 References

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7 Annex

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