UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

SEASONAL INFLUENCE IN THE SUCCESSION OF ENTOMOLOGICAL FAUNA ON CARRIONS OF *CANIS FAMILIARIS* IN LISBON, PORTUGAL

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Abstract

"Seasonal influence in the succession of entomological fauna on carrions of *Canis familiaris* in Lisbon, Portugal"

In the last years, there has been a significant increment on the study of sarcosaprophagous insects and other arthropods as an important tool in the resolution of crime processes. This branch of forensics is known as forensic entomology, and one of its many applications is to provide estimates of the time elapsed since death by taking into consideration the entomofauna present on a decomposing carrion. Although relatively well documented in human forensics, there is still a lot of room for research and improvement in the veterinary field.

The present study represents the first research on the insect succession on carrions of dogs (*Canis familiaris*) performed in Portugal, enabling the acquisition of information concerning its entomological succession and the documentation of forensically relevant Diptera and Coleoptera species occurring in the region of Lisbon, Portugal. This study also aimed to investigate differences regarding both quantity and quality of the entomofauna occurring along the decomposition process and between different seasons. The entomological material was collected in Lisbon (Portugal) during the winter and summer seasons by using a modified version of the "Malaise" trap associated with a "Pitfall". Identification and analysis focused mainly on the Diptera and Coleoptera orders. As anticipated, climatic factors had a major influence in the carrions' decomposition rate and entomological succession. The quantity of specimens collected differed greatly between seasons, with twice the amount of Diptera and four times more Coleoptera in summer. As for quality, both seasons had Calliphoridae as the predominant family of the Diptera order, with *Calliphora vicina, C. vomitoria* and *Pollenia* sp. predominating in winter, and *Chrysomya albiceps* in summer. The species *Pollenia vagabunda* was recorded for the first time in Portugal. Regarding the Coleoptera order, Staphylinidae was the most prevalent family in both seasons, while Dermestidae and Histeridae were mostly present in summer.

Key-Words: Forensic; Insect succession; Diptera; Coleoptera; Dog; Lisbon; Portugal

Resumo

"Influência sazonal na sucessão da fauna entomológica em cadáveres de *Canis familiaris* em Lisboa, Portugal"

Nos últimos anos tem-se vindo a assistir a um aumento no interesse pelo estudo de artrópodes associados a cadáveres como ferramenta coadjuvante na resolução de processos criminais. Este ramo das ciências forenses é designado por entomologia forense e permite, entre inúmeras aplicações, a realização de estimativas do intervalo pós-morte tendo como base a entomofauna presente no cadáver. Embora já relativamente bem documentada no Homem, existe ainda muito espaço para investigação e desenvolvimento desta área no sector veterinário.

O presente estudo representa a primeira investigação relativa à sucessão de insectos em cadáveres de cão (*Canis familiaris*) efectuada em Portugal, possibilitando a aquisição de informação relativa à sua fauna entomológica, bem como a documentação taxonómica de dípteros e coleópteros de interesse forense presentes na região de Lisboa, Portugal. Este estudo teve ainda como objectivo a investigação de diferenças tanto na quantidade como qualidade da entomofauna presente ao longo do processo de decomposição e entre diferentes estações do ano. Todo o material entomológico foi recolhido em Lisboa ao longo das estações de Inverno e Verão, recorrendo a armadilhas do tipo "Malaise" e "Pitfall". A identificação e análise dos espécimes focou-se principalmente nas ordens Diptera e Coleoptera.

Tal como esperado, os factores climáticos exerceram uma grande influência no processo de decomposição e sucessão entomológica nos cadáveres. A quantidade de espécimes diferiu grandemente entre estações, com duas vezes mais dípteros e quatro vezes mais coleópteros no Verão. A família Calliphoridae, da ordem Diptera, foi a mais prevalente em ambas as estações, com as espécies *Calliphora vicina, C. vomitoria* e *Pollenia* sp. predominando no Inverno, e *Chrysomya albiceps* no Verão. Uma das espécies identificadas, *Pollenia vagabunda,* foi relatada pela primeira vez em Portugal. Em relação à ordem Coleoptera, a família Staphylinidae foi a mais prevalente em ambas as estações, enquanto as Dermestidae e Histeridae ocorreram principalmente no Verão.

Palavras-chave: Forense; Sucessão entomológica; Diptera; Coleoptera; Cão; Lisboa; Portugal

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Abbreviations

DW ..... Dog placed in Winter
DS .....Dog placed in Summer
IPMA.....Instituto Português do Mar e da Atmosfera (Portuguese Institute for Sea and Atmosphere)
PMI ..... Post Mortem Interval

I. Literature Review

1. Forensic entomology

As suggested by the name, forensic entomology consists in the investigation of insects and other arthropods recovered from crime scenes in order to aid legal investigations – it is present whenever there is an interaction between insects and the law (Sharma & Singh, 2015). Although contained in the broad field that is forensics, forensic entomology is too a large field by itself, with numerous applications and areas of interest. There are currently three main accepted areas of interest: urban, stored products and medicocriminal. Urban entomology focuses on problems concerning pest insects in the human environment, generally in man-made structures. Examples include damage in buildings by termite infestation, or the breeding of flies in livestock (Byrd & Castner, 2010). Stored products entomology concerns for the presence of arthropods or arthropod traces in stored edible commodities, causing both quantitative and qualitative losses to the owner (Caneparo, Corrêa, Mise & Almeida, 2012). As some cases may result in prosecution, special attention must be taken as sometimes false evidence may be "planted" on an attempt to defraud a business (Byrd & Castner, 2010). Medicocriminal entomology, the context of this thesis, is related with the use of arthropod evidence in solving crimes such as physical abuse or murder. As ubiquitous beings, arthropods are found in a widespread range of locations, including crime scenes. This particularity opens way to a great variety of applications, specifically in the investigation of arthropods recovered from a carrion and its surroundings (Benecke, 2008). It's primary and most known application is to provide Post Mortem Interval (PMI) estimations, i.e. the time elapsed since death. Other less obvious situations include determining whether or not the body has been moved, detection of abuse and neglect by infestation of tissues with fly larvae (myiasis) in hospital patients, nursing homes and pets (Anderson & Huitson, 2004), among many others.

2. History of forensic entomology

Tracing back in the history of human society, the first evidence of some interest in forensic science dates back to as early as 4000-3000 BC, when there were already written records related with legal-medicine problems (Biswas, 2015).

Nevertheless, it was not until the 13th Century that the first known treatise in forensics was written, entitled "His Yüan Lu" or, as translated by McKnight (1981), "*The Washing away of Wrongs"*. The book, written by criminalist Song Ci, intended to guide Chinese bureaucrats in the investigation of questionable deaths, and contains a series of descriptions of post mortem examinations, forensic cases experienced by the author and, most notably, the first reported historical case of the use of forensic entomology. The case goes as follows: a murder in a village near a rice field was reported. The coroner in charge of the investigation determined that the wounds had been inflicted by a sharp object, possibly a sickle. He thus required every villager to lay their sickles down on the ground before him. After a short while, numerous flies were attracted to a single sickle among seventy others, conceivably allured by invisible traces of blood. Daunted with such an indubitable sight, the owner of the sickle confessed his crime (McKnight, 1981; Benecke, 2008).

Around the Middle Ages, there was a strong artistic interest in the decomposition of corpses. Numerous detailed illustrations of human bodies colonized by maggots were found from that time, some even emphasizing the effects of the feeding maggots in decomposition (Benecke, 2008).

In the 17th century, Redi performed the so called "Blowfly experiment", proving for the first time that maggots in decomposing meat were a result of oviposition by adult flies. This was an important breakthrough in decomposition studies, finally disproving the idea of spontaneous generation of maggots from meat (Sharma & Singh, 2015).

Around the 18th and 19th centuries, mass exhumations in France and Germany allowed the observation that buried bodies were colonized by many kinds of arthropods and that maggots played an important role in the decomposition process (Benecke, 2008). However, it was only by the mid-1800 that the first use of forensic entomology in the West was recorded, credited to the French physician Bergeret. In this case, the mummified remains of an infant had been found inside a house. Based on the insect fauna found on the remains, Bergeret concluded (although with some alleged inaccuracies) that the child had been dead for roughly 2 years, hence absolving the suspects, which had been

residing the house for a shorter period of time (Byrd & Castner, 2010). Around the same years, the French veterinarian Mégnin popularized the subject of forensic entomology with his work "La faune des cadavres", developing a theory on which the insect fauna colonized corpses in a predictable series of eight waves, whose study would enable PMI estimations. Later inspired by Mégnin, a number of systematic entomological studies started being performed on human corpses (Benecke, 2008). Though revolutionary in the history of forensic entomology, climatic discrepancies around the globe do not allow the integral application of the entomological succession pattern as described by Mégnin, both due to variations in the decomposition rate, and differences on the insect species found (Oliveira-Costa, 2013a). It is nevertheless considered to be a significant development in forensic entomology, paving the way for further studies on entomological succession.

On the first half of the 20th century, lists of forensically important species were published. In addition, there was a growing interest on the fields of pest control and maggot therapy which, while not directly related to forensics, supported the establishment of a foundation for interpretation of insect evidence. On the second half of the century, Leclerq, considered one of the most noteworthy pioneers in the application of entomology to forensic sciences, provided a major contribute to the diffusion of this science in Europe with his many publications on the subject (Benecke, 2008).

The current advancements in the field of molecular biology have enabled a whole new array of possibilities, with prospects of great improvements in the accuracy of conclusions derived from the collected specimens, as it supports the most crucial stage in forensic entomology: specimen's identification.

3. Current situation in Portugal

Until the recent years there was a lack of entomological data regarding the main species of forensic relevance in Portugal (Cainé et al, 2009). Only recently has there been an actual endeavor in databasing the entomological fauna, specifically the fauna associated with carrions and their morphological and molecular features (Rebelo, Meireles, Moreira & Pereira da Fonseca, 2014). Since then, more studies have been performed, most focused in the central region of Lisbon (Marques, 2008; Prado e Castro, 2011; Farinha et al, 2014), but also in other parts of the country like Serra da Estrela (Gusmão, 2008) and Aveiro (Lopes, 2012). Not rarely, new species until then not described in Portugal are found, a further indication that there is still a lot of room for research in this area.

Regarding veterinary forensics in particular, research is also bound to improve, with an increased concern on animal welfare being observed all around the globe in the latest years. Specifically, in Portugal, a number of laws protecting companion animals from abandonment and mistreatment were approved (e.g. Lei nº 69/2014, 29 de Agosto), inevitably directing people to an increased awareness in all possible legal resources that may assist in acquiring evidence in crime situations. There is, for this reason, a growing interest and involvement of scientific communities on this matter.

4. Decomposition process

A decomposing carrion is a complex dynamic ecosystem. Defined by its intrinsic autolytic and putrefactive processes and the interrelationships with its environment, it acts as a specialized habitat for several organisms (Carter, Yellowlees & Tibbett, 2007). The decaying process may vary from environment to environment, from animal to animal (Stokes, Forbes & Tibbett, 2013), and even from one part of the carrion to another.

4.1 Factors that influence decomposition

Numerous factors may influence the decomposition rate, be it intrinsic or extrinsic.

Regarding the intrinsic factors, it has been observed that obese and diabetic individuals will decompose faster, as well as if sepsis was present before death. Conversely, decay will proceed slower in infants and thin individuals (Spitz & Spitz, 2006). Carrion integrity, e.g. presence of wounds, is another important factor that accelerates decomposition.

The major extrinsic factor affecting decay is temperature. In general, decaying processes are accelerated by higher temperatures, both due to the faster biochemical processes involved in putrefaction, and to the increased insect development rate. Additionally, bodies located in direct sunlight will heat up more rapidly, and thus decompose faster (Byrd & Castner, 2010).

The next most important extrinsic factor is insect colonization, whose contribution lies with the acceleration of tissue breakdown and by facilitating the propagation of bacteria. It is also related with temperature in the sense that the presence of larval masses generate heat, both with their normal metabolism and friction movements. This supplementary increment on the carrion's internal temperature further accelerates bacteria metabolism, hastening the decomposition process even more (Sharanowski, 2004).

Other relevant factors include humidity (decomposition bacteria and fly larvae require moisture to survive, otherwise dehydration and mummification of the soft tissues occur), soil type and scavenging by vertebrate predators, which accelerate decomposition by consuming tissues and disarticulating the remains (Galloway, 1997).

4.2 Stages of decomposition

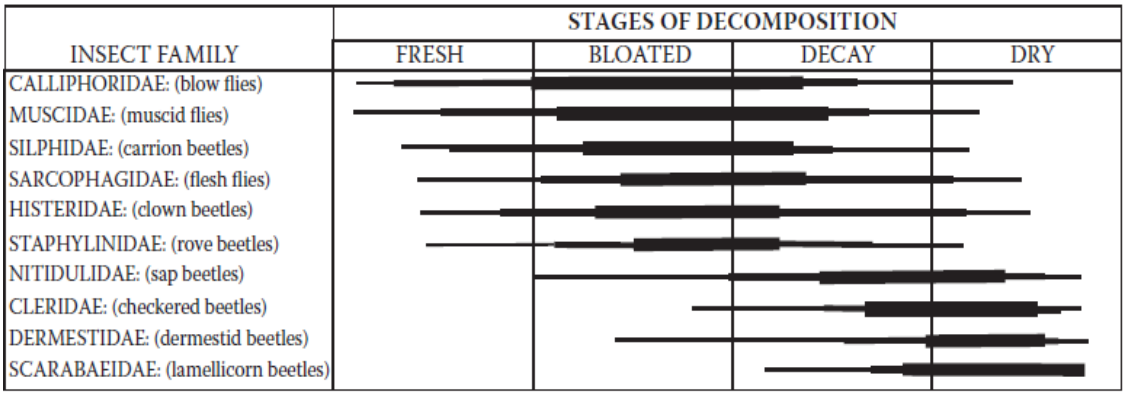
Although a continuous process, for convenience in discussion and by taking into consideration observable physical variations, it is common to split the decomposition process in distinct stages (Goff, 2009). The length of each stage is variable, and is dependent on intrinsic and extrinsic factors that affect decomposition rate (as described above). Distinct stages attract different arthropod species by providing a particular habitat for feeding and oviposition, resulting in a predictable entomological succession (Benecke, 2004).

Numerous divisions have been proposed by various authors. For the context of this thesis, the following five stages are described: Fresh, Bloated, Active decay, Advanced decay, and Dry/Remains.

- Fresh: Initiates at the time of death, i.e. cessation of heartbeat and depletion of internal oxygen, and ends when inflation of the carrion becomes evident (Goff, 2009). The usual duration of this stage is one to three days, but can be stretched in time to some extent depending on the conditions surrounding the carrion. Throughout this stage, early post mortem changes occur, e.g. rigor mortis (stiffening of the muscles), algor mortis (cooling of the body), among others. As the cells are deprived of oxygen, their structural integrity begins to deteriorate, leading to the release of enzymes that will degrade the body's tissues in a process known as autolysis (Carter et al, 2007). Simultaneously, putrefaction processes begin, with bacteria digesting the internal tissues and generating gases that provide odor cues (typically not noticeable to humans) which attract the first insects. Fly attraction occurring as soon as a few minutes after death has been reported. Under most circumstances, the first insect families to arrive are Calliphoridae, followed by Sarcophagidae (Byrd & Castner, 2010). Due to flies being very sensitive to humidity levels, there is an earlier occurrence of oviposition in the presence of bodily excretions or blood. For this reason, oviposition generally starts on natural body openings, namely the eyes, nose, mouth, ears, anus, genitals and eventual wounds present on the carrion (Goff, 2009). Moreover, later in this stage the laid eggs begin to hatch into first instar larvae, which start feeding on the soft tissues and fluids of the carrion. As primary agents in the

consumption of dead tissues, larvae are a fundamental piece on the progress of a carrion's breakdown (Carter et al, 2007). In addition to flies, some adult individuals of the Coleoptera order may begin to appear (Table 1).

Table 1 – Distribution of the most relevant families occurring on each decomposition stage, as observed on human remains in spring/summer, in Tennessee. Adapted from Rodriguez and Bass by Byrd & Castner (2010).



* Each stage of decomposition is given the same amount of space in this table.

Indicates a small number of individuals present.
Indicates a moderate number of individuals present.
Indicates a large number of individuals present.

- Bloated: Oxygen depletion initiated on the fresh stage produces an ideal environment for anaerobic microorganisms, particularly bacteria originated from the gastrointestinal tract (e.g. *Clostridium* sp.). In the putrefactive processes, anaerobic bacteria digest tissues and produce, among others, byproduct gases such as hydrogen sulfide, methane, cadaverine and putrescine, which are known to cause a strong decay odor. Accumulation of these gases lead to an increased internal pressure, which causes inflation of first the abdomen, and eventually the whole body. The increased pressure forces fluids out of cells and blood vessels, into the body cavity, eventually seeping out through the natural openings. Being rich in ammonia, the fluids alter the pH of the soil beneath the carrion, resulting in invasions by new sets of organisms, along with a shift in the soil fauna (Carter, et al, 2007). The seeping of fluids and increased odor strongly attracts adult Calliphoridae, Sarcophagidae and Muscidae whose numbers usually peak at this stage (Byrd & Castner, 2010), as observable in the study summarized in Table 1. Though maggot masses are visible on and near natural openings or wounds, large populations that cannot be seen are also present internally, benefiting from communal heat and shared digestive secretions. In some cases, larval movements are observable through the layers of skin. The carrion's internal temperature increases, both due to the metabolic processes taking place and the formerly mentioned maggot activity, with reports of temperatures reaching more than

50ºC (Goff, 2009). Concerning Coleoptera, Histeridae and Staphylinidae families increase in numbers (Table 1), along with larvae (Rebelo et al, 2014). The bloated stage ends when there is skin rupturing and the carrion starts to deflate.

- Active Decay: As the skin is breached by the combined effort of bacterial putrefactive processes, gas pressure and feeding by larvae, trapped gases and fluids escape and the body starts to deflate (Goff, 2009). As such, a very strong odor of decay is usually present and the carrion acquires a wet appearance (Sharanowski, 2004). Maggot activity is at its peak, with large masses present externally, internally and even spilling to the soil. This results on a peak of the carrion's internal temperature, along with a major loss of soft tissues due to maggot feeding. In addition to the flies present, Coleoptera continue to increase in numbers, specifically predators that feed on other insects such as Staphylinidae and Histeridae (Goff, 2009).

- Advanced Decay: The beginning of this stage is marked by a mass migration of fly larvae in order to pupate (Carter et al, 2007). Due to the decrease in maggot activity inside the carcass, there is also a decrease in the internal body temperature and the rate at which body mass is lost. Odor is substantially decreased (Sharanowski, 2004). With the exception of wet habitats (e.g. swamps and rainforests), Diptera are no longer the predominant arthropods, being mostly replaced by Coleoptera, represented by the Dermestidae family (Goff, 2009). By the end of this stage, both fly larvae and coleopterans will have removed most of the flesh, leaving only skin, cartilage and bone.

- Dry/Remains: The carcass consists mostly of dried skin, cartilage and bones. Little to no odor is detected (Sharanowsky, 2004). Coleoptera remains the predominant order, specifically families that feed on the dry remains (Dermestidae) (Byrd & Castner, 2010). As time passes, there is a gradual return of the soil's composition to normal. In the long term, most of the taxa will gradually disappear, leaving mites and Collembola as the main PMI indicators. There is no definite end point to this stage (Goff, 2009).

5. Medicolegal entomology applications

The investigation of arthropods, whether on a carrion and its surroundings, or related to a crime scene can assist in providing specific information on various topics, including:

- Time of death;
- Crime location;
- Presence of abuse or neglect;
- Detection of illicit substances (entomotoxicology);
- Identification of human remains (molecular analysis).

5.1 Post Mortem Interval (PMI)

Upon death, a carrion undergoes a series of changes brought about by endogenous putrefactive reactions, predation by diverse types of animals (of various sizes, ranging from ants to foxes), and bacterial and fungal attack, culminating in the return of nutrients to the food chain (Saukko & Knight, 2004). These changes are of particular interest when predicting the time elapsed after death. Pathological findings are often a main source of data for PMI estimations, with one of the most reliable factor at the very early stages of death being body temperature variations (Payne-James, Jones, Karch & Manlove, 2011). However, some of these changes (e.g. *livor mortis*) are usually not immediately perceivable in animals with a dense hair coating such as dogs, as opposed to animals with sparse hair such as pigs and humans. To some extent, this feature hinders the observation of some pathological changes in companion animals, further leading to the need of resourcing to different methods. In addition, pathological data is generally most useful at early stages of death (Abdulazeez & Noordin, 2010), specifically on the first two to three days. Forensic entomology thus proves to be of great use in the context of PMI estimation, since it manages to provide relatively accurate predictions from the first to several weeks after death (Hall & Amendt, 2007). These estimations can either be based on a generally predictable succession of arthropod species, or on the determination of the maturation stage of an insect found in, on, or surrounding the carrion (Saukko & Knight, 2004).

The first method consists in analysing the entomological succession colonizing a carrion at a given time, and rests on the principle that different arthropod species will be attracted to distinct decomposition stages. As soon as a few minutes after death, numerous sarcosaprophagous arthropods begin to colonize a carrion. Calliphoridae (Diptera) are usually the first family, being attracted over great distances by the carrion's odor. Braack

& Retief (1986) caught radioactively labeled flies of the genus *Chrysomya* (Diptera: Calliphoridae), on traps up to 63,5 km away from the release point. The time of the day may also influence when oviposition takes place. Since blowflies are heliotropic, unless under artificial light they do not typically lay eggs at night. As decomposition progresses, the odor emanated from a carrion will also change, becoming more or less attractive to other species (Byrd & Castner, 2010). The state of the carrion is thus a deciding factor on the attraction of each species, e.g. Calliphoridae have a preference for fresh tissues, whereas Dermestidae beetles (Coleoptera) favor the more advanced stages, feeding mainly on hair and skin.

According to Smith (1986), arthropods colonizing a carrion can be divided into four ecological groups:

- Necrophagous, which feed on the carrion itself, e.g. Diptera: Calliphoridae and Sarcophagidae; Coleoptera: Dermestidae and Scarabaeidae;
- Necrophile, which predate or parasite the necrophagous species, e.g. Diptera: Genus *Chrysomya* (Calliphoridae) and *Hydrotaea* (Muscidae). Coleoptera: Staphylinidae and Histeridae;
- Omnivorous, which feed on the carrion and its inhabitants, e.g. Hymenoptera: Ants and wasps;
- Adventive, which use the carrion as an extension of its environment, e.g. Collembola, Araneae and Isopoda.

In addition, some species may occur accidentally, for example dropped on a carrion from the surrounding vegetation.

The conjunction of these ecological groups serve their purpose not only as PMI indicators, but also as seasonal and environmental indicators. The first two groups (necrophagous and necrophile) are the ones which provide the most valuable information in PMI predictions, and mainly include species of the order Diptera and Coleoptera (Smith, 1986). This method, based on entomological succession, is particularly advantageous when investigating deaths that occurred for more than three months, as there will already be numerous species of both Diptera and Coleoptera present, which will enable a more precise prediction (Rebelo et al, 2014). In order to maximize its accuracy, it is essential to gather information on the typical arthropod species colonizing a carrion at a given region and in different times of the year, along with each species' feeding habits and preferences on each particular decomposition stage.

The second method is based on the determination of the maturation stage of insects collected from the carrion. The majority of insects are oviparous and go through drastic morphological changes until their adult stage is reached, in a complex process designated as metamorphosis. This process involves periodic molts that allow the insects to escape their chitinous exoskeleton and continue to grow. Diptera, generally the first and one of the most prevalent order to colonize a carrion, go through four distinct stages: egg, larva (with 3 instars, generally termed as L1, L2 and L3), pupa and adult. The larval instar can be identified by examining the spiracular plate, length and, more recently, by analyzing the composition of hydrocarbons present on the cuticle's outer layer, which have been known to change along the insect's development. The oldest stage found on the carrion should be the one used in the calculations (Oliveira-Costa, 2013a).

As poikilothermic organisms, an insect's biological cycle is strongly influenced by temperature. For each species, there is a threshold temperature below which growing stops, known as the minimum threshold. In order to develop to the next stage, the insect needs to spend a certain amount of time above this threshold, defined as accumulated degree hours or days (ADH or ADD, respectively). PMI estimation is thus possible by correlating developmental data from a particular species found on a carrion at a given temperature (e.g. how many hours it takes to reach L2 at 20º C) with the meteorological data on the crime scene (Joseph, Mathew, Sathyan & Vargheese, 2011). However, with the exception of the Calliphoridae family, there is currently a lack of literature available all around the globe regarding this topic. In Portugal, the absence of data is even more flagrant, as even Calliphoridae data must be sourced from other regions, further limiting its applicability. Citing the words from Krikken & Huijbregts (2001), "Regional calibration is urgently required".

Limitations of this method include the fact that most calculations are based on laboratorial studies developed in constant temperatures, which do not occur in nature (Rebelo et al, 2014) and that the larval development rate varies according to the species or even organ on which they fed on. In a study performed by Clark, Evans & Wall (2006), it was observed that larvae grew significantly faster when reared on pig organs, when compared to the cow's, and on lung and heart tissues when compared to liver, thus highlighting the importance of additional care when extrapolating data from standard curves, since these are often developed with larvae reared on a single medium.

5.2 Season of death

The entomological fauna of a region is strongly influenced by seasons, which lead to variations not only in its abundance in general but also in the presence of individual species. Season-related data has been used to assist in situating the season of death even of pre-historical remains (Germonpre & LeClercq, 1994). For example, presence of puparia of a species known to occur only in the winter season harshly reduces the possibility that death took place in summer.

Seasonal influence may impact the time of colonization and the species life cycle as well. This means that, in order to develop solid databases for a particular region and to provide accurate PMI estimations, carrion studies should be performed throughout the various seasons and in different years (Byrd & Castner, 2010).

5.3 Location

In order to maximize resource partitioning, evolution has led to distinct habitat preferences among arthropod species. Some species favor urban areas, others rural, some are more active in direct sunlight, while others prefer shade, and so on. Knowledge of each species preferences is yet another valuable tool in a forensic context, particularly when determining whether or not the body has been moved.

Among the broad family Calliphoridae, rural, urban and ubiquitous species have been described. *Calliphora vomitoria* Linnaeus, 1758 is often considered to be a rural species (Byrd & Castner, 2010), while *Calliphora vicina* Robineau-Desvoidy, 1830 and *Lucilia sericata* Meigen, 1826 seem to favor urban areas (Anderson, 1995; Hwang & Turner, 2005). However, problems arise in regions with mixed environments (both urban and rural) or when rural areas are close to human habitations, which can lead to an accidental transport of urban species to the rural environment and vice-versa. For this reason, special caution should be taken when determining if a body has been moved from a rural to an urban area or vice-versa solely based on the entomological fauna present. Corroboration with other pieces of evidence is always advisable.

Similarly, some species may serve as indicators of indoor or outdoor environments. By analyzing cases from British Columbia along a five-year period, Anderson (1995) observed that *Lucilia sericata* was commonly found inside buildings, while *Calliphora vomitoria* was only collected outdoors. However, this behavior is also dependent on region, and again care should be taken when attempting to extrapolate data from different

biogeoclimatic regions. An example of this can be observed in Goff's study (as cited by Byrd & Castner, 2010) where it was concluded that *Stomoxys calcitrans* Linnaeus, 1758 (Diptera: Muscidae) served as an accurate indicator of indoor situations in Hawaii. In other geographic regions however, it is a species commonly found outdoors (Dominghetti, Barros, Soares & Cançado, 2015). In addition, insects will naturally take longer to colonize a carrion inside a building, and the decomposition rate will consequently be slower. Direct sun exposure may also heat a carrion more rapidly, thus accelerating decomposition. All these factors will necessarily affect eventual PMI estimations.

Hanging, particularly when there is no contact with the soil, can also affect the decomposition process and insect colonization in a unique way. The reduced number of larvae exploiting the carcass and the inability of ground-dwelling arthropods to access the carrion (Shalaby, Carvalho & Goff, 2000) leads to, not only a decreased number and diversity of insects, but also to a slower decomposition process.

Whether or not and when a body has been buried is yet another question on which the investigation of insect evidence can contribute. Insect colonization and decomposition rate vary greatly even when the body is shallowly buried (Byrd & Castner, 2010). An interesting situation was described by VanLaerhoven & Anderson (1999), on which there were no larval masses observed when the body was buried immediately, as opposed to when burial was delayed. This is due to the inability of Calliphoridae to lay their eggs on a buried carrion (unless very shallowly buried or having parts exposed during the bloated stage). On the other hand, delaying burial opens a window of opportunity for Calliphoridae oviposition, whose eggs will later hatch into larvae underground.

5.4 Abuse and neglect

Forensic entomology has been employed to determine whether there has been abuse or neglect on a living person or animal, as well as how long it has taken place. Blowflies generally start colonizing a carrion at the natural openings of the body (i.e. mucous membranes). In the case of sites of trauma and wounds not taken care of, or in the presence of unhygienic conditions, insect colonization may occur on living people or animals, and thus assist in providing further evidence in possible abuse situations or serve as indicators of neglect. As for wounds, a greater attraction has been observed if the

wound was inflicted prior to death, most likely due to the presence of blood flow, in opposition to post mortem wounds (Goff, 2009).

Situations of neglect can eventually lead to myiasis, an infestation of a live animal's tissues with larvae of flies. Adult flies may lay their eggs on unbreached skin, but are usually more attracted to the presence of body fluids. Untreated wounds, accumulation of feces and urine, all pose factors that increase the chances of insect colonization.

Besides collection of specimens from the animal itself, the surrounding area should also be searched for pre-pupae and pupae, as these stages occur off of the host and can sometimes be overlooked. By analyzing the specimens and their current cycle stage, it is possible to estimate how long the person or animal has been neglected, which can assist in strengthening the prosecutor's case (Anderson & Huitson, 2004).

5.5 Entomotoxicology

When feeding on a living animal or a carrion, arthropods will indirectly ingest substances present on its organism. These substances may influence the arthropod's development or delay their colonization on a carrion (e.g. animal that died due to pesticide ingestion), resulting in an altered PMI. Situations such as these must always be taken into account when attempting to make PMI estimations (Introna, Campobasso & Goff, 2001).

On another topic, there has been an increasing number of deaths related to drug consumption in the last two decades and, not rarely, the corpse is already decomposed or skeletonized by the time it is found (Byrd & Castner, 2010). On this matter, arthropods may serve as alternative toxicological samples for drug detection, specifically when the conventional matrices normally taken for such purposes, e.g. blood and urine, are no longer available (Dayananda & Kiran, 2014). Currently, it's possible to detect a wide variety of substances on arthropods feeding on a carrion, including heavy metals such as arsenic, mercury and lead; illicit substances such as cocaine, heroin and marijuana; and pesticides such as malathion, all of which may assist in identifying the cause of death (Gomes, 2010).

5.6 DNA analysis

Due to its remarkable sensitivity, evidence based on DNA is more and more becoming popular in forensic circumstances (Rabêlo et al, 2015), playing a crucial role both on investigations and in the conclusive resolution of crimes. In the context of forensic entomology, it can be beneficial in two distinct fields: identification of an insect species, and analysis of the insect's meal in order to identify the person or animal on which it fed on. Traditional insect identification methods are mainly based on morphological features. Although recommended for the identification of adult specimens, some species are morphologically very similar and thus difficult to identify accurately, e.g. *Lucilia caesar* Linnaeus 1758 and *Lucilia illustris* Meigen 1826 (Diptera: Calliphoridae); females of *Pollenia* sp. (Diptera: Calliphoridae). Identification of immature stages is likewise very challenging, particularly when it comes to taxonomic levels lower than family (Thyssen, Lessinger, Azeredo-Espin & Linhares, 2005). It often requires their rearing into adults in order to be able to use the available taxonomic keys for adults, leading to a significant delay in the progress of the criminal investigation. Furthermore, when collecting evidence from a carrion it is not unusual that only sections of arthropods are found, hampering or even precluding an accurate identification. When it comes to the analysis of an insect's meal, applications range from the identification of human remains, by extracting DNA from tissue feeding maggots, beetles, etc. to the placement of a suspect at an indoor crime scene, which can be done by correlating the suspect's DNA (obtained, for example, from his saliva) with the DNA extracted from the blood-meal of hematophagous mosquitoes occupying a closed indoor crime scene (Rabêlo et al, 2015).

6. Arthropod collection methods on a crime scene

Forensic entomology starts at the crime scene. Accuracy of the conclusions reached upon analysis of entomological evidence largely depends on how well collection was done (Oliveira-Costa, 2013b). For this reason, procedures to follow on these situations should be acknowledged by the professionals regularly present at the crime scene, as it is unlikely that a forensic entomologist will be present in every situation (Byrd & Castner, 2010).

As soon as it is collected, every evidence (be it entomological or not) should be labeled and accompanied by the proper documentation and any relevant information. Establishment of a chain of custody (Gomes, 2010), ensures that there is an appropriate tracing of the evidences.

6.1 Preliminary procedures and area assessment

When arriving at a crime scene and before approaching the carrion to proceed to any collection actions, safety precautions should be assured and a thorough description of the crime scene should be registered. The major procedures to be taken are summarized as follows:

- Wearing of protection equipment (e.g. overalls) in order to avoid contamination of both the crime scene (Oliveira-Costa, 2013b) and oneself;
- Photographic record of the whole area, the carrion itself, and the arthropods present both on and around the carrion.
- Description of the area, including natural and/or artificial characteristics of the habitats. This documentation assists in the determination of the usual insect species present on that environment, which may assist in establishing whether the carrion has been moved (Byrd & Castner, 2010);
- Presence of arthropod infestations on and surrounding the carrion, noting numbers, life cycle stages present, e.g. eggs, larvae, pupae and adults (Oliveira-Costa, 2013b) and location of the major sites of colonization. Insect documentation is of great importance as some insects will inevitably move on or hide before they can be collected.
- Registering of current meteorological parameters during the scene assessment, including environmental temperature, temperature of the carrion surface and at the center of the larval masses, and relative humidity. If buried larvae or pupae were harvested, then soil temperature should be measured at the same depth on which the specimens were collected. A retrieval of the historical climate data from the nearest meteorological station should also be made. Meteorological information is crucial when estimating PMI based on insect activity.

6.2 Arthropod collection

Human activity can significantly impact the presence of arthropods on and around the carrion. As such, it should be disturbed as little as possible prior to entomological collection, e.g. by limiting the number of people near the carrion. On the other hand, even with the utmost care and caution, the act of collecting evidence will inevitably result in minor disturbances on the area. A good practice to minimize these disturbances without

missing on valuable information would be to split the collection process into three steps (Byrd & Castner, 2010):

1. Start with a collection of the superficial and flying specimens present on the carrion;
2. After removal of the body, collect specimens that were located under it, as well as up to 6 meters of distance from the carrion location;
3. Finally, collection of arthropods from inside the carrion during the necropsy.

In order to obtain a representative sample of the entomological fauna present, an attempt to collect as many individuals as possible should be made. All entomological evidence should be stored on labeled vials. In order to avoid ink removal, labeling should be done with a graphite pencil.

With the exception of live samples, all arthropods can be preserved in vials containing alcohol 70-95%. By preserving a specimen, development of the arthropod will cease and it's possible to preserve the exact stage of development at the time for posterior identification.

6.2.1 Flying specimens

The first insects to be collected should be the flying adults, and usually require a greater degree of experience and practice. Specimens should be collected from both the carrion itself and the surrounding area. Some methods that can be executed involve using an insect net to either perform a figure of 8 sweeping motion in the air, or to swat the arthropods from above, causing them to fly upwards to the end of the net (Byrd & Castner, 2010). When possible, placement of sticky traps is yet another method that can be used for the collection of flying arthropods (Bilaniuk & Beresford, 2010).

6.2.2 Eggs, crawling insects and pupae

As with flying specimens, collection of crawling individuals has to be performed fast since human activity will often cause them to disperse and attempt to hide. Relevant orders to take into consideration include Diptera (larvae or newly emerged flies), Coleoptera (larvae and adults) and Hymenoptera (predators such as ants and wasps). Representatives of all life stages should be collected and preserved, including eggs, larvae, pupae and adults, taking special care to retrieve the samples from different parts of the carrion and the surrounding area, storing them in separate, adequately labeled vials. Dipteran larvae typically comprise three instars (L1, L2 and L3), meaning that they will

shed their skin, or molt, two times. The excess skin is denominated exuviae, and is yet another indicator of potential use in PMI estimations. After L3, the larva enters a pre-pupal stage, on which they migrate away from the carrion in order to seek a suitable pupation site, usually burying themselves in the soil or under the carrion.

The natural openings of the body, as well as sites that are tainted with feces or blood, are usually the most attractive to arthropods and should be carefully examined. Searching up to 6 meters of the area around the body (including under the soil up to 10cm of depth) is also of great importance because that is where older life stages, the most valuable when estimating PMI, usually are (Oliveira-Costa, 2013b). Forceps or even one's own hands (with gloves) can be used for collection.

6.2.3 Live specimens

An accurate larvae identification with non-DNA methods can be extremely challenging. As such, in addition to the preserved specimens, some live samples should also be collected and allowed to develop to the adult stage.

Rearing of both eggs and larvae can be done in plastic containers containing a substrate, such as soil and a rearing medium, such as raw meat. Pieces of moist paper should be added at the bottom of the container in order to avoid desiccation. The substrate should be in enough quantity to allow the larvae to wander and bury themselves when they reach the pre-pupal stage. The rearing medium should always be added in excess, as a way to prevent cannibalism due to the lack of food. Care should be taken not to mix live dipteran larvae with live coleopteran larvae, as several species of the latter will predate on the former (Byrd & Castner, 2010).

Live pupae can be stored in plastic containers containing only soil, as this stage no longer requires to feed. Empty puparіums can be placed on empty containers and transported at ambient temperature (Oliveira-Costa, 2013b).

II. Seasonal influence in the succession of entomological fauna on carrions of *Canis familiaris* in Lisbon, Portugal

1. Objectives

By comparing the entomofauna collected in controlled studies with the entomofauna encountered on a carrion at a given crime scene, it is possible, among other applications, to estimate the time elapsed since death. As such, the more data available regarding various biogeoclimatic regions, the more precise the estimate will be, as it becomes possible to compare arthropod succession patterns in similar environmental conditions. In Portugal, various forensic entomology studies have recently started to emerge, the majority performed on pig carcasses, with the goal of extrapolating the results to humans (e.g. Marques, 2008; Prado e Castro, 2011; Prado e Castro, Arnaldos, Sousa & García, 2011) and some concerning wildlife (e.g. Gusmão, 2008; Centeio, 2011). In the field of veterinary forensic entomology, specifically regarding companion animals, there is still a lack of data and a lot of room for further research, expansion and improvement.

This study was designed with the following main objectives:

- To investigate the diversity and succession patterns of sarcosaprophagous insects colonizing carrions of *Canis familiaris*;
- To examine quantitative and qualitative differences in the entomological fauna encountered on two distinct seasons (winter and summer);
- To contribute to the documentation of forensically relevant insects occurring in Lisbon, gathering data that can be employed in this specific biogeoclimatic region or in similar environments.

2. Material and Methods

2.1 Location

The present study took place at the Faculty of Veterinary Medicine, of the University of Lisbon (Portugal). The trap was set in a fairly secluded place as a way to minimize disturbance from the Faculty's everyday activities, yet at the same time exposed to the various abiotic factors, i.e. precipitation, sun and wind. Prior to the carrion placement, the vegetation present on the surrounding area was cut, though both its quantity and height sharply rose as the study progressed. The environment of the site can be classified as a large urban area when taking into consideration the density of human population and residue presence, but also as a rural environment to some extent, since it is located near the Monsanto forest park.

IPMA (Portuguese Institute for Sea and Atmosphere) classifies the climate in Lisbon as Temperate Mediterranean, characterized by hot dry summers and mild rainy winters. On the year on which the study took place, 2015, the maximum mean temperatures registered were superior to the usual values, being the 2º highest since 1931 (IPMA, 2015).

2.2 Carrions

Two individuals of C*anis familiaris* species, which had been euthanized due to medical reasons, were used in this experiment. They were placed in traps at different times of the year for a period of approximately five months, in an effort to better analyze and compare the entomological fauna and succession pattern during different seasons.

The first dog (DW) was a mongrel male with approximately 2 years old, medium size (about 20 Kg of weight) and long hair. It was euthanized at a dog shelter and had its liver removed for research purposes, thus left with a large abdominal gash. In order to reduce the influence of bodily fluids in arthropod attraction, the abdomen was carefully sutured and the outer fur washed with current water before placement in the trap. It was placed in the winter and left through spring, from the 13th January to the 29th May of 2015.

The second dog (DS) was a mongrel female with approximately 7 years old, medium size (about 15 Kg of weight) and short hair. On the abdomen, a large tumor was visible. It was euthanized at the Faculty's hospital, and placed in the trap from the summer season through autumn, from the 7th August to the 22nd December of 2015.

Due to logistic reasons, both dogs were stored in the refrigerator for one day before being placed in the trap.

2.3 Trap description

A modified version of the “Malaise” trap associated with a “Pitfall” were employed for this study, as depicted in Figure 1.

Fig. 1 - Frontal (A) and lateral (B) view of the trap used to capture the entomological material (original photo).

This version of the “Malaise” trap consisted of an iron frame covered with two metallic nets. The lower net had a mesh size of approximately 1 cm^2, allowing the attracted insects to easily go in. The upper net had a tighter mesh, with approximately 1 mm^2, preventing the insects from going out. The original “Malaise” was first developed by the Swedish entomologist René Malaise (1937) and resembled an open tent. The idea came to him after observing that insects consistently accumulated at the top of his tent while trying to escape, pressing their way through small holes in the fabric instead of simply leaving through the opened tent door. Since then, this trap has been continuously modified and adapted into various models, but the original principle remains the same, i.e. flying insects have a tendency to go up while trying to escape, finding themselves directed to the upper side of the structure where a capturing medium is placed (Sheikh, Thomas, Bhandari & Meshram, 2016), in this case a glass bottle. The main advantages of this type of traps are its low cost both in construction and maintenance, and that it may stay unattended for an extended period of time (Malaise, 1937). A limitation often pointed out is that it is restricted to the collection of flying specimens, e.g. Diptera (Zou, Feng, Xue, Sang & Axmacher, 2012). Ground-dwelling insects may climb the structure and eventually fall into the bottle, but that ensues on occasional events, and is not the rule.

The “Pitfall” trap consisted of a small plastic container with a cylindrical shape, buried on the soil but with its rim at surface level. It was located inside the structure referred above, near the posterior end of the carrions. This trap is meant to fill the gaps of the “Malaise” type traps, enabling the capture of specimens that typically dwell on the ground (Laub, Youngman, Love & Mize, 2009), e.g. Coleoptera. Some disadvantages of the “Pitfall” trap are that it is not suitable to capture airborne arthropods (a limitation nullified by associating with the previously referred “Malaise” trap) and that, being highly unspecific, it may also capture slugs, small mammals and amphibians, which will rot quickly and affect the composition of arthropods captured (Zou et al, 2012)

Both containers were 25% to 50% filled with a solution consisting of half ethylene glycol, an antifreezer used to lower the rate of evaporation, and half ethanol 70%, used to both kill and preserve the captured specimens (T. Rebelo, personal communication, 2015). Due to natural losses by evaporation or dilution due to precipitation, these containers where regularly refilled as needed.

In addition, a thermometer was permanently hung on the structure (Fig. 1 - B), in order to register the minimum and maximum temperatures between the times of collection.

2.4 Arthropod sampling procedure

In general, decomposition advances at a swifter rate on the first stages, slowing down progressively. For this reason, the interval of days on which harvest took place was continuously adapted, i.e. initially every day, but only weekly by the last stages. In order to reduce unnecessary variation in temperature and humidity measurements, harvests were always performed at the same hour: 13:00. Besides the collection of the specimens that had been captured in both traps, a manual collection of eggs, larvae and other crawling arthropods on the carrion was also performed. Pre-pupal and pupal stages buried under or away from the carrion were also either collected, or had their overall quantity registered.

Except for larvae, all specimens were preserved directly in alcohol 70%. When placed alive directly in alcohol, the larvae’s skin darkens and the tissues contract, hindering posterior identification. In order to avoid this phenomenon, larvae were first blanched with hot water (approximately 80ºC) and only then placed in the 70% alcohol vials for preservation.

On each harvest day, the following procedures were performed:
- Registering of the minimum and maximum temperatures recorded by the thermometer permanently set on the trap (Fig. 1 - B).
- Measurement of the environmental temperature and humidity.
- Measurement of the temperature inside the carrion (starting after skin breaching takes place).
- Photographic documentation of the carrion.
- Collection of dead specimens captured in the "Malaise" and "Pitfall" traps.
- Collection of relevant live specimens creeping on the carrion or found on the terrain surrounding it. The latter included larvae, pupae, newly emerged adults of Diptera and some Coleoptera.

2.5 Specimen identification

Identification was done by direct observation and through a magnifier x20 and x40 (ocular x10, objectives x2 and x4), and was fully based on morphological features. Identification keys and morphological descriptions were sourced from the following authors:
- Diptera: Oldroyd (1954); McAlpine et al (1981); McAlpine et al (1987); Whitworth (2006); Carvalho & Mello-Patiu (2008); Grzywacz (2010); Thyssen (2010); Marshall, Whitworth & Roscoe (2011); Zeegers (2011); Jewiss-Gaines, Marshall & Whitworth (2012); Akbarzadeh, Wallman, Sulakova & Szpila (2015); Szpila (n.d.-a); Szpila (n.d.-b); Szpila (n.d.-c); Szpila (n.d.-d);
- Coleoptera: Peacock (1993); Choate (2003); Gomes (2010).

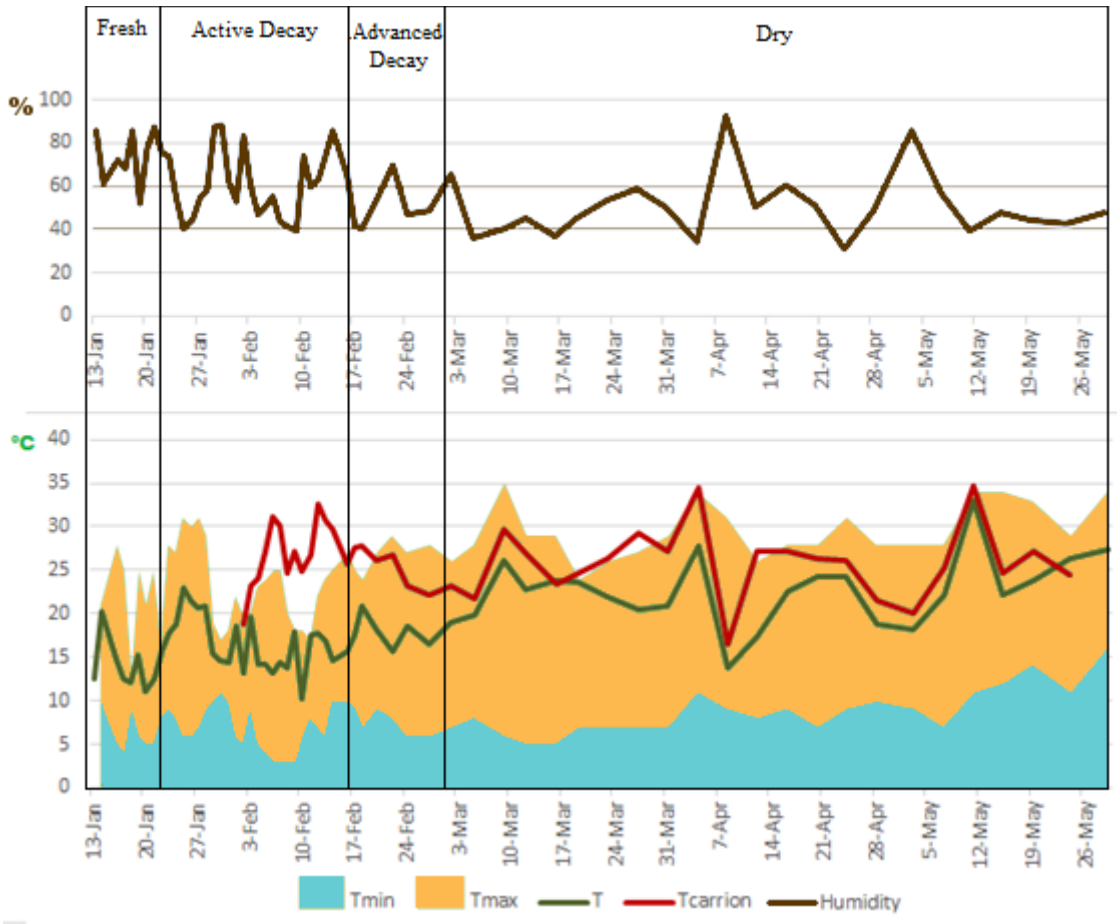
In addition, a dichotomous key to the main families collected was created based on keys from other authors and personal observations (appendix IV). Due to time restrictions and the thoroughness required for some of the families, identification to the species level was only performed on the families Calliphoridae, Sarcophagidae, Muscidae and Rhinophoridae in the Diptera order, and Dermestidae in the Coleoptera order.
In the case of adults recently emerged from the pupae, identification was based on hairs and bristles, by carefully unfolding their wings (when possible), or by analyzing the male's genitalia. The specimen's color should not be considered, as they differ greatly from the color of fully developed adults (Joseph, Mathew, Sathyan & Vargheese, 2011).

3. Results

3.1 Climatic conditions

Figures 2 and 3 depict the minimum and maximum temperatures registered along the study for DW and DS, respectively, as well as the environmental temperature, humidity and temperature inside the carrion, measured at the time of arthropod collection.

Fig. 2 – Relative Humidity and Temperature measurements in winter. The temperature inside the carrion (red) reflects the temperature of the larval masses, when present. The dark green line represents the environmental temperature measured at the time of harvest.

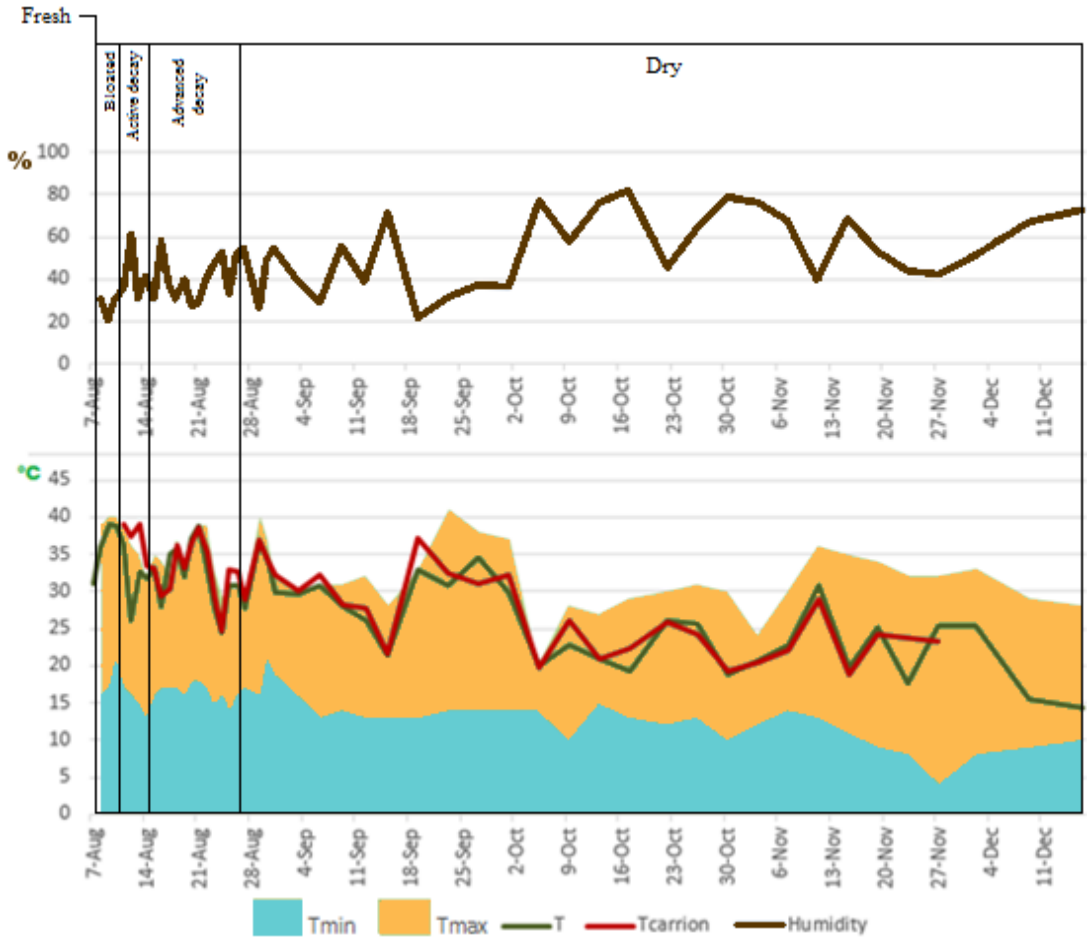


The highest temperature registered on DW was 35ºC, an isolated point on the 9th March. The lowest temperature was 3ºC, registered on the second week of February. The mean temperature along the whole process was 18,6ºC, with the warmer period occurring at the end of the decomposition process, from the middle of April onwards. The carrion's internal temperature largely exceeded the environmental temperature during the month of February, reaching 17,9ºC above the environmental temperature on the second week. This point overlapped with the active decay stage, when large larvae masses were present.

From the month of March onwards, this temperature remained roughly equal to the environmental temperature.

As for humidity, the peak was reached on a rainy day on the 8th April, with 92,8% and the minimum on the 24th April, with 30,7%. The overall mean humidity was 58%, with the moister period occurring between January and February, and the driest in May. The rainy days were mostly restricted to the beginning of the study (until the middle of February), with only sporadic precipitation occurring afterwards. The relatively high humidity contributed to a substantial growth in vegetation, observable from the beginning of the decomposition process.

Fig. 3 – Relative Humidity and Temperature measurements in summer. The temperature inside the carrion (red) reflects the temperature of the larval masses, when present. The dark green line represents the environmental temperature measured at the time of harvest.



The highest temperature registered on DS was 41ºC in September, and the lowest was 4ºC in November, with an overall mean of 28,4ºC. Temperature inside the carrion was the highest at the 12th August, reaching 11,3ºC above the environmental temperature. Like in DW, this point coincided with the active decay stage.

Regarding humidity, the highest point was reached on the 17th October, a rainy day with 82,3% and the lowest point on the 9th August, with 20,8%. The mean humidity was 47,9%, considerably brought up by the moister days that occurred from October onwards. The increased humidity on the last third of the experiment resulted on a considerable growth of vegetation around the carrion (Fig. 4 – B4).

3.2 Decomposition stages

Along the whole decomposition process, a photographic record was taken, enabling a more careful analysis and distinction of each decomposition stage (Fig. 4 and Table 2).

Fig. 4 - Main changes occurring along the decomposition process in winter (A) and summer (B) (original photos).

A: Carrion at 3, 15, 30 and 70 days post mortem, respectively.
B: Carrion at 3, 5, 15 and 70 days post mortem, respectively.

Table 2 - Periods on which each decomposition stage took place.

	Days post mortem	
Decomposition Stage	**DW**	**DS**
Fresh	0 - 9	0
Bloated	NO	1 - 3
Active Decay	10 – 34	4 - 8
Advanced Decay	35 – 45	9 - 18
Dry	46 +	19 +

NO: Not observed

DS clearly followed the five stages of decomposition, as described on page 5. In DW, however, the bloated stage was not observed, contrasting with the exuberant bloating of DS (Fig. 4 – B1). Table 2 summarizes the temporal points of each decomposition stage, assuming the colocation date of the carrion as day 0. As observed, DW had all stages considerably stretched in time, while DS progressed remarkably fast: by day 7, the lateral side of the body touching the ground (the head in particular) was already skeletonized. The active decay stage (Fig. 4 – A2/A3) was the longest on DW, corresponding with the greatest morphological changes and abundant larval masses, while the advanced decay stage (Fig 4 – B3) was the longest on DS. At the early dry stage on DW (day 50) and late dry stage on DS (day 80), large clumps of fungi were observable under the carrions.

3.3 Entomological fauna and seasonal variances

Although only specimens of the Diptera and Coleoptera orders were subject to identification, a great variety of entomological fauna was captured. The "Malaise" was a more specific trap, mostly addressed at Diptera adults, but the "Pitfall" was highly unspecific, netting both crawling and flying insects including Diptera and Coleoptera (along with their larvae), Hymenoptera (ants and wasps), Lepidoptera (moths), Orthoptera (grasshoppers), and other arthropods such as Arachnida (spiders), Diplopoda (millipedes) and Malacostraca (woodlice). Non-arthropods of the Gastropoda class (snails) and even vertebrates (e.g. small reptilians) were also captured. Directly or indirectly, these animals may interact with the carrion or its environment. Among the Hymenoptera order, for example, numerous Formicidae (in winter) and some Vespidae (in summer) were seen preying on both dipteran larvae and adults.

3.3.1 Overall quantities

Due to time restrictions, only the two orders considered most relevant on a forensic context were analyzed: Diptera, with particular emphasis on the brachyceran suborder, and Coleoptera. A total of 1488 adult specimens were subject to identification, of which 1137 dipterans and 351 coleopterans. Other stages of the biological cycle of these insects were also taken into consideration: a total of 981 dipteran larvae (of which the third instar was identified to family or, when possible, to species) and 569 coleopteran larvae (not subject to further identification) were collected. Table 3 depicts the total number of adults

and larvae collected on each carrion. To see a compilation of all the families and species collected on each day, refer to appendices I and II.

Table 3 - Total number of adults and larvae collected on each season during the study.

		DW	DS	Total
Diptera	**Adults**	400	737	1137
	Larvae	851	130	981
Coleoptera	**Adults**	70	281	351
	Larvae	9	560	569

Overall, DS attracted almost twice the amount of adult Diptera, and four times more Coleoptera than DW. In contrast, there was a larger number of dipteran larvae in DW (about 6 times more). However, when it comes to Coleoptera larvae there was an even greater difference, with DS yielding 60 times more specimens. On DW, Coleoptera adults and larvae only began to rise in numbers by the dry stage, but never reached the proportions found on DS. Both orders were generally dispersed along the whole sampling period on DS, but concentrated on the final stages on DW.

3.3.2 Diptera

In Figure 5, the families with the most specimens captured for each season are depicted, and Table 4 represents the overall quantities of species from the families Calliphoridae, Sarcophagidae and Muscidae. Many of the female Sarcophagidae could only be identified to the subfamily Sarcophaginae. Calliphoridae were present in high numbers in winter and summer alike. It was the first family to colonize the carrions, with adults observable from the fresh to the end of the dry stage. The greatest quantities, however, were captured on the dry stage for DW, and advanced decay stage for DS. Two waves of adult Calliphoridae occurring at different temporal points could be observed, although much more noticeable on DS.

Fig. 5 - Abundance of the most represented Diptera families, captured on both winter and summer. Only the families that include more than 10 individuals on at least one of the seasons were considered.

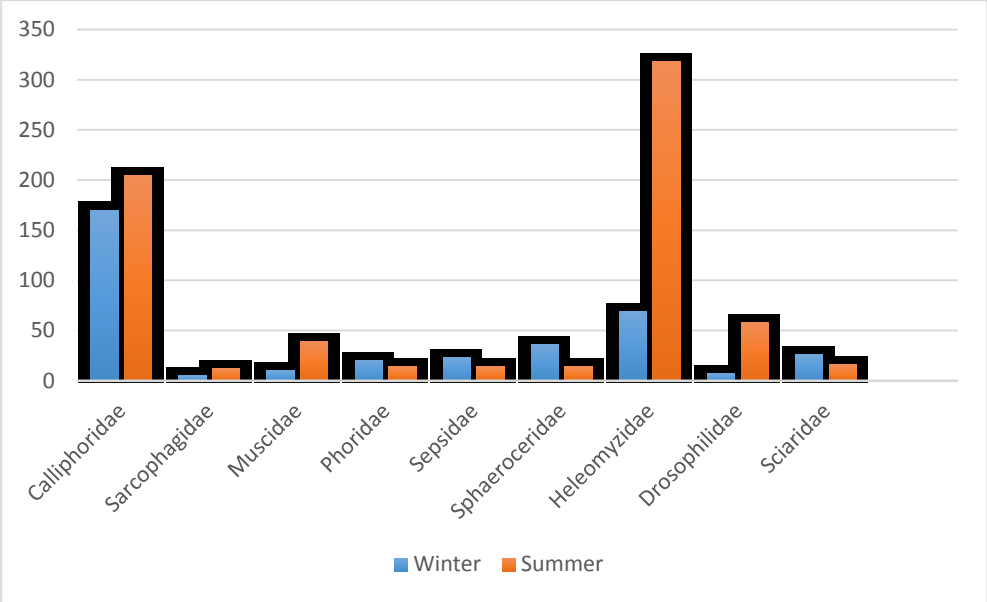
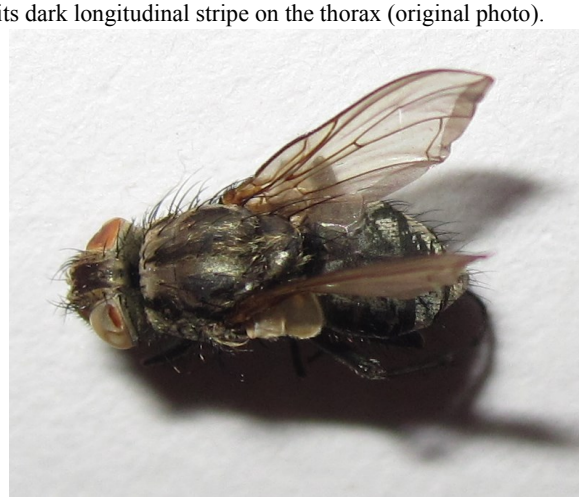


Table 4 – Number of specimens captured for each species of the families Calliphoridae, Sarcophagidae and Muscidae.

Family	Species	DW	DS	Total
Calliphoridae	*Calliphora vicina* Robineau-Desvoidy, 1830	29	2	31
	Calliphora vomitoria Linnaeus, 1758	111	0	111
	Chrysomya albiceps Wiedemann, 1819	0	201	201
	Pollenia sp. Robineau-Desvoidy, 1830	29	1	30
	Pollenia vagabunda Meigen, 1826	1	0	1
Sarcophagidae	*Ravinia striata* Fabricius, 1794	0	1	1
	Sarcophaga africa Wiedemann, 1824	0	4	4
	Sarcophaga teretirostris Pandellé, 1896	0	1	1
	(Sarcophaginae sp. ♀ *)*	5	6	11
Muscidae	*Helina* sp. Robineau-Desvoidy, 1830	1	2	3
	Hydrotaea aenescens Wiedemann, 1830	0	1	1
	Hydrotaea dentipes Fabricius, 1805	1	0	1
	Hydrotaea ignava Harris, 1780	4	2	6
	Musca domestica Linnaeus, 1758	0	29	29
	Muscina levida Harris, 1780	1	0	1
	Mydaea sp. Robineau-Desvoidy, 1830	1	0	1
	Phaonia sp. Robineau-Desvoidy, 1830	2	5	7

The species collected from both carrions were *Calliphora vomitoria, C. vicina, Pollenia* sp. and *Chrysomya albiceps* Wiedemann 1819. While discriminating the various species of the Calliphoridae family in both carrions, a notable dichotomy of genera could be observed, indicating a clear seasonal preference. In winter, there was a strong prevalence of the genus *Calliphora* (comprising both *C. vomitoria* and *C. vicina*), while in summer *Chrysomya albiceps* almost monopolized the carrion, vastly outnumbering all the other species. *Pollenia* was mostly present in winter, with only one specimen being captured on DS (during the latter month, when the temperatures were already lower). The majority of specimens collected for all species was female, but in the *Pollenia* genus an even greater female-biased sex ratio was observed, as all the collected specimens were female. One of the *Pollenia* specimens could be identified as *Pollenia vagabunda*, a species previously unreported in Portugal (Fig. 6).

Fig. 6 – Pollenia vagabunda (Calliphoridae), characterized by its dark longitudinal stripe on the thorax (original photo).



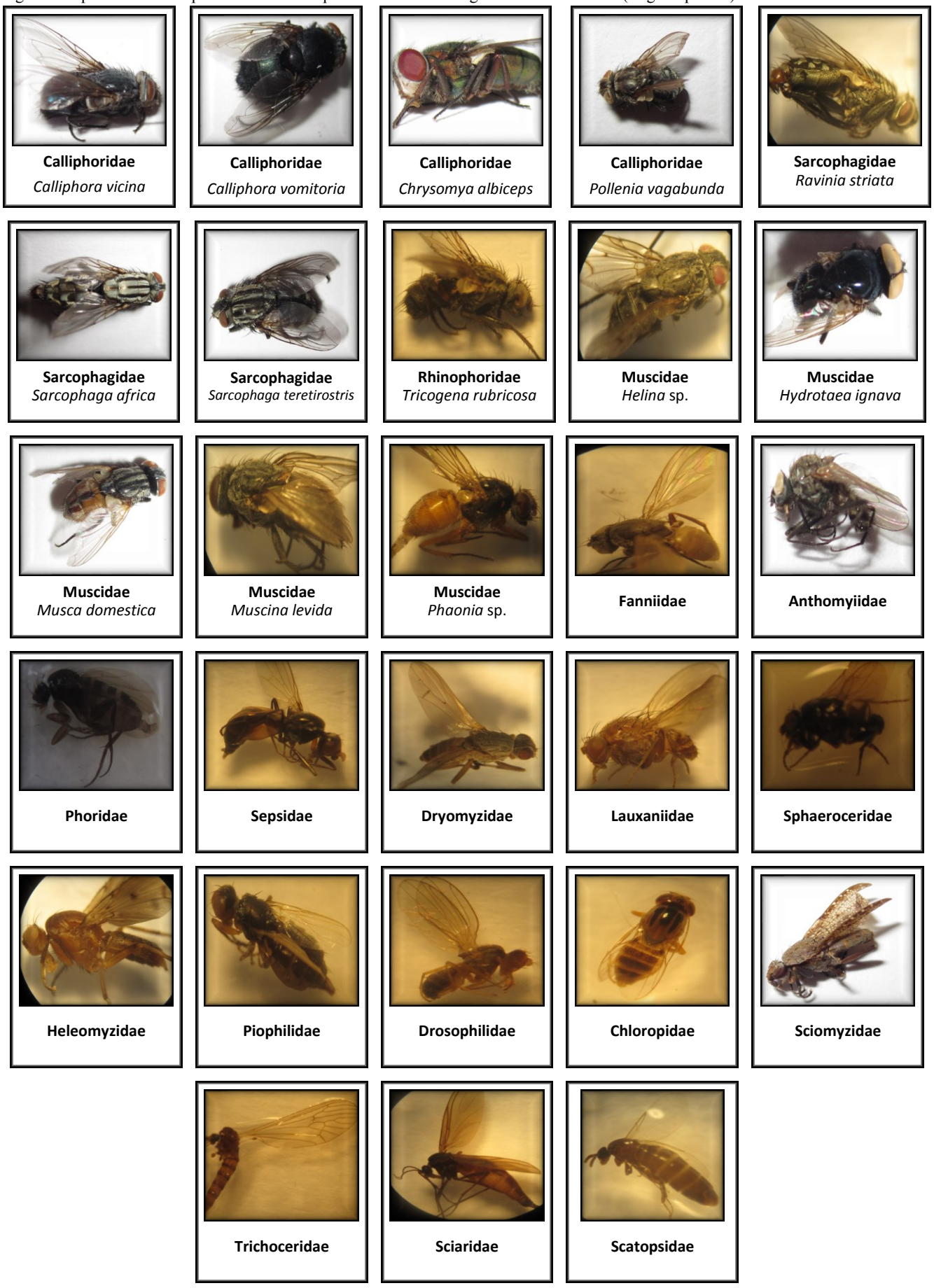
Sarcophagidae adults were present mostly at the active and advanced decay stages in the summer season. In winter, the amount of specimens from this family was minor, and mostly comprised to the dry stage. Among the species that could be identified to the species level, *Sarcophaga africa* Wiedemann 1824 was the most prevalent.

Specimens of the Muscidae family were likewise mostly present in summer, mainly by the end of the bloated stage and beginning of the active decay stage. The most prevalent species was *Musca domestica* Linnaeus 1758, comprising about 60% of the total Muscidae, but occurring exclusively in the summer season. The genera *Helina, Hydrotaea* and *Phaonia* were represented in both seasons, although in minor quantities.

Heleomyzidae and Drosophilidae, though numerous, were concentrated at the end of decomposition process (see appendix I), on which there had already been a considerable growth of grass around the carrion (Fig. 4 – A4/B4) and fungi development.

As opposed to the other families mentioned, Phoridae, Sepsidae, Sphaeroceridae and Sciaridae were present at higher numbers in winter. Most species and families collected on both carrions are illustrated in Fig. 7.

Fig. 7 – Depiction of the Diptera families and species collected during Winter and Summer (original photos).



3.3.3 Coleoptera

As depicted in Fig. 8, the Coleoptera order was mostly represented in summer, with the predominating families being Staphylinidae, Dermestidae, Histeridae and Tenebrionidae. In both seasons, the majority of the specimens collected belonged to the Staphylinidae family, occurring mainly at the most advanced stages of decomposition. Whereas in winter the remainder families were present only in insignificant quantities, summer featured reasonable amounts of Dermestidae, Tenebrionidae and Histeridae, the latter being the first colonizer on this season. Fig. 9 depicts the most relevant families and species of the Coleoptera order.

Dermestidae occurred at an early stage of decomposition in Summer. Two species could be identified: *Dermestes frischii* Kugelann, 1792 and *D. undulatus* Brahm, 1790.

D. frischii, with 67 adults captured, markedly outnumbered *D. undulatus*, with only 3 individuals collected.

Fig. 8 - Abundance of the most represented Coleoptera families, captured on both winter and summer. Only the families that include more than 10 individuals on at least one of the seasons were considered.

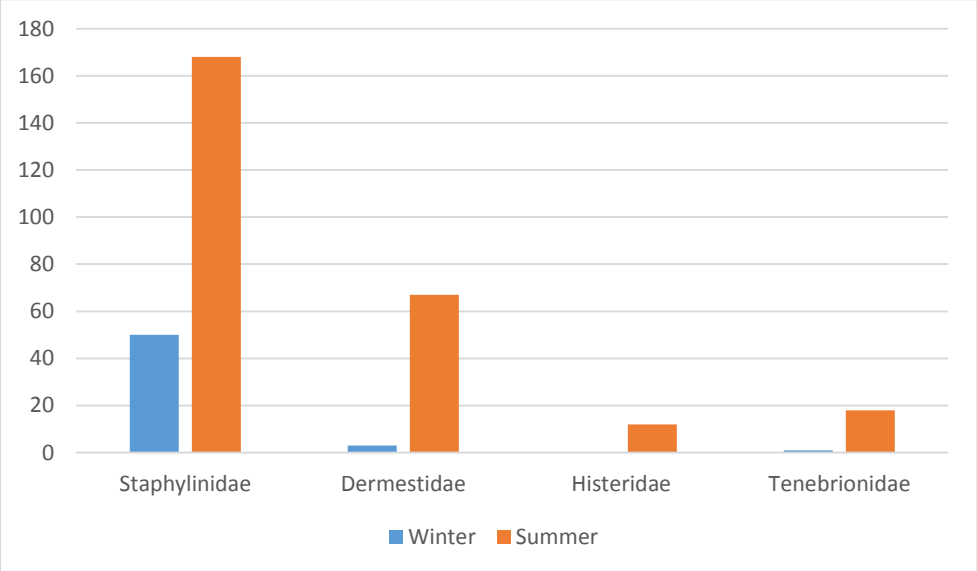
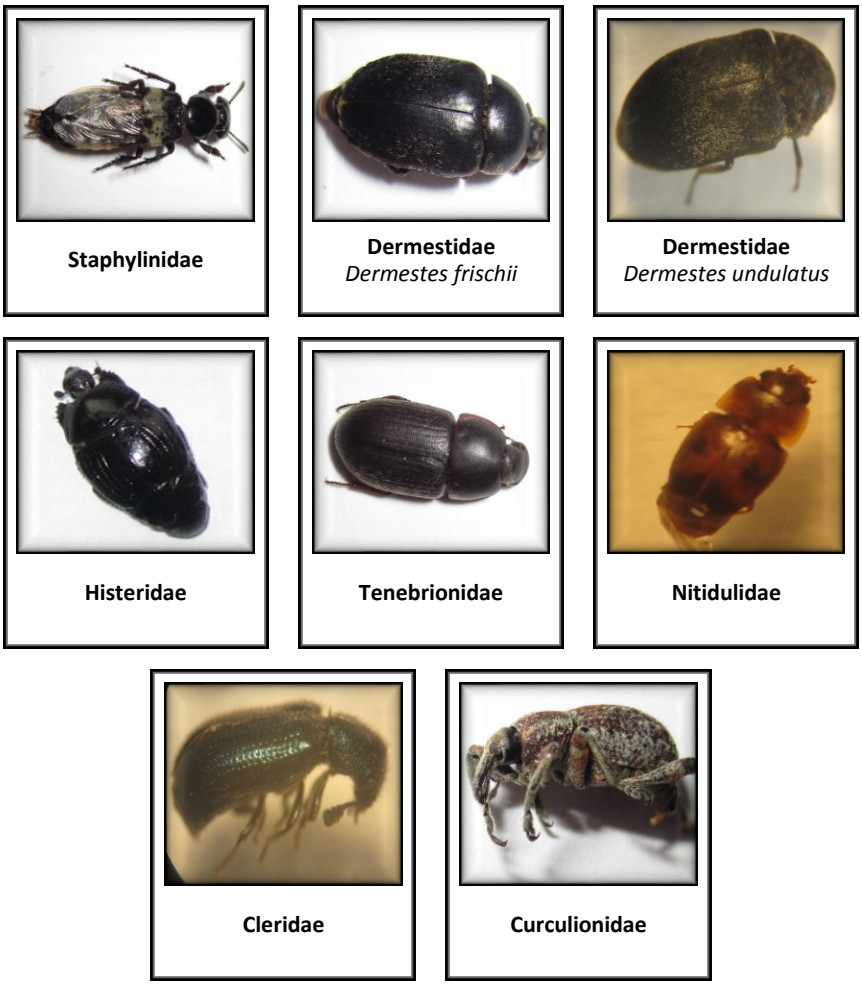


Fig. 9 – Depiction of the main Coleoptera families and species collected during Winter and Summer (original photos).



3.3.4 Life cycle stages

Along the whole decomposition process, various life cycle stages were observed and harvested. Table 5 summarizes the days after death on which each particular life stage of the biological cycle was present. For a complete table, including the number of larvae and young adult flies present on each day, refer to appendix III.

The first target spots for oviposition were the natural openings, starting with the oral, and followed by the anal cavity (in winter). Egg presence was noted for an extended period of time in DW, while in DS they were only observed on the first 2 days, and restricted to the oral cavity.

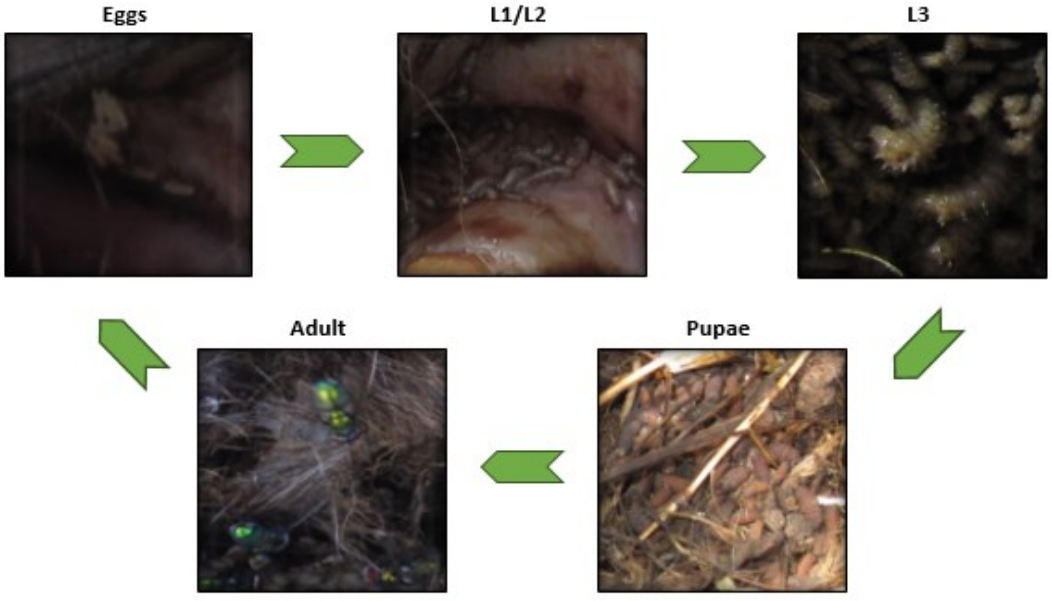
Around day 7 on DW and day 3 on DS, the first larvae were observed, hatched from the eggs that were laid at the oral cavity. Though occurring earlier in DS, the larval population was present for a very short period of time, as opposed to DW which had larvae present for roughly a month, hence yielding a surprisingly high amount of all larval instars. The peak of larval activity was observed along the active decay stage on both carrions.

Table 5 - Periods on which each life stage of specimens from Diptera and Coleoptera occurred.

Order	Life Stage	Larvae Instar	Days Post Mortem	
			Winter	Summer
Diptera	Eggs		3 - 6 (mouth) 5 - 11 (anus)	1 - 2 (mouth)
	Larvae	L1	7 - 29 (peak at 16)	3
		L2	9 - 34 (peak at 28)	3 - 7 (peak at 3)
		L3	12 - 45 (peak at 28)	4 - 9 (peak at 7)
	Pupae		-	Peak at 10
	Young Adults		42 - 78	12 – 15 (peak at 14)
Coleoptera	Larvae		49 - 106 (minor quantities)	6 - 112 (peak at 30 - 33)

As mentioned earlier, larvae identification resourcing only to morphological features is often problematic, particularly when it comes to the species level. An exception to this statement was *Chrysomya albiceps*, whose L3 could be easily identified by its characteristic long fleshy projections present on each body segment (Fig. 10 – L3). As such, it was possible to determine that all dipteran larvae found on DS were of this species, while DW had mostly larvae of the *Calliphora* genera (the precise species of each individual could not be determined accurately). Identifications performed on the larval stages were greatly supported with the observation of the adult population, whose taxa matched the conclusions reached.

Fig. 10 - Depiction of the various blowfly life cycle stages observed on the carrions (original photos).



By the 8th day, larvae in summer had already crept away from the carrion and barely moved, an indication that they were getting ready for pupation. By the 14th day, a great quantity of pupae was observed below the carrion (Fig. 10 - Pupae). In winter however, this behavior was not observed and no pupae were found.

A number of newly emerged adults was collected. These were specimens that had recently arose from the pupae, hence still having shriveled wings, a dull grey/brownish color and their chitinous exoskeleton not yet properly hardened (Fig. 11). All newly emerged adults collected in summer were *Chrysomya albiceps*, and began to emerge 4 days after the first pupae were observed. In winter, the newly emerged adults were all either *Calliphora vicina* or *Calliphora vomitoria* and occurred in far lesser amounts. Overall, the species of the various life cycle stages identified were in consonance with the adult population captured.

Fig. 11 - Adult *Chrysomya albiceps* (Diptera: Calliphoridae) at two different states of development (original photos).

A: Newly emerged *Chrysomya albiceps*, with shriveled wings, dull color and soft body.
B: Full-fledged adult *Chrysomya albiceps*, with its characteristic metallic color and hard chitinous exoskeleton.

Regarding Coleoptera life stages, only larvae and adults were observed. On DW, larvae occurred only by the dry stage and were present in minor quantities. DS on the other hand had significant numbers of both larvae and exuviae, present from the advanced decay stage onwards, with the peak occurring by the 30th day, when the carrion was already at the dry stage of decomposition.

4. Discussion

The present study enabled the acquisition of information concerning the entomological succession on carrions of *Canis familiaris* and the observation of numerous differences regarding both quantity and quality of the entomofauna occurring along the decomposition process and between different seasons. This study also contributed to document forensically relevant species of Diptera and Coleoptera occurring in the region of Lisbon, Portugal.

4.1 Trap efficiency

The traps employed in this study were both considered effective in their own purpose: the "Malaise" netted mostly flying specimens, while the "Pitfall" plugged the gaps, capturing the ground-dwelling individuals. This highlights the importance of a combination of different methods in forensic studies, thus ensuring an accurate representation of the entomological fauna around the carrion. It has been suggested by some authors that adding a funnel to the "Pitfall" trap would improve the rate of capture, as arthropods may back away from an abrupt hole (Capinera, 2004). A gradual slope would be less daunting and also prevent the captured specimens from escaping and other small animals from inadvertently falling into the trap (Laub et al, 2009). Attaching a cover to the top of the trap could also potentially minimize losses by evaporation and dilution by rainwater, allowing unattendance for more extended periods of time. However, addition of funnels or covers has been shown to negatively affect the number of arthropods captured (Cheli & Corley, 2010), and this fact should be taken into account when such devices are used. The preservative solution employed in the traps was considered effective both as a killing and as a preservative agent. The use of ethylene glycol in open areas has however been discouraged due to its high toxicity and its characteristic sweet flavor, which heightens the ingestion by vertebrate animals. Propylene glycol has been suggested as a less toxic alternative, with the disadvantage of being more expensive (Gossner et al, 2016). Adding a few drops of detergent to break the surface tension has been performed in previous studies (Gusmão, 2008; Prado e Castro, 2011) and may be beneficial in order to prevent specimens from floating and escaping, but have been found to repel some arthropods, e.g. Staphylinidae (Pekár, 2002). In some cases, a dry version of the "Pitfall" trap can be employed, particularly if live specimens are needed. It will however require more

frequent collections in order to avoid destruction of the captured individuals from other live animals entering the trap (Capinera, 2004) and to prevent escaping of the specimens.

4.2 Temperature and Humidity

Abiotic parameters such as Temperature and Humidity are the main factors affecting decomposition rates and the presence of insects on a carrion. It was observed that 2015 was a particularly hot year, with the maximum mean temperatures situated above the average in recent years. These observations are in concordance with the now widely accepted global climate change. Warming predictions range from 1ºC to 5ºC by 2080 (Hulme et al, 2002), and these changes are bound to cause impacts on the biodiversity of ecosystems, either by enhancing or hindering the viability of particular insect species. An example could be observed in this study, with *Chrysomya albiceps* greatly predominating in warmer conditions.

The highest temperatures were registered on the first weeks of summer, contributing to the initial boost on both decomposition and quantity of insects. As observable in Figures 2 and 3, the internal temperature of both carrions in general evolved slightly higher, but fairly close to the environmental temperature, measured at the time of harvest. The temperature differential reached its peak at the active decay stage, coinciding with the time on which maggot masses were more active. This peak was reached much earlier in summer than in winter, as was expected when taking into consideration the rate at which both decomposition and insect colonization evolved. The higher temperature differential occurred for a prolonged period of time on DW, which is also in consonance with the amount of larvae associated with this carrion in an extended period of time. The presence of long hair on DW may have also helped prevent the loss of heat. These results further substantiate that a carrion's internal temperature may be a good indicator of the activity of larval masses, particularly when they are not readily identified by observation.

The rise in humidity levels was accompanied by a significant growth of vegetation around the carrion. Similarly, the occurrence of fungal growth at the more advanced stages of decomposition, observed on both carrions, was most likely largely influenced by humidity. DW had fungi clumps observable much earlier than on DS, and this discrepancy in time may be related to the increased moisture levels on the former, while on the latter the fungi's ideal ecological conditions were only met when the rainy season arrived. While not the focus of this study, fungal growth can also potentially be used as tools for PMI estimation, particularly when forensic entomology cannot be applied (Hitosugi et al,

2006). Both the occurrence of fungi on the carrion and vegetation growth around it consist of additional factors that will inevitably affect the entomofauna present, and should always be taken into account in forensic studies and have its presence registered in crime scene situations.

4.3 Decomposition

All of the decomposition stages observed on DW were considerably stretched in time, evolving in general at a much slower rate when comparing with DS. Absence of the bloated stage on DW could be due to a combination of a variety of factors. A very mild bloating could have been present, but concealed by the presence of long hair, which necessarily limits the observation and distinction of the characterizing features of each decomposition stage. In addition, presence of the abdominal incision (even though previously sutured) may have allowed some air to escape, as well as the absence of the liver, which would be a great medium for bacterial proliferation. Finally, the lower temperatures have led to the stages being displayed with a decreased intensity, in contrast with the exuberant changes observed on DS, since putrefactive reactions and insect development proceed slower. Rupturing of the abdominal wall occurred much earlier on DS, which is also related to both its increased bloating and the greater initial boost in fly larvae.

The considerable difference observed in the decomposition rate may be associated not only with the lower temperatures, which decelerate the putrefactive reactions and slow down or even halt the biological cycle of insects, but also with the increased rainfall at the initial stages, which is known to delay a carrion's colonization by insects (Kyerematen, Boateng, Haruna & Eziah, 2013). The presence of long hair on DW may be yet another hindering factor on early insect colonization and consequently on the progress of decomposition. It has been reported that younger individuals will have a lower decomposition rate (Spitz & Spitz, 2006). Being 5 years younger than DS, this factor may have also contributed to the slower decomposition process.

Similarly, to the documented in previously performed forensic studies (Prado e Castro, 2011), decomposition progressed at distinct rates in different parts of the carrion. The head decomposed considerably faster than the rest of the body, which was to be expected since this was the preferred site for initial colonization.

4.4 Entomological fauna

All four groups of insects usually associated with carrions were observed: necrophagous, necrophile, omnivorous and adventive. The first two groups include the majority of the dipterans and coleopterans identified, and typically provide the most valuable information regarding PMI estimations. However, other orders that were not analyzed may also deliver additional information and assist the case at hand. Omnivorous specimens such as Hymenoptera, for instance, influence a carrion's ecological system not only as predators of necrophagous arthropods, but also as having sarcosaprophagous habits themselves (Prado e Castro, García, Palma & Martínez-Ibáñez, 2014). Predation on Diptera was observed in this study, with a large number of ants (winter) and some wasps (summer) seen preying on dipteran larvae and adults, respectively. Presence of these arthropods in large numbers possibly reduced the number of necrophagous insects and could have therefore had an impact on the decomposition process and total specimens collected.

The increased amount of specimens captured in summer may be justified by the increased temperatures in general, which are known to attract more insects, e.g. due to an increased odor (Joseph et al, 2011) and to boost their development. It was initially thought that traces of blood that would inevitably be present from the abdominal wound (even if previously sutured and washed) on DW would lead to an additional attraction to insect colonization. However, no effects were observed on this matter. The absence of liver, however, could have contributed to a decreased odor on DW, limiting insect attraction.

4.4.1 Diptera

Previous forensic studies performed in Portugal (Gusmão, 2008; Marques, 2008; Cainé, 2010; Centeio, 2011; Prado e Castro, 2011; Farinha et al, 2014) have contributed to the databasing of the dipteran fauna in Portugal, and have listed numerous families associated with carrions. Calliphoridae is the common denominator on all studies, typically being the predominating family and usually followed by other forensically relevant families such as Sarcophagidae, Muscidae and Fanniidae. In this study, with the exception of Calliphoridae, none of the other families were represented in particularly high numbers. Additionally, as opposed to the previous studies mentioned, the bloated stage did not yield the highest quantity and diversity, the first place being relegated to the dry stage on DW, and active decay and dry stages on DS.

As the first colonizers of carrions and being present throughout the entire decomposition process, Calliphoridae was by far the most representative family. Two waves of adults

from this family could be observed on both carrions. The first wave consisted of the first colonizers, attracted to the carrion as a medium for food and oviposition. The second wave consisted mostly of adults that had emerged from the pupae, as verified by the fact that many of them still displayed several features that characterize newly emerged adults, such as a soft body, dull color and shriveled wings (Fig. 11). In summer, the population followed a typical pattern, with the first wave of calliphorids occurring only a few days after placement of the carrion in the trap, and the second wave occurring a mere four days after the peak of pupae was observed. In winter however, they occurred only in small quantities at the beginning of decomposition, only starting to rise in number by the end of the decomposition process, as opposed to what was expected of the normal behavior of this family (Oliveira-Costa, 2013a). Taking into consideration the large amount of calliphorid larvae present, however, it is inferred that a number of adults were in fact present for oviposition, but were not captured at the traps.

In relation to the seasonal characterization of Calliphoridae species, it was observed that winter blowfly populations were represented by *Calliphora vomitoria, Calliphora vicina* and *Pollenia sp.*, as was expected when taking into consideration these species' preferences for moderately cooler climates (Schröder, Klotzbach & Püschel, 2003; Horenstein, Linhares, Rosso & García, 2007; White, 2016). Conversely, the thermophile species *Chrysomya albiceps* clearly dominated the summer season. These results are in consonance with previous studies performed in Portugal (Cainé, 2010; Prado e Castro, 2011; Farinha et al, 2014), with the *Calliphora* genus being mostly encountered during the spring and winter seasons, and *Chrysomya* more prevalent in summer and autumn. *Pollenia,* a genus known to parasite or predate earthworms (Rognes, 1987) was mostly present in winter. No earthworms were observed at the time of capture, so it is supposed that they were attracted by the carrion itself. However, the purpose of this genus on the carrion's decomposition process is still unclear (Bas, Cifrián, Díaz-äranda & Martín-Vega, 2007). While easily discernible from all other calliphorids by the absence of metallic shine and presence of long golden hairs on the thorax, the taxonomic classification of the Pollenia genus to species remains problematic, often requiring the examination of the male's genitalia (Parchami-Araghi, Gilasian & Rognes, 2014). As commonly perceived in other Diptera species, a clear female-biased sex ratio has been observed associated with carrions in various experiments (Bas et al, 2007), most likely due to its biological urge for oviposition. The present study was no exception: all the captured *Pollenia* were female, and it was thus not possible to identify all specimens to

species level with a satisfactory degree of certainty. *Pollenia vagabunda* was the sole species that could be identified, thanks to the presence of a characteristic dark stripe and anterior postpronotal setae (Jewiss-Gaines et al, 2012). Though originally Palearctic, this species has been already reported in the Iberian peninsula (Martínez-Sánchez, Rojo, Rognes & Marcos-García, 1998), but has not been yet reported in Portugal.

In relation to the preferred habitat, *Calliphora vomitoria* is often considered a rural species (Anderson, 1995), while *C. vicina* (Hwang & Turner, 2005) and *Chrysomya albiceps* (Moretti & Godoy, 2013) were found to have a preference for urban environments. As such, both rural and urban species have been collected in this study, which may be explained by the environment on which it took place, characterized as being both urban and rural to some extent. A curious finding among the calliphorid species found was the total absence of the genus *Lucilia* on both carrions, as this was a commonly found genus on previous forensic studies performed in Portugal (Cainé, 2010; Centeio, 2011; Farinha et al, 2014) and in other European countries (Schröder, 2003). On the other hand, the summer population was largely dominated by the thermophile species *Chrysomya albiceps*. Even if its presence in the hotter season was to be expected, the high numbers on which it occurred in relation to the other species, along with the total absence of *Lucilia* were still surprising. *Chrysomya albiceps* is known to exhibit cannibalistic behaviors in the maggot stage, feeding on the other calliphorid species and even displacing native species (Faria, Orsi, Trinca & Godoy, 1999). This phenomenon, associated with the particularly hot year that took place (IPMA, 2015), may consist of a possible explanation for such high numbers of this species, as well as the absence of *Lucilia*, i.e. the rise in temperature allowed the propagation and exacerbated proliferation of the thermophile *Chrysomya albiceps*. This species occupied an ecological niche, in this case the carrion, and impaired the proliferation of other calliphorids, both by depletion of the same food, and by direct attack on the larval stages. In addition, by being the first to arrive to the carrion, they were necessarily bigger by the time other larvae ecloded, thus gaining a natural competitive advantage.

Sarcophagidae, a family often associated with the colonization of carrions and as strong indicators in forensic cases, were present in relatively low numbers. Its temporal preference, however, was in correlation with the expected, occurring at the more advanced stages of decomposition (Oliveira-Costa, 2013a). For the most part of the females captured, identification to the species level was not possible. Complications surrounding the identification of female Sarcophagidae (as well as several other females

in general) are one of the hindering factors to an accurate characterization of the faunistic composition of several countries, including Portugal (Prado e Castro, García, Arnaldos & González-Mora, 2010). By analyzing the composition of the male population it is expected that the specimens that could only be identified to the subfamily Sarcophaginae would consist predominantly of *Sarcophaga africa*, as this was the most represented species not only in this study, but also in other Portuguese reports (Prado e Castro et al, 2010).

Flies of the Muscidae family were likewise present in low numbers, especially in the winter season. Commonly found in a great variety of biogeographical regions, this family comprises various species with sarcosaprophagous habits, thus being of potential forensic importance. The most common species in this study, *Musca domestica*, occurred only in summer, although it has been reported in other seasons by other studies performed in Portugal (Gusmão, 2008; Prado e Castro, 2011; Farinha et al, 2014). *Hydrotaea ignava* had more individuals in winter, though as observable in Table 4 and similarly to the other muscid species, the low numbers encountered are not sufficient to draw conclusions.

By the end of the experiment, a significant increment on the number of specimens of the Heleomyzidae and Drosophilidae families could be observed, particularly on DS. Heleomyzidae has been often associated to both animal carcasses and fungi (Oliveira-Costa, 2013a; McAlpine, 1987), and are known to prefer forested, cool areas (Woznika & Klasa, 2009). Though often associated with animal carrions, Drosophilidae are considered phytophagous (Dupont, Champlain, Cyrille & Félix, 2011), with most species feeding on flowers, overripe and rotting fruits (Walsh et al, 2011) and other decaying organic matter (McAlpine, 1987), or mycophagous, feeding on fungi (Gottschalk et al, 2009). Information on the prevalence of phytophagous species can be important in determining if a carrion has been moved, e.g. between a highly vegetated area to one with no vegetation, and vice-versa.

The families Phoridae, Sphaeroceridae and Sciaridae differed from the previous families mentioned for being present at higher numbers in winter. These findings are in concordance with observations from some other authors, with Phoridae having been observed mostly in winter by Schröder (2003), and the latter two families by Gusmão (2008).

4.4.2 Coleoptera

The greater quantity of Coleoptera in summer may be related to not only the warmer temperatures, but also because of the stretching of the latter stages of decomposition, which are generally preferred by this order in opposition to the initial stages.

The predominance of Staphylinidae on the most advanced stages of decomposition is in consonance with its predatory feeding habits and preference for higher moisture levels (Márques, 2003) (Appendix II and Figures 2 and 3) and other studies previously performed in Portugal, on various carrion species (Marques, 2008; Prado e Castro, 2011; Lopes, 2012).

Dermestidae occurring mostly in summer and its nearly absence in winter may be related to this family's natural predilection for warmer temperatures (Klok & Harrison, 2013). Its feeding preferences for dry and leathery tissues was initially not thought to be in concordance with the stage at which it was found in higher numbers, i.e. the active decay stage. However, due to the remarkably rapid decomposition process that occurred in this season, it was observed that some parts of the carrion were at times in a much more advanced state than others (as previously referred, the side of the carrion touching the ground was already skeletonized while the active decay stage was still occurring in other parts of the carrion). This fact would help explaining the substantial presence of Dermestidae at this period.

Finally, the discrepancy in Coleoptera larvae may understandably be due to the overall greater number of adult Coleoptera in summer, which will necessarily produce more larvae.

4.4.3 Life cycle stages

The rate on which a life cycle develops is greatly dependent on temperature. The higher the temperature, the faster the rate of development. This premise was observed in this study when comparing the life stages present on the two carrions, as each stage was considerably extended for a longer period of time in the winter season. For instance, eggs were observable in summer for only two days, with the first larvae already present by the 3rd day. On the other hand, eggs were observable in winter for 9 days, with the first larvae occurring only by the 7th day. For this reason, estimates of the post mortem interval based on the insect's life cycle should have as a foundation studies performed in different climatic conditions.

The greater quantity of larvae on DW may be correlated with the elongated decomposition process, particularly the active decay stage which lasted 2 weeks longer than on DS. The difficulty in uncovering pupae on this carrion could be due to the great amount of vegetation and hair detached from the carrion at the time, associated with the low numbers of specimens present.

The newly emerged adults, though sometimes difficult to identify to the species, could prove to be of great use in the estimation of the post mortem interval as they are the latest development stage of a fly's life cycle and mark a specific moment in time, after the fly emerges from the pupa.

5. Final considerations

The present study represents the first research on the entomological succession on carrions of dogs (*Canis familiaris*) performed in Portugal. Through this work it was possible to investigate its entomological fauna, to report *Pollenia vagabunda* for the first time in Portugal and to observe various seasonal differences on the decomposition rate and entomofauna present.

A growing awareness concerning animal welfare has been observable all around the globe. It is thus becoming more and more relevant that various professionals from various sciences acquire competences which will assist in the detection of possible situations of abuse and neglect, along with the ability to provide informed opinions backed with good quality data. Forensic entomology in particular, has had in the latest years an increasing acceptance as a valid tool in medicolegal investigations. Currently in the veterinary field, it is seldom used when investigating cases of animal abuse and neglect. Raising awareness on its numerous applications and providing veterinarians with knowledge on how to properly handle documentation and collect entomological evidence would certainly increase the quality of information obtained from a crime scene and greatly benefit the construction of a stronger case in court.

The numerous factors that affect a carrion's decomposition and insect colonization however, make it necessary to perform studies in distinct regions and under different conditions. To generalize and extrapolate data are always options, but entail the possibility of erroneous or inaccurate conclusions, detrimental to the forensic case at hand. Knowledge of the rate at which the biological cycle of each particular species takes place, particularly in distinct temperatures and humidity, along with the record of the

usual entomofauna present on a given region and season, all may prove to be valuable forensic tools in a crime scene situation. A current record of the common entomofauna of a given region is therefore essential, both from a forensic perspective, and as a tool to preserve public health.

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Appendix I: Daily Diptera species and families

Winter

Suborder	Family	Species	Days post mortem																																																											TOTAL
			0	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	34	35	36	38	40	42	45	49	52	56	59	63	66	70	74	78	82	86	90	94	98	102	106	111	115	119	123	127	132	137	
Brachycera	Calliphoridae	Calliphora vomitoria			1									1			1	1			1		1			1		4							2							1		1	5	44	39	6		2															111
		Calliphora vicina				1	1					1			1		1				1			1																1		2		4	4	7	4																		29
		Pollenia vagabunda																								1																																							1
		Pollenia spp.																																								1		3	2	4	1			1	1	3		4	5	4									29
	Sarcophagidae	(Sarcophaginae spp.)																																																2	1												1	1	5
	Muscidae	Phaonia spp.																																													1								1											2
		Helina spp.											1																																																					1
		Hydrotaea ignava																																																					1	3									4
		Hydrotaea dentipes																																													1																		1
		Mydaea spp.																																																				1											1
		Muscina levida																																															1																1
	Fanniidae																	3																	1													1			1	1									1				8
	Anthomyiidae																									1																						1		2										1		1			6
	Phoridae					3				1				1			2							1																				5											1	1	1			2			1	1	20
	Sepsidae																																		1															6		3		1		1	1	6	1	2	1				23
	Lauxanidae																																													1																	1	3	5
	Sphaeroceridae					1	1										1				2												1		2				1	9		3		6	1							2					3		2	1					36
	Heleomyzidae									1	1	1																																				3	5	5		11	8	17		4	8	2	1			1	1		69
	Piophilidae																																						1							1																			2
	Drosophilidae									1	1							1																																1		1								2					7
	Chloropidae																									3																																							3
Nematocera	Trichoceridae					1	1		1																																																								3
	Sciaridae																	1					3			1									1				1								1					1		4		2	8	1				1		1	26
	Ceratopogonidae																							1																																									1
	Cecidomyiidae																																1						2																										3
	Scatopsidae																																																											2		1			3

Summer

Suborder	Family	Species	Days post mortem																																																		Total
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	23	24	27	30	33	36	39	43	47	51	55	59	63	67	71	76	80	84	88	92	96	100	104	108	112	117	124	131	
Brachycera	Calliphoridae	*Chrysomya albiceps*			4	11	11								4	42	117	12																																			201
		Calliphora vicina																																			1								1								2
		Pollenia spp.																																							1												1
	Sarcophagidae	*Sarcophaga africa*								1			1																		1		1																				4
		Ravinia striata														1																																					1
		Sarcophaga																														1																					1
		(Sarcophaginae spp.)								1			1			1													2						1																		6
	Rhinophoridae	*Tricogena rubricosa*			2																																																2
		Stevenia deceptoria																											1																								1
	Muscidae	*Musca domestica*				8	9	4				1										1					1			1							2	2															29
		Phaonia spp.				1			1																															2											1		5
		Helina spp.																																											1	1							2
		Hydrotaea ignava					1	1																																													2
		Hydrotaea aenescens					1																																														1
	Fanniidae					3																																															3
	Anthomyiidae					1																																	1	1	1						2	1		1			8
	Phoridae														1														1					1				1	1		1						1	1	3	2		1	14
	Sepsidae																																													1			3	6		4	14
	Dryomyzidae																																															1					1
	Sciomyzidae																																												1								1
	Lauxaniidae																																1			1		1					1												4
	Sphaeroceridae										1						1																												8	1		1	1	1			14
	Heleomyzidae																																1							3	1				19	93	37	43	24	15	40	42	318
	Piophilidae								2	2		2																																									6
	Drosophilidae					1																								4		1	2	1		1	1					1		2	28	5	3	3		1	1	3	58
	Carnidae						1	3	1			1																1																									7
Nematocera	Sciaridae																																						1				2					1			1	11	16
	Mycetophilidae																																											2	2									4
	Scatopsidae															1																													1	1	1	2	2		1		9
	Cecidomyiidae																																													2							2

Appendix II: Daily Coleoptera families

Winter

Family	Species	Days post mortem																																			
		0	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	34	35	36	38
Staphylinidae											1																										
Dermestidae	*Dermestes frischii*																																				
	Dermestes undulatus																																				
Tenebrionidae																																					
Chrysomelidae																																					
Cleridae																																					
Silvanidae																																					
Curculionidae																																					
Nitidulidae																																					
Coccinellidae																																					
Cryptophagidae																																					

Family	Species	Days post mortem																										TOTAL
		40	42	45	49	52	56	59	63	66	70	74	78	82	86	90	94	98	102	106	111	115	119	123	127	132	137	
Staphylinidae		4	3				8						2		2		6			5		2	2		1		14	50
Dermestidae	*Dermestes frischii*															1												1
	Dermestes undulatus																						1	1				2
Tenebrionidae															1													1
Chrysomelidae																			1									1
Cleridae									1				1		1													3
Silvanidae																						2			1			3
Curculionidae																						1			2			3
Nitidulidae													1								1		1					3
Coccinellidae							1																					1
Cryptophagidae																					1		1					2

Summer

Family	Species	Days post mortem																				
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Staphylinidae								2		1					2							
Dermestidae	*Dermestes frischii*						1	20	4	1	1		2	9	3	5		5	2		3	3
	Dermestes undulatus																					
Histeridae					3		3	2		1	1		1		1							
Hydrophilidae																						
Tenebrionidae																						
Chrysomelidae																						
Cleridae													1					1				
Nitidulidae																						
Silvanidae					1																	
Ptiliidae							1															
Curculionidae																						
Carabidae																						

Family	Species	Days post mortem																												Total
		22	23	24	27	30	33	36	39	43	47	51	55	59	63	67	71	76	80	84	88	92	96	100	104	108	112	117	124	131	
Staphylinidae						1									1		2			1	1	1	31	10	7	14	63	24		7	**168**
Dermestidae	*Dermestes frischii*				3	1		2				1																			**66**
	Dermestes undulatus		1																												**1**
Histeridae																															**12**
Hydrophilidae																							1								**1**
Tenebrionidae						12	2	2				1	1																		**18**
Chrysomelidae																				1											**1**
Cleridae										1																					**3**
Nitidulidae										2					1								3								**6**
Silvanidae																														1	**2**
Ptiliidae																															**1**
Curculionidae																														1	**1**
Carabidae																							1								**1**

Appendix III: Daily Blowfly and Coleoptera life stages

Winter

Order	Life Stage	Larvae instar	Days post mortem																																																												TOTAL
			0	1	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	34	35	36	38	40	42	45	49	52	56	59	63	66	70	74	78	82	86	90	94	98	102	106	111	115	119	123	127	132	137	
Diptera	Eggs				+	++	++	++	+	+	+	+	+																																																				-
	Larvae	L1							13	7	9	18	4		2		14	71	12	11	1	4			2		1	1	1		1																																		172
		L2									17	42	21	1	12	3	6	16	24	22	13	5	7	3	5	2	13	4	43	65	9	1			18																														352
		L3												5		6		1	8	9	6	2	13	7	3	15	16	12	52	54	43	12	4		38		7		7	3	4																								327
	Young adults																																							1		1		2	5	17	7	1		1															35
Coleoptera	Larvae																																									1		2								2	1			1	1							1	9

+: <20
++: 20-100

Summer

Family	Life Stage	Larvae instar	Days post mortem																																																		**Total**
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	22	23	24	27	30	33	36	39	43	47	51	55	59	63	67	71	76	80	84	88	92	96	100	104	108	112	117	124	131	
Diptera	Eggs			+	+																																																-
	Larvae	L1				2																																															2
		L2				37	7			1																																											45
		L3					8	15	7	27	17	9																																									83
	Pupae										+	++	+++																																								-
	Young Adults														2	34	117	11																																			164
Coleoptera	Larvae								1					1					7		3	33	23	27	6	3	55	136	103	49	23	7	49	8	2			2					6	3	6	3	2	1	1				560

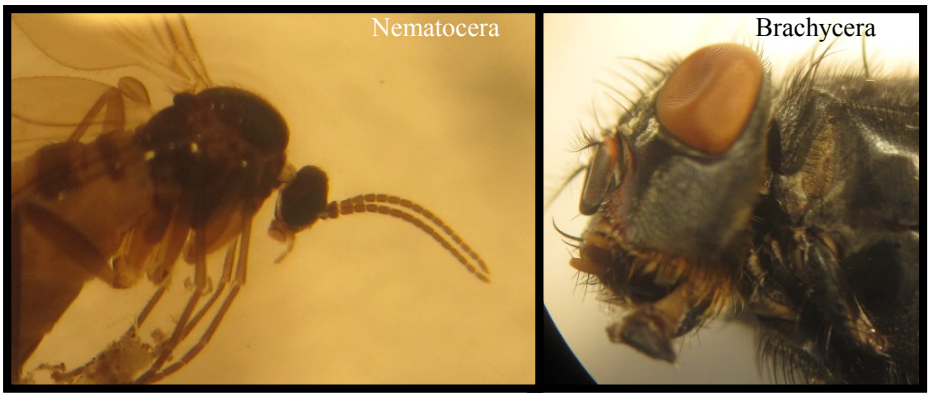
+: < 20
++: 20-100
+++: > 100

Appendix IV

Dichotomous key of the main Diptera families collected from *Canis familiaris* carrions

(adapted from Carvalho & Mello-Patiu (2008), Szpila (n.d.-d), Oldroyd (1954), McAlpine (1981) and based on personal observations. Original photos)

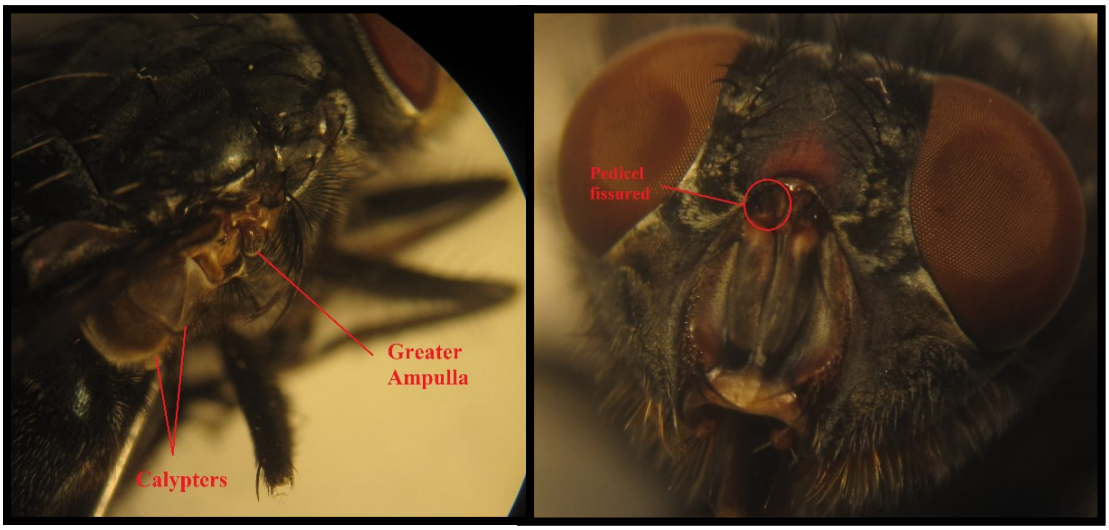
1. Antenna with more than 6 flagellomeres. Body and legs usually elongated……...…….... suborder NEMATOCERA
   Antenna with less than 6 flagellomeres, with the apical segments often modified into an arista. Body and legs usually sturdy…..…….. suborder BRACHYCERA…..2



2. Small humpbacked flies. Subcosta incomplete. Costa, subcosta and radial veins tickened, with the remainder four veins faint and straight. Cross veins absent……..... Phoridae
   Features not as described above ……... 3

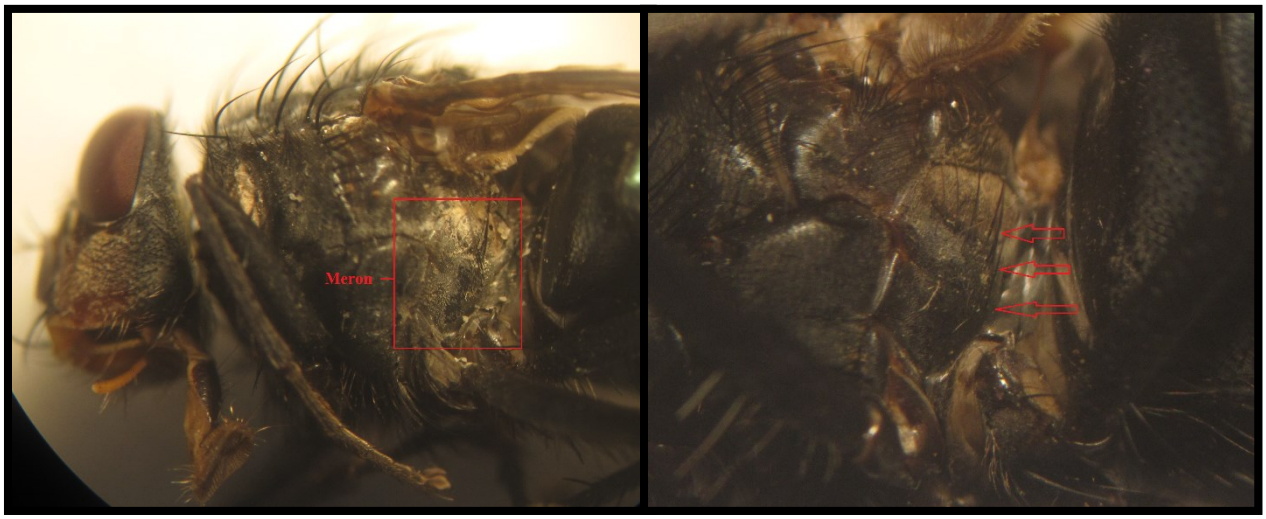
3. Calypters well developed. Second antennal segment (pedicel) fissured. Greater ampulla present.....4

   Calypters small or absent. Greater ampulla absent.....9

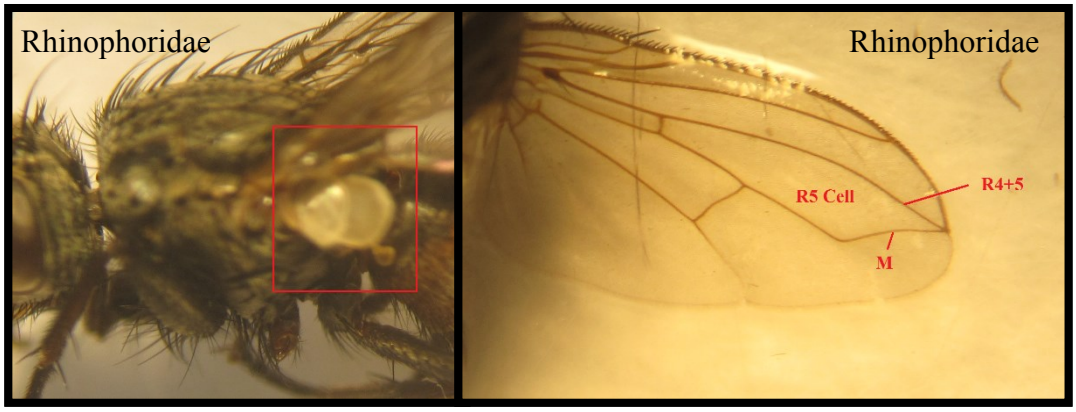


4. Meron with a row of strong bristles near the hind margin……..... 5

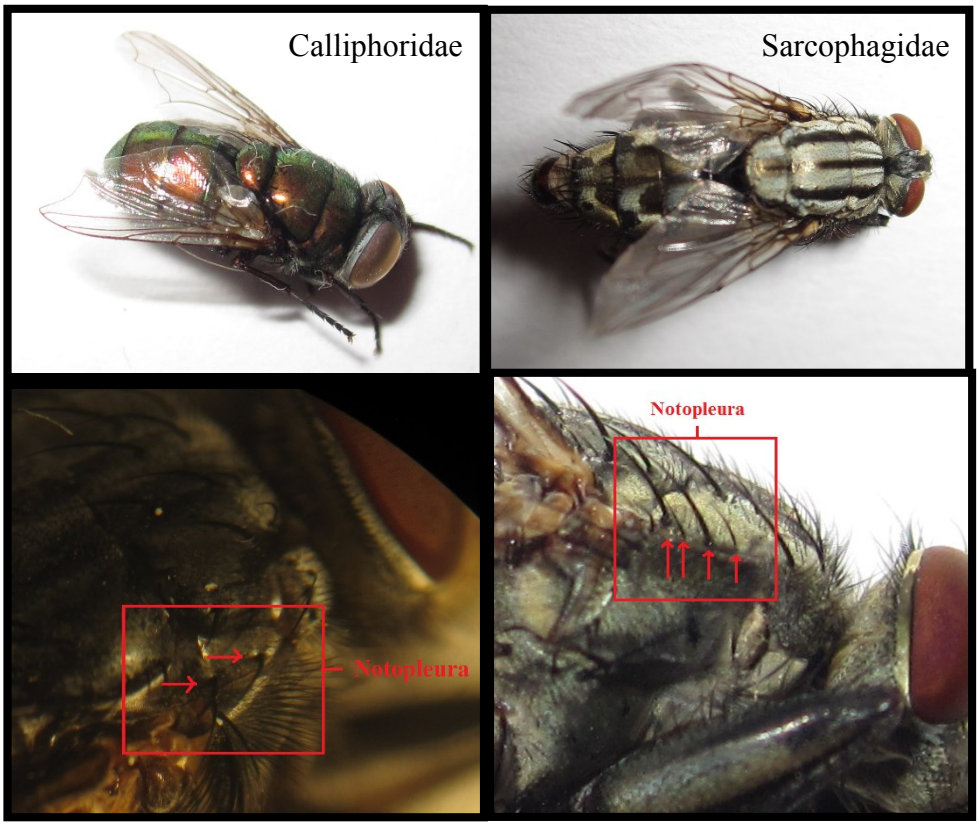
   Meron without a row of bristles, or rarely with a few scattered weak setae 7



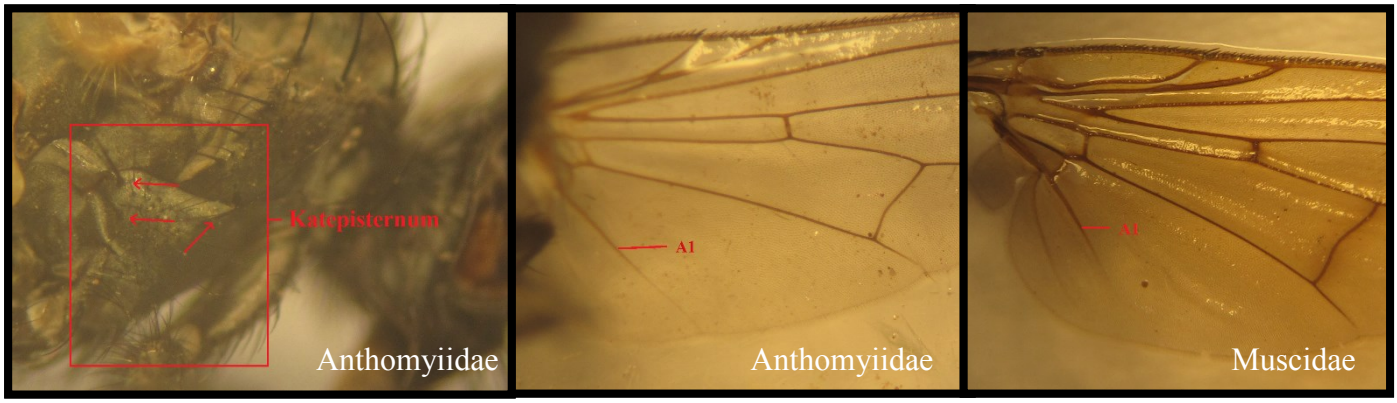
5. Calypters narrow and diverging from the sides of the scutellum. M joining R4+5 (cell R5 closed)……. Rhinophoridae
   Features not as described above……. 6



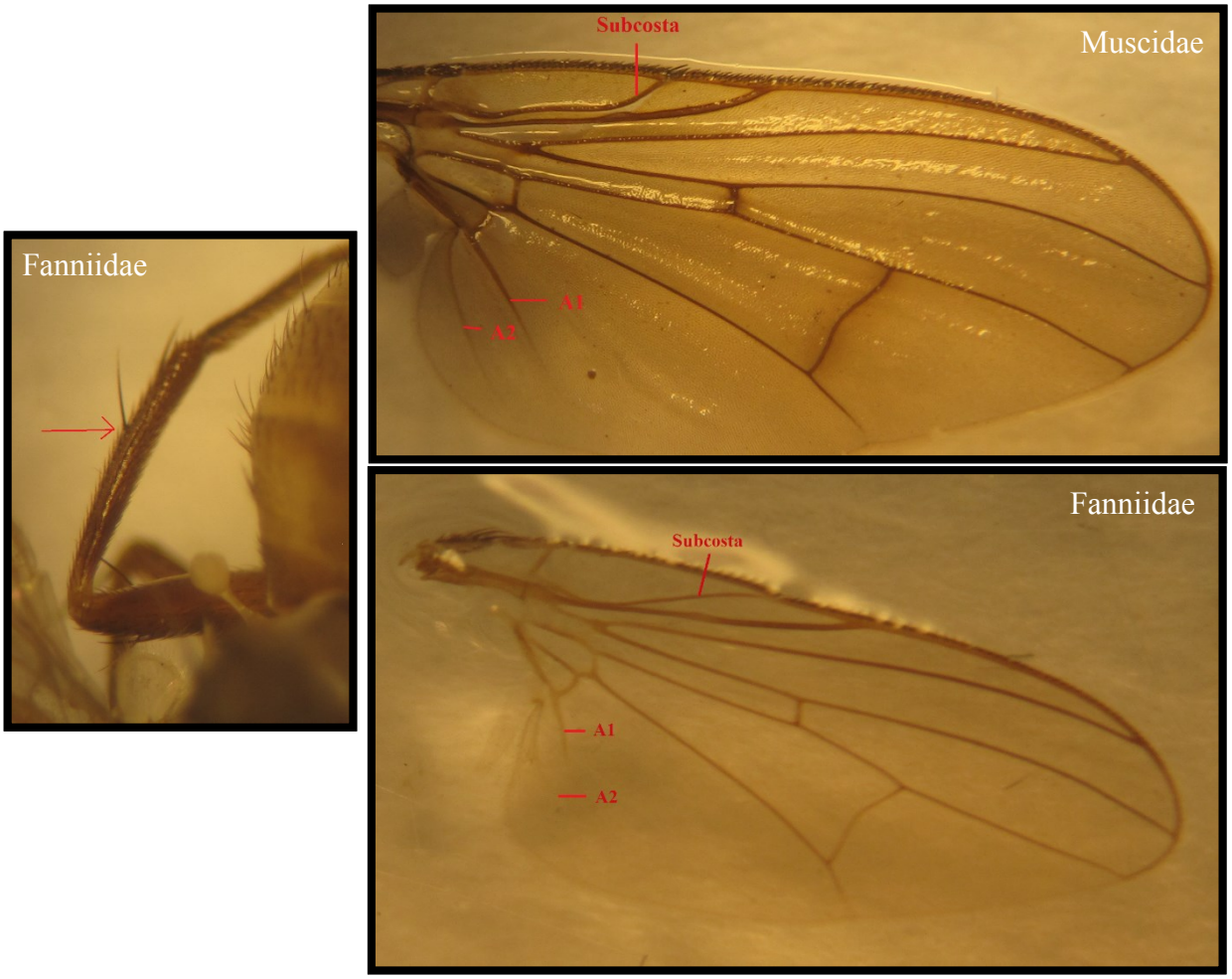
6. At least abdomen with metallic colour (green or blue). If abdomen dull, presence of golden curly setae. Notopleura with 2 strong bristles ……..... Calliphoridae
   Thorax with three longitudinal black and grey stripes. Abdomen checkered or with black spots. Notopleura with 2 strong bristles intercalated with 2 smaller ones ……..... Sarcophagidae



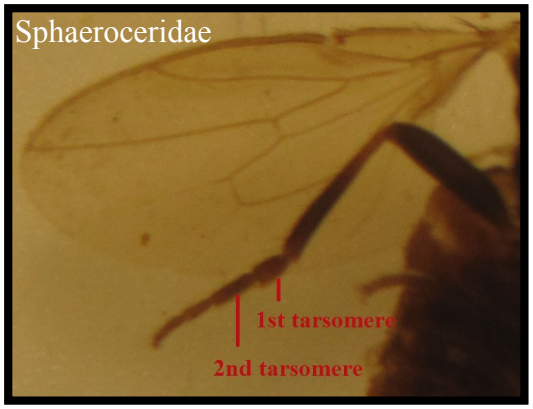
7. Anal vein (A1) reaches wing margin. Two to four katepisternal bristles present. Scutellum usually with setae on ventral apex……...……..... Anthomyiidae
Anal vein (A1) ending before wing margin ……..... 8



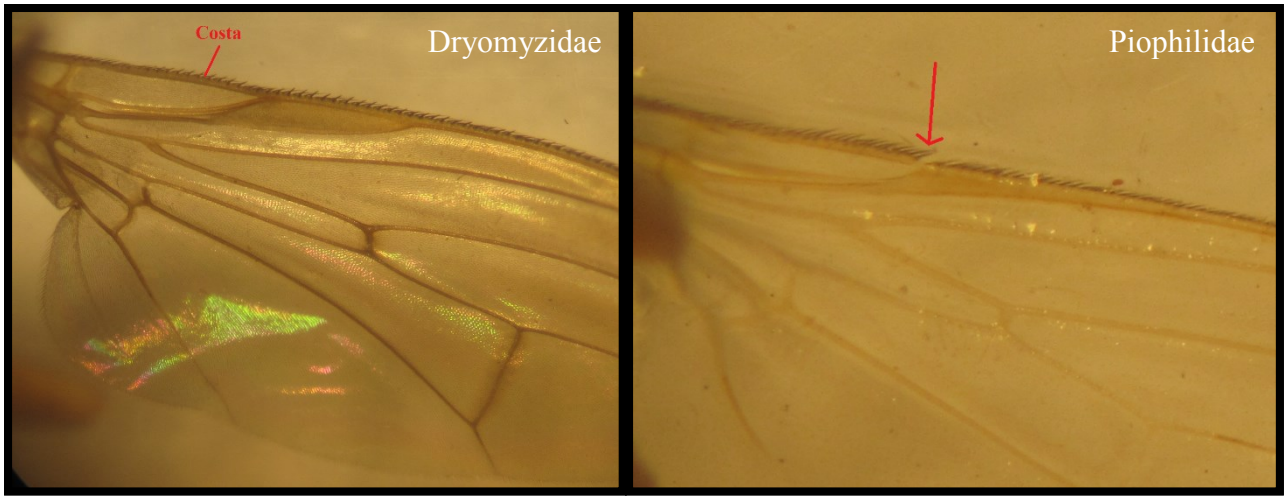
8. A1 shortened and A2 bent towards A1. Subcostal vein nearly straight on apical two thirds. Hind tibia with a strong bristle near middle of dorsal surface…….. Fanniidae
A1 not shortened and A2 straight. Subcostal vein distinctly curved on the apical third, nearly in a straight angle……..... Muscidae



9. First tarsomere of hind leg distinctly swollen and shorter than the second. Wing venation often reduced ……..... Sphaeroceridae
   First tarsomere of hind leg longer than the second ……..... 10

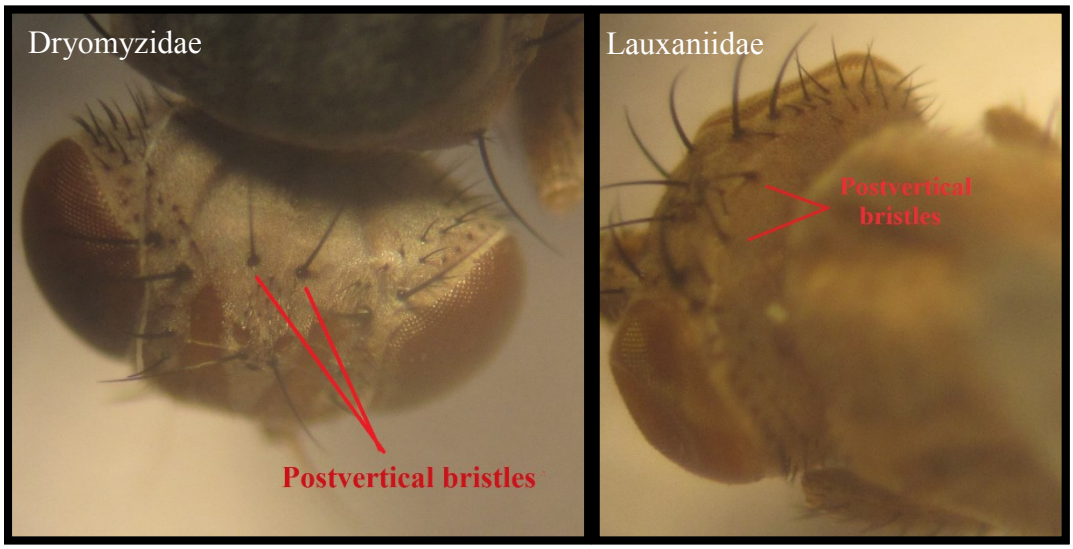


10. Costal vein complete …….... 11
    Costal vein interrupted at least once ……... 13

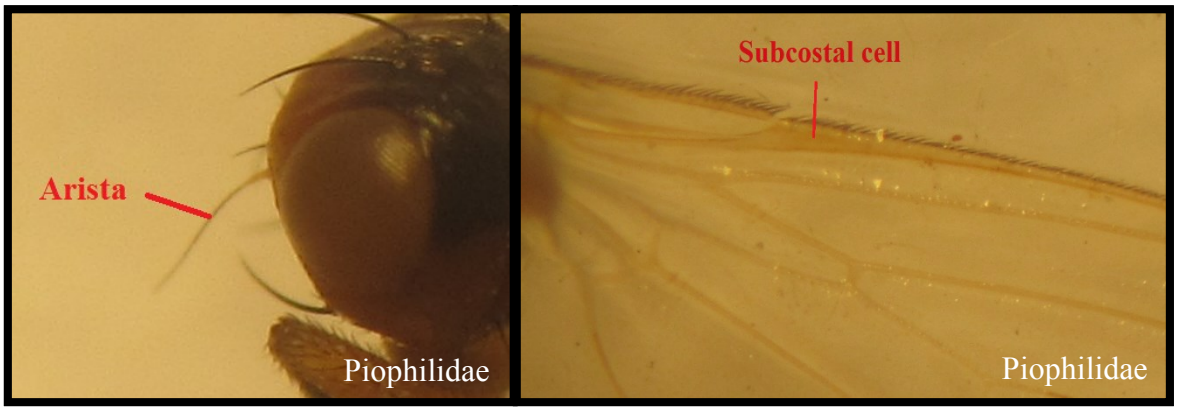


11. Small flies resembling ants, with a rounded head and constricted abdomen. Palpi vestigial. Dark spot often present near wing apex ……..... Sepsidae
    Features not as described above ……..... 12

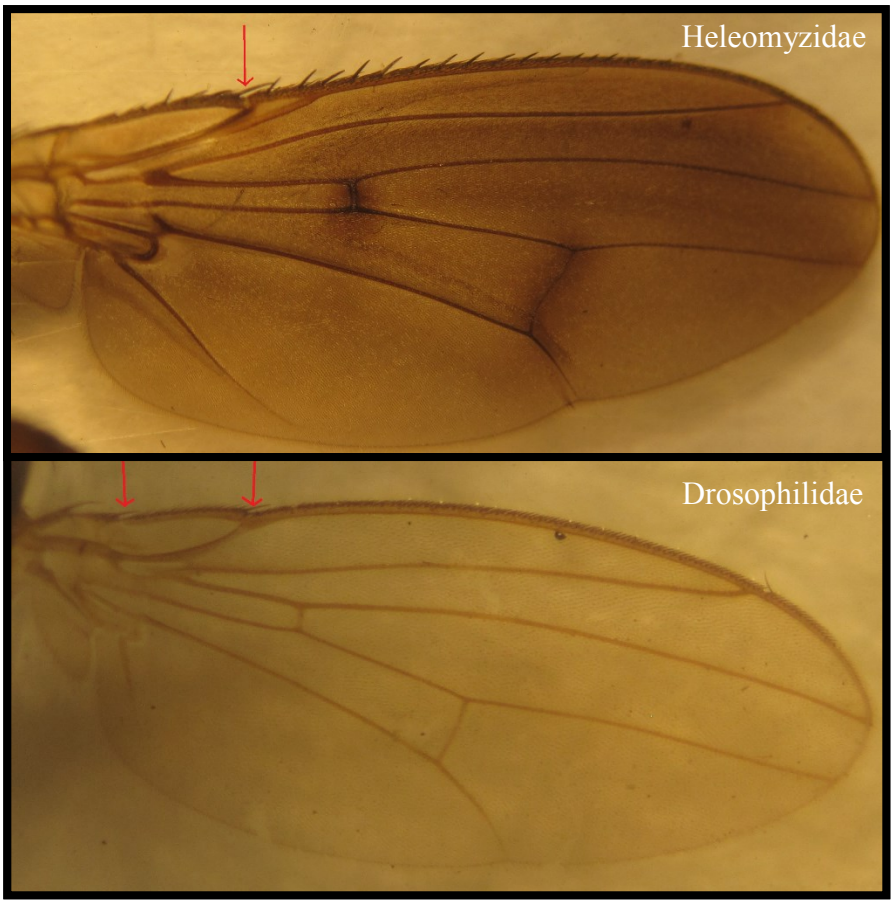
12. Postvertical bristles parallel or divergent ……...…….... Dryomyzidae
    Postvertical bristles convergent, often crossed …….…….... Lauxaniidae



13. Arista bare. Postvertical bristles absent, parallel or divergent. Subcostal cell usually pigmented ……..…….. Piophilidae
Features not as described above ……..... 14



14. Costal vein interrupted only once, and with strong spines intercalated with smaller bristles. Cross veins often clouded …….…….....……..... Heleomyzidae
Costal vein without such spines and interrupted twice. Arista with long hairs and a forked tip …….....……...……...……... Drosophilidae



Diptera fauna associated with dog and cat carcasses and their possible role as vectors of several agents of animal and human diseases

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Introduction

Diptera is one of the largest insect orders and is almost ubiquitous in its geographical distribution. A significant number of Diptera species play an important role as decomposers and scavengers. Among them, different species possess distinct adaptations to the environment and therefore occupy different ecological niches in the decomposition process. As the decomposition of a carcass takes place, so do insect species appear, colonize the remains, and eventually move on when conditions are no longer favorable. The relative predictability of this faunal succession is an object of study in the field of forensic entomology, as it enables the estimation of a post mortem interval for human or animal remains[1].

Dipterans' attraction to carrion, their adaptability to a wide variety of conditions, plus their great capability for dispersion all converge to make them an efficient means of propagation of infectious agents from a contaminated source, such as an animal carcass, to nearby animals and humans.

Diptera have been implied in the mechanical transmission of several pathogens, among them *Salmonella*, *Mycobacterium*, *Escherichia coli*, *Vibrio cholerae*, *Shigella*, *Staphylococcus aureus* and *Campylobacter*. Transmission of protozoans such as *Giardia*, *Entamoeba* and *Cryptosporidium* has also been documented, as well as of helminths, namely *Taenia*, *Ancylostoma*, *Dipylidium*, *Trichuris* and *Ascaris*[2]. In addition to their role as mechanical vectors, certain dipterans are also known to cause myiasis – the infestation of a live host's tissues by maggots. Consequences to the host can range from simple irritation, discomfort and pruritus to death on account of extensive tissue damage and secondary bacterial infection[3].

Objectives

To contribute for the study of the entomological succession in domestic animal corpses, whilst also highlighting some of these species' potential as vectors of infectious diseases agents. The present report is part of a wider study on forensic entomology, meant to compare and contrast faunal succession in different seasons and in animals with different causes of death.

Material and Methods

- This study was performed at the Faculty of Veterinary Medicine of the University of Lisbon, Portugal.
- Modified Malaise traps were placed along the years 2014 and 2015 in order to capture insects attracted to cat and dog carrion (Fig.1; Fig. 2).
- A total of 4967 Diptera were collected throughout the decomposition process at regular intervals until the remains were fully skeletonized.
- Identification of the specimens was based on morphological features.

Figure 1: A modified version of the Malaise trap used to capture insects attracted to a carrion. A glass bottle was coupled to the upper net to capture flying specimens, and a plastic container was placed at the bottom to capture crawling insects.

Figure 2: Detail of the bottle used to collect flying insects. Antifreeze was used to preserve the specimens.

DNA of the specimens being reported for the first time in Portugal was sequenced in the following protocol: the front legs of each specimen were collected and had their DNA extracted using the Chelex® method. The Cytochrome Oxidase I gene (COI) was then amplified by PCR. The resulting products were checked by gel electrophoresis and sent to StabVida for sequencing. Additionally, the Maximum Likelihood algorithm was used to build a phylogenetic tree in order to illustrate the phylogenetic relationships of these specimens.

Results and Discussion

The specimens collected were predominantly of the Brachyceran infraorder Muscomorpha. By comparison, the amount of Nematocera collected was almost negligible and mostly limited to the final stages of decay.

The graph in Fig.3 depicts the pattern of entomological succession on one of the cats, whilst also summarizing the most frequent families collected. Two peaks are present: the first was at a very early decay stage, mainly comprising flies of the Calliphoridae and Muscidae families. The second took place in a more advanced decay state, at which point three species not yet reported in Portugal were found, those being *Lucilia cuprina* (Wiedemann, 1830), *Sarcophaga aratrix* Pandelle, 1896 and *Pollenia leclercqiana* (Lehrer, 1978).

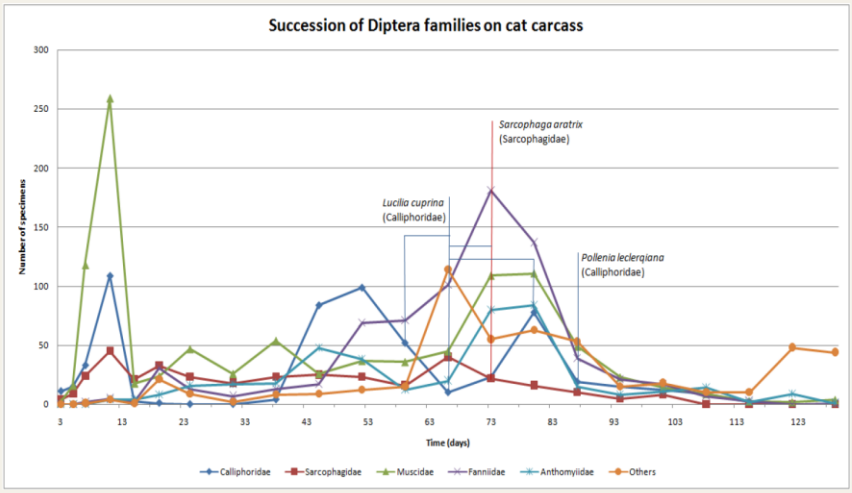

Figure 3: Entomological succession in a cat carcass from August to December 2014, displaying a few of the most significant and representative Diptera families collected. Specimens belonging to the three new species being reported were all found when the carcass was in a state of advanced decay.

Lucilia cuprina has since long been implicated in the development of myiasis, causing a condition known as sheep blowfly strike, particularly in warmer climates such as Australia and southern Africa[4]. Barring Spain, this is the first time it's reported in Europe[5]. These facultative ectoparasites lay large batches of eggs on the host's fleece or open wounds and the resulting larvae feed extensively on the epidermal tissues and skin secretions, causing weight loss, strong odor (which attracts even more flies) and even death by ammonia poisoning[4]. Additionally, the extensive use of pesticides for both treatment and prevention is leading to an increasing insecticide resistance, creating the need for new measures to counter this condition.

Interestingly, upon analysis of the eight specimens sequenced, it was observed (Fig. 4) that *L. cuprina* samples split into two clusters, suggesting a certain degree of phylogenetic difference. One possible explanation lies on the capability of *L. cuprina* to produce hybrids, specifically with *L. sericata*. These two species are very similar in morphology and, due to allele dominance, hybrids tend to inherit the physical traits of *L. cuprina*, easily leading to misidentification[6]. It should also be noted that *L. cuprina* exhibits some degree of intraspecific morphological variability dependent on geographic location, enough that two subspecies are acknowledged – *L. cuprina cuprina* and *L. cuprina dorsalis*. These subspecies also interbreed and their intermediate forms are believed to be common[3], which may also help to explain the phylogenetic difference found.

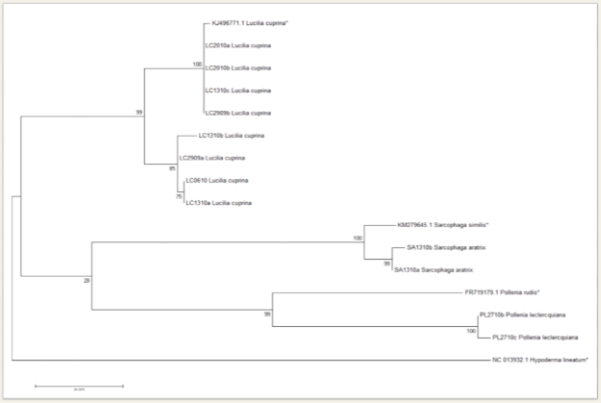

Figure 4: Phylogenetic tree obtained by the Maximum Likelihood algorithm, with statistical support by bootstrapping with 1000 replicates. The specimens marked with * were obtained from GenBank (www.ncbi.nlm.nih.gov) for a comparative analysis. *Hypoderma lineatum* was used as an outgroup.

Conclusion

Among the specimens collected and identified, three Diptera species that were previously unreported in Portugal were found: *Lucilia cuprina*, *Pollenia leclercqiana* and *Sarcophaga aratrix*.

L. cuprina in particular is notable both as an indicator in the field of forensic sciences and as an important myiasis agent in domestic animals, sheep being the most commonly affected. Also known to be resistant to several insecticides used in its control and capable of interbreeding with the pre-existing *L. sericata*, this species has the potential to cause a significant negative impact on both productivity and animal welfare, should it happen to gain a foothold and disseminate throughout the country.

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