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Blowflies's (Diptera: Calliphoridae) nocturnal oviposition in Southern Europe (Portugal)

Cláudia Sofia Fontes Fernandes

Dissertação conducente ao Grau de Mestre em Ciências e Técnicas Laboratoriais Forenses

Gandra, 21 de outubro de 2022

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Trabalho realizado sob a orientação de:
Professora Doutora Catarina Barros de Prado e Castro

Declaração de Integridade

Eu, Cláudia Fernandes, declaro ter atuado com absoluta integridade na elaboração deste trabalho.

Confirmo que em todo o trabalho conducente à sua elaboração não recorri a qualquer forma de falsificação de resultados ou à prática de plágio (ato pelo qual um indivíduo, mesmo por omissão, assume a autoria do trabalho intelectual pertencente a outrem, na sua totalidade ou em partes dele).

Mais declaro que todas as frases que retirei de trabalhos anteriores pertencentes a outros autores foram referenciadas ou redigidas com novas palavras, tendo neste caso colocado a citação da fonte bibliográfica.

O estudante,

____

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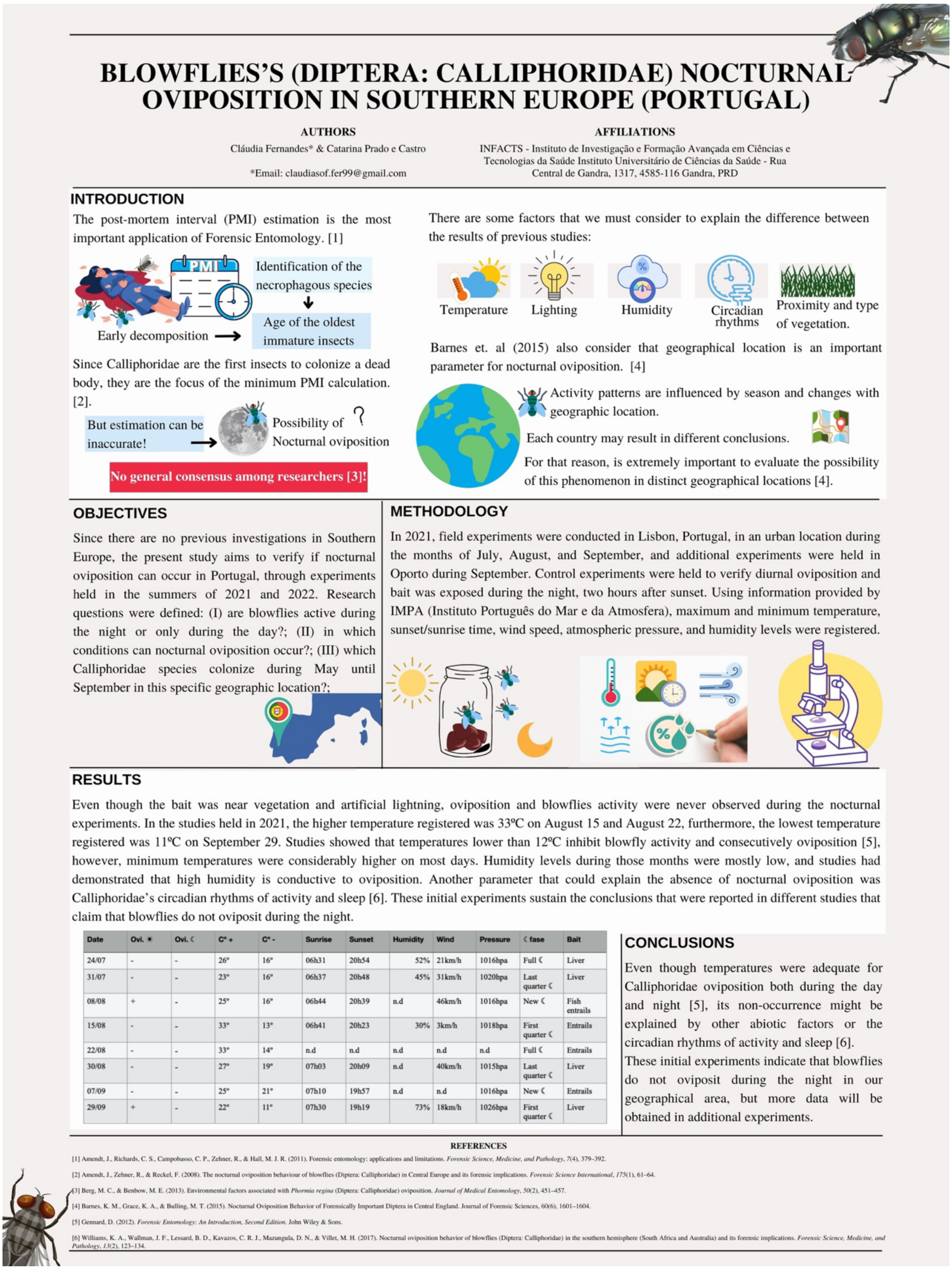

BLOWFLIES'S (DIPTERA: CALLIPHORIDAE) NOCTURNAL OVIPOSITION IN SOUTHERN EUROPE (PORTUGAL)

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INTRODUCTION

The post-mortem interval (PMI) estimation is the most important application of Forensic Entomology. [1]

Since Calliphoridae are the first insects to colonize a dead body, they are the focus of the minimum PMI calculation. [2].

There are some factors that we must consider to explain the difference between the results of previous studies:

Barnes et. al (2015) also consider that geographical location is an important parameter for nocturnal oviposition. [4]

Activity patterns are influenced by season and changes with geographic location.

Each country may result in different conclusions.

For that reason, is extremely important to evaluate the possibility of this phenomenon in distinct geographical locations [4].

OBJECTIVES

Since there are no previous investigations in Southern Europe, the present study aims to verify if nocturnal oviposition can occur in Portugal, through experiments held in the summers of 2021 and 2022. Research questions were defined: (I) are blowflies active during the night or only during the day?; (II) in which conditions can nocturnal oviposition occur?; (III) which Calliphoridae species colonize during May until September in this specific geographic location?;

METHODOLOGY

In 2021, field experiments were conducted in Lisbon, Portugal, in an urban location during the months of July, August, and September, and additional experiments were held in Oporto during September. Control experiments were held to verify diurnal oviposition and bait was exposed during the night, two hours after sunset. Using information provided by IMPA (Instituto Português do Mar e da Atmosfera), maximum and minimum temperature, sunset/sunrise time, wind speed, atmospheric pressure, and humidity levels were registered.

RESULTS

Even though the bait was near vegetation and artificial lightning, oviposition and blowflies activity were never observed during the nocturnal experiments. In the studies held in 2021, the higher temperature registered was 33ºC on August 15 and August 22, furthermore, the lowest temperature registered was 11ºC on September 29. Studies showed that temperatures lower than 12ºC inhibit blowfly activity and consecutively oviposition [5], however, minimum temperatures were considerably higher on most days. Humidity levels during those months were mostly low, and studies had demonstrated that high humidity is conductive to oviposition. Another parameter that could explain the absence of nocturnal oviposition was Calliphoridae's circadian rhythms of activity and sleep [6]. These initial experiments sustain the conclusions that were reported in different studies that claim that blowflies do not oviposit during the night.

Date	Ovi. ☀	Ovi. ☾	C° +	C° -	Sunrise	Sunset	Humidity	Wind	Pressure	☾ fase	Bait
24/07	-	-	26°	16°	06h31	20h54	52%	21km/h	1016hpa	Full ☾	Liver
31/07	-	-	23°	16°	06h37	20h48	45%	31km/h	1020hpa	Last quarter ☾	Liver
08/08	+	-	25°	16°	06h44	20h39	n.d	46km/h	1016hpa	New ☾	Fish entrails
15/08	-	-	33°	13°	06h41	20h23	30%	3km/h	1018hpa	First quarter ☾	Entrails
22/08	-	-	33°	14°	n.d	n.d	n.d	n.d	n.d	Full ☾	Entrails
30/08	-	-	27°	19°	07h03	20h09	n.d	40km/h	1015hpa	Last quarter ☾	Liver
07/09	-	-	25°	21°	07h10	19h57	n.d	n.d	1016hpa	New ☾	Entrails
29/09	+	-	22°	11°	07h30	19h19	73%	18km/h	1026hpa	First quarter ☾	Liver

CONCLUSIONS

Even though temperatures were adequate for Calliphoridae oviposition both during the day and night [5], its non-occurrence might be explained by other abiotic factors or the circadian rhythms of activity and sleep [6].
These initial experiments indicate that blowflies do not oviposit during the night in our geographical area, but more data will be obtained in additional experiments.

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RESUMO

A estimativa do intervalo post-mortem (IPM) é a aplicação mais importante da Entomologia Forense. Durante os estágios iniciais de decomposição, a estimativa do IPM pode ser determinada através da identificação das espécies necrófagas presentes no cadáver, calculando a idade dos insetos imaturos mais velhos. Como as moscas da família Calliphoridae são os primeiros insetos a colonizar um cadáver, são os principais indicadores do cálculo do IPM mínimo. No entanto, existem vários fatores que podem tornar essa estimativa imprecisa, especificamente, a possibilidade de oviposição noturna. A comunidade científica tem assumido que as moscas varejeiras são inativas durante a noite e incapazes de pôr ovos, no entanto vários estudos recentes observaram que a oviposição noturna pode ocorrer. No entanto, os resultados são controversos e não há consenso entre os investigadores.

Uma vez que não existem investigações anteriores no Sul da Europa, o presente estudo pretende verificar se a oviposição noturna pode ocorrer em Portugal, através de experiências realizadas nos verões de 2021 e 2022. As experiências foram conduzidas em Lisboa e no Porto, Portugal, num local urbano durante os meses de maio a outubro. Experiências de controlo foram realizadas para verificar a oviposição diurna e os iscos foram expostas durante a noite, duas horas após o pôr do sol. Utilizando informações fornecidas pelo IMPA (Instituto Português do Mar e da Atmosfera), foram registados a temperatura máxima e mínima, horário do pôr do sol/nascer do sol, precipitação, velocidade do vento, níveis de humidade e pressão atmosférica.

A atividade das moscas varejeiras e oviposição nunca ocorreu durante as experiências noturnas, mas ocorreu em 29 das 35 experiências diurnas. Os adultos que foram capturados e posteriormente identificados durante as recolhas de 2022 foram *Calliphora vicina* Robineu-Desvoidy, 1830, *Calliphora vomitoria* (Linnaeus, 1758), *Lucilia caesar* (Linneus, 1758), *e Lucilia sericata* (Meigen, 1826).

Embora as temperaturas tenham sido adequadas para a oviposição destas espécies tanto durante o dia quanto à noite, a sua não ocorrência pode ser explicada por outros fatores abióticos ou pelos ritmos circadianos de atividade e sono. Este estudo sugere que é improvável a ocorrência de oviposição noturna em Portugal.

Palavras-chave: entomologia forense; oviposição noturna; intervalo post-mortem; Calliphoridae; Portugal.

ABSTRACT

The post-mortem interval (PMI) estimation is the most important application of Forensic Entomology. During the initial stages of decomposition, the minimum PMI can be estimated by identifying the necrophagous species present in the corpse and then calculating the age of the oldest immature insects. Since flies belonging to the Calliphoridae family are the first insects to colonize a dead body, they are the main indicators of the minimum PMI calculation. However, there are several factors that can make this estimation inaccurate, specifically, the possibility of nocturnal oviposition. Although it is assumed that blowflies are inactive during the night, several studies remarked that nocturnal oviposition can occur. However, the results are controversial and there is no consensus among researchers.

Since there are no previous investigations in Southern Europe, the present study aims to verify if nocturnal oviposition can occur in Portugal, through experiments held in the summers of 2021 and 2022.

Field experiments were conducted in Lisbon and Oporto, Portugal, in an urban location during the months of May to October. Control experiments were held to verify diurnal oviposition and baits were exposed during the night, two hours after sunset. Using information provided by IMPA (Instituto Português do Mar e da Atmosfera), maximum and minimum temperature, sunset/sunrise time, rainfall, wind speed, humidity levels and atmospheric pressure were registered.

Oviposition and blowfly activity was never observed during the nocturnal experiments, but occurred in 29 out of the 35 diurnal experiments - the adults that were caught and posteriorly identified during the 2022 experiments were *Calliphora vicina* Robineau-Desvoidy, 1830, *Calliphora vomitoria* (Linnaeus, 1758), *Lucilia caesar* (Linnaeus, 1758), e *Lucilia sericata* (Meigen, 1826).

Even though temperatures were adequate for Calliphoridae oviposition both during the day and night, its non-occurrence might be explained by other abiotic factors or the circadian rhythms of activity and sleep. This study suggests that nocturnal oviposition is unlikely to occur in Portugal.

Keywords: forensic entomology; nocturnal oviposition; post-mortem interval; Calliphoridae; Portugal.

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PART I - INTRODUCTION, STATE OF THE ART AND OBJECTIVES OF THE DISSERTATION

Introduction - How can insects be used for forensic purposes?

Forensic entomology is the science that collects and analyzes insect evidence in cases with legal repercussions (Amendt et al., 2007). There are three different areas in forensic entomology: (I) urban entomology, (II) stored product entomology, and (III) medico-criminal entomology (Rivers & Dahlem, 2014).

Urban entomology involves insects and arthropods that affect human habitation, environment, and structures, leading to material damage or loss. Some specimens include termites, cockroaches, among others (Prado e Castro, 2005). Stored product entomology approaches legal cases that involve insects and other arthropods found in food products, either their presence, their body parts, or signs of their activity (Rivers & Dahlem, 2014).

Medico-criminal entomology focuses on the insects that colonize decaying organic matter - essentially human remains. Its main application is the estimation of the post-mortem interval (PMI) (Amendt et al., 2007), which refers to the time between death and discovery of the corpse (Amendt et al., 2011). In certain circumstances, colonization can happen in living humans or live vertebrates. This phenomenon, denominated myiasis is usually evidence of neglect or abandonment (Amendt et al., 2011).

The postmortem interval is a key question in criminal investigations since it can help the reconstruction of events and circumstances of death (Amendt et al., 2007). Further, it can provide information that helps in the identification of the victim (Rivers & Dahlem, 2014), it may associate a suspect to the crime scene, and establish testimony credibility (Amendt et al., 2007). The time since death is also important in civil matters such as in cases of insurance or inheritance (Rivers & Dahlem, 2014).

There are two methods to estimate the postmortem interval: (I) during the initial stages of decomposition, the minimum post-mortem interval can be estimated by identifying the necrophagous species present in the corpse and then calculating the age of the oldest immature insect (Amendt et al., 2008); (II) during the late postmortem period, the estimation is based on the successional patterns of the arthropod community present at the site (Amendt et al., 2007).

This first methodology applies the calculated time period needed for larval development. This depends on the crime location temperature (Amendt et al., 2011), and the temperature required for development fluctuates between species. For that reason, the identification of the species collected is a first and fundamental step in the analysis of insect evidence (Amendt et al., 2007).

The reconstruction of the crime scene's temperature using the nearest weather station is also essential. However, since there may be differences between the crime scene temperatures and the temperatures recorded by the weather station, usually a data logger is placed in the crime scene for several days to then perform a correction on the temperature reports (Amendt et al., 2007, 2011).

Subsequently, with this information, it is possible to estimate the rate of development of the immature insects collected, and obtain a minimum PMI.

The first insects to colonize a body are, in most circumstances, the blowflies (Diptera: Calliphoridae). Thus, several necrophagous species belonging to this family are the focus of forensic entomologists in the calculation of the minimum PMI. Besides having a marked seasonality, forensically important species differ from region to region (Prado e Castro et al., 2012), so local studies need to be conducted in this forensic area.

Colonization by blowflies can occur within minutes, hours, or days after death (depending on several factors such as temperature or accessibility of insects to the corpse). However, there are several parameters that can delay the colonization and lead to an underestimate of the minimum post-mortem interval (Amendt et al., 2011). Some of these factors can be related to low temperatures, unfavorable weather (e.g.rain; wind speed), physical disturbance of the body (e.g. if it was wrapped up; if it was buried); chemicals, or traces of drugs on the body (Zurawski et al., 2009). These limitations need to be taken into account when performing a forensic entomology expertise (Rivers & Dahlem, 2014).

Establishing the post mortem interval one, two, or more months after death means finding a large community of insects present on the body (Gennard, 2012). The study of insect succession makes it possible to associate each species or group of species with a stage of decomposition (Amendt et al., 2007), since each stage is attractive to different arthropod species (Prado e Castro et al., 2012, 2013).

Smith (1986) proposed four categories of insects: (I) necrophagous, that feed directly on the body (Diptera: Calliphoridae, Sarcophagidae; Coleoptera: Silphidae, Dermestidae); (II) predators and parasites, of the colonizing fauna (Diptera: Calliphoridae *(Chrysomya),* Muscidae *(Hydrotaea);* Coleoptera: Silphidae, Staphylinidae, Histeridae); (III) omnivores, that can feed on the corpse and related inhabitant fauna (Hymenoptera: Formicidae, Vespidae; Coleoptera) and (IV) adventive species, which use the corpse as an extension of their environment (Araneae; Acarina; Collembola; Chilopoda; Diplopoda).

As the decomposition progresses, successive waves of insects colonize the body (Rivers & Dahlem, 2014) in a more or less predictable chronological order. Usually, Diptera (in particular, Calliphoridae) are attracted to a fresh body, while Coleoptera are attracted to the final stages of decomposition.

The second method of PMI estimation is based on the insect community present on the corpse. With this method, used in advanced stages of decomposition, insects collected from a forensic case are compared to the expected successional patterns, specifically from the same geographical area (Amendt et al., 2007, 2011).

To sum up what has been discussed so far, the importance of forensic entomology is mainly associated with the determination of the post-mortem interval. Yet, forensic entomology expertise can also provide answers to questions regarding the location of death and possible postmortem displacement; circumstances of death; drug detection; and even cases of negligence and abandonment (Amendt et al., 2007). The insects are in fact very useful sources of information. Forensic entomology presents several benefits during an investigation, namely its cost-benefit and accuracy that others methods not always can provide. Nonetheless, it's mandatory to have adequate entomological knowledge.

The beginning and evolution of forensic entomology

A myriad of researchers have briefly summarized forensic entomology history and developments in the last centuries. In this section, we will be looking at an overview of key historic events that led to the evolution of the discipline and present-day knowledge. Even though ancient civilizations showed some understanding of the life cycles of insects and recognized the scavenger activity of some of them (Rivers & Dahlem, 2014), the first record of the use of entomological knowledge in a criminal investigation comes from China in the thirteenth century. In Sung Tz'u medico-legal book "Washing Away of Wrongs", the author claims that, during a murder investigation, blowflies were attracted to the murder weapon - a sickle - which led the perpetrator to confess the murder of a fellow farm worker.

However, in Europe, Forensic Entomology only began to develop around the seventeenth century, with biology studies that had an impact on the field. Redi (1668), performed several experiments using the flesh of different animals and demonstrated that larvae emerged from eggs laid by flies (Gennard, 2012). These studies were meant to test the abiogenesis theory which was the belief that maggots spontaneously formed on meat (Rivers & Dahlem, 2014).

In the eighteenth century, Linnaeus (1775) established a binomial naming system for classifying species of animals and plants. The naturalist also collected and identified thousands of animals, including two thousand insects - among them some forensic relevant species (Gennard, 2012). Redi and Linnaeus' research contributed to the better understanding of insect life stages and their duration. Yet, the groundwork for modern forensic entomology occurred in the nineteenth century (Rivers & Dahlem, 2014).

The first record of modern forensic entomology being used in a legal case happened in France. Bergeret (1855), applying his knowledge in both pathology and entomology, was capable of solving a case regarding the remains of a newborn baby (Gennard, 2012). The investigator found, during the autopsy, larvae of *Sarcophaga carnaria* (Linnaeus, 1758) (Diptera: Sarcophagidae) and moth's pupae. Even though he wrongly estimated that a fly development would take eight to ten months, it was the first transcript of entomological evidence used to establish a postmortem interval (Rivers & Dahlem, 2014).

Regarding the postmortem interval, Mégnin (1894), author of *La faune des cadavres: application de l`entomologie a la médecine légale*, stated that it could be established by analyzing different arthropod species present in a decomposing body. After performing several experiments, the author claimed that insect succession was predictable (Gennard, 2012).

Related to this information, the german physician Hermann Reinhard (1882) conducted the first systematic research in forensic entomology, focusing his investigation on insect succession of buried bodies. His work allowed the identification of Diptera: Phoridae, associated with buried or enclosed corpses. Motter (1898) also studied exhumed remains, which provided information on the insect fauna and burial conditions (depth and soil type). The United States Civil War allowed the direct observation of thousands of human and animal remains and there were reports of bodies covered in maggots. It was at this time that maggot therapy was first noticed as beneficial for removal of necrotic tissues (Rivers & Dahlem, 2014).

There was a moment between the end of the nineteenth century to the middle of the twentieth that forensic entomology stagnated. The causes associated were (I) the distancing between entomologists and medico-legal professionals; (II) a small number of cases where entomologists were needed and (III) a lack of entomologists specialized in biology and taxonomy of cadaver colonizing species (Prado e Castro, 2005). Topics such as corpse skeletonization or modifications caused by insects were investigated in this period. However, new data was not being applied to forensic cases (Rivers & Dahlem, 2014).

It was from the fifties that a new cycle of advances in Forensic Entomology was established. Bornemissza (1957), Reed (1958) and, Payne (1965) focused their investigation on the succession of arthropods and the association of different stages of decomposition to characteristic groups of insects. A.S Kamal (1958) characterized thirteen species of scavenger flies from the families Calliphoridae and Sarcophagidae. This research provided data on the development of each life cycle stage at different temperatures (Prado e Castro, 2005).

It was since the 1980s that forensic entomology grew as a discipline and as a valuable tool used in legal investigations (Rivers & Dahlem, 2014). Several researchers started to dedicate to the discipline, particularly in Europe and the U.S. at that time, and later, in the 1990s, research groups started to be created mainly in universities (Tomberlin & Benbow, 2015). In 2002, the European Association for Forensic Entomology was created, promoting the recognition of this field. The association encourages information exchange between laboratories and entomologists, as well as establishing common practices and stimulating the implementation and development of techniques (EAF E, n.d.).

Diptera: Calliphoridae - Morphology, life cycle, and characteristics

While conducting an investigation, it is essential to have knowledge about which insects are colonizing the body and their particularities (Gennard, 2012). This section is dedicated to the study of flies (Order: Diptera), particularly the morphology, life cycle, and characteristics of the Calliphoridae family - the group focused in this thesis.

The Diptera order, universally denominated as flies, is one of the largest insect orders, with over eighty-six thousand species. Due to its diversity, the group is classified into several families, and just in Europe, there are one hundred and thirty-two recognized families.

Flies have an essential role in decomposing dead organic matter and recycling nutrients. These insects are also important in plant pollination and can serve as food for other animals. However, despite these essential functions, there are some species which are parasites and disease transmitters, as in many insect groups (Byrd & Tomberlin, 2019).

Adult *Diptera* possesses an exoskeleton distributed by three regions - head, thorax, and abdomen (Fig.1), with three dimensions - dorsum, sternum, and pleuron.

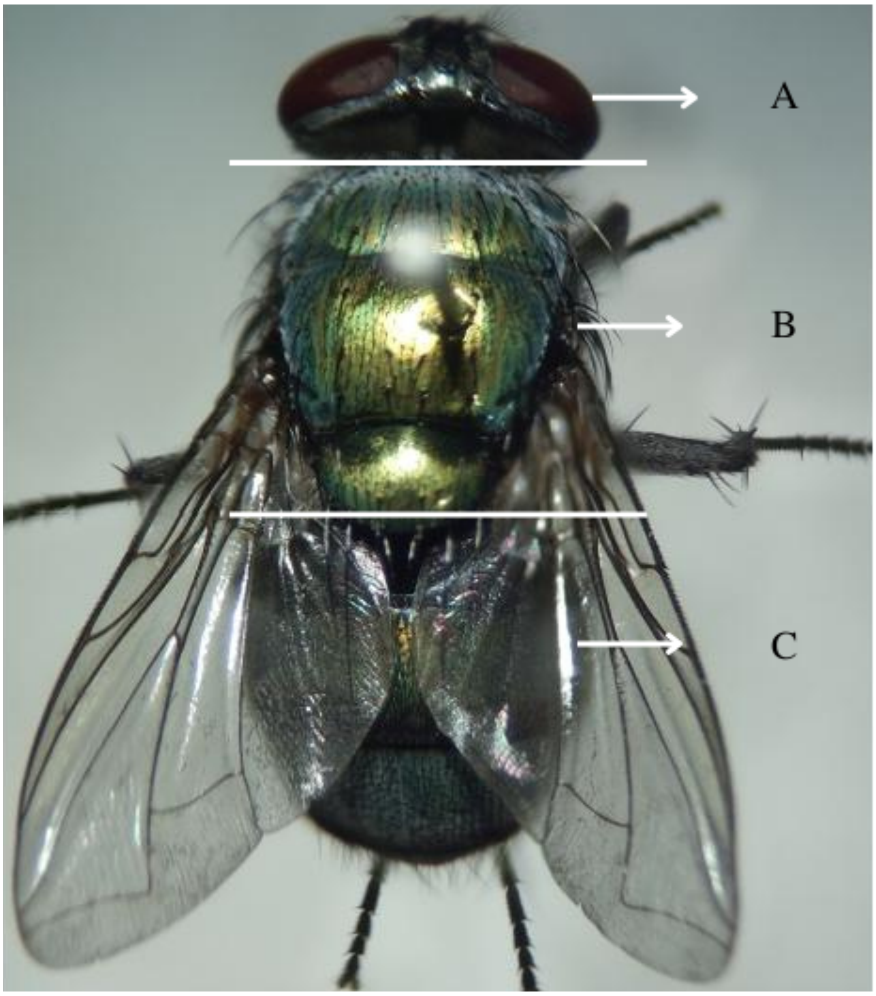

Figure 1: Dorsal view of *Calliphoridae Hemipyrellialigurrien* divided by three regions. (A) Head. (B) Thorax. (C) Abdomen. Adapted from Bunchu et al. (2012)

The thorax is distributed by the prothorax, the mesothorax, and the metathorax. In each segment of the thorax, there is one pair of legs - with coxae, femur, tibia, and tarsus (Gennard, 2012). Present in the mesothorax flies possess a pair of membranous wings (Rivers & Dahlem, 2014), and, in the metathorax, a second pair labeled halters, that are used to stabilize the flight (Byrd & Tomberlin, 2019). The membranous wings are supported by veins, particularly important for species identification.

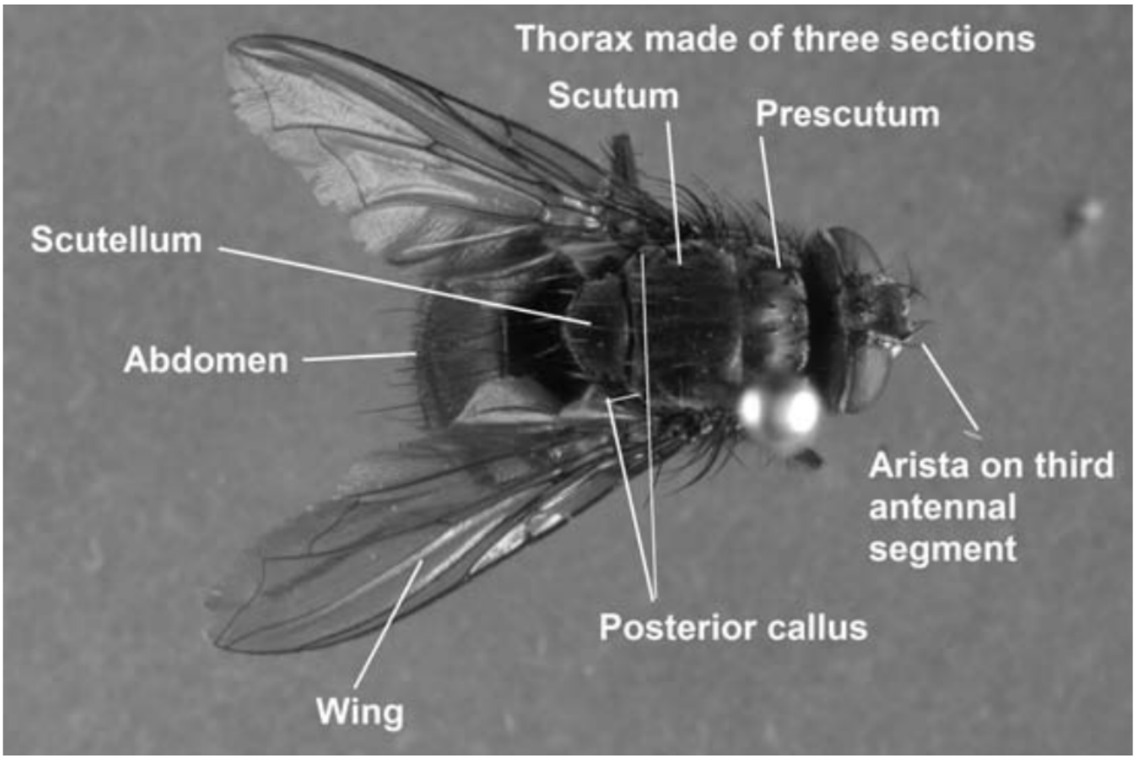

Figure 2: Diptera morphology with description. Taken from Gennard (2012)

In the insect's head, there is the antennae (Figs. 3 and 4), a sensory organ, also useful for species identification. (Gennard, 2012)

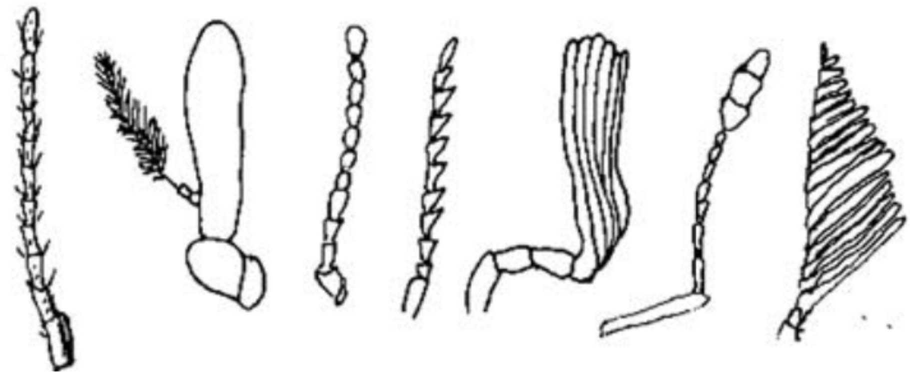

Figure 3: Representation of multiple insect antenna. Taken from Gennard (2012)

Figure 4: Antennae of a Diptera. Taken from Byrd & Tomberlin (2019)

Diptera mouthparts differ according to the insect's diet. In the case of flies associated with decomposing matter, these present sponging mouthparts (Fig. 5) (Byrd & Tomberlin, 2019).

Figure 5: Bronzebottle fly sponging mouthpart in rotting fruit. Taken from Byrd & Tomberlin (2019)

One of the Diptera families is Calliphoridae, a broad family with around 70 species present in Europe and approximately 1000 described worldwide (Prado e Castro & Ameixa, 2021).

Blowflies range from six to fourteen millimeters in size, although the adult dimensions depend on the species and available food source (Byrd & Tomberlin, 2019). Visually, most Calliphoridae display a metallic shine with colors ranging from bright green or blue (Rivers & Dahlem, 2014) to bronze or lustrous black (Fig.6).

Figure 6: Calliphoridae different colors. Taken from Gennard (2012)

Calliphoridae are mainly drawn to decomposing remains, excrement, and some vegetal material. Most of the time, they are the first to reach a dead body, and since eggs are frequently laid on open cavities and wounds, this group is particularly important to forensic entomology (Byrd & Tomberlin, 2019).

The eggs are the first stage of a blowfly life cycle and are typically laid around 150 to 200. Shiny and white, the egg morphology (Fig. 7) is formed by the chorion (the outer, textured coating) (Gennard, 2012), the micropyle (a hole at the end of the egg that allows access of the spermatozoa) (Amendt et al., 2010), the plastron (that allows respiration even when submerged in water) and the eclosion / hatching line (Gennard, 2012).

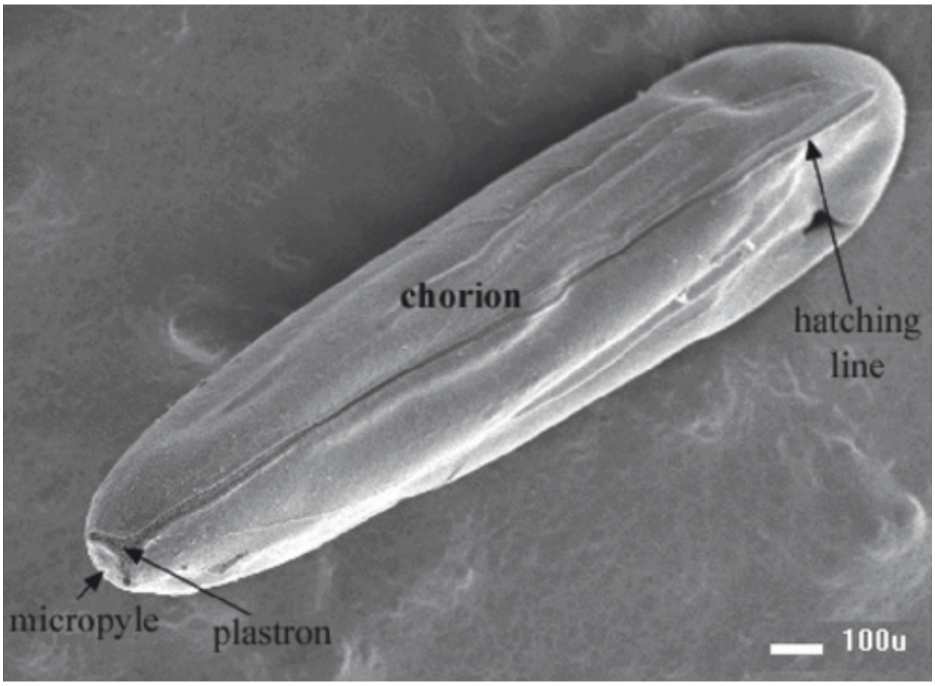

Figure 7: Morphology of a calliphorid egg. Taken from Amendt et al. (2010)

Larvae have a cylindrical form and a cephalopharyngeal skeleton whose shape changes according to the larval stage. Maggots are constituted by twelve segments, a slightly pointed head, followed by a prothoracic, a mesothoracic, a metathoracic segment, and eight abdominal segments (Fig 8.) (Amendt et al., 2010).

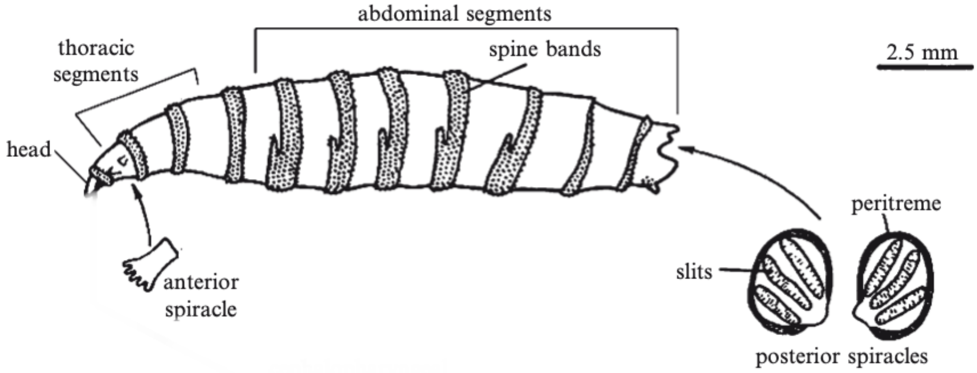

Figure 8: Larvae Morphology. Adapted from Amendt et al. (2010)

Present in the last abdominal segment, the posterior spiracles (Fig.9) are important indicators in species identification. At the same time, each spiracle has a number of slits that provide information about the larval stage. It is possible to identify the mature larval stage, either by the presence of anterior spiracles (Fig. 9) or, as mentioned above, by the number of slits in the posterior spiracles (Gennard, 2012). Anterior spiracles are positioned on each side of the prothoracic segment and stick out from the body (Amendt et al., 2010). Further, the terminal segment of third-stage larvae commonly has six or more tubercles around its perimeter (Gennard, 2012).

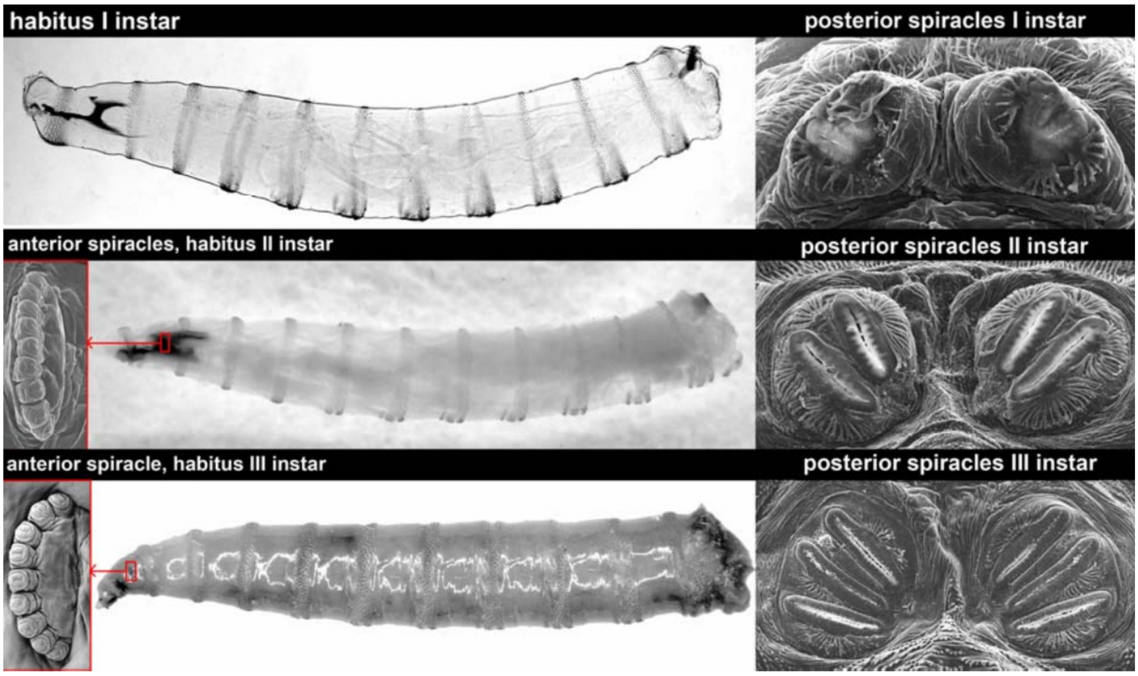

Figure 9: Spiracles and instar identification. Taken from Szpila (2012)

The puparium morphology is identical to the preceding stage, whereas smaller in size. It has a structure shaped like a barrel (Fig. 10), developed by the hardening of the third instar larval cuticle (Amendt et al., 2010).

Figure 10: Puparium found on soil. Taken from Byrd & Tomberlin (2019)

Key features in adult Calliphoridae are important to differentiate between species. In the wings, the forensic entomologist may observe the stem-vein (if it has hairs or not) (Fig.11), the anterior margin, and may remark the color of the basicosta (Fig. 12).

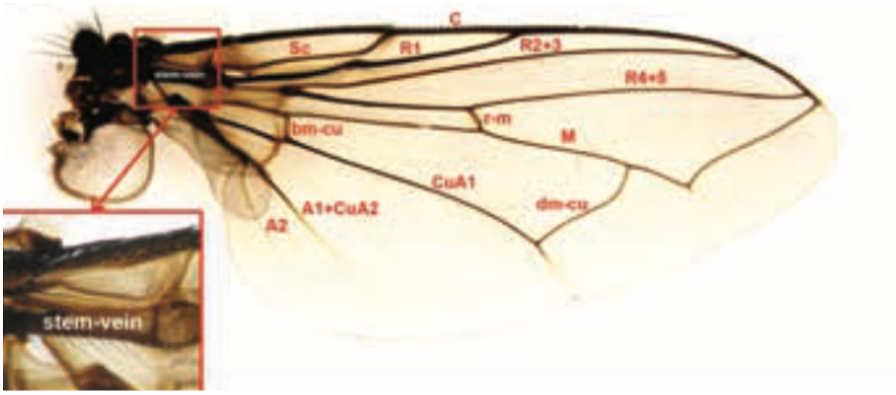

Figure 11: *Photophormia terraenovae* (Robineay-Desvoidy, 1830) wing. Taken from Szpila (2012)

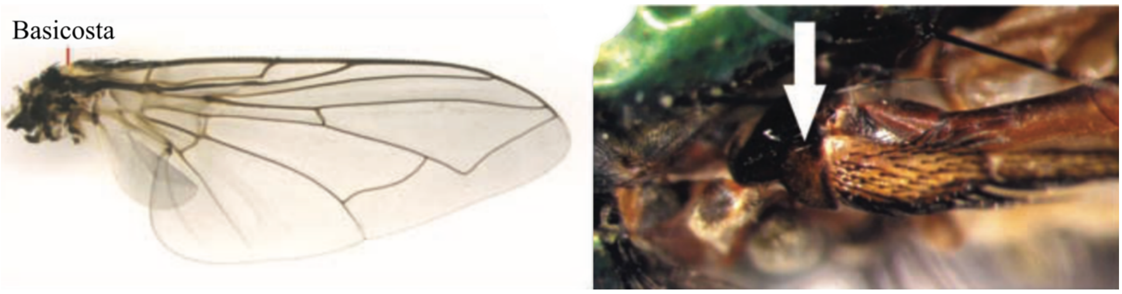

Figure 12: *Lucilia sericata* (Meigen, 1826) wing and basicosta. Taken from Szpila (2012)

In the thorax, it is important to observe the color of the fly and, if present, the color of the abdominal bands. It is important to confirm the presence of a coxopleural streak, as well as the number of acrostichal bristles (Fig. 13) on the post sutural area and the color of the anterior thoracic spiracle. The color and bristliness of the upper and lower calypter (Fig.14), as well as the bristliness of the greater ampulla, may be registered.

Figure 13: The thorax showing the position of the acrostichal bristles. Taken from Gennard (2012)

Figure 14: Lateral view of a Calliphoridae adult. (A) Calypter. Adapted from Bunchu et al. (2012) and Szpila (2012)

In the Calliphoridae head it is important to notice the color of facial ridges and mouth edge, along with the number of bristles in central occipital area. Additionally, the color of the postgena and genal dilation need to be taken into account (Fig. 15).

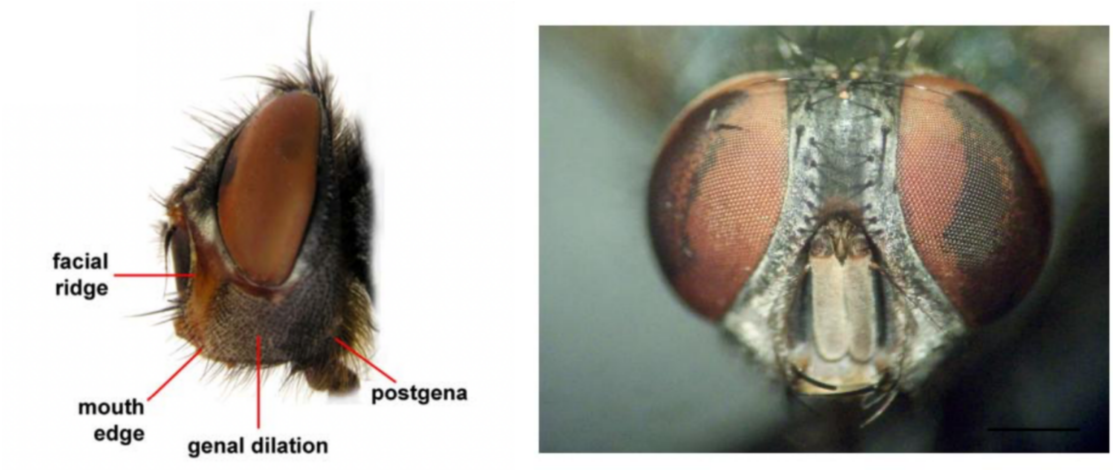

Figure 15: Calliphoridae head morphology. Adapted from Bunchu et al. (2012) and Szpila (2012)

The blowfly genitalia (Fig. 16) is also an important feature in the identification of the species (Gennard, 2012).

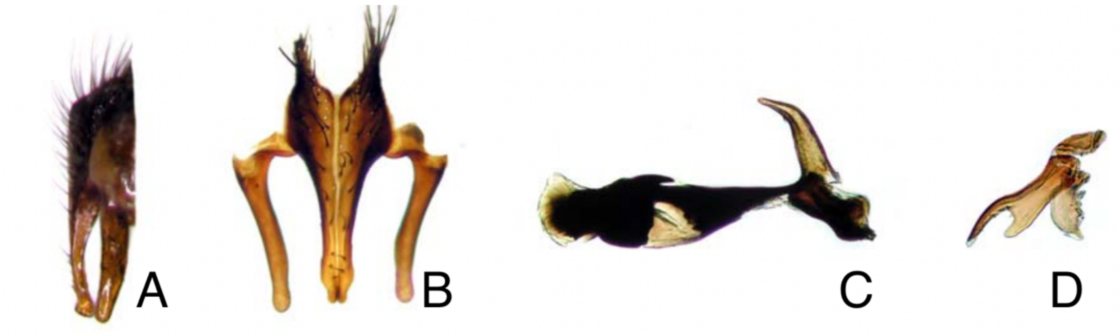

Figure 16: Male genitalia of *Chrysomya albiceps* (Wiedemann, 1819). (A) Cerci and Surstyli, lateral view; (B) Cerci and surstyli, dorsal view; (C) Phallus, lateral view; (D) Pregonite and postgonite, lateral view. Taken from Szpila (2012)

As stated previously, Calliphoridae is the most important insect family in the establishment of the minimum post-mortem interval. Species identification is crucial to calculate the age of the immatures feeding on the body, since different species have different rates of development. Different species have also distinct ecological roles in a cadaver (from necrophagous to predators, parasites and omnivores), as well as seasons of the year with different patterns of activity according to each species. For that reason, it is imperative to perform a proper species identification. There are two possible methods: morphological identification, with the application of dichotomous keys or molecular identification, being up to the forensic entomologist to decide which method is the most adequate, taking into account the case specificity.

Nocturnal Oviposition: what we know so far

A bibliographic review of previous research was performed in this section, focusing on nocturnal oviposition, taking into account what variables were studied, which species were identified, and the conditions of the investigation.

The moment of oviposition triggers a biological clock, becoming a key factor in every calculation of the minimum post-mortem interval (Berg & Benbow, 2013). It is generally assumed that Calliphoridae flies are inactive at night (Amendt et al., 2008) and, because of that, post-mortem interval calculations considered that blowflies do not oviposit at night. Thus, if a body is deposited at night, it is assumed that it will not attract flies until the following day (Amendt et al., 2010).

However, several studies have addressed the possibility of nocturnal oviposition under a variety of conditions. This is one of the factors that can lead to an inaccurate estimation of the post-mortem interval: if blowflies oviposit at night there can be a discrepancy of up to twelve hours (Zurawski et al., 2009). This topic has been controversial among researchers since there is no general consensus (Berg & Benbow, 2013).

Greenberg (1990) was the first to report that blowflies can oviposit during the night. The author performed his experience in a Chicago residential area, in June, July, and August 1988. The experience was repeated in the following year, with the only difference being the type of bait used. In the first year, the author used rat carcasses and in the subsequent year ground beef, however, both baits were in similar conditions, placed at ground level and near sodium vapor lamps. Greenberg measured both temperature and humidity thirty centimeters away from the bait. In 1988, the bait was exposed at sunset and later until four in the morning. Whilst in 1989, baits were exposed on six nights from midnight to three in the morning during July and August. Results showed nocturnal oviposition in both experiments. Recorded temperatures were around 20ºC, with a minimum temperature of 17ºC and a maximum of 24ºC. Relative humidity was more distributed, with a minimum of 40% and a maximum of 100%. The species of ovipositing flies were *Calliphora vicina* Robineau-Desvoidy, 1830, *Lucilia sericata* (Meigen, 1826), and *Phormia regina* (Meigen, 1826) (Greenberg, 1990).

Later experiments conducted by Singh and Bharti (2001), established that Greenberg's results weren't a simple coincidence. However, the authors stated that Greenberg committed a mistake when placing the bait on the ground. For that reason, Singh and Bharti placed the bait - mutton - 1.85 meters above the ground, on top of a wood beam protected with tape preventing flies from crawling instead of flying. Their experiments were held in Patiala, India, during a full week in both March and September 1999 from 10 pm to 3 am near artificial lighting. The minimum temperature registered was 16ºC and the maximum was 27ºC and relative humidity fluctuated from 75% to 85%. Results supported Greenberg's conclusions that *Calliphora vicina*, *Lucilia sericata,* and *Phormia regina* oviposited at night (Singh & Bharti, 2001).

Additionally, in Baldrige's et. al (2006) study, oviposition was recorded once. The author's research was conducted in Texas, in both urban and rural areas. They used a variety of baits, such as beef liver, rodents, and pigs. In the experiments held at a rural location with artificial lighting, a bloated pig carcass attracted oviposition around 9 pm. The species that oviposited were *Lucilia coeruleiviridis* (Macquart, 1855), *Cochliomya macellaria* (Fabricius, 1775)*, and Musca domestica* (Linnaeus, 1758). The authors recognized that generally blowflies' interest in the baits stopped after sunset, and in the majority of cases no activity was recorded until after sunrise (Baldridge et al., 2006).

Furthermore, in Kirkpatrick and Olson's (2007) experiments, oviposition was recorded in the Summer and Spring of 2003. The studies were performed in a Texas rural area under artificial lighting. The bait - ground beef - was positioned in the soil and both temperature and humidity were recorded at the site. Results demonstrated that blowflies oviposited under artificial lighting and at temperatures above 26ºC. The ovipositing species were *Lucilia eximia* (Wiedemann, 1819) and *Cochliomya macellaria* (Kirkpatrick & Olson, 2007).

Most recently, William's et al. (2017) established that nocturnal oviposition is, in fact, possible and does occur. Distinctly from other studies, these authors executed three field experiments and three laboratory experiments. Two experiments were held in South Africa but different cities, although both in urban areas. In the field experiment in Grahamstown, South Africa, there was no artificial lighting and the only source of illumination was moonlight. The baits used were laboratory rats. In this experiment, it was only recorded one nocturnal oviposition event by *Lucilia sericata*. This oviposition occurred under the last quarter of the moon at a temperature of 19.7°C and relative humidity of 86.5%. The experiment conducted in Durban, South Africa had security lighting and chicken liver was used as bait. Nocturnal oviposition occurred three nights under the last quarter, new and full moon phases. Temperatures were above 20°C and the minimum relative humidity was 79%. The species that oviposited were *Chrysomya megacephala* (Fabricius, 1794) and *Lucilia cuprina* (Wiedemann, 1830).

The last field experiment was executed in Australia and piglet carcasses were used as bait. LED lights were placed above each carcass to study the effect of artificial lighting. However, nocturnal oviposition did not occur in these experiments.

The first laboratory experiment was performed in two constant environment rooms with a temperature of 20ºC. One room was set at a normal photoperiod, while the other was reversed. In this experiment, *Lucilia sericata*, *Chrysomya putoria* (Wiedemann, 1830), and *Chrysomya chloropyga* (Wiedemann, 1818) laid in the dark once each species. The focus of the second experiment was to measure oviposition under different temperatures for two hours. The authors concluded that a positive correlation exists between temperature and oviposition.

The third and last experiment monitored both nocturnal and diurnal oviposition in light and darkness. The authors remarked that there was no significant association between the percentage of diurnal and nocturnal oviposition for *Lucilia cuprina*, however, there was a significant association for *Calliphora augur* (Fabricius, 1775). For the latter, nocturnal oviposition was higher when exposed to artificial lighting (Williams et al., 2017).

In other studies, particularly Amendt‘s et al. (2008), it was concluded that nocturnal oviposition can happen but only under specific conditions. The experiments were carried out in Germany during August 2004 and from May until September 2005. In the first year, hedgehog carcasses were placed at both urban and rural locations from 10 pm until sunrise. The following year, the bait used was beef liver and the experiments took place in an urban location. In both field experiments, there was no oviposition. Nonetheless, the authors performed laboratory experiments where a colony of *Lucilia sericata* was placed under artificial diurnal rhythm with ten hours of darkness at a constant temperature around 25ºC. Bait was provided from nine pm to seven am. In these conditions, nocturnal oviposition was registered two times (Amendt et al., 2008).

With similar results, George's et al. (2013) held experiments at a semi-rural location in Australia. The bait - ox liver, was placed in a plastic bowl with drainage holes, placed in a platform filled with water to avoid ants. Temperature, humidity, rainfall, wind speed, light intensity, and atmospheric (barometric) pressure were measured at each location. In these experiments, nocturnal oviposition was not recorded and blowflies were not active during that time. The latest oviposition documented was at sunset under mean temperatures of 18ºC and 19ºC. However, caged experiments were performed to verify if blowflies colonize baits during the night when close enough to crawl instead of flying. In this case, nocturnal oviposition did occur four times and the species registered were *Calliphora augur*, *Calliphora stygia* (Fabricius, 1775), *Calliphora dubia* (Macquart 1855), and *Lucilia sericata* (George et al., 2013).

Ultimately, in other studies, oviposition was never documented. Zurawaski et al. (2009) conducted experiments in Michigan during the year 2006 and repeated them in the following year. The authors placed pig carcasses elevated into a fifteen centimeters platform until two hours after the sunset. Ambient temperature was measured in the location and results showed that after the sunset the minimum temperature recorded was 12.4ºC and the maximum temperature was 23.4ºC. The authors also performed laboratory tests to verify if blowflies would fly and oviposit in the dark. The conclusion was that blowflies would not oviposit in complete darkness and nocturnal oviposition did not occur (Zurawski et al., 2009).

Likewise, Stamper et al. (2009) held experiments in both 2006 and 2007. The experiments were performed in Ohio but in four different locations, three were urban and the other was rural. Rat carcasses were used as bait. Even though three of the experiment sites had artificial lighting, unlike other studies, nocturnal oviposition was not observed (Stamper et al., 2009).

Apart from testing nocturnal oviposition, Berg and Benbow (2013) also performed experiments to assess the effects of artificial lighting and bait height in oviposition. In these experiments, held in Ohio, beef liver was placed each month within three or four days of a new moon. The authors declared that they didn't find any significant effects of artificial light on diurnal oviposition, however, the bait on the ground was more attractive for diurnal oviposition under high luminosity conditions. Regarding the nocturnal oviposition experiments, there was no documented oviposition during the night. At the same time, the authors remarked that blowflies activity would cease one to two hours after sunset (Berg & Benbow, 2013).

In addition, Barnes et al. (2015) developed their experiments in 2011 and 2013 in an urban environment, Central England. Liver was placed in a hanging trap at 1.61 meters from the ground. A wildlife camera with night vision was used to record blowfly activity around the trap. Temperature and humidity were recorded in the site with a data logger. The authors reported no consistent nocturnal oviposition nor blowflies activity during the night (Barnes et al., 2015).

As we can see, there is substantial variability in the results of the studies (Berg & Benbow, 2013), which have considered: (I) ambient temperature (some species are not active in low temperatures); (II) lighting (whether it is artificial or natural); (III) humidity levels (high humidity is conductive to oviposition); (IV) circadian rhythms of activity and sleep; (V) proximity and type of vegetation; (VI) bait height (Williams et al., 2017).

Ambient temperature is the most important factor affecting oviposition and larval development (Faucherre et al., 1999), being a critical parameter for post-mortem interval determination (Amendt et al., 2010). Since insects are poikilothermic, they resort to the ambient temperature as a source of warmth. Studies showed that temperatures lower than 12ºC inhibit blowfly activity and consecutively oviposition. For example, *Phormia regina* activity is inhibited by temperatures around 12.5ºC (Gennard, 2012). Temperatures during the night are lower than temperatures during the day and even in the summer, the temperature drops considerably. There are some Calliphoridae species that have a higher resistance to cold such as *Calliphora vicina* and *Calliphora vomitoria* (Linnaeus, 1758). *Calliphora* species in general have a minimum temperature for muscular activity much lower than *Chrysomya* species (Amendt et al., 2010).

Several studies have shown that a positive correlation exists between light intensity and flies activity. Investigators believe that artificial lighting is capable of neutralizing circadian rhythms and prolonging blowflies' activity. Moonlight is also an important aspect to observe during the experiments since it is a source of natural light - depending on the lunar phase (Williams et al., 2017). However, it is important to note that flies often oviposit in dark places during the daytime, so researchers speculate that is not the darkness associated with the nighttime that inhibits oviposition, but their diurnal rhythms (Byrd & Tomberlin, 2019).

Calliphoridae require minimum levels of humidity for oviposition and high levels of humidity are considered to be conductive to oviposition.

Circadian rhythms of activity and sleep in blowflies affect the probability of the occurrence of nocturnal oviposition during normal conditions (Williams et al., 2017). Circadian rhythms are described by investigators as "clock-like cycles in physiological processes" and they can affect processes like oviposition, larval growth, eclosion, and adult activity (Amendt et al., 2010). However, these rhythms can be disturbed by the weather, the presence of artificial lighting, and even human activity.

Some articles suggest that the presence of vegetation can be a resting place for flies, and it may be an important factor in the occurrence of nocturnal oviposition. This is deeply related to the bait height in the experiments since there is a possibility that blowflies crawl into the bait instead of flying (Williams et al., 2017).

To sum up, even when these factors are considered, there are still discrepancies between studies and no consensus between the authors. Therefore, the possibility of nocturnal oviposition should not be excluded when investigating a crime (Gennard, 2012).

Objectives

Barnes et. al (2015) consider that the geographical location is an important parameter for nocturnal oviposition and that Calliphoridae activity patterns are influenced by season and changes with geographic location (Gennard, 2012).

The particularities of each country, such as temperature, rainfall, humidity, and vegetation may result in different conclusions regarding nocturnal oviposition. Lutz et. al. (2022) state that there is a high correlation between seasons and abiotic factors. For that reason, it is extremely important to evaluate the possibility of this phenomenon in distinct geographical locations with different seasonalities.

This research aims to establish if nocturnal oviposition may occur in Portugal. No studies were performed so far in Southern Europe, being the results from England (Barnes et al., 2015) the ones geographically closest to our region.

Research questions were defined: (I) are blowflies active and able to lay eggs during the night or only during the day?; (II) in which conditions can nocturnal oviposition occur?; (III) which Calliphoridae species colonize during May until October in this specific geographic location?;

The following specific objectives were outlined:

- Verify the weather conditions (temperature, rainfall, wind speed, atmospheric/barometric pressure, and humidity levels) at the location of the experiment and associate the data with possible nocturnal and diurnal oviposition events;
- Understand if lighting (either artificial or natural) affects nocturnal oviposition;
- Observe and monitor Calliphoridae's behavior after the sunset and interpret their circadian rhythms;
- Rear the collected eggs for species identification;

PART II - METHODOLOGY, MATERIALS AND EXPERIMENTAL PROCEDURES

Materials and Methods

Localization description

Portugal is a Southern European country situated in the Iberian Peninsula. By the Köppen-Geiger classification, Portugal's climate is classified as Temperate Mediterranean, characterized by dry summers and rainy winters (Mora & Vieira, 2020). Furthermore, it was defined the following variations of the Cs sub-type: (I) Csa, hot and dry summers in the interior regions of Douro, as well as in the southern regions of Montejunto-Estrela (with the exception of the west coast of Alentejo and Algarve); (II) Csb, dry and mild summer, in almost all regions north of Montejunto-Estrela and in the west coast of Alentejo and Algarve (Instituto Português do Mar e da Atmosfera, n.d).

Portugal's rainy season lasts from October to March, while in the months of April to September the weather is considerably dry, with relatively no precipitation in July and August. Portugal's precipitation varies across the country, reflecting factors of latitude, continentality, and topography. Similarly, annual mean temperatures also vary across the country and its regions. This has to do with the Atlantic and Mediterranean influences of each region, as well as the typology - the northern and central regions are mountainous, while the south is characterized by plains and hills. Additionally, there are several mountains parallel to the coast which decreases the precipitation and increases thermal contrasts in the interior (Mora & Vieira, 2020).

The experiments were carried out during the warmer months, from July 2021 to August 2022 in Lisbon and Oporto, Portugal. Lisbon climate is classified as type Csa, while Oporto climate is classified as type Csb (Instituto Português do Mar e da Atmosfera, n.d).

In 2021, experiments were conducted in Lisbon, Portugal, an open field (Fig. 17) at Quinta do Conventinho (38.81930457649809, -9.167792869943836) near a residential area. The study site was in the shade and near low vegetation, at night, there was no direct artificial lighting other than street lights near the buildings. Even thought it was located nearby a neighbourhood, the location chosen was away from anthropological and animal activities.

In 2022, experiments were held in Lisbon, Portugal, in a residential building flat roof (Fig. 17), inserted on the same residential area (38.81986847942101, -9.167226918420987). The rooftop had no human intervention during the day nor the night, and due to it's height, there was no direct artificial lighting during the night.

Figure 17: Location of the Lisbon experiments. 2021 and 2022 respectively.

Additionally, in both 2021 and 2022 other experiments were held on a rooftop in an urban area (Fig.18) in Oporto, Portugal (41.18623333760805, -8.650453065442656). The roof is located on the third floor of a residential building and there is no artificial illumination during the night.

Figure 18: Location of the Oporto experiments. The bait was placed on the shade side during the diurnal experiments.

Experimental procedures

2021 experiments were executed during the months of July, August, September, and October, while 2022 experiments were held during the months of May, June, and July. Control experiments were carried out to verify diurnal oviposition and during the night, bait was exposed two hours after sunset then removed before sunrise. In both control and nocturnal tests, the experiments were replicated three times, in the same location and using the same type of bait.

In 2021, the dates of the experiment were chosen based on the lunar cycle: first quarter, full moon, last quarter and new moon. The baits used were fresh beef liver, acquired on the date of the experiment. After the exposure period, the bait was inspected for eggs and, when oviposition was positive, the eggs were collected using a plastic spoon and preserved in plastic containers for posterior rearing and identification.

In 2022, the experiments were not held on specific dates because of the limitations associated. Nonetheless, the lunar cycle was still taken into account and registered. The bait used was a mixture of fresh beef liver, acquired on the date of the experiment and fish viscera provided by a local fish market. This time, as a result of the previous year difficulty with larvae development that culminated in unsuccessful identification of the 2021 ovipositing species, similarly to Barnes et al. (2015), the type of trap was adapted with the intention of capturing the adults for identification. The traps were created following the Hwang and Turner (2005) method (Fig. 19) in which the trap was divided in two different compartments using water bottles –the bait was placed in the bottom, and the top chamber was dedicated for the blowfly collection (Fig. 20).

Figure 19: Visual diagram of the plastic bottle trap. Design adapted from Hwang & Turner (2005)

Figure 20: Trap used during the 2022 experiments. Model from 7th August 2022 - nocturnal experiments. Picture taken with Flash True Tone to better capture the trap.

In both 2021 and 2022, the data registered was the maximum and minimum temperature, sunset/sunrise time, rainfall, wind speed, and humidity levels. Such information was provided by IMPA (Instituto Português do Mar e da Atmosfera) at their weather forecast official website.

Identification: All collected blowflies were identified to the species level. For this assignment, a stereomicroscope and dichotomous keys (Szpila, 2012) corresponding to this Diptera family were used.

Data analysis

General Linear Mixed Model GLMM fitted by the Restricted Maximum Likelihood (REML) estimation method, was used to test the effects of the different environmental variables on diurnal oviposition (response variable). Fixed environmental variables were selected after running a previous selection model (GLM) using the ‘vif’ function from the “car” package (Lin et al., 2011) to check for collinearity among them. The “locality” was used as random factor in the GLMM model to discard the geographical influence on the response variable. Significance of the fixed effects was tested using the function “anova” performed on the response variable and the environmental factors. GLMM was performed using the R packages “nlme” version 3.1–128 (Pinheiro et al., 2016), implemented in the Statistical software R, version 1.4.2 (R Core Team 2009-2021).

PART III - RESULTS, DISCUSSION, CONCLUSION

Results

Table 1: A summary of the environmental conditions recorded during the experimental periods in 2021 and 2022 and registry of diurnal and nocturnal oviposition.

DATE	LOCATION	MOON PHASE	WEATHER	MAX TEMP (ºC)	MIN TEMP (ºC)	SUNRISE	SUNSET	HUMIDITY (%)	WIND (Km/h)	RAINFALL (mm)	PREASSURE (hPa)	DIURNAL OVIPOSITION	NOCTURNAL OVIPOSITION
24/07/2021	Lisbon	Full Moon	Sunny	26	16	06:31H	20:54H	52	21	0	1016	No	No
31/07/2021	Lisbon	Third Quarter	Partially Clouded	23	16	06:37H	20:48H	45	31	0	1020	No	No
08/08/2021	Lisbon	New Moon	Partially Clouded	25	16	06:44H	20:39H	?	46	0	1016	Yes	No
15/08/2021	Fradizela	First Quarter	Sunny	33	13	06:41H	20:23H	30	3	0	1018	No	No
22/08/2021	Fradizela	Full Moon	Partially Clouded	33	14	06:40H	20:25H	?	?	0	1019	No	No
30/08/2021	Lisbon	Third Quarter	Cloudy	27	19	07:03H	20:09H	?	40	0	1015	No	No
07/09/2021	Lisbon	New Moon	Partially Clouded	25	21	07:10H	19:57H	?	?	0	1016	No	No
29/09/2021	Oporto	Third Quarter	Sunny	22	11	07:30H	19:19H	73	18	0	1026	Yes	No
13/10/2021	Oporto	First Quarter	Sunny	27	14	07:45H	18:56H	24	18	0	1018	Yes	No
20/05/2022	Oporto	Waning Gibbous	Cloudy	25	11	06:11H	20:50H	90	6	0	1018	Yes	No
28/05/2022	Oporto	Waning Crescent	Sunny	28	16	06:05H	20:57H	45	11	0	1013	Yes	No
29/05/2022	Oporto	Waning Crescent	Cloudy	20	14	06:05H	20:58H	70	5	0	1009	No	No
05/07/2022	Lisbon	Waxing Crescent	Sunny	28	17	06:17H	21:04H	76	5	0	1018	Yes	No
06/07/2022	Lisbon	First Quarter	Sunny	29	17	06:17H	21:04H	55	14	0	1020	Yes	No
07/07/2022	Lisbon	First Quarter	Sunny	37	21	06:18H	21:03H	32	18	0	1019	Yes	No
14/07/2022	Lisbon	Full Moon	Sunny	32	18	06:23H	21:00H	47	10	6	1014	Yes	No
15/07/2022	Lisbon	Waning Gibbous	Sunny	33	19	06:24H	21:01H	45	10	0	1018	Yes	No
18/07/2022	Lisbon	Waning Gibbous	Sunny	30	18	06:26H	20:59H	80	6	0	1017	Yes	No
19/07/2022	Lisbon	Waning Gibbous	Sunny	26	17	06:25H	21:00H	75	8	0	1018	Yes	No
20/07/2022	Lisbon	Third Quarter	Sunny	28	18	06:26H	20:58H	70	15	0	1017	Yes	No
21/07/2022	Lisbon	Third Quarter	Sunny	29	18	06:28H	20:56H	74	21	0	1018	Yes	No
22/07/2022	Lisbon	Waning Crescent	Sunny	31	18	06:29H	20:57H	72	17	0	1019	Yes	No
23/07/2022	Lisbon	Waning Crescent	Sunny	33	17	06:31H	20:59H	70	19	0	1018	Yes	No
24/07/2022	Lisbon	Waning Crescent	Sunny	32	17	06:35H	20:58H	80	21	0	1019	Yes	No
02/08/2022	Lisbon	Waxing Crescent	Sunny	31	18	06:38H	20:45H	81	23	0	1014	Yes	No
03/08/2022	Lisbon	Waxing Crescent	Sunny	28	18	06:39H	20:46H	83	20	0	1014	Yes	No
04/08/2022	Lisbon	Waxing Crescent	Sunny	27	18	06:40H	20:44H	85	21	0	1014	Yes	No
05/08/2022	Lisbon	First Quarter	Sunny	28	18	06:41H	20:43H	86	10	0	1015	Yes	No
06/08/2022	Lisbon	Waxing Gibbous	Sunny	24	18	06:43H	20:42H	80	10	0	1017	Yes	No
07/08/2022	Lisbon	Waxing Gibbous	Sunny	25	17	06:41H	20:40H	79	8	0	1016	Yes	No
08/08/2022	Lisbon	Waxing Gibbous	Sunny	26	16	06:44H	20:39H	79	5	0	1015	Yes	No
09/08/2022	Lisbon	Waxing Gibbous	Sunny	29	17	06:45H	20:38H	50	18	0	1014	Yes	No
10/08/2022	Lisbon	Waxing Gibbous	Sunny	26	18	06:45H	20:42H	47	16	0	1014	Yes	No
11/08/2022	Lisbon	Waxing Gibbous	Sunny	26	17	06:47H	20:36H	48	15	0	1015	Yes	No
12/08/2022	Lisbon	Full Moon	Sunny	30	18	06:48H	20:35H	47	16	0	1014	Yes	No

Calliphoridae activity and oviposition was never observed during the nocturnal experiments. During the diurnal control experiments, there was 29 sampling days with positive oviposition and 7 with no oviposition. It did not occur on the following occasions: on the experiments held on 24th of July 2021 (with temperature ranging from 16ºC to 26ºC, mean humidity of 52%, wind speed of 21 km/h and 0mm of rainfall); 31st of July 2021 (temperature ranging from 16ºC to 23ºC, mean humidity of 45%, wind speed of 31 km/h and 0mm of rainfall); 15th of August 2021 (temperature ranging from 16ºC to 33ºC, mean humidity of 30%, wind speed of 3km/h and 0mm of rainfall); 22nd of August 2021 (temperature ranging from 14ºC to 33ºC and 0mm of rainfall); 30th of August 2021 (temperature ranging from 19ºC to 27ºC, wind speed of 40 km/h and 0mm of rainfall) and 7th of September 2021 (temperature ranging from 21ºC to 25ºC and 0mm of rainfall). In 2022 there was no oviposition verified on 29th of May (temperature ranging from 14ºC to 20ºC, mean humidity of 70%, wind speed of 5km/h and 0mm of rainfall). (Table 1)

The lowest temperature verified during the experiments was 11ºC (on both 29/09/2021 and 20/05/2022) and the higher temperature verified was 37ºC (on 07/07/2022).

The lowest relative humidity recorded was 24% (on 13/10/2021) and the highest was 90% (on 20/05/2022). In most of the experiment dates there was no rainfall and it was only verified a light rainfall (6mm) on 14th of July 2022. However, rainfall only occurred during the night and not during the diurnal experiments. Windspeed ranged 3-46km/h during the sampled periods.

Regarding the atmospheric (barometric) pressure, the lowest level documented was 1009 hPa (on 29/05/2022) and the highest level was 1026 hPa (on 29/09/2021) (Table 1).

Over the 2021 experiments, even though there was positive oviposition during the day, it was not possible to identify the ovipositing species. This was due to the fact that the larvae from the eggs reared died before reaching the second instar.

During the May 2022 diurnal experiments, the adults that were caught and posteriorly identified were *Lucilia caesar* (Linneaus, 1758) and *Calliphora vomitoria* (Linnaeus, 1758). In July and August 2022, the species captured were *Lucilia caesar*, *Calliphora vicina* Robineau-Desvoidy, 1830 and *Lucilia sericata* (Meigen, 1826) (Table 2).

Table 2: Summary of the species identification during the 2022 experiments.

SPECIES	LOCALITY	YEAR	MONTH	☀	☾
Lucilia caesar (Linneaus, 1758)	Oporto Lisbon	2022	May July August	✔	
Calliphora vomitoria (Linnaeus, 1758)	Oporto (Senhora da Hora)	2022	May	✔	
Calliphora vicina Robineau-Desvoidy, 1830	Lisbon (Quinta do Conventinho)	2022	July August	✔	
Lucilia sericata (Meigen, 1826)	Lisbon (Quinta do Conventinho)	2022	July August	✔	

Data analysis with GLMM using the locality as random factor showed that, among the selected variables, the effect of weather (cloudy vs. sun) was the only one significantly explaining diurnal oviposition (Table 3), with the sunny weather influencing positively the occurrence of oviposition in relation to the cloudy weather (t-value= 3.341, P= 0.003).

Table 3: Summary of GLMM analyses showing the fixed effects of selected environmental variables on diurnal oviposition. Statistically significant results are in bold with F-values presenting the numerator (nDF) and denominator (dDF) degrees of freedom. Random effects variables: Local.

Response variable	**Fixed effects**	**nDF, dDF**	**F**	**p value**
Diurnal oviposition	Weather (sun/cloudy)	1, 24	25.49	**<0.001**
	Maximum temperature (ºC)	1, 24	1.348	0.257
	Moon (fases)	5, 24	1.455	0.241
	Sunrise (time)	1, 24	0.899	0.353

Discussion

In the course of the 35 experiments conducted in the 2021 and 2022 sampling periods, no nocturnal oviposition was recorded. These experiments reinforce the observations reported in different studies, namely the ones held by Spencer (2002), Amendt et al. (2008) and Barnes et al. (2015) in Europe, where all field experiments so far never reported blowfly oviposition during the night.

The specimens collected during the diurnal experiments provide a characterization of the Calliphoridae community present in the experimental site and which could oviposit during the day and eventually also during the night. *Calliphora vicina, C. vomitoria, L. caesar* and *L. sericata* were captured from May to August 2022. While *C. vicina* and *L. sericata* are widespread species mainly associated with urban locations, *C. vomitoria* and *L. caesar* are more associated with rural and shaded areas, yet *L. caesar* can also be considered an intermediate species on Mediterranean climate regions with dispersed trees (MacLeod & Donnelly, 1956; Smith, 1986). Finding these group of species together might be explained due to the experiments being held in an urban area, but relatively isolated and near to an open field with a lot of vegetation and small trees. Prado e Castro et al. (2011, 2012) also collected these species in forested "islands" inside urban locations in Coimbra and Lisbon.

From this group of 4 species, there are no records of nocturnal oviposition for *L. caesar* and *C. vomitoria*. Studies held by Matuszewski (2013), reported *L. caesar* activity from 14ºC and oviposition was always recorded in the first 12h of the experiments when temperatures were above 17 °C.

Calliphora vomitoria is capable of tolerating lower temperatures, above 3ºC (Niederegger et al., 2010). However, in previous research, activity of this species was only recorded during diurnal experiments (Zurawski et. al, 2009; Stamper et al., 2009; Barnes et al., 2015). Furthermore, Wooldridge et al. (2007) concluded that both *C. vomitoria* and *L. sericata* decreased the flight activity when the luminosity levels were lower. Hence, the authors concluded that – nocturnal oviposition could occur in situations when the full moon or artificial lighting was able to provide adequate illumination.

Lucilia sericata has preference for high temperatures (Gennard, 2012), even though its activity is only inhibited from temperatures below 9ºC (Niederegger et al., 2010). Moreover, research has found that oviposition was always recorded in the first 12h of the experiments when temperatures were above 18 °C (Matuszewski et al., 2013). Greenberg (1990) found that this species was able to oviposit during the night under temperatures of 23.5ºC and 24ºC and under mean humidity of 40%, 100% and 87%, however, the location was illuminated by street lights. Amendt (2007) was able to record nocturnal oviposition in laboratory trials using a sample of *L. sericata* females in temperatures of 25ºC. However, Zurawski et al. (2009) research reported that this species was not capable of flying in total darkness. Stamper et al. (2009) reported no nocturnal oviposition as well. Yet, in George et al. (2013) experiments, nocturnal oviposition of *L. sericata* under low levels of light was documented – but never under complete darkness – and in temperatures above 20ºC. Furthermore, during Williams et.al (2017) experiments, *L. sericata* nocturnal oviposition was recorded in field experiments under mean temperatures of 19.7ºC and mean humidity of 85.6% and laboratory experiments with an established temperature of 20ºC.

Concerning *Calliphora vicina,* comparatively to *Lucilia* species, it is capable of tolerating lower temperatures. Research has found that *C. vicina* activity was documented in temperatures from 2º C (Niederegger et al., 2010). In Finland, there were reports of the species until November (Gennard, 2012). As such, for some authors it is anticipated that nocturnal oviposition occurs due to its cold-adapted nature. However, again, previous research was not conclusive. On Greenberg's (1990) experiments, *C. vicina* oviposited during the night under conditions of 70% relative humidity and temperature of 20ºC. It was also registered low levels of light. Faucherre et al. (1999) investigation of the behavior of this species under extreme conditions has found that, even though there is activity in temperatures from 2ºC, oviposition stops in temperatures below 8ºC. Furthermore, in Singh and Bharti's (2001) research, *C. vicina* nocturnal oviposition occurred in temperatures between 16ºC to 23ºC and relative humidity of 75% as well as low levels of light. Nonetheless, during Zurawski et al. (2009) research, there was only diurnal activity recorded in temperatures of 28ºC. George et al. (2013) had a similar result, as *Calliphora vicina* only oviposited during the diurnal experiments and under temperatures of 18.1Cº and 23.9ºC, 46.8% and 41.9% of relative humidity, low windspeed and atmospheric (barometric) pressure of 1016.9 and 1014.2. Barnes (2015) also did not record any nocturnal oviposition nor nocturnal activity of this species.

The data analysis performed found that, during the experiments held, the effect of weather (cloudy vs. sunny) was the only variable significantly explanatory regarding diurnal oviposition, with the sunny weather influencing positively the occurrence of oviposition in relation to the cloudy weather. This information corroborates with Smith (1986) findings that, in general, blowflies prefer sunlight. Payne (1965) research established that heavy cloud cover was a condition that lowered blowfly activity. Furthermore, Mohr & Tomberlin (2014) experiments sustained such findings as the presence of heavy cloud cover reduced significantly Calliphoridae activity. During Ament (2008) and Stamper (2013) experiments regarding nocturnal oviposition, cloud cover was also analyzed as a condition that affected natural lighting both during the day and the night, however, no relevant findings were noted during their research. Additionally, Scala & Wallace (2009) found that temperature is affected by cloud cover and it might explain the decrease of activity. Barnes et al. (2015) followed the same linear model, and the results obtained during their research indicated that temperature was the only significant variable affecting both diurnal activity and oviposition.

Wooldridge et al. (2007) stated that nocturnal blowfly activity was often related to ambient temperature. In general, temperatures lower than 12ºC inhibit Calliphoridae activity and consecutively oviposition (Amendt et al., 2011), however, minimum temperatures were considerably higher in most of our experimental nights. Only during the 29^{th} of September 2021 and 20^{th} of May 2022 experiments that the minimum temperature was lower than 12ºC (11ºC). Furthermore, *C. vicina* is able to oviposit in temperatures lower than 8ºC (Faucherre, 1999). The minimum temperature range reported was 11ºC to 21ºC – yet, no nocturnal oviposition was registered. Even though the temperatures were viable for nocturnal oviposition, to *C. vicina* and *L. sericata*, both species in which this phenomenon has been previously described under field experiments in Chicago (Greenberg, 1990) – United States of America, Punjab – India (Singh & Barthi, 2001), Durban - South Africa (Williams et al., 2017), it never occurred in Portugal. Hence, this suggests that not only temperature, but other factors influence nocturnal oviposition.

Regarding the humidity levels, in 2021 values recorded in the experimental period were lower than the levels recorded during 2022, which, overall, were more successful than the experiments held in the previous year. Studies have demonstrated that high humidity is conductive to oviposition. The minimum humidity recorded was 24% on 13^{th} of October 2021, however, diurnal oviposition still occurred. Barnes (2015) also reported diurnal oviposition under the mean humidity of 26.7%. *Calliphora vicina* oviposited under humidity levels of 41.9% and 46.8% (George et al., 2013) and *L. sericata* oviposited under mean humidity of 40% (Greenberg, 1990). Nonetheless, there was also oviposition in conditions of relative humidity levels above 70% (Greenberg, 1990; Williams et al., 2017; Singh & Bharti, 2002) and even 100% (Greenberg, 1990). By itself, we cannot consider that the humidity alone is able to influence nocturnal oviposition due to the broad range of different results.

Digby (1958) performed an investigation related to the flight activity of the blowflies in relation to the wind speed. The author suggested that wind speed above 25.2 km/h inhibits the ability of *Calliphora vicina* to fly. Yet, George et al. (2013) states that the lack of wind, can as well have an inhibiting effect. As Lutz et al. (2022) refers, the wind is able to provide orientation information for blowflies. The data recorded during this study complies with Digby (1958) remarks as, in 2022 experiments, wind speed was always below 22km/h. On 8^{th} of August 2021, there was oviposition recorded during the experiments even though the wind speed was 46km/h. However, this can be explained with the fact that blowflies could have crawled from the ground into the bait due to the proximity of vegetation.

During the experiments, atmospheric (barometric) pressure had relatively standard values (1013.25hPa) with light fluctuations. Wellington (1946) investigation suggests that Calliphoridae's antennal aristae is sensitive to variations in pressure due to the baroreceptors. Lutz et al. (2022) refer that changes in pressure are related to the weather conditions – hence, an increase in atmospheric (barometric) pressure anticipates favorable conditions to blowfly activity. Still, there is no clear and extensive investigation regarding the effects of atmospheric (barometric) pressure in oviposition (Lutz et al., 2022).

A parameter that could explain the absence of nocturnal oviposition is related to Calliphoridae's circadian rhythms of activity and sleep (Amendt et al., 2008). Literature states that circadian rhythms arise from "endogenours, temperature-compensated genetic oscillator that is naturally entrained by light and temperature cycles each day". These circadian rhythms affect all the process of a blowfly life cycle – especially the oviposition. Urban areas maintain human activity during the night, however, on the location of the experiments, and due to the fact of being a relatively small and isolated neighborhood, human activity during the night time was especially low. Perhaps, if we considered a busier neighborhood – with nightclubs, bars, and night entertainment - Calliphoridae rhythms of activity and sleep could be easily more disturbed. However, circadian rhythms can be reestablished by exposure to light (artificial), heat (Amendt et al, 2010); by the weather and human activity (Williams et al., 2017). The presence of low levels of artificial lighting from the experimental site also did not provide any results in the nocturnal oviposition. As such, and during the course of the present study, there was no condition that disrupted the normal circadian rhythms – hence, no nocturnal activity was recorded.

Associated limitations and future investigation

In the process of conducting this research there were several circumstances recognized as limitations.

Due to the nature of the study and the results we were trying to obtain, the weather conditions were crucial to accomplish the most successful results. For that reason, the experiment dates were, in general, carefully chosen to have the highest chance of success – hence, the studies were held during the warmest months and rainy days were avoided. At the same time, limiting the study to a certain season and conditions narrowed the possible sampling days and excluded completely almost half of the year. For instance, during the 2022 experiments, there were no experiments during June due to the rainfall experienced as Greenberg (1990) stated that calliphorids do not fly in the rain.

Also noted by Lutz et al. (2022), additional constraints to this study were the fact that, instead of on-site measurements, the data was obtained from nearby weather stations. Therefore, small discrepancies could arise between the reports and the real conditions of the site.

Other limitation was due to the rearing and posterior identification of the hatched blowflies. During the 2021 experiments, one of the most difficult challenges faced was regarding rearing the eggs until the adult stage. The hatching phase occurred without any issue after one to three days depending on the temperature in the following days of the experiment. However, the larvae died before reaching the second instar and made it impossible to identify the correspondent species. Due to this situation, following Barnes et.al (2015) method, the 2022 experiments focused on catching adult flies to posterior identification, however, it is important to note that this method had its constraints since it is difficult to access if every species caught would in fact oviposit on the bait.

Furthermore, there was the issue with some omnivorous species that end up colonizing the bait. In 2021 there was a colony of ants (Formicidae) that efficiently took over the liver that was being used for the experiments – and as a result, end up disturbing the Calliphoridae activity.

As well, there were different occasions when wasps (Vespidae) would appear in the middle of the experiments, disrupting the process and possibly feeding in any laid eggs.

At last, and as previously suggested by Berg and Benbow (2013), the bait used in the experiments is not able to replicate accurately what would happen in a forensic investigation – a human dead body can be more attractive to necrophagous species than a few grams of a mixture of liver and fish entrails. Subsequently, in my point of view, future investigation should take into account the type of bait used during the experiments and attempt to use a carcass comparable to humans – such as, adult pig remains.

Future investigation regarding nocturnal oviposition in Southern Europe should also take into account other variables – besides environmental - for example the presence of blood and drugs in the crime scene, the number of victims, presence and type of wounds and the cause of death. In such case, and as Spencer (2002) suggested, investigators could replicate the conditions of real forensic cases, in order to provide more accurate information regarding this subject.

Furthermore, it is also suggested to perform separate experiments in controlled circumstances, similarly to the studies held by Williams et. al. (2017) in order to provide a more certain range in which conditions oviposition occurs. However, we also have to keep in mind that even though laboratory experiments are able to provide accurate limits for each variable, in a field experiment and consequently in a forensic investigation all other variables are taken into account at the same time, and such conditions are not easily replicated on a lab.

To sum up, is understood that future experiments regarding nocturnal oviposition should take into account a myriad of other variables besides abiotic factors and explore different possibilities that were not studied before. This study can be perceived as an initial investigation for a more extensive analysis of Calliphoridae nocturnal oviposition in Southern Europe.

Conclusion

In order to respond to the questions outlined, field experiments were executed during 2021 and 2022. The following conclusions were defined:

Are blowflies active and able to lay eggs during the night or only during the day? (I) In Portugal, and during the 35 experiments performed, only diurnal oviposition was verified.

In which conditions can nocturnal oviposition occur? (II) Bibliographic analysis of previous research indicates that oviposition was possible under certain conditions. Under natural conditions, Greenberg (1990) recorded nocturnal oviposition under temperatures from 17ºC to 24ºC and humidity above 40%. Singh and Barthi (2001) found that nocturnal oviposition occurred on artificial lighting sites, under high humidity levels and temperatures above 16ºC. Kirkpatrick and Olson (2007) found that nocturnal oviposition occurred under artificial lighting and temperatures above 26ºC. At last, Williams et al. (2017) found that oviposition occurred under temperatures above 19.7ºC and high humidity levels. Still, although conditions were suitable for nocturnal oviposition, no positive results were found during the experiments.

Which Calliphoridae species colonize during May until October in this specific geographic location? (III) The species that were identified as a result of the control experiments held in 2022, were *Lucilia caesar*, *Calliphora vomitoria, Calliphora vicina* and *Lucilia sericata.*

In this study, the influence of the environmental factors that could possibly affect the occurrence of diurnal oviposition was tested. Results demonstrated that the weather (cloudy vs. sunny) was the only variable significantly explaining diurnal oviposition, with the sunny weather influencing positively the occurrence of oviposition in relation to the cloudy weather.

In conclusion, this study suggests that nocturnal oviposition is unlikely to occur in Portugal. These experiments reinforce the observations reported in the previous studies performed in Europe. However, as previously referred, more extensive investigation regarding this process is advisable, including experiments in different conditions and regions from the country to be able to reach a broader consensus.

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