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Necrophagous species living in Calabria and their application in forensic field

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To my family

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SUMMARY

INTRODUCTION

Forensic entomology is a new field of applied entomology that studies the insects involved in human activities. This science is applied in three branches: *urban entomology*, which focuses on the insects that live in a human environment and infest private houses and public buildings. This branch also investigates the species that cause myiasis, diptera larvae infestation in living human beings and animals; *stored products entomology*, which studies the insects breeding in badly preserved foodstuff; *medico-legal (or forensic) entomology*, which focuses on the insects that thrive on human and animal corpses[1].

The medico-legal entomology pays particular attentions to the insects that occur on corpses discovered in unknown circumstances, indoors and outdoors. At the point of death, a corpse becomes a trophic resource for several species of insect, that start to settle on it until decomposition is completed. The insect activity on a corpse is important to study in order to establish how much time has passed between death and the discovery, i.e.

[1] Byrd&Castner, 2010

evaluate the PMI (*post mortem* interval)[2], as each species infects the corpse at different stages of decomposition. Thus, since the body appears in the environment, several ecological categories start to colonize it, following a normal ecological succession[3]. Necrophagous species (above all Diptera Calliphoridae) start the insect succession; in fact they can reach a corpse from distances far away, through smelling the decomposition odor. This family begins by laying eggs in natural cavities of the body in order to have available food for the hatching larvae. In general Calliphoridae lay eggs immediately after death, so the age of their coincides with PMI. As the body continues the decomposition process, several other species appear and each one having a different ecological role. Following this necrophagous, saprophagous, predator and parasite species occur on the cadaver, and insect activity accelerates the decomposition process. When skeleton stage is reached, only a few coleopteran and mites species remain, using the corpse to feed on dry skin[4,5,6].

Time of colonization, duration of settlement, species composition and duration of decomposition process are influenced by several factors, such as environmental temperature and humidity, season, geographic position,

[2] Hall, 1990 in Byrd&Castner, 2010
[3] Megnin, 1894
[4] Megnin, 1894
[5] Smith, 1986 in Amendt *et al*., 2004
[6] Campobasso *et al*, 2001

habitat type, corpse exposure (shade, sunlight, indoor, outdoor), presence of competitors species[7].

AIM OF THE STUDY

In Italy this science is still rarely applied, only little knowledge about necrophagous species is available. No data concerning South Italy is known, above all, for the Mediterranean areas. Moreover, the influence of environmental factors and species competition are unknown, thus there isn't an official data base for necrophagous species in Italy.

The aim of this study is to discover which insect species play the role of necrophagous in Cosenza province, focusing on the Calliphoridae family as main indicators of early PMI. We investigated the ecological, seasonal and biological preferences of Calliphoridae species.

The first step undertaken was to study which species colonize a corpse in the Cosenza area, using animal models to simulate human corpses, over the course of several seasons. This study was carried out to understand the difference in species composition over different seasons and also to gain better knowledge concerning the influence of seasonal variation on decomposition time insects activity and also if there are other factors, such

[7] Byrd&Castner, 2010

as interspecific competition or predation, which could affect a normal succession process. Moreover this study looks to learn if insect activity can also cause thanatological changes on the bodies.

The second step was to study if Calliphoridae species have different ecological preferences, at which points of the seasons the species appear, trapping them in three different areas with the help of bait bottle traps over a period of two years.

The final goal of this study is to have a global knowledge about Calliphoridae habits that live in the Cosenza province, in order to create the first database for South Italy that can be applied to real cases.

MATERIALS AND METHODS

Entomological successions

In order to study insect succession and time of colonization, four pig carcasses (*Sus scrofa* L.) were used as animal models, each presented for each season. They were taken from a pig farm near the study area and immediately after death placed in the Botanical Garden of University of Calabria. Carcasses have simulated human bodies dead in an outdoor environment. Since the death, daily inspections took place in each seasonal

experiment. Environmental data (temperature, humidity), thanatological changes and insect activity were noted every two hours every day. Adults and larvae were collected in order to identify the species. When the skeleton stage was reached, the remains were moved and entomological finds were collected from surrounding area. The collected samples were identified and ecological categories were defined for each season.

Sampling of necrophagous species

For the sampling, three areas with different urbanization/naturalness level were chosen for this study. The *wild area* was represented by a wood of *Fagus* sp, situated at about 900 meters above sea level; *rural area*, represented by the Botanical Garden of University of Calabria, was a transition area because its naturalness is influenced by surrounding urbanization. The *urban area*, represented by the city of Cosenza- Rende, is a none-natural area. In each area, eight bait bottle traps were positioned and replaced each month. In each replacement, samples were separated from adults and larvae, and adult families were sorted. The Calliphoridae family was identified at species level. Data analysis for Calliphoridae species were performed with IBM SPSS Software and Past Software.

RESULTS

Seasonal successions

In the autumn, summer and spring experiments four stages of decomposition were identified (fresh, bloated, decay and dry) (figures 22, 26, 36). In the winter experiment five stages of decomposition were recognized (fresh, bloated, decay, adipocere and dry) (figure 31). In each season, the entire decomposition process had different durations, due to the influence of environmental conditions. Ecological categories occurred on the carcasses presenting different species composition according to the phenology of the species. For the necrophagous category, the Calliphoridae species were the dominant taxon, represented by different species for each season. According to Dominance Index, calculated for the sampled individuals (table 1), *Calliphora vicina* Robineau-Desvoidy 1830, *Calliphora vomitoria* (L.) and *Lucilia caesar* (L.) were dominant in the autumn experiment; in the summer investigation *Chrysomya albiceps* (Wiedeman 1819) was the most abundant species; in the spring investigation *L. caesar* and *Lucilia sericata* (Meigen 1826) were the dominant species; in the winter experiment a very low number of *Calliphora* spp were observed because of the low environmental

temperature, thus the role of necrophagous in this investigation was played by Coleoptera Silphidae *Thanatophilus rugosus* L. and *Thanatophilus sinuatus* (Fabricius 1775). Furthermore species composition of the other ecological categories and their time of arrival were different in each investigation, as reported in the figures 25, 29, 35, 41. We observed the important role of Hymenoptera Formicidae that acted as predator towards the Calliphoridae larvae and also caused *post mortem* artifacts on the carcasses skin (figure 30).

Sampling of necrophagous species

In two years, several Diptera families were trapped in the three areas, as reported in table 2. The Calliphoridae family was the most abundant for *wild area* (36,57%) and *urban area* (30,86%). In *rural area* Calliphoridae represented the 18,99% of sampled families, and in this area the most abundant was the Platystomatidae family (27,63%).

Data analysis were performed for Calliphoridae because this family is the most important in PMI estimation. Calliphoridae species are identified as *Calliphora vicina* Robineau-Desvoidy 1830, *Calliphora vomitoria* (L.), *Chrysomya albiceps* (Wiedeman 1819), *Lucilia ampullacea* Villeneuve 1922, *Lucilia caesar* (L.) and *Lucilia sericata* (Meigen 1826).

From Correspondene Analysis (CA) the species-habitat associations can be observed in graphic 8. As shown, *C.vicina* and *L.caesar* are related to *urban area*; *L. ampullacea*, *Ch. albiceps* and *L.caesar* are related to *rural area*; *C.vomitoria* is the most representative for *wild area*.

According to Synanthropyic Index (table 10), the most synanthropic species is *L. sericata* that was never trapped in the *wild area*, instead *C. vomitoria* is not related to the *urban area* but is strongly related to the *wild area*.

Concerning the temporal variability, phenology of each species, it is different in each area (as shown in the graphs 9, 10 and 11), probably because the average temperatures for each area are different. In general *Calliphora* spp are more active during the cooler months, but not in the severe winters of *wild area* (winter average temperature 3,56 ± 1,08°C). In this area *Calliphora* species shift their activity in the spring and autumn months. *L. caesar* is the most abundant species for *rural area* for almost the entire sampling period except for winter months when the *C. vicina* becomes the dominant species. *L. sericata* is the most abundant in summer months in *urban area*, the rest of year, *C. vicina* is the dominant urban species. *L. ampullacea* is related to *rural area* during spring, *Ch. albiceps* represents the species with the lowest number of individuals, although it had colonized the carcass in the summer investigation. Also, *Lucilia*

species show a strong correlation with environmental temperatures, as reported by the Pearson Correlation in table 25. From this analysis, it appears that the abundances of *Lucilia* spp are positively related with temperatures, instead for the other species there isn't a correlation. Maybe *Ch. albiceps* is represented by a very low number of individuals and *Calliphora* spp are more influenced by the habitat then the temperature.

CONCLUSIONS

This research has collected important data for forensic entomology in Calabria. Now we are able to know which species we expect to find in human corpses and which ones are affected by the surrounding environment and possible competitors.

The important role of Calliphoridae was evaluated, along with the ecological and biological factors that affect species colonization were observed and analyzed. We also observed the important role of Hymenoptera Formicidae in *post mortem* artifact creation.

We studied the ecological habits of Calliphoridae of forensic importance, and have a general knowledge concerning the habitat preferences and the period of species activity.

Future studies will focus on the life cycle of species, breeding dipteral colonies in laboratory conditions in order to time the development cycle duration at different temperatures, and complete the biological aspects useful in forensic investigations.

AIM OF THE STUDY

Medico-legal entomology (or forensic entomology), is a developing science in Italy, although in the rest of Europe and in America, it is already fully applied. This science investigates in both medical and forensic fields. The insects involved in forensic cases, above all Diptera Calliphoridae, are attracted to the decomposing organic matter, thus allowing to colonize human and/or animals corpses. Forensic entomology can be applied through several ways because with the presence of the insects on the corpses, it is also possible to understand if the body was displaced or if it had suffered traumas or mutilations *ante mortem*. Moreover, lots of the necrophagous species are involved in myiasis cases, infestations of diptera larvae on living individuals that are neglected in hygienic conditions. Entomological finds represent important investigative tools in cases of unknown death and in myiasis in vulnerable individuals (elderly, children and animals). Insect evidence is considered valid proof to present in court for the correct interpretation of a death scene or for negligence cases.

Necrophagous species activity is strongly affected by geographical position, due to the fact that species composition is different according to their spatial distribution.

In Italy this science is rather new, totally unknown for Southern Italy. The lack of information represents the starting point of this study. Nowadays there is not an official data base to use as reference, thus the aim of this research was to evaluate which insect species living in the Cosenza province are involved in corpses colonization and myiasis cases. This study was carried out following two steps:

1. Studying which species are involved in cadavers colonization in several season, using animal models;
2. Studying the environmental preferences of necrophagous species through a two-years sampling in three different areas characterized for a different level of urbanization.

The final aim of this study is to realize the first official data base facilitating the application of experimental data in real cases.

Chapter 1

INTRODUCTION

Forensic entomology is a rather new branch in the forensic and medico-legal field. The science that applies the entomological knowledge in this field is **forensic entomology**, which studies the species of insects and other arthropods involved in civil and criminal disputes and/or involved in human environment infestation. In turn, this science is divided into three branches: *stored products entomology* that investigates arthropods that infest badly preserved foodstuffs; *urban entomology* that studies the species infesting human environment. Myiasis cases also belong to this branch. Negligence on vulnerable individuals (elderly, children and animals) in public or private building, when hygienic conditions are unsatisfactory, is pursued by the law and myiasis gives proof of the time of larvae infestation, along with the time during which the individual was neglected[8] along with information concerning the duration of neglection; *medico-legal entomology* (or *forensic entomology*) that studies the insect species involved in criminal investigation such as homicide, suicide, sudden death

[8] Anderson and Huitson, 2004 in Byrd&Castner, 2010

etc., and in criminal offenses (physical abuse, smuggling, drugs)[9]. This field mainly investigates the duration of time a corpse was exposed to the environment (*post mortem* interval or PMI) and the area in which the corpse was discovered, using insect evidence found on it and in the surrounding areas[10].

Forensic entomology represents an important investigative tool in case of sudden death or discovery of corpses in advanced stages of decomposition, because since the moment of death several insect species colonize the corpse feed on the decomposing organic matter, putrefactive liquids or preying other species.

THANATOLOGICAL METHODS TO EVALUATE TIME OF DEATH

The decomposition of a body is a continuous process that starts with the death and ends when the corpse reached the skeleton stage. The time of death (*post mortem* interval or PMI) estimation is usually calculate according to thanatological changes occurred on the body after death[11]. The fisical-chemical changes that start the decomposing process appear immediately or shortly after death and progress in an orderly manner until

[9] Byrd&Castner, 2010
[10] Hall, 1990 in Byrd&Caster, 2010
[11] Goff, 2009

complete disintegration. The duration of thanatological changes is strongly influenced by unpredictable endogenous or environmental factors[12]. PMI evaluation is an estimation based on putrefactive phenomena that occur on the body, not an absolute value, so its precision decreases with the passing of time[13,14].

Thanatological changes which occur on the body after death are mainly *algor mortis* (body cooling), *rigor mortis* (cadaverous rigidity), *livor mortis* (lividity) and putrefaction.

Algor mortis

Body cooling is the more useful element to estimate time of death during the first *post mortem* 24 hours. With the end of metabolic activities that produce heat (thermogenesis), the body undergoes a progressive lowering of the temperature, which is dispersed in the form of heat through the surface of the body. The lowering of the body temperature continues until it reaches the same value as the environment. The PMI estimation can be made knowing the speed of lowering, namely the gradient. This parameter can be calculate if it is possible to make temperature measurements at

[12] Pounder, 1995
[13] Goff, 2009
[14] Puonder, 1995

precise time intervals. The temperature gradient starts when the inner part of the body begins to cool, and this parameter can vary from an instantaneous time to several hours. The cooling speed of the body depends on several factors, such as the size of the body (larger bodies dissipate heat quickly because the dispersion surface is greater), the presence of clothes (which slow down the process because they retain heat), air humidity and the immersion of the body in water (which instead promote the cooling rate). After this step, the body temperature will increase again because of metabolic activity of bacteria and other organisms present inside the body[15].

Rigor mortis

The cadaveric rigidity is due to the chemical reactions that occur in the muscles. After death, the muscles relax completely, and because the muscle tissues become anoxic, the cells work anaerobically to produce ATP. The final product of anaerobiosis is the lactic acid that decreases the cell pH level and causes the glycogen desegregation and ATP diminution. The ATP function in the muscles is to inhibit the link between actine and myosin. After anaerobiosis, the ATP diminution causes the actine-myosin link,

[15] Pounder, 1995

inducing the muscle rigidity. The rigor mortis appears 2-3 hours after death, the time to resolution vary from 24 to 84 hours, according to the factors which influence the duration. Generally, the rigidity occurs firstly in the face muscles and continues in a cranio-caudal sense extending first to the upper limbs and then to the lower limbs. The total rigidity of the body remains stationary for about 36-48 hours from death and finally the muscles return to relax following the same cranio-caudal order.

In general, *rigor mortis* duration is affected by environmental temperature and muscle activity before death[16,17].

Livor mortis

The post mortem lividity consists of the brownish coloration of the body, it's a physical event due to the interruption of blood circulation. The blood doesn't receive the heart's thrust, so it starts to migrate to the lower parts of the body according to the gravity, forming clots defined *hypostasis*. The hypostasis pass through an initial phase during which the blood is still semiliquid and they can migrate if the body is displaced. When the clot becomes permanent, the hypostasis become fixed and do not follow the

[16] Goff, 2009
[17] Pounder, 1995

body movements. The *livor mortis* appears from half an hour to two hours after death and ends about 9-12 hours after death[18,19].

Putrefaction

The putrefaction is the most important process degrading the organic matter after death, due to the activity of bacteria and enzymes. The cellular autolysis leads to the gradual dissolution of tissues in gasses, liquids and mineral salts[20,21,22]. As reported by several authors, the decomposition process has several stages, which occur gradually. In outdoor environments, or in uncontrolled conditions, a decomposing body represents a source of food and shelter for many species of insects and arthropods, which contribute to the decay of organic matter, sometimes accelerating the process. When the corpse reach an advanced decomposition stage, the thanatological methods are not sufficient to estimate the PMI and insect evidence is essential in determining the time of death[23]. The body decomposes through several stages, each one attractive

[18] Goff, 2009
[19] Pounder, 1995
[20] Campobasso *et al*., 2001
[21] Gennard, 2007
[22] Pounder, 1995
[23] Byrd&Castner, 2010

for different insect and arthropod groups[24,25]. The stages of decomposition which occur on the corpse can be reassumed in:

Fresh stage: it starts at the moment of death and ends with the first signs of bloating. This stage attracts necrophagous species which use natural cavities of the body as oviposition sites. In general the first colonizers are the Diptera Calliphoridae and Sarcophagidae[26,27];

Bloated stage: it starts the putrefaction. Anaerobic bacteria living inside the body start the tissues digestion. The gasses emitted by their metabolism confer the state of swelling of the body. Diptera Calliphoridae are mainly attracted by the putrefaction smell, and also Coleoptera occur in this stage preying diptera larvae[28].

Active decay stage: it starts when the skin lacerates and gasses are released outside. The decomposition process goes on leading to the formation of butirric and caseic acids that attract Diptera Piophilidae. During this stage the maggot mass is conspicuous on all the body and predator Coleoptera are more abundant (Staphylinidae, Histeridae, Silphidae)[29,30].

[24] Gennard, 2007
[25] Goff, 2009
[26] Gennard, 2007
[27] Goff, 2009
[28] Gennard, 2007
[29] Gennard, 2007
[30] Goff, 2009

Post-decay stage: the body reduces in skin, cartilage and bones. Diptera are no more the dominant taxon, the majority of them have already completed their life cycle. During this stage the corpse is attractive for several species of Coleoptera which feed on the decomposing remains (Dermestidae, Cleridae)[31,32].

Skeletal stage: only bones and hair remain on the body, in general this stage is not colonized by specialist insects but by accidental species that seek refuge in the dry remains.

The time of decomposition vary strongly with the size of the body, with environmental factors and the conditions in which the body stay. PMI estimation becomes more difficult and less precise using only thanatological methods[33].

ENTOMOLOGICAL METHODS TO EVALUATE PMI

The time since death (post mortem interval or PMI) is the most important information to obtain in cases of homicides or in unknown death investigations[34]. Even in cases of natural death, this information is useful

[31] Gennard,
[32] Goff,
[33] Byrd&Castner,
[34] Catts, 1990; Gebert, 1996 in Byrd&Castner, 2010

for inheritance and insurance reasons[35]. Often, forensic investigations concentrate on maximum PMI determination, or the time interval between the discovery of body and the moment that the deceased was seen alive for the last time. This method can be applied only if the deceased can be identified[36]. Legal medicine can only estimate the time of death accurately during the first 72 hours after death, because after this period the body undertakes drastic changes and thanatological inspections are not sufficient to determinate the PMI. When a corpse is discovered in the advanced stage of decomposition, it is not simple to establish the time of permanence of the body in the environment. Moreover, with traditional investigations, it is not always possible to know if the place of death coincides with the place of discovery[37]. The use of insect evidence turns out of fundamental importance in determination of time of death because, as observed through a lot of research, the necrofauna activity shows PMI-dependent processes[38]. Necrophagous insects are attracted to decomposing organic matter, not by living individuals, except in particular cases. From their presence on a body, it is possible to estimate **minimum PMI**, or the time elapsed between the moment of death and the moment of discovery of the body[39].

[35] Henssge *et al.*, 1995 n Byrd&Castner, 2010
[36] Henssge *et al.*, 1995 in Byrd&Castner, 2010
[37] Marchenko, 2001
[38] Hall, 1990 in Byrd&Castner, 2010
[39] Smith, 1986 in Byrd&Castner, 2010

Entomological method to evaluate PMI is based mainly on two approaches. The first one concerns the succession with which several ecological categories colonize the body, replacing themselves as the decomposition goes on. This method is especially utilized when the discovered corpse is in advanced stage of decomposition, considering the ecological category present at a precise stage. The succession method is used to estimate both minimum and maximum PMI[40].

The second method concerns the estimation of larval age of immature that feed on the body tissues. The first colonizers occur almost immediately after death, laying the offspring in the natural cavities of the body or in the open wounds. The young feed on the tissues for the entire larval cycle, so the age of samples indicates the time of the body exposure in the environment. This method is more accurate and is very useful for estimation of minimum PMI[41].

Entomological successions

In natural environments, the ecosystems modify as the time passes changing the species composition of communities that live in it. The same

[40] Shoenly *et al*., 1992 in Byrd&Castner, 2010
[41] Smith, 1996 in Byrd&Castner, 2010

happens with the cadaveric resource, that modify with the decomposition process, attracting gradually different groups of species. Since death, the corpse undergoes several physical, chemical and biological changes, modifying its thanatological conditions through several stages of decomposition until the complete skeletonization[42]. Each stage is attractive for different insect species which colonize the body at different times but always related to the stage of degradation of the trophic resource. The experiments carried out by Francesco Redi[43], that refuted the aristotelian theory about spontaneous generation, focused the attention on the existence of necrophagous species attracted by decomposing organic matter. The relation between insects and corpses, although known since the ancient Egyptians, already applied in China during XIX century and observed during mass exhumations in France and Germany during XVIII and XIX centuries[44], starts to be studied in detail by P. Mégnin at the end of 1800[45]. In 1894, in fact, after the publication of the book *La faune des cadavres*, it is highlighted how different insect species are attracted to decomposing bodies and how they are replaced as the decomposition continues, due to the fact that each one is trophically related at a precise thanatological

[42] Coe and Curran, 1980; Henssge *et al*., 1995; Van den Oever, 1976 in Byrd &Castner 2010
[43] Redi, 1875
[44] Benecke, 2001
[45] Mégnin, 1894

condition. Mégnin observed which species occur on the corpses and which role they play, attributing them to several ecological categories grouped in eight teams of *travailleurs de la mort*[46](figure 1).

Figure 1. The *travailleurs de la mort* described by Mégnin (from Benecke, 2002).

From the Mégnin observations, several studies about insect colonization were carried out, and still today the successions study represents the starting point for entomological investigations in forensic field[47,48]. The eight teams of *travailleurs de la mort* described by Mégnin, defined

[46] Mégnin, 1894
[47] Byrd&Castner, 2010
[48] Gennard, 2007

successional waves[49], are nowadays counted in four main ecological categories *necrophagous, predators & parasites, omnivorous, accidental*[50] (figure 2). The first colonizers, pioneers in the cadaveric succession, are represented by necrophagous, in which belong Diptera Calliphoridae and Sarcophagidae families, which lay eggs or directly larvae in natural cavities of the body or in the wounds. Larvae hatch and feed on the decomposing tissues. As the decomposing process continues, other Diptera families occur in the body, such as Muscidae, Fannidae, Piophilidae, Phoridae, attracted by advanced decay stages. The last stages of decomposition are attractive for Coleoptera Dermestidae and Cleridae that feed on dry rests of skin and bones[51].

Predators and parasites feed on other insect or arthropod species that are present on the corpse. The most important representatives are Coleoptera Staphylinidae, Silphidae, Nitidulidae, Histeridae, although some Silphidae species can act also as necrophagous in particular circumstances[52,53]. Also schyzophagous species belong to this category, which first feed on the body and when they reach mature larval stages feed on other larval species,

[49] Tuschetto&Vanin, 2004
[50] Smith, 1986 in Amendt *et al*., 2004
[51] Campobasso *et al.*, 2001
[52] Bonacci *et al.*, 2010
[53] Campobasso *et al*., 2001

such as *Chrysomya* genus (Calliphoridae)[54]. Omnivorous species, which include Hymenoptera Formicidae, Vespidae and some Coleoptera species, feed on both the body and other colonizers.

Finally, accidental species, such as Springtails and Arachnids, use the body as extra resources or occasional shelter[55].

Figure 2. Entomological succession on an animal carcass (from Klein, 2005).

Based on the presence of a certain ecological category on the body, it is possible dating the period of the exposure in the environment, this method is used above all in cases of discovery of bodies exposed for a long period

[54] Campobasso *et al.*, 2001
[55] Smith, 1986 in Amendt *et al*., 2004

of time and/or in cases of death happened in unknown circumstances[56]. Although the modalities of replacement of categories are rather predictable, specie composition varies for each category because it affected by the environment in which the corpse is exposed and time of arrival of each species and time of stay depend on their ecological requirements[57].

A correct identification of the species found on the body represents the starting point in a crime scene interpretation and PMI evaluation[58]. Several variables affect the process of corpse colonization, in particular the modalities of succession vary depending on geographical position, the body exposure (sunlight, shade) and the kind of habitat in which the body is placed[59].

Geographical position is one of the most principal factors that affect specie composition of ecological categories in a succession. The biogeoclimatic zone defines a particular habitat, the vegetation, the soil type and meteorological conditions of the area. This affects both the presence and the phenology of the colonizer species. Moreover, thanatological processes are different according to the same factors, along with the time of arrivals of the involved species, which vary between regions[60].

[56] Byrd&Castner, 2010
[57] Byrd&Castner, 2010
[58] Campobasso *et al*., 2001
[59] Byrd&Castner, 2010
[60] Byrd&Castner, 2010

The season has a strong influence on weather conditions and on the typical flora and fauna characterizing a region, in other words from season depends phenology of the species. Many necrophagous species have different abundance depending on the season because their activity takes place in a determinated period of the year. Some species are tipically summer species due to being related to high temperature, others in contrast are active in cooler months[61,62]. The presence of a species or of its tracks on the rests depends on its phenology and on the ability to withstand seasonal weather conditions[63].

The position of the corpse has a decisive effect on remains colonization. The main effect is the difference of heat between corpses directly exposed in sunlight and those in shade. The direct exposition to sunlight determinates an increase of temperature and this fact accelerates the decomposition process. The biomass reduces more rapidly than a corpse in shade, accelerating the passage from a decomposition stage to another. Heliophilous species are more attracted by sunlight exposed corpses and the increase of heat caused by sun radiation, which also accelerates their

[61] Arnaldos *et al*., 2001
[62] Arnaldos *et al*., 2004
[63] Byrd&Castner, 2010

activity. Sciaphilous species are instead attracted by shade corpses, as in this case the decomposition process is slower[64,65].

The kind of habitat in which a body is discovered has fundamental importance because necrophagous species have a spatial distribution affected by their ecological preferences. Some species have cosmopolitan distribution, because they are present worldwide and in every habitat, others are specialist and they can only be found in rural or woodland habitats. Synanthropic species live in urban environments, closely with humans. The synanthropy level of a particular species is important to know during a forensic investigation, for example when entomological finds belonging to a urban species on a body discovered in a woodland are found, it could be surmised that the body was displaced after death[66].

The environment surrounding the body (city, countryside, indoor or outdoor), the conditions in which it is (buried, burnt, on a beach, in a woodland or at sunlight exposed), the microclimatic conditions (temperature, humidity, weather) represent elements to consider for the entomological research and an accurate PMI estimation[67].

[64] Joy *et al*., 2006
[65] Byrd&Castner, 2010
[66] Byrd&Castner, 2010
[67] Byrd&Castner, 2010

Post mortem artifacts

The cadaveric resource is colonized by a variety of insect species, some of them occur feeding on the body tissues, others occur instead preying on other species attracted by the remains[68,69]. Many species are omnivorous, playing the double role of necrophagous and predators. The species that occur on the body leave traces of their activity, such as larval exuviae, empty puparia, peritrophic membranes, all elements useful in forensic investigations[70]. But, often, their activity can also alterate the thanatological conditions of the body, causing ***post mortem* artifacts** difficult to interpret with the only medico-legal surveys[71].

In outdoor or uncontrolled environments the exposed corpse can be preyed by macrofauna (rodents, carnivores, other animals) and also by microfauna (insects in particular) that living the area[72,73]. The artifacts caused by macrofauna are quite distinguishable from *ante mortem* injuries because they don't show bleeding or redness in the tissues adjacent the lesion margins and also have well-defined boundaries based on the shape of the

[68] Rodriguez&Bass, 1983 in Campobasso *et al.*, 2009
[69] Smith, 1986 in Campobasso *et al*., 2009
[70] Byrd&Castner, 2010
[71] Byard, 2005
[72] Tsokos, 2005
[73] Byard *et al*., 2002

teeth[74]. The microfauna activity instead is more difficult to identify because *post mortem* artifacts inflicted by insects are often confused with wounds or lesions suffered *ante mortem* (figure 3).

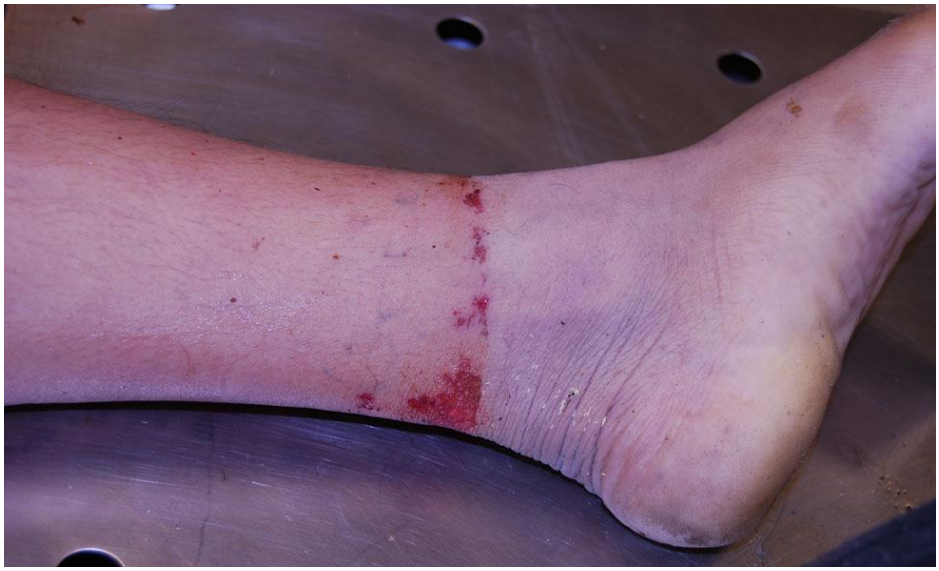


Figure 3. *Post mortem* artifacts caused by insect activity (from Batalis & Cina, 2012).

For example, Calliphoridae larvae or other necrophagous Diptera, pass through the skin of the corpse making the small circular holes often confused with gunshot wounds suffered before death. Other opportunistic species, such as cockroaches, produce abrasions on the skin surface that mimic skin deseases[75]. Hymenoptera Formicidae, as well as being predators of immature stages of Diptera, can also cause lesions on the

[74] Tsokos, 2005
[75] Tsokos, 2005

cadaver skin through corrosive secretions, easily confused as strangulation marks or excoriation suffered *ante mortem*[76,77,78].

The Formicidae intervention on bodies is immediate and often impedes or hinders the access to other ecological categories. The lesions caused by Formicidae on a body represent their interference with Diptera Calliphoridae colonization, therefore the identification of *post mortem* artifacts contributes to a better PMI interpretation[79,80]. Sometimes, Hymenoptera Formicidae represents the numerically dominant taxon among the all colonizers and occasionally they can establish a colony on the colonized corpse. Goff (1997) reports a case of minimum PMI estimation calculated according to the period of establishment of an ant colony *Anoplolepsis longipes*, found on a human corpse remains in Oahu island (Haway)[81].

Using animal models in bodies succession studies

The decomposition processes and the modalities of species colonization are strongly influenced by environmental and other factors inherent in both the

[76] Tsokos, 2005
[77] Bonacci *et al*., 2011
[78] Byard, 2005
[79] Bonacci *et al*., 2010
[80] Bonacci *et al*., 2011
[81] Goff, 1997

position and condition of exposed bodies. Therefore it is essential to study the necrophagous fauna in a determinated area in different experimental conditions. The studies of seasonal variations, habitat influence and the effect of bodies exposition in several environments have been carried out by several researcher worldwide, thanks to the use of animal models[82,83,84]. Nowadays, international works include several models used for forensic purposes[85], but the model considered most valid in application of data to real cases is the domestic pig *Sus scrofa* (L.). The experimental model shows decomposition times and modes quite similar to those of a human corpse. The domestic pig, in fact, is an omnivorous species which has an intestinal bacterial flora similar to the human, has a very thick skin and modes of decomposition correspond approximately to the human body with similar size[86,87].

Life cycle of necrophagous Diptera

Necrophagous Diptera, mainly represented by Calliphoridae and Sarcophagidae families, are the principal elements for PMI estimation

[82] Byrd&Castner, 2010
[83] Bonacci *et al*., 2010
[84] Tomberlin *et al*., 2012
[85] Tomberlin *et al*., 2012
[86] Campobasso *et al*., 2001
[87] Tomberlin *et al*., 2012

because they occur on a cadaver immediately after death[88,89]. The adults, attracted by decomposition smell even from long distances, colonize the corpse laying eggs (or directly larvae as in Sarcophagidae) in the natural cavities of the body or in the open wound, in order to ensure the trophic resource for the offspring. Immature feed on the decaying tissues completing the entire larval cycle on the body. Once the third larval stage is reached and completed, they migrate from remains and pupate in surrounding soil waiting for metamorphosis[90,91] (figure 4).

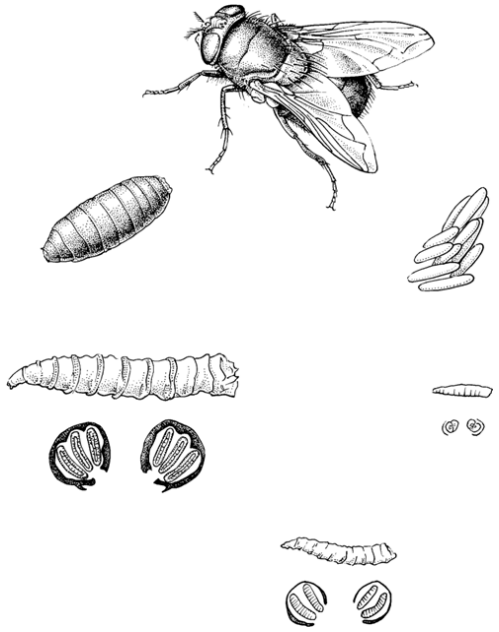


Figure 4. Life cycle of a Diptera Calliphoridae (from Goff, 2000).

[88] Byrd&Castner, 2010
[89] Donovan *et al*., 2006
[90] Byrd&Castner, 2010
[91] Nuorteva in Tedeschi *et al*., 1989

The larval size, usually indicated by their length, is related to their age and (it, not needed) is expressed as a function of time and temperature. Taking into account the environmental temperature, the larval age estimation of an older sample found on or near the body, represents the minimum PMI[92]. Necrophagous flies don't show the same time of development, because each species has optimal ranges of temperature dictated by their ecological needs. So, the larval development is a temperature-dependent process, each species has a thermal history defined as the amount of heat necessary to complete the cycle [93]. In general an increase of temperature contributes to a decrease of development time, in contrast lower temperatures increase it, in any case the amount of heat necessary to complete the cycle is constant[94]. The identification of the species found on a corpse represents the first step during the entomological investigations[95]. For each species, every development stage needs a certain amount of heat, that is quantified as *accumulated degree/hours* (ADH) or *accumulated degree/days* (ADD), necessary to reach the pupa stadium[96,97,98]. The heat accumulation, in hours or days, for any temperature is calculated as[99]:

[92] Donovan *et al*., 2006
[93] Joseph *et al*., 2011
[94] Wilson *et al*., 1993; Greenberg&Kunich, 2002 in Niederegger *et al*., 2010
[95] Joseph *et al*., 2011
[96] Donovan *et al*., 2006
[97] Joseph *et al*., 2011
[98] Ames&Turner, 2003
[99] Niederegger *et al*., 2010

$ADH = (T - T_{LDT}) * \text{hours to develop}$

$ADD = (T - T_{LDT}) * \text{days to develop}$

When:

T = temperature (or means temperatures) gained on the discovery location

T_{LDT} = *Lower Development Treshold*, low temperature at which development stops

The difficulty in this method is to establish the minimum temperature of development, different for each species but also for the same species, this parameter can vary according to other factors. For example, the geographic position influences the phenology, whereby the same species shows a greater or lesser tolerance to extreme temperatures according to the environment in which lives. Several European authors report a LDT different for the same studied species[100,101].

Another approach to estimate the larval age is by comparing the length of the older found samples with *isomorphen* and *isomegalen* diagramas[102,103].

[100] Ames&Turner, 2003
[101] Donovan *et al*., 2006
[102] Reiter, 1984
[103] Grassberger&Reiter, 2001

From *isomorphen diagrams*, it is possible to predict how much time it takes a species to reach a particular stadium at a certain temperature (figure 5). This graph is useful above all when samples are found in *post feeding* or pupa stages, namely when the length is not more an useful criterion to estimate larval age[104].

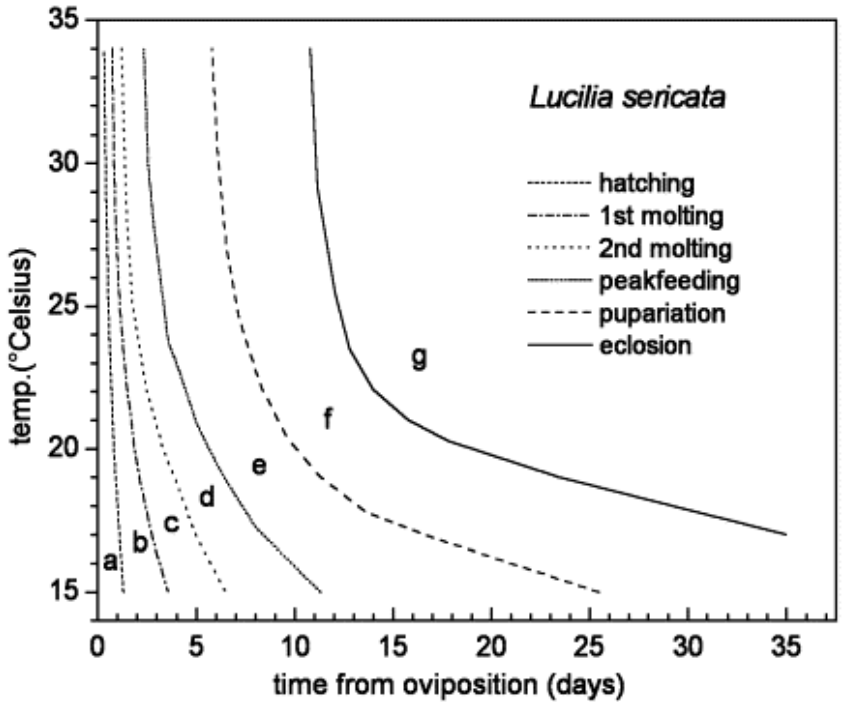


Figure 5. Isomorphen diagram for the species *Lucilia sericata* (Calliphoridae). All the immature stage are shown, from eggs to adults emergence at different temperatures. The spaces between the lines represent the same morphological stages at different temperatures. a: eggs; b: L1; c: L2: d: L3; e: postfeeding (prepupae); f: pupae; g: adults (from Grassberger & Reiter, 2001).

From *isomegalen diagrams*, it is possible to estimate the larval age based on the length of the samples, assuming that it developed at constant temperature (figure 6).

[104] Grassberger&Reiter, 2001

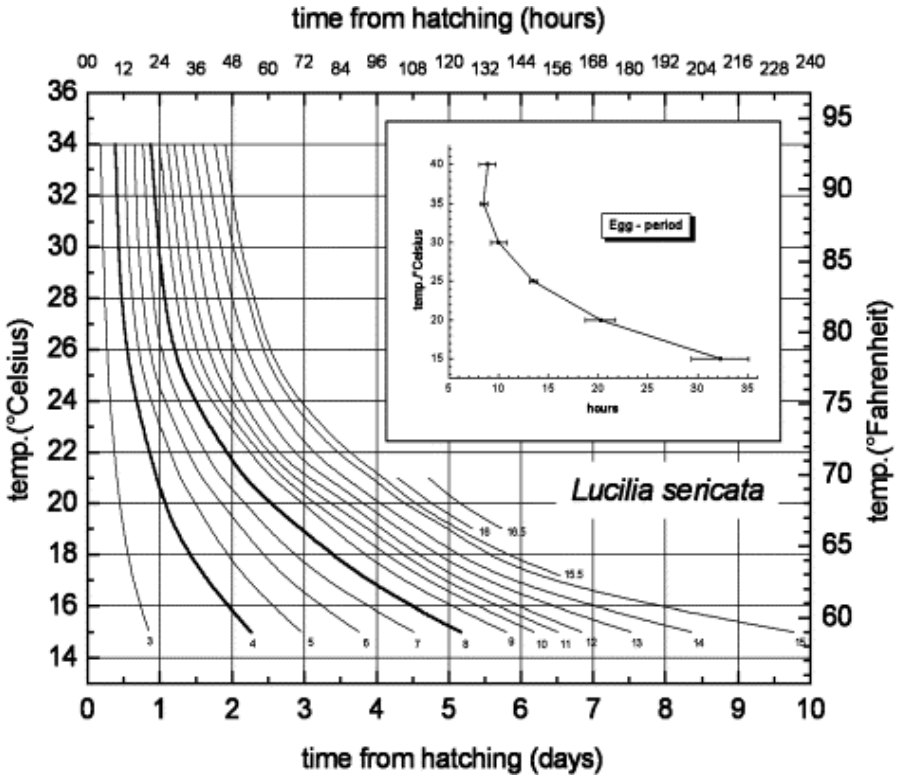


Figure 6. *Isomegalen diagram* for the species *Lucilia sericata* (Calliphoridae) from the hatching to third larval stage. The temperature is represented as a function of the time, each line in the graph represents the same measure of length in mm. In the upper right, the graph shows the duration of egg stage before the hatching at different temperatures (from Grassberger & Reiter, 2001).

However, the *isomegalen diagrams* are developed through constant temperatures and this fact can provide a loss of information, because in real cases it could be calculated as a means of environmental temperatures[105].

[105] Donovan *et al*., 2006

MYIASIS

In some particular situations, necrophagous species which generally colonize corpses can also infest living individuals causing **myiasis** in natural cavities or in open wounds[106]. The correct definition of myiasis is given by Zumpt (1965) defining them as infestation of diptera larvae on living human and other vertebrates that at least for a certain period feed on living or dead tissues, corporal liquids or ingest food of the host[107]. The most important criterion of this definition is that infesting larvae complete, or continue at least for a certain period, their normal development on the host body[108].

Myiasis agent Diptera belong to three main groups, divided according to their habits[109,110]:

1. *Obligatory parasites*: which develop exclusively on living tissues. To complete their cycle they must find a living host. To this group belong myiasis caused by Oestridae and species of Sarcophagidae families, which infest livestock (figure 7);

[106] Byrd&Castner, 2010
[107] Zumpt, 1965
[108] Zumpt, 1965
[109] Zumpt, 1965
[110] Hall, 1991

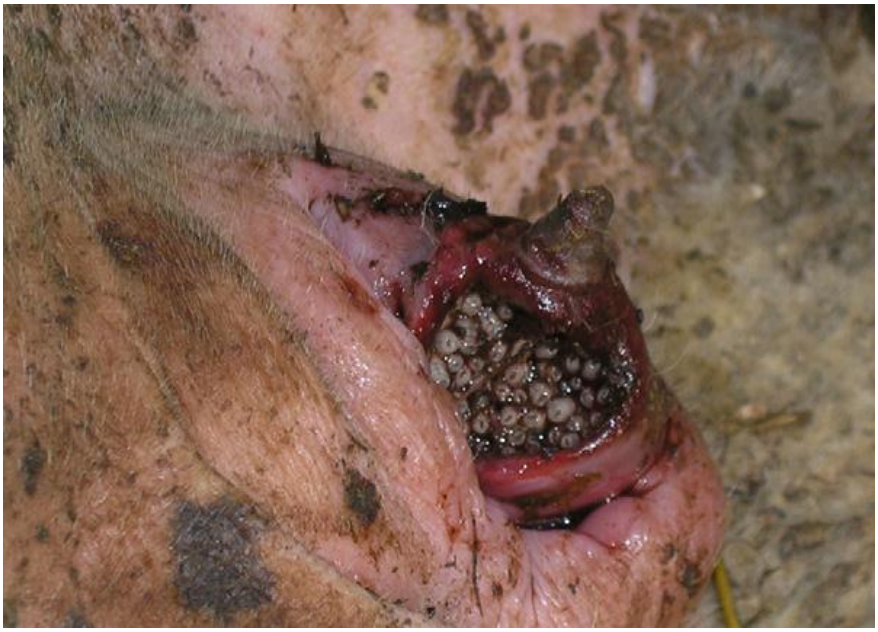


Figure 7. Urogenital myiasis in a sheep farmed caused by *Wohlfahrtia magnifica* (Sarcophagidae) (from Giangaspero et al., 2011).

2. *Facultative parasites*: which are usually free living, in general are represented by necrophagous species that feed on dead bodies and only occasionally infest living individuals. Diptera Calliphoridae, main PMI indicators, belong to this group. Facultative parasites can cause *primary* myiasis if they start the infestation; *secondary* myiasis if they occur only when the infestation was already started by other species; *tertiary* myiasis is the host is close to death[111,112];
3. *Accidental parasites*: the infestations in this case are defined *pseudomyiasis* because larvae don't infest the host directly but they are ingested accidentally, for example for food contamination, and

[111] Hall, 1991
[112] Cruz, 2004

pass through the digestive tract. In this case, the larvae ingestion is almost always passive and often the passage in the digestive tract causes their death. Larvae can be expelled through the feces or establish in the intestine causing enteric disorders[113].

Myiasis can be classified also according to the site of infestation or the site of next development in the host. From the medical point, the infestations are defined as *gastrointestinal*, *urogenital*, *nasopharyngeal*, *auricular* and *cutaneous* based on the anatomical area selected by infesting larvae[114,115] (figure 8).

[113] Amendt *et al*., 2010
[114] Hall, 1991
[115] Cruz, 2004

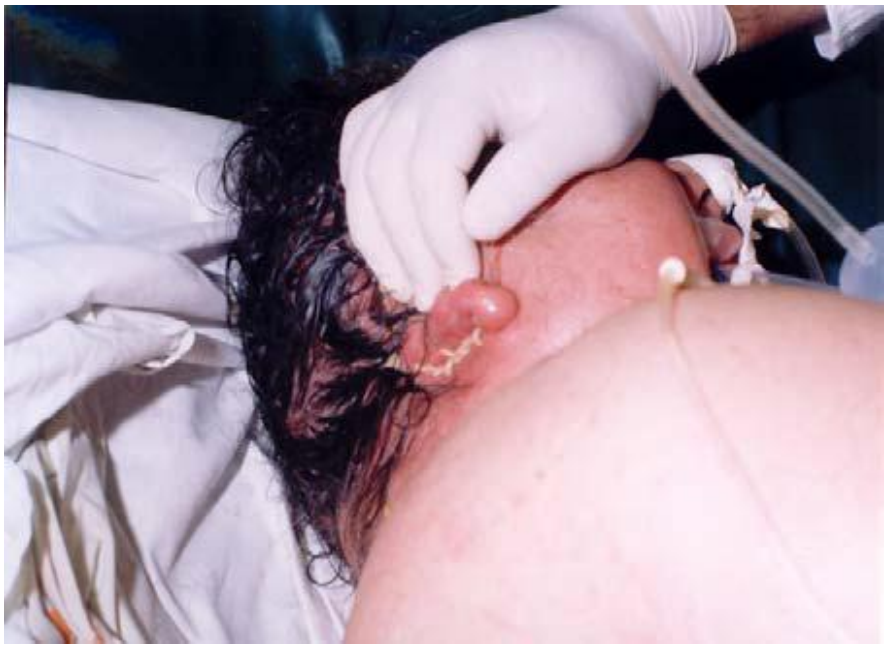


Figure 8. Auricolar myiasis in a 62 aged female caused by *Lucilia sericata* (Calliphoridae) (from Yaghoobi et al., 2005).

In the forensic field, myiasis are related more communally to facultative parasites of Calliphoridae, Sarcophagidae and Muscidae families, generally used for PMI estimation. In some particular cases, myiasis can represent an element of confusion because provide to a longer PMI estimation then the real time elapsed from death (such as can occur in *tertiary* myiasis cases). In other situations, for example in the case of infestation on living individuals, myiasis represent a valid help to solve negligence cases on vulnerable individuals[116].

[116] Amendt *et al*., 2010

Chapter 2

MATERIALS & METHODS

1. ENTOMOLOGICAL SUCCESSIONS

The study of entomological successions was carried out over four experiments, taking place in each season (autumn, summer, winter, spring). The autumn experiment was carried out from November; the summer one in July; the winter one from February; the spring experiment from May.

Entomological researches were carried out in the same study area (in the Botanical Garden of University of Calabria) using specimens of domestic pigs, *Sus scrofa* (L.), as animal models.

Animal models

For the seasonal experiments, four specimens of *Sus scrofa* (figure 9) weighing 20-25 kg were used, one for each season. The models were taken from a farm located close to the study area and euthanised by lethal injection with the assistance of a veterinarian. Following their death, the

models were positioned on the soil covered by a wire netting fine mesh and enclosed in a fence, thus avoiding avoid a possible macrofauna attack. The experimental procedure's aim was to simulate situations of death in an outdoor environment and evaluate the seasonal influence (and accordingly the climatic factor influence) on both the presence and activity of colonizer species and on thanatological changes of exposed carcasses.

Figure 9. Animal model, *Sus scrofa* (L.), used for the experiments (from Bonacci T.).

Study area

The carcasses were placed in an area of Botanical Garden of University of Calabria (figure 10), suitably selected respecting to hygiene and health standards established by law.

The Botanical Garden is developped on a hilly terrain at 180-230 meters a.s.l. and covers an area of about 8 hectares. Geographic coordinates are included between 39°18’ and 39°24’ of latitude N and 16°10’ and 16°20’ longitude E. The boundaries that trace the perimeter of the garden are determined by a concrete wall with a wire netting masked by creepers and bordered to the south and west sides by a row of cypress trees. The wall stops at five gates of which only one is used as an input to (the) visitors. The garden encloses semi-natural areas with a significant sample of flora Calabria: more than 4000 species of about 2500 total census for the whole of Calabria. The geological substratum of the area consists of sand and conglomerates and cement-crystalline metamorphic from brown to reddish clays with interbedded gray to blue-gray. The presence of these layers of clay permits the formation of a discrete water system at the surface.

The botanical garden in fact, besides being crossed from the west to east by a stream that goes in the direction of the river Crati includes four wells and two small perennial springs.

The vegetation in the area consists mainly of plant communities established on former cropland and fragments of training subspontaneous represented by an oak grove and a riparian poplars grove and willows along the creek.

Figure 10. Entrance of the Botanical Garden of University of Calabria (da Greco S.).

The potential vegetation of the area is represented by deciduous oaks classifiable in *Querceta ilicis* (thermophilic elements species-rich evergreen) and *Querceta robori-petraea* (mesophilic elements). The dominant species is *Quercus virgiliana*, oak belongs to the cycle of *Q.*

pubescens (downy oak). It features a little disturbed forest formation, where there are many characteristic species of thermophilous woods (*Erica arborea*, *Cornus sanguinea*, *Clinopodium vulgare*, *Brachypodium sylvaticum*, *Knautia calycina*, *Helleborus bocconei*).

Part of the site is occupied by a thicket of willows (*Salix caprea* and *S. triandra*), in which undergrowth is abundant *Equisetum telmateja*. Other hygrophilous species are represented by *Dorycnium rectum*, *Potentilla reptans*, *Mentha suaevolens*. This vegetation is related in this area to a perennial source. The valley floor is characterized by a grove of trees in riparian *Populus alba* belonging to the order *Populetalia albae*. In the undergrowth there are abundant species typical for hygrophilous woods such as *Equisetum telmateja*, *Lamium flexuosum*, *Lythrum junceum*.

Part of the area is characterized by secondary vegetation abandoned cropland with scattered fruit trees and now converted into meadow hay (*Avena barbata*, *Agrostis castellana*, *Chrysanthemum segetum*, *Echium plantagineum*) [117].

The experiments were carried out in an area characterized by *Quercus pubescens* Willd. 1805, *Pirus* spp (L.) and *Olea europea* (L.) (figure 11).

[117] Gangale&Uzunov in Tenuta *et al*., 2007

Figure 11. The area of the Botanical Garden surrounding the experiment site (from Greco S.).

Experimental procedure

The carcasses were transported to the study area immediately after death, placed on the soil inside a fence and covered with a metallic netting in absence of researcher (figure 12).

Figure 12. Animal model placement in the study area (from Bonacci T.).

From the moment of placement, two digital data logger were activated, one with a probe inserted in the carcass the other outside, for continuous recording of the temperature and humidity. Data concerning rainfall related to the studied period were found on the official website of ARPACAL.

Daily inspections are made, more times a day until the thanatological conditions were established. When the dry stage was reached, the inspections were less frequent, waiting for the complete body skeletonization. Daily, direct catches of necrofauna were made through an entomological net, also larvae were sampled with tweezers. Procedures for the collecting and preserving the samples followed the guidelines proposed by Amendt *et al*., 2007.

Thanatological changes of the body and the necrofauna activity related to them, were photographed, recorded and noted on appropriate experimental protocols (figure 13).

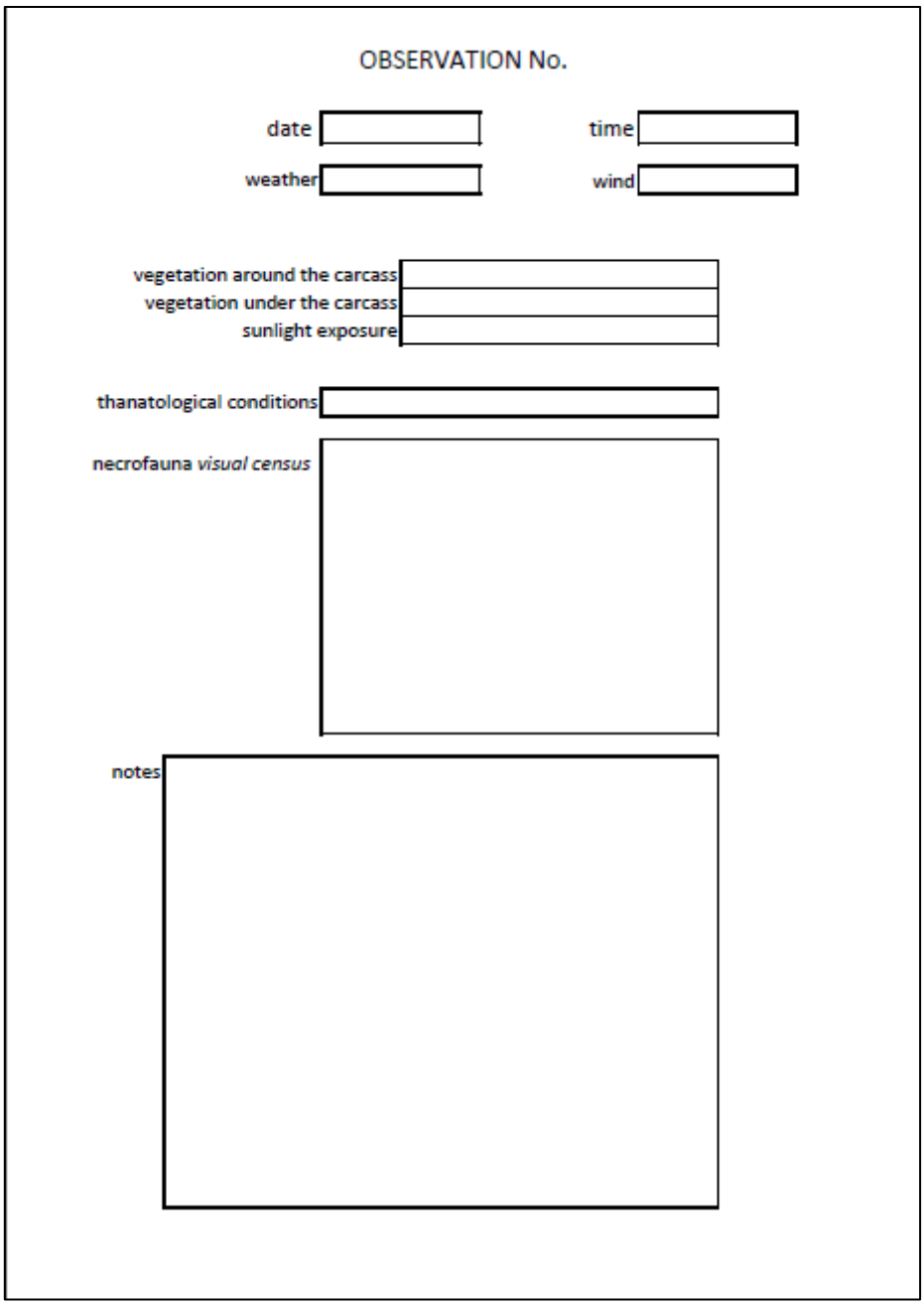


Figure 13. Experimental protocol used for data notation in the field.

When carcasses were completely dry, the remains were removed and buried and entomological finds were collected in the soil under and around the carcasses site (figure 14).

Figure 14. Collecting of entomological finds after the carcass removal (from Greco S.).

The adults samples collected during the experiments were identified using international literature[118,119,120], sorted by ecological category and stored in the Entomological Collection of Ecology Department (University of Calabria).

[118] Chynary, 2004
[119] Oosterboek, 2006
[120] Rivosecchi, 2000

2. SAMPLING OF NECROPHAGOUS SPECIES

The sampling of necrophagous species was carried out using *bottle traps*. Three areas with different degrees of nature were selected, in order to evaluate the influence of the habitat and climatic factors on the species distribution.

Traps design

The traps were made following the model proposed by Hwang & Turner (2005).

A trap is realized with the aid of two plastic bottles of 1,5 l, of the same shape and dimensions. The traps are positioned in order to create two chambers: the *collecting chamber* situated at the top of the trap and the *bait chamber* represented by the lower part. The collecting chamber is made with the two upper parts of both bottles, one pushed in the other (figure 15).

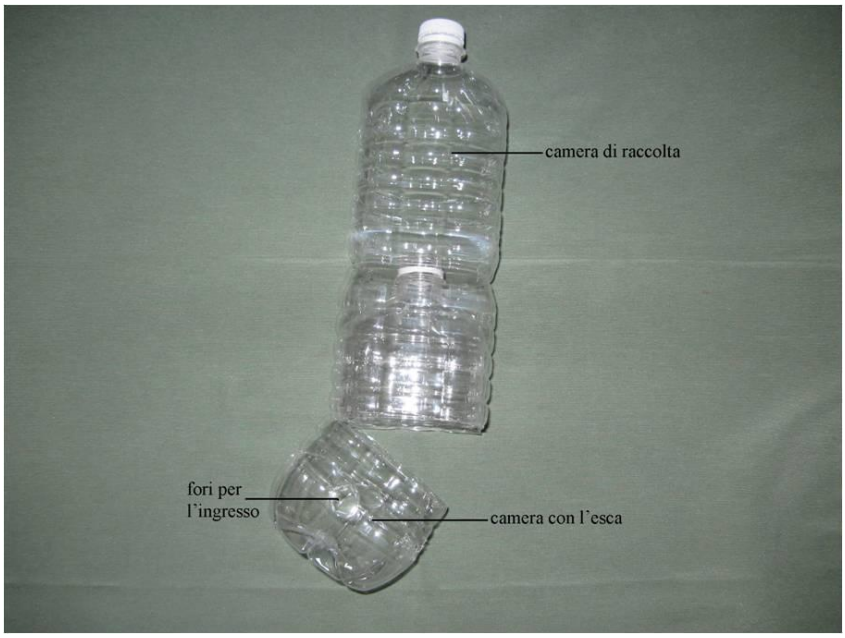


Figure 15. Design of the traps used for the sampling (from Greco S.).

Inside the bait chamber were positioned two plexiglas containers, one with mince and the other with NaCl solution in order to avoid the rapid dryness of the bait.

After the bait position, the two chambers are closed with sellotape and the traps are moved in the study areas (figure 16).

Figure 16. Bottle traps placement in one of the study areas (from Greco S.).

Study areas

The sampling of necrophagous species were carried out in three different areas of the Cosenza province, characterized by a different level of nature and defined *wild area*, *rural area* and *urban area*. Geographic localization of the areas is indicated in figure 17.

Figure 17. Geographical position of the study areas (from *Google Earth*).

Wild area

The natural site considered (figure 18) is located at about 15 Km from the University of Calabria, situated near San Fili village, at 985 m of elevation. Geographical coordinates are 39°19'12.37'' latitude N and 16°6'45.86'' longitude E.

The potential vegetation of the area is represented by a macrothermal beechwood linked to a humid tempered bioclimate markedly oceanic. These climatic conditions favor the spread of the beech on the Catena Costiera even at relatively low altitude at which the other Calabrian mountains are more thermophilous forest types (oak, chestnut and pine forests). The presence of beech at low altitudes is explained, in fact, by the

extreme nebulosity which also occurs in the summer period starting from about 650-700 m above sea level. This climatic peculiarity favoring the development of beech forests are characterized by a more complex structure with a rich shrub layer with mostly evergreen shrubs such as holly (*Ilex aquifolium*), butcher's broom (*Ruscus aculeatus*), Dafne laurella (*Daphne laureola*). This type of beech wood is *Anemono apenninae-Fagetum*, association *Fagetalia sylvaticae*, endemic to the mountains of the southern Apennines. The beech wood is generally governed by high forest, pure, in good vegetative conditions and with a full density.

Figure 18. *Wild area* (from Greco S.).

The area is crossed by numerous rivers that create valleys characterized by forest vegetation in lindens and maples typical for moist ravines. These woods form a continuous band which characterizes the sides of the valley and come into contact with the hydric wood at the bottom and at the top of the beech wood. The undergrowth is rich in species nemoral such as *Hedera helix*, *Vinca major*, *Acanthus mollis*, *Helleborus bocconei*, etc.. These formations are quite rare in the province. They are framed in *Ostryon carpinifoliae* alliance, which brings together mainly mixed forests with maples, lindens and elms linked to a rather wet and cool microlimate, located in ravines and valleys[121].

Rural area

The rural site is represented by the Botanical Garden of the University of Calabria. The description of the area is already reported at page 33. The traps were positioned in four sub-habitats: two oak wood, broom wood, poplar wood (figure 19).

[121] Gangale&Uzunov in Tenuta *et al.*, 2006

Figure 19. *Bottle trap* positioned in the *rural area* (from Greco S.).

Urban Area

The urban site of Rende-Cosenza (figure 20) is located in a hilly area at an elevation between 185-575 m a.s.l.; the geographical coordinates are 39°18’39’’60 latitude N and 16°15’3’’60 longitude E. the site is situated in a totally man-made environment in which the potential vegetation, represented by thermophilous deciduous oak forest, has totally disappeared. The few unbuilt areas are characterized by ruderal vegetation, weeds, trees and flower beds with non-native species[122].

[122] Gangale, personal communication

Figure 20. *Urban area* (from Albano R.).

Experimental procedure

The three sampling areas have the same extension, in order to ensure comparability of the results. For each area 8 bottle traps were used, positioned at a distance of about 15 meters one from other.

The investigation started in February 2010 with the first traps location in all the areas at the same time. Traps were replaced each months, for two years, until January 2012. In each replacement the traps were moved to the laboratory, empty and sorted, separating adults from larvae. Also in this

case we used the guidelines from Amendt *et al*., 2007 for the samples treatment.

Larvae were preserved in 90% ethanol post boiling, thus ensuring the necessary procedure for the removal of bacteria and proper maintenance of the samples. In each sampling date, for all the area, the adults were sorted by families and stored in 70% ethanol. Taxa were counted and finally Calliphoridae species were identified using international identification keys[123,124,125,126] (figure 21).

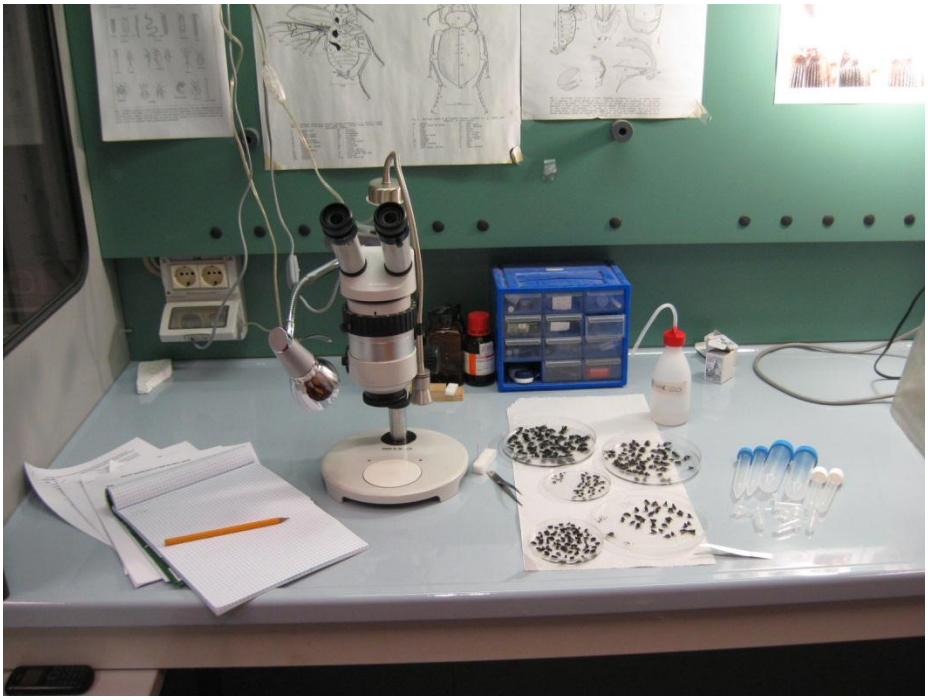


Figure 21. Sorting and identification of entomological material in each sampling date (from Greco S.).

[123] Rognes, 1980
[124] Wallmann, 2001
[125] Whitworth, 2006
[126] Marshall *et al*, 2011

Data concerning environmental temperatures and rainfall for the sampling date were found on the official website of ARPACAL.

Data analysis

Statistical analysis for sampling data of necrophagous species, on their phenology and abundance comparison in the three area, were performed with *IBM SPSS Statistic v. 20* and *Past v.2.17* softwares.

Pearson's Chi-square and Kruskall-Wallis tests were calculated in order to evaluate the statistically significance of the species abundance.

Correspondence Analysis (CA) were performed to evaluate the species distribution in the areas, comparing also the seasonal abundances of the species.

Moreover, the *Synanthropy Index* (SI), proposed by Nuorteva (1963) was calculated to evaluate which species were more related to a human environment, with the formula:

$$I.S. = \frac{(2a + b - 2c)}{2}$$

When:

a = abundance (%) of species sampled in *urban area*

b = abundance (%) of species sampled in *rural area*

c = abundance (%) of species sampled in *wild area*

The value of the index is between +100 (species totally synanthopic) and -100 (species totally forestry).

Finally correlations between abundance of species and environmental temperatures were made in order to evaluate the relation between these variables.

Chapter 3

RESULTS

ENTOMOLOGICAL SUCCESSIONS

Autumn investigation

During the autumn investigation, four thanatological stages were identified: fresh, bloated, decay and dry (figure 22).

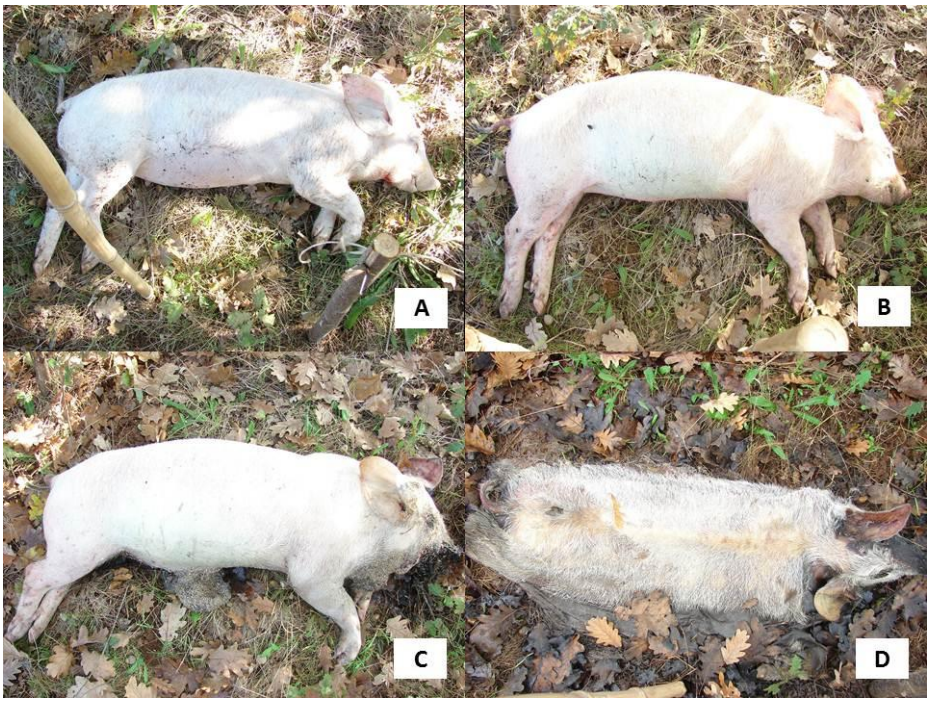
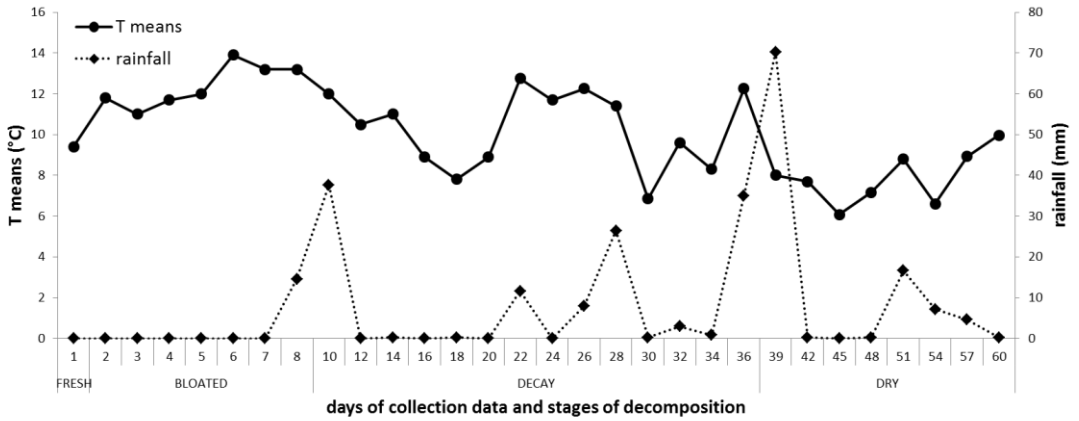


Figure 22. Stages of decomposition of the carrion in autumn. A) Fresh; B) Bloated; C) Decay; D) Dry (from Bonacci T.).

The entire decomposition process lasted 60 days, influenced by environmental conditions. The graph 1 shows the trend of average daily temperatures and rainfalls during the experimental period.



Graph 1. Trend of temperatures and rainfall during autumn experiment.

First colonizer occurred on the carrion were Diptera Calliphoridae, represented by *Calliphora vicina* Robineau-Desvoidy 1830, *Calliphora vomitoria* (L.), *Chrysomya albiceps* (Wiedeman 1819), *Lucilia caesar* (L.) and *Lucilia sericata* (Meigen 1826), although with different abundance. Calliphoridae colonized the carcass five minutes after the corpse placement, invading the natural orifices lying eggs (figure 23). After 21 hours from placement, other egg depositions were found in the right eye, exposed to sunlight. After 95 hours from placement, larvae in the mouth cavity started to migrate inside the body; after 170 hours other egg

depositions were found on the right foreleg and in the abdominal part; after eight days egg depositions were also found in the anal cavity.

Figure 23. Mouth cavity colonization and first spawns from Diptera Calliphoridae after few minutes from carcass placement (from Bonacci T.).

After nine days, a conspicuous maggot mass was observed in the anterior part of the body and after ten days the maggot mass reached the abdominal part. After 22 days the entire carcass were colonized by Calliphoridae larvae. First pupae were collected after 20 days from carcass placement.

The other ecological categories occurred gradually as the decomposition process proceeded. Diptera Muscidae were collected after ten days from the carcass placement, when Calliphoridae were less abundant. Hymenoptera Vespidae, represented by *Vespula germanica* (Fabricius 1973) were

observed during the bloated stage, preying Calliphoridae larvae and other adult Diptera. Other predators, represented by *Nicrophorus humator* (Gleditsch 1767) (Coleoptera: Silphidae) (figure 24), *Creophilus maxillosus* (L.) (Coleoptera: Staphylinidae) and *Nitidula carnaria* (Schaller 1783) (Coleoptera: Nitidulidae) occurred after 30 days from carcass placement.

Figure 24. *Necrophorus humator (*Coleoptera: Silphidae) occurred after 30 days from carcass placement (from Bonacci T.).

Hymenoptera Formicidae, belonging to *Camponotus* Mayr 1861 genus, showed a predator behavior towards the Diptera larvae, occurring in the body between the 9th and the 22nd day.

Interestingly there was a total absence of Coleoptera Dermestidae, usually colonizers during the dry stage. Maybe the rainfall occurred over the last days (see the graph 1), impeded the Dermestidae activity, more attracted by dry remains of the corpses. Figure 25 shows in detail the relative abundance and the period of activity (arrivals and departures) of ecological categories occurred on the carcass for the entire experimental period.

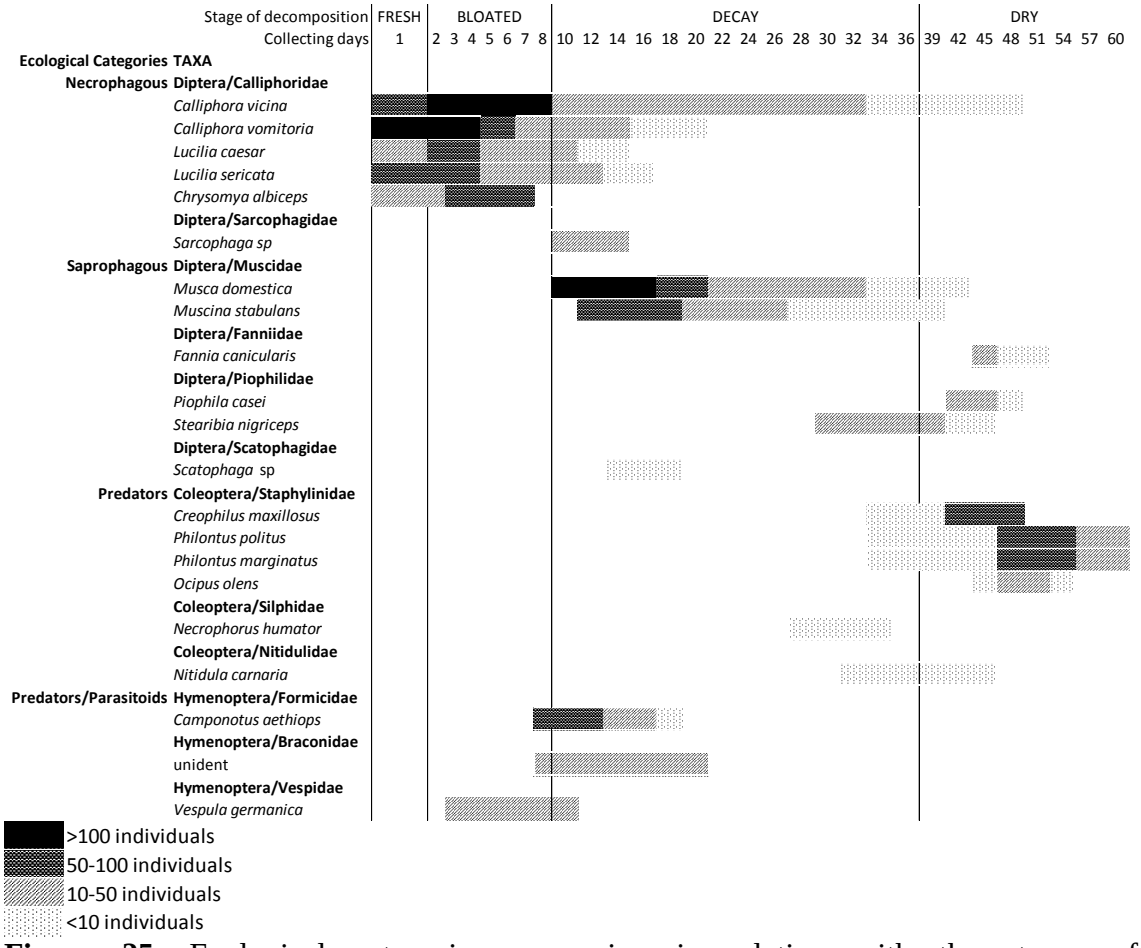


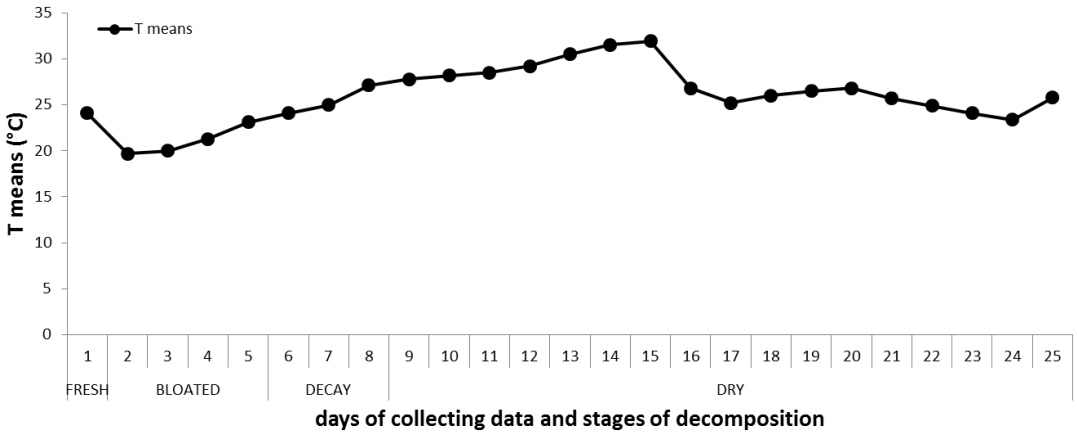
Figure 25. Ecological categories succession in relation with the stages of decomposition during autumn experiment.

Summer investigation

During the summer experiment, four decomposition stages were identified, identical to autumn; fresh, bloated, decay and dry (figure 26), although the decomposition process accelerated due to high environmental temperatures.

Figure 26. Stages of decomposition of the carcass during summer experiment. A) Fresh; B) Bloated; C) Decay; D) Dry (from Bonacci T.).

The carcass decomposed in 25 days, due to the high temperatures and the lack of rainfall (graph 2). The graph 2 also shows the duration of each decomposition stage related to the average temperature of the experimental period.



Graph 2. Trend of temperatures and rainfall during summer experiment.

Thanatological changes were faster and in only 9 days the carcass reached the dry stage. The dominant species of Calliphoridae was *Chrysomya albiceps*, followed by less abundant *Lucilia caesar* and *L. sericata*. No *Calliphora* spp were found in this season. But Calliphoridae weren't the first colonizers, because for the first 24 hours Hymenoptera Formicidae *Crematogaster scutellaris* (Olivier 1792) colonized the carcass impeding the egg deposition of blowflies. Formicidae predated adults and larvae occupying the preferencial sites of Calliphoridae (figure 27). After 24 hours Formicidae left the resource and Calliphoridae started to laying eggs.

Figure 27. Colonization of *Crematogaster scutellaris* (Hymenoptera: Formicidae) during the first 24 hours from carcass placement (from Bonacci T.).

First Calliphoridae eggs were found after 27 hours from carcass placement. Larvae of *Chrysomya albiceps* at the first stage were found after 45 hours, instead the third larval stage was reached after four days from carcass placement (figure 28).

The first pupae were collected from the 9th day of the carcass placement.

Figure 28. Third larval stage of *Chrysomya albiceps* after four days from carcass placement (from Bonacci T.).

During the dry stage, the carcass was colonized by Coleoptera Cleridae *Necrobia* sp (De Geer 1775) and Dermestidae *Dermestes (Dermestinus) maculatus* De Geer 1774, occurring on the 6th day.

In this season Coleoptera Staphylinidae *Creophilus maxillosus* were also found on the carcass. The entire summer succession of the ecological categorie occurred is reported in figure 29.

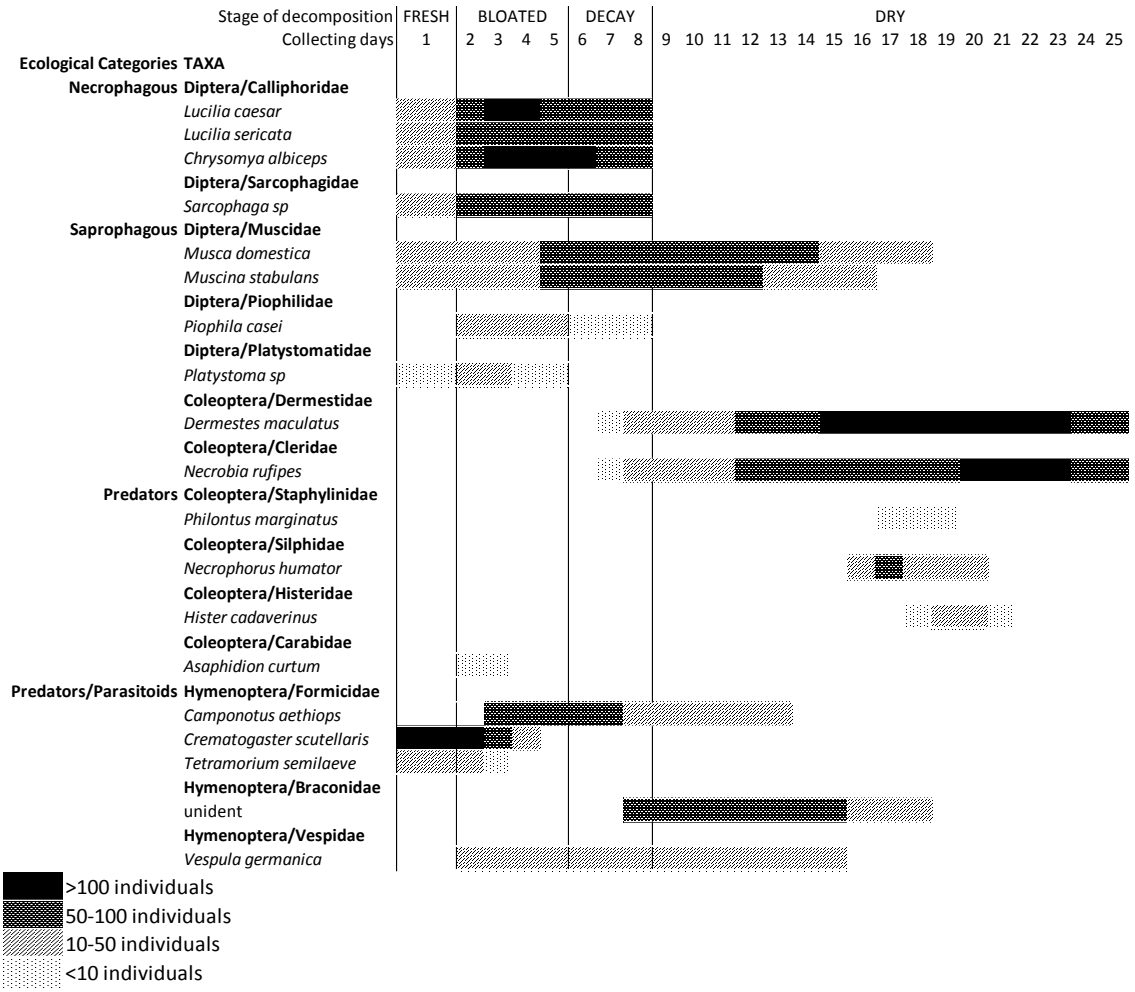


Figure 29. Ecological categories succession in relation with the stages of decomposition during the summer experiment.

Interesting in this investigation was the Formicidae activity which, as well as delaying the Calliphoridae colonization, it also caused particular lesions on the pig skin identified as *post mortem* artifacts (figure 30).

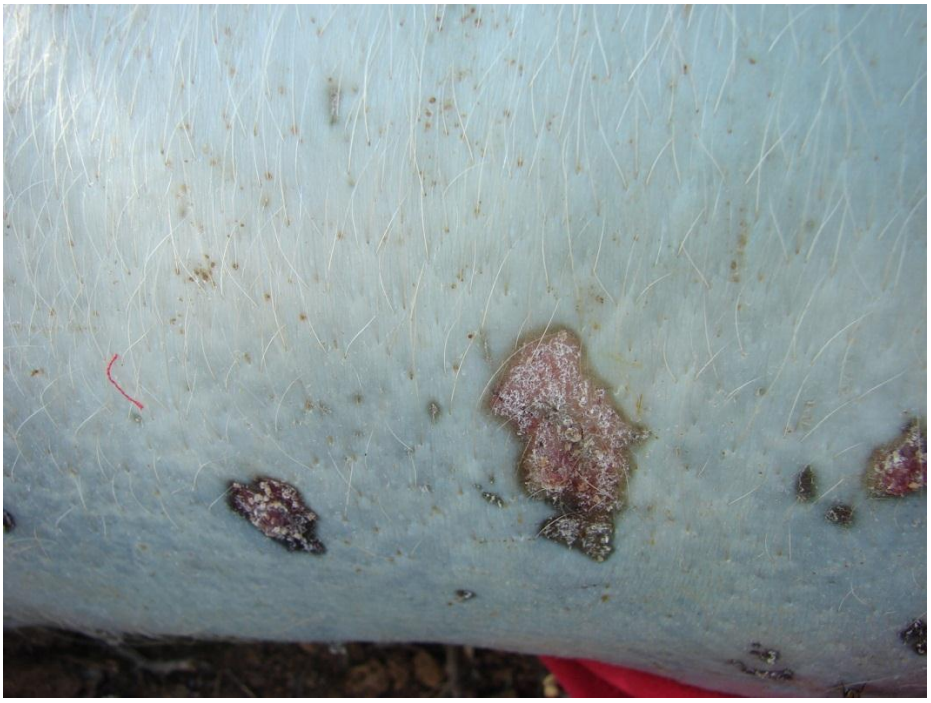
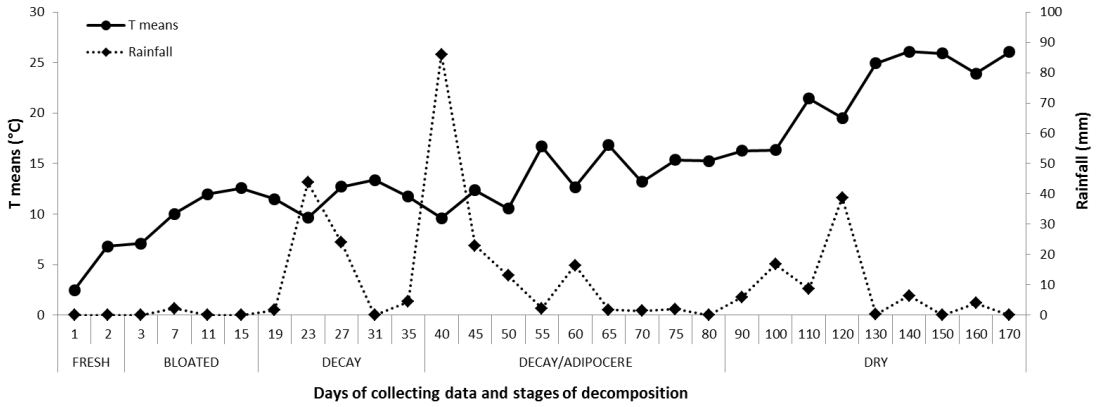


Figure 30. *Post mortem* artifacts caused by *Crematogster scutellaris* during the first hours from carcass placement (from Bonacci T.).

Winter investigation

In this investigation both the thanatological changes and necrophagous insect activity were slowed down due to the climatic condition of the period. The low temperatures and abundant rainfall (graph 3), in addition to high soil humidity, created the right condition to *saponification* (or *adipocere*), an anomalous form of decomposition that maintained the carcass in a preservative waxy stage that increased the time of decay. Five stages of decomposition were identified (fresh, bloated, decay, adipocere and dry) which occurred slowly and gradually (figure 31).



Graph 3. Trend of temperatures and rainfall during winter experiment.

Figure 31. Stages of decomposition of the carcass during winter experiment. A) Fresh; B) Bloated; C) Decay; D) Adipocere, E) Dry (from Bonacci T.).

Among first colonizers, *Calliphora* spp were found on the carcass (totally absent during the summer investigation). First egg depositions were found after 3 hours from carcass placement, in the left eye; the hatching of eggs occurred after 3 days, due to low temperatures in the first days (T_{mean} = 4,65±3,04°C).

The Calliphoridae, Muscidae and Fanniidae activity increased during the bloated stage, from the third day of carcass placement; at the same time predators activity also increased, above all for Hymenoptera Braconidae and Formicidae represented by *Camponotus (Tanaemyrmex) aethiops* (Latreille 1798). The trophic action of Calliphoridae larvae caused the rapid degradation of the pig head before the rest of the body that has been held intact for a long time during the adipocere stage (figure 32). The complete skeletonization of the head caused the intervention of Coleoptera Cleridae and Dermestidae after 18 days of carcass placement, which usually occur during the dry stage (figure 33).

Figure 32. Thanatological condition of the body after 20 days from carcass placement. The head is completely skeletonized instead the rest of the body is preserved in adipocere stage (from Bonacci T.).

Figure 33. Detail of the skeletonized head and colonization by *Necrobia* sp (Coleoptera: Cleridae) after 20 days from carcass placement (from Bonacci T.).

The rainfalls (76 mm at the end of the bloated stage) together with the low temperatures (Tmean = 10,42±2,47) moved away the egg depositions of the next days and hindered the Calliphoridae colonization which had a poor activity on the carcass resource. After about 30 days from carcass placement, as the decomposition process reached the adipocere stage, the carcass was totally colonized by Coleoptera Silphidae *Thanatophilus rugosus* (L.) and *Thanatophilus sinuatus* (Fabricius 1775), which used the pig cadaver as breeding site and trophic resource, sharing the carcass without interspecific competition (figure 34).

Figure 34. Colonization by *Thanatophilus* spp (Coleoptera: Silphidae), after 30 days from carcass placement (from Bonacci T.).

The dry stage was reached about 85-90 days following carcass placement. During this stage, the carcass was colonized by Coleoptera Dermestidae *D. maculatus* and Cleridae *Necrobia* spp. In figure 35 the entomological succession for this investigation is reported in detail.

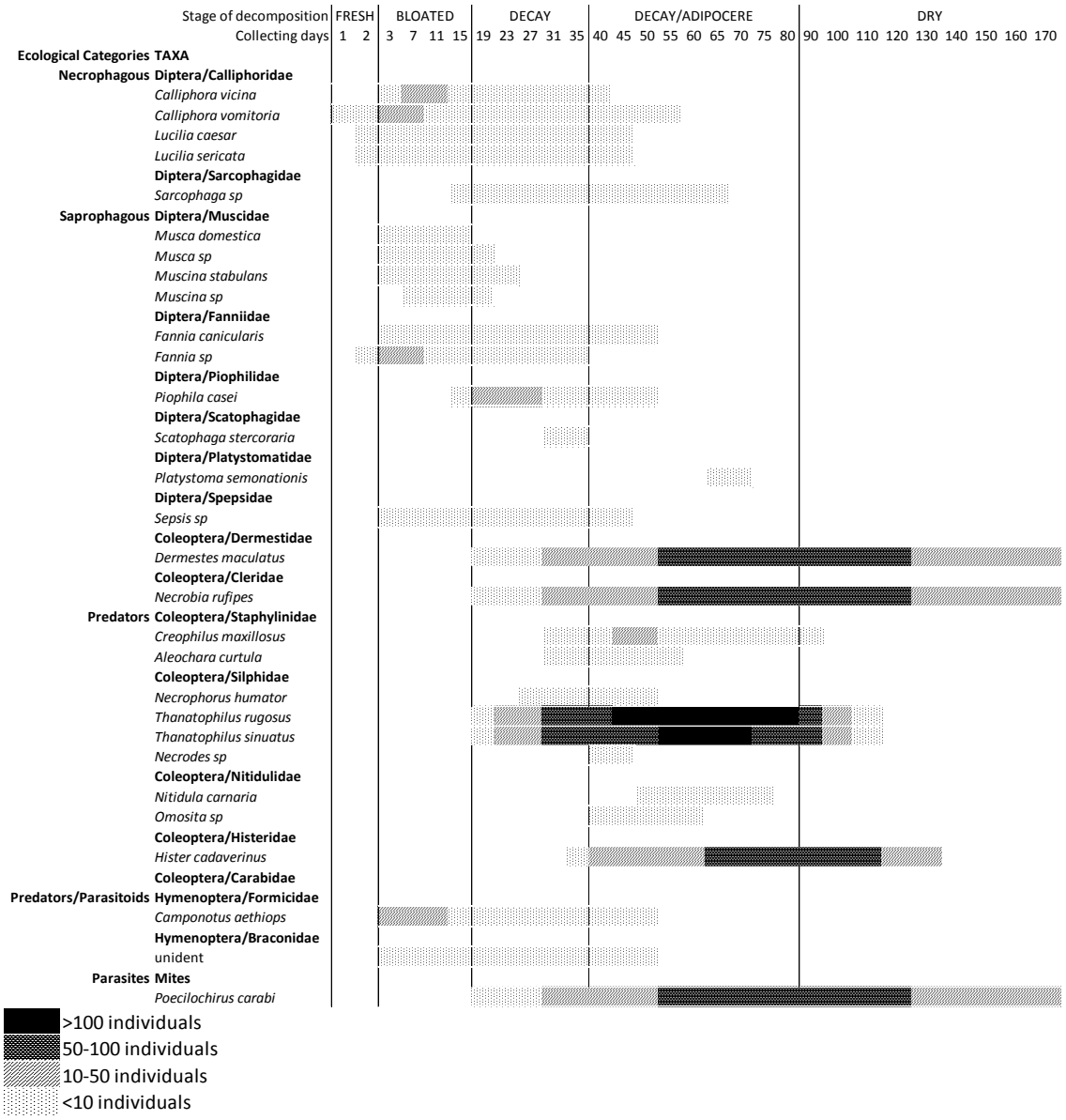
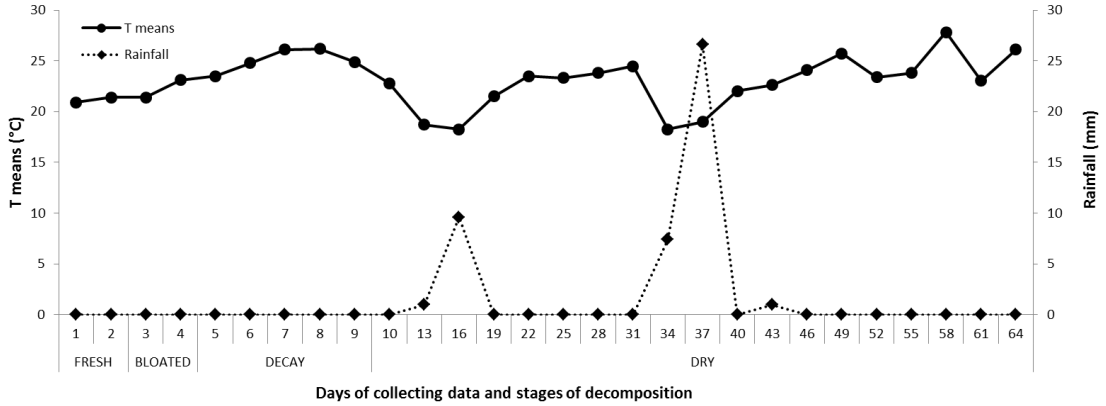


Figure 35. Ecological categories succession in relation with the stages of decomposition during the winter experiment.

Spring investigation

Four stages of decomposition were identified in this period (fresh, bloated, decay and dry) (figure 36). The complete decomposition process of the carcass lasted 64 days. Furthermore in this investigation, both thanatological changes and insects activity were influenced by environmental conditions (graph 4).

Figure 36. Stages of decomposition of the carcass during spring experiment. A) Fresh; B) Bloated; C) Decay; D) Dry (from Greco S.).



Graph 4. Trend of temperatures and rainfall during spring experiment.

The carcass was colonized by Calliphoridae represented in this season by *Lucilia Caesar* and *Lucilia sericata*, which occurred immediately after the carcass placement. The first egg depositions were found in the mouth after 3 hours from carcass placement, in the next hours and days other egg depositions were found in the other cavities of the body. Larvae at the first stage were discovered in the mouth after 22 hours. Larvae at the third stage started to feed inside the body after 5 days and by the 6th day, they were observed migrating in the surrounding soil. First pupae were collected after 13 days from carcass placement.

The interesting part of the investigation was the Diptera Platystomatidae activity, which occurred on the carcass simultaneously with Calliphoridae, although no information in scientific literature about their role as forensic indicators is available (figures 37 and 38).

Figure 37. *Platystoma* spp (Diptera: Platystomatidae) in the anal region where there are eggs of *Lucilia* spp (Diptera: Calliphoridae) after 48 hours from carcass placement (from Greco S.).

Figure 38. *Lucilia* spp e *Platystoma* spp in the anal region after 48 hours from carcass placement (da Greco S.).

Also in this investigation, predator behavior of Hymenoptera Formicidae was observed. The Formicidae *Camponotus* spp and *Formica (Serviformica) gagates* Latreille 1798 predated eggs and the larvae of Calliphoridae (figure 39, A and B). In particular, the trophic activity on the carcass skin by *F. gagates* created *post mortem* artifacts on abdominal area and on the limbs (figure 39, C and D). Unlike the summer investigation, in this case Formicidae didn't impede the Calliphoridae colonization which settled on the resource from the carcass placement.

Figure 39. Predatory behavior (A and B) and *post mortem* artifacts (C and D) caused by Hymenoptera Formicidae occurred on the carcass during spring experiment (from Greco S.).

Among Coleoptera, we observed the Silphidae *Thanatophilus* spp activity which in this case gave up to necrophagous role and played the predators role against the Calliphoridae larvae , as reported in figure 40.

Figure 40. *Thanatophilus sinuatus* (Coleoptera: Silphidae) predates a Calliphoridae larva (from Greco S.).

In figure 41, the succession of the spring investigation is reported in detail.

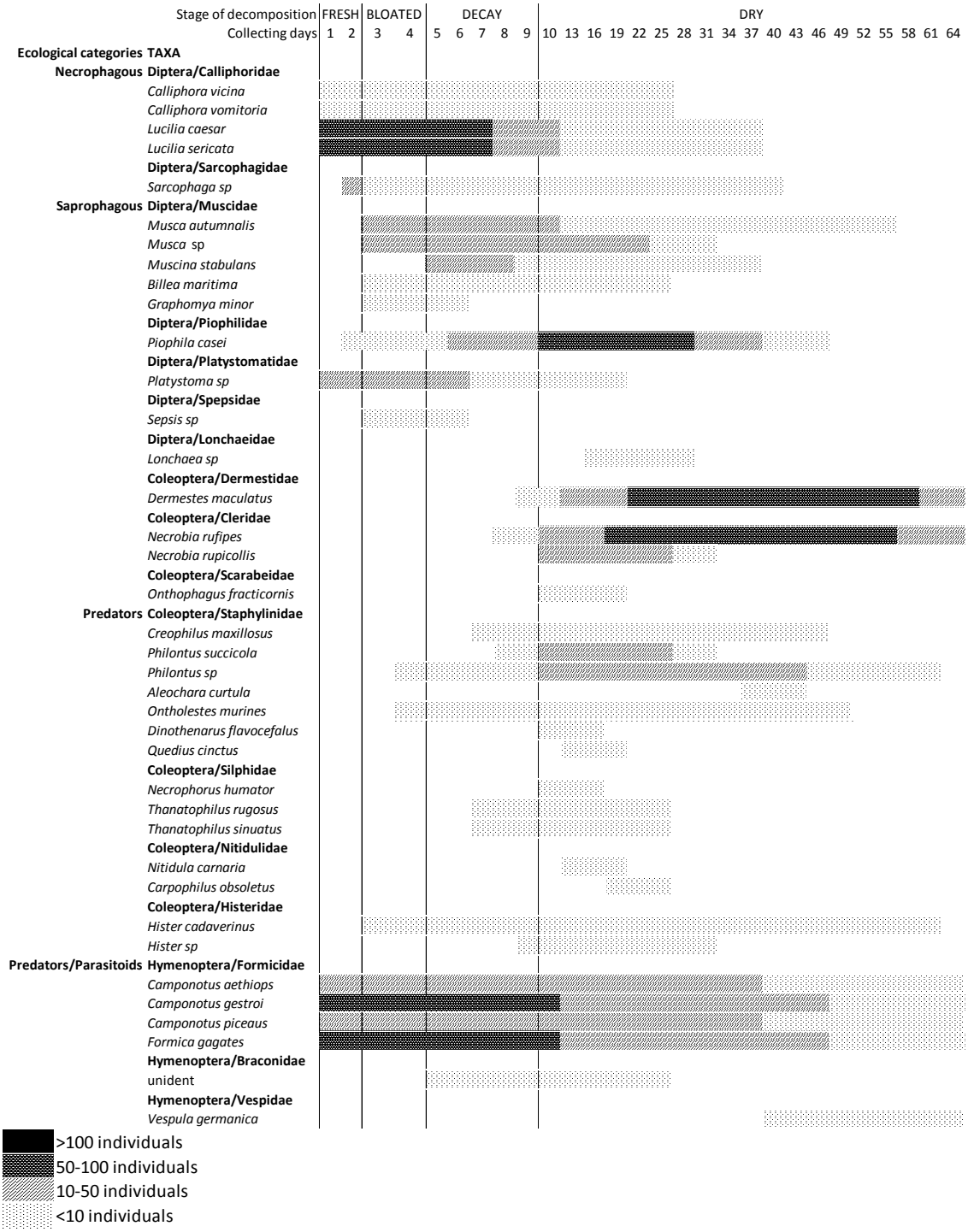


Figure 41. Ecological categories succession in relation with the stages of decomposition during the spring experiment.

During each season, carcasses were colonized by different species, occurred on the resources during their optimal periods, influenced by their phenology; in fact, during the investigations, the ecological categories were represented by different species with the same ecological role. In particular for Diptera Calliphoridae (main necrophagous) a different seasonal species composition was observed, as confirmed by the Dominance Index (DI) reported in table 1. The abundances of the species were estimated based on the visual observations and the caught samples. In autumn, the most abundant species was *C. vomitoria* (DI = 0,52), in summer *Ch. albiceps* (DI = 0,74), in winter *C. vomitoria* (DI = 0,54) and *C. vicina* (DI = 0,23), although they weren't the main necrophagous, and in spring *L. caesar* (0,70) and *L. sericata* (DI = 0,20) were the most abundant.

Table 1. Dominance Index for Calliphoridae species which colonized the carcasses in the seasonal experiments.

	AUTUMN	**SUMMER**	**WINTER**	**SPRING**
Calliphora vicina	0,15	-	0,23	0,07
Calliphora vomitoria	**0,52**	-	**0,58**	0,02
Chrysomya albiceps	0,1	**0,74**	-	-
Lucilia caesar	0,19	0,09	0,1	**0,7**
Lucilia sericata	0,04	0,17	0,09	0,2

SAMPLING OF NECROPHAGOUS SPECIES

In two years of sampling several Diptera families were trapped and identified, a portion of them were already known as corpse invaders, others accidentals. Also predators Coleoptera (Silphidae, Staphilinidae) and saprophagous Coleoptera (Dermestidae) were caught but excluded from data analysis due to statistically poor representative.

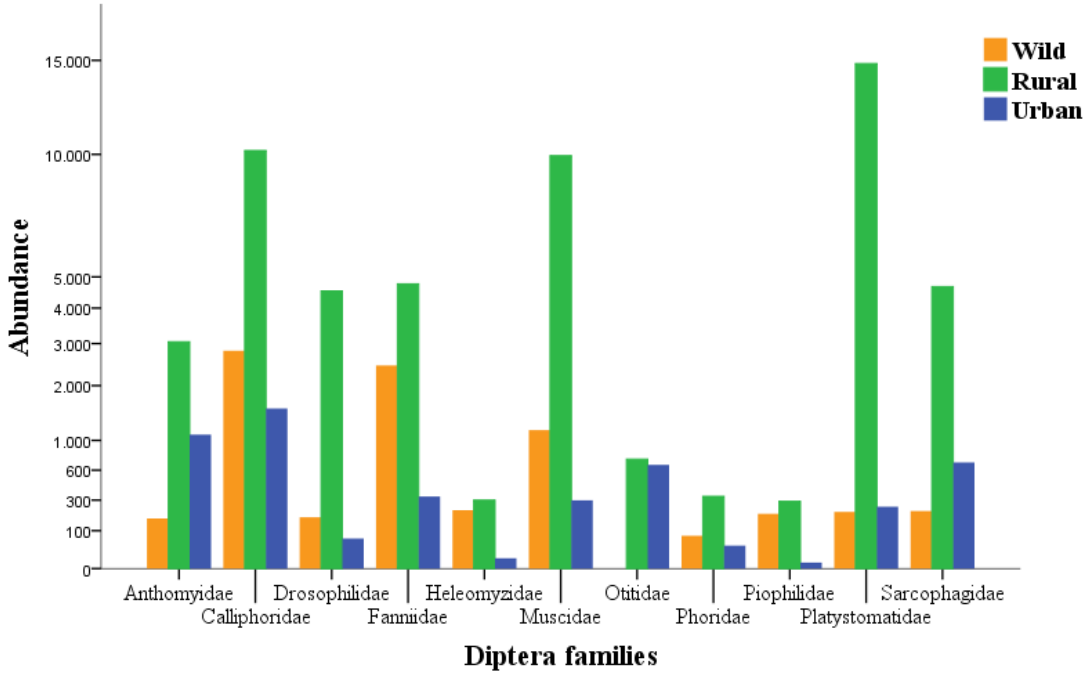
In table 2 the most interesting families sampled in two years are reported, along with their percentage of abundance for each study area.

In general, the Calliphoridae family represent the taxon with the greater abundance in the *wild* (36,57%) and *urban* (30,86%) areas, while in rural areas, only representing 19,99%. Regarding the other families, in *wild area* it is interesting to note the abundance of Faniidae (31,88%) and Muscidae (15,10%); in *rural area* the most abundant families are Platystomatidae (27,63%) and Muscidae (18,53%); for *urban area* interesting is the result about Anthomyidae (21,84%) and Otitidae (13,31%) families.

Table 2. Diptera families sampled during the two-years investigation and percentage of abundance.

	% abundance		
TAXA	**WILD**	**RURAL**	**URBAN**
Anthomyidae	2,17	5,68	21,84
Calliphoridae	36,57	18,99	30,86
Drosophilidae	2,27	8,47	1,32
Fanniidae	31,88	8,90	6,64
Heleomyzidae	2,90	0,56	0,22
Muscidae	15,10	18,53	5,98
Otitidae	0,00	1,37	13,31
Phoridae	1,00	0,62	0,82
Piophilidae	2,57	0,54	0,10
Platystomatidae	2,73	27,63	4,97
Sarcophagidae	2,81	8,72	13,94

In graph 5 the abundance values of the families for each study area are reported. Pearson's Chi-square was applied in order to test the statistical significance of the families distribution (table 3). From the report of the test explained in table 3 we can see the high significance of the Diptera families' spatial distribution sampled in two years.



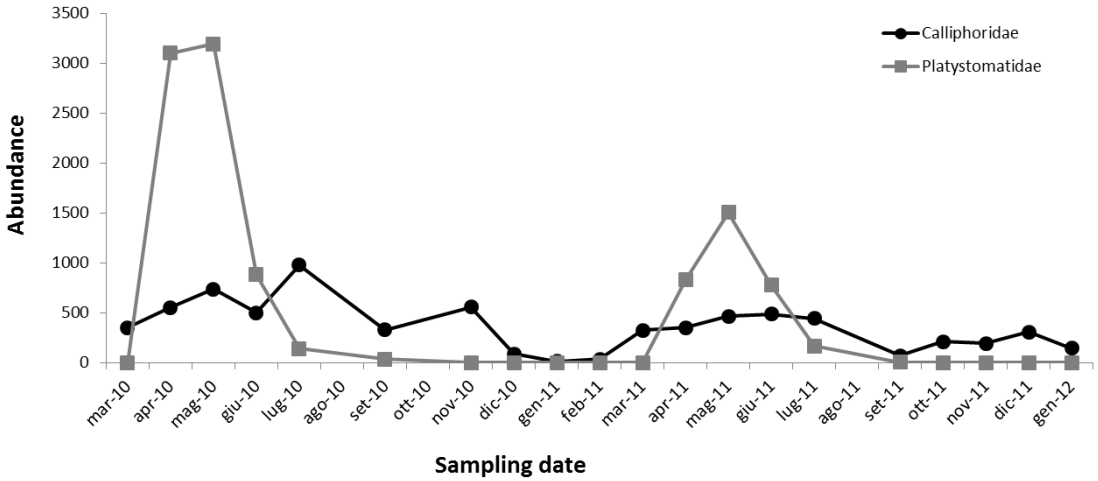
Graph 5. Abundance values of sampled families in the three study areas.

Table 3. Pearson's Chi-square for the abundance values of sampled Diptera families. Spss Software.

	Value	df	Sig.
Pearson's Chi-square	14725,853a	20	0,000
Likelihood ratio	12910,35	20	0,000
Linear-linear association	336,018	1	0,000
N. of valid cases	66300		

It is interesting to observe the dominance of Platystomatidae family in the *rural area* compared to other families. We identified three Platystomatidae species, *Platystoma seminationis* (Fabricius 1775), *Platystoma gemmationis* (Rondani 1869) and *Platystoma lugubre* (Rubineau-Desvoidy 1830), which only occurred in the traps dominating the Calliphoridae

during spring months. Graph 6 compares the phenologies of Calliphoridae and Platystomatidae during the experimental period.



Graph 6. Compared phenology of Platystomatidae and Calliphoridae families sampled in *rural area*.

The abundances variations between the two families are statistically significative, as confirmed by the Chi-square test (table 4).

Table 4. Chi-square test for abundance of Calliphoridae (N1) and Platystomatidae (N2). Past Software.

Calliphoridae vs. Platystomatidae	
N1:	9029
N2:	14811
Deg. freedom:	22
Chi^2:	9576,6
p(same):	0
Monte Carlo p(same):	0,0001
Fisher exact p(same):	N/A

Data on Platystomatidae can be taken into account for future investigations, as in international literatures there is a lack of information concerning about the food preferences of adults and larvae of the sampled species.

For this work, the Calliphoridae family was analyzed in details because their species are usually the first colonizers on a body and thus the most important PMI indicators. Overall six species were sampled and identified, listed below:

Calliphora vicina Robineau-Desvoidy 1830

Calliphora vomitoria (L.)

Chrysomya albiceps (Wiedemann 1819)

Lucilia ampullacea Villeneuve 1922

Lucilia caesar (L.)

Lucilia sericata (Meigen 1826)

Among the six species, almost all occurred on the carcasses during the successional experiments, except for *L. ampullacea* which has also forensic important due to its' involvement in myiasis.

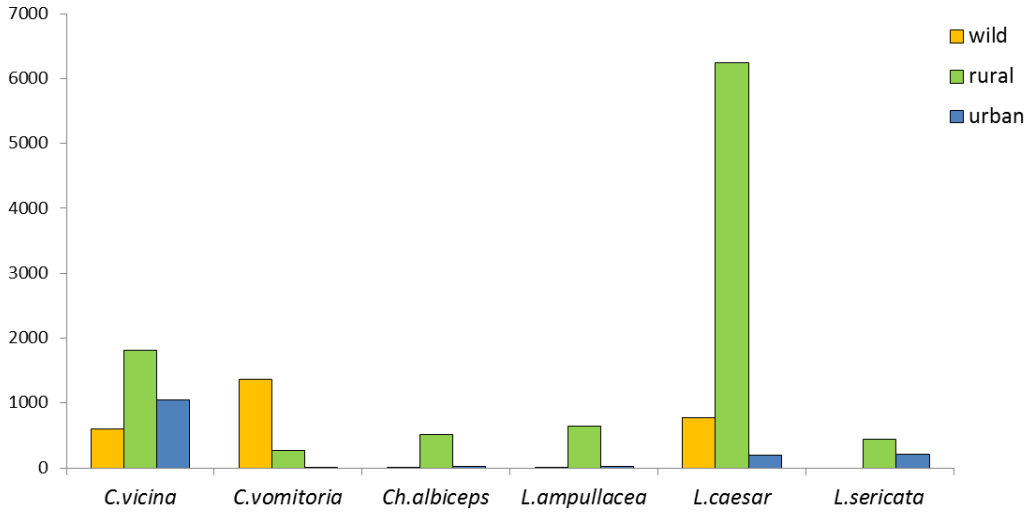
Spatial variability

From the data concerning Calliphoridae species, it is possible to observe their distribution, checking their habitat preferences.

The abundances of the sampled species in the three areas in two years of sampling are reported in table 5 and in graph 7.

Table 5. Contingence table with the abundance values for Calliphoridae species sampled in the three study areas in two years of investigation.

	WILD AREA	RURAL AREA	URBAN AREA
Calliphora vicina	597	1815	1054
Calliphora vomitoria	1369	273	8
Chrysomya albiceps	2	512	27
Lucilia ampullacea	5	649	15
Lucilia caesar	779	6248	202
Lucilia sericata	0	439	213



Graph 7. Abundance of the species of Calliphoridae in the three study areas.

The species distribution in the three study areas has statistical significance, as confirmed by Pearson's Chi-square (table 6) and Kruskall-Wallis tests (table 7; figure 42).

Table 6. Pearson's Chi-square for the abundance of Calliphoridae sampled in the three areas.

	Value	**df**	**Sig.**
Pearson's Chi-square	7499,355	10	0,00
Likelihood ratio	6389,89	10	0,00
Linear-linear association	34,362	1	0,00
N. of valid cases	14207		

Table 7. Result of the Kruskall-Wallis test, using the habitats as grouping variable. SPSS Software.

	C.vicina	***C.vomitoria***	***Ch.albiceps***	***L.ampullacea***	***L.caesar***	***L.sericata***
Chi-square	8,643	19,079	7,874	24,077	23,881	20,473
df	2	2	2	2	2	2
Sig..	0,013	0,000	0,02	0,000	0,000	0,000

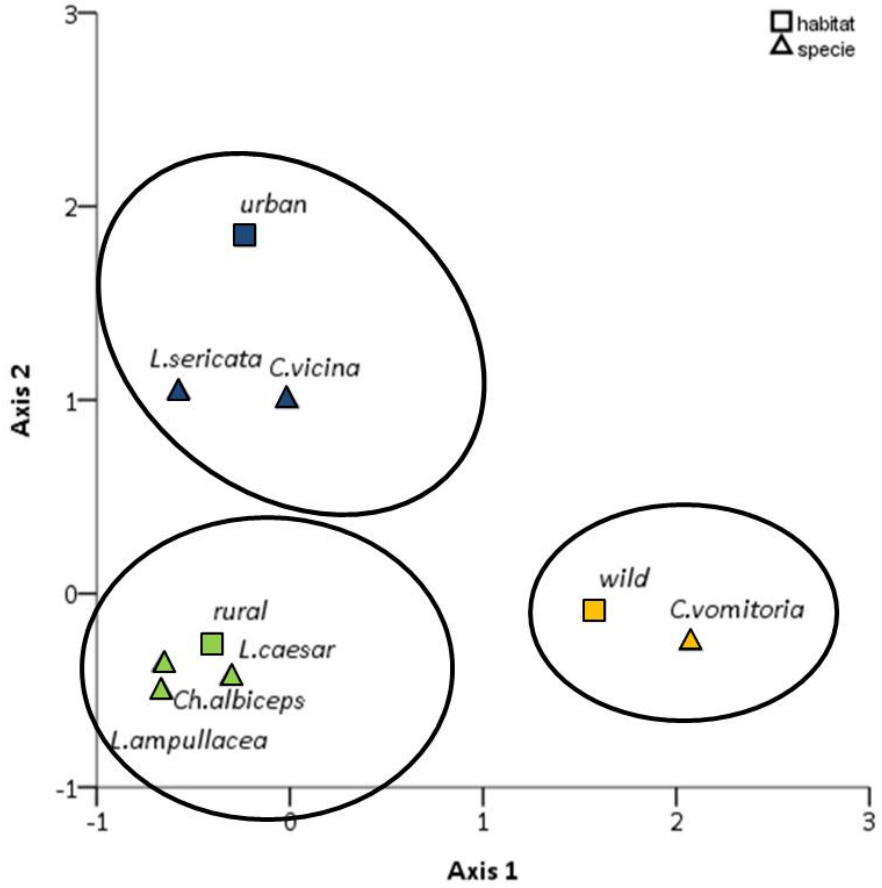
Figure 42. Result of the Kruskall-Wallis test. SPSS Software.

The species with wide distribution are *C. vicina* and *L. caesar*, sampled in all the three areas, instead *C.vomitoria* is related to the wooded environment and very rare in the *urban area*. *Ch. albiceps* and *L. ampullacea* are related to *rural area*, in fact in the other areas these species presented poor activity. Finally *L. sericata* is strongly related to *urban area*, this species wasn't found in the *wild area*.

Correspondence Analysis (CA) (table 8; graph 8) shows the habitat preferences of the sampled species.

Table 8. Result of Correspondence Analysis. SPSS Software.

Dimension	Singular value	Inertia	Chi-square	Sig.	Proportion of inertia		Confidence of singular value	
					Explained	Cumulated	Standard deviation	Correlation 2
1	0,597	0,357			0,676	0,676	0,008	0,049
2	0,414	0,171			0,324	1	0,008	
Total		0,528	7499,355	0,000	1	1		



Graph 8. *Correspondence Analysis* shows the spatial distribution of the species sampled in the study areas. SPSS Software.

From CA showed in graph 8, it is apparent that the Calliphoridae species have habitat preferences. In fact, the most representative species for *urban area* are *L. sericata* and *C. vicina*; for *rural area Ch. albiceps, L. caesar* and *L. ampullacea* are the most representative; the *wild area* is dominated by *C. vomitoria*.

Furthermore the Synantropy Index confirms the habitat preferences, in particular reports as *L. sericata* is the most synanthropic in contrast with *C. vomitoria* which is the most wooded species. Table 9 reports the abundance

(in percentage) of the species and table 10 orders the species according to their synanthropy values.

Table 9. Abundance % for Calliphoridae species sampled in the three study areas.

	% WILD	% RURAL	% URBAN
Calliphora vicina	17,87	50,82	31,31
Calliphora vomitoria	84,98	14,53	0,50
Chrysomya albiceps	0,37	94,64	4,99
Lucilia ampullacea	0,75	97,01	2,24
Lucilia caesar	10,78	86,43	2,79
Lucilia sericata	0,00	67,33	32,67

Table 10. Synanthropy Index for each species sampled in the study areas.

specie	valore I.S.
Lucilia sericata	+66,33
Chrysomya albiceps	+51,94
Lucilia ampullacea	+50,00
Calliphora vicina	+38,85
Lucilia caesar	+35,23
Calliphora vomitoria	-77,22

Temporal variability

Calliphoridae species show different abundance based on the sampling months, according to their phenology. The abundance values relative to the sampling date for each area have statistical significance, as reported in tables 11, 12 and 13.

Table 11. Pearson's Chi-square for abundance values of Calliphoridae during the sampling date in *wild area*.

WILD AREA	Value	df	Sig.
Pearson's Chi-square	1542,538	80	,000
Likelihood ratio	1577,015	80	,000
Linear-linear association	198,405	1	,000
N. of valid cases	2752		

Table 12. Pearson Chi-square for abundance values of Calliphoridae during the sampling date in *rural area*.

RURAL AREA	Valore	df	Sig.
Pearson's Chi-square	9821,463	140	,000
Likelihood ratio	8479,910	140	,000
Linear-linear association	573,414	1	,000
N. of valid cases	9936		

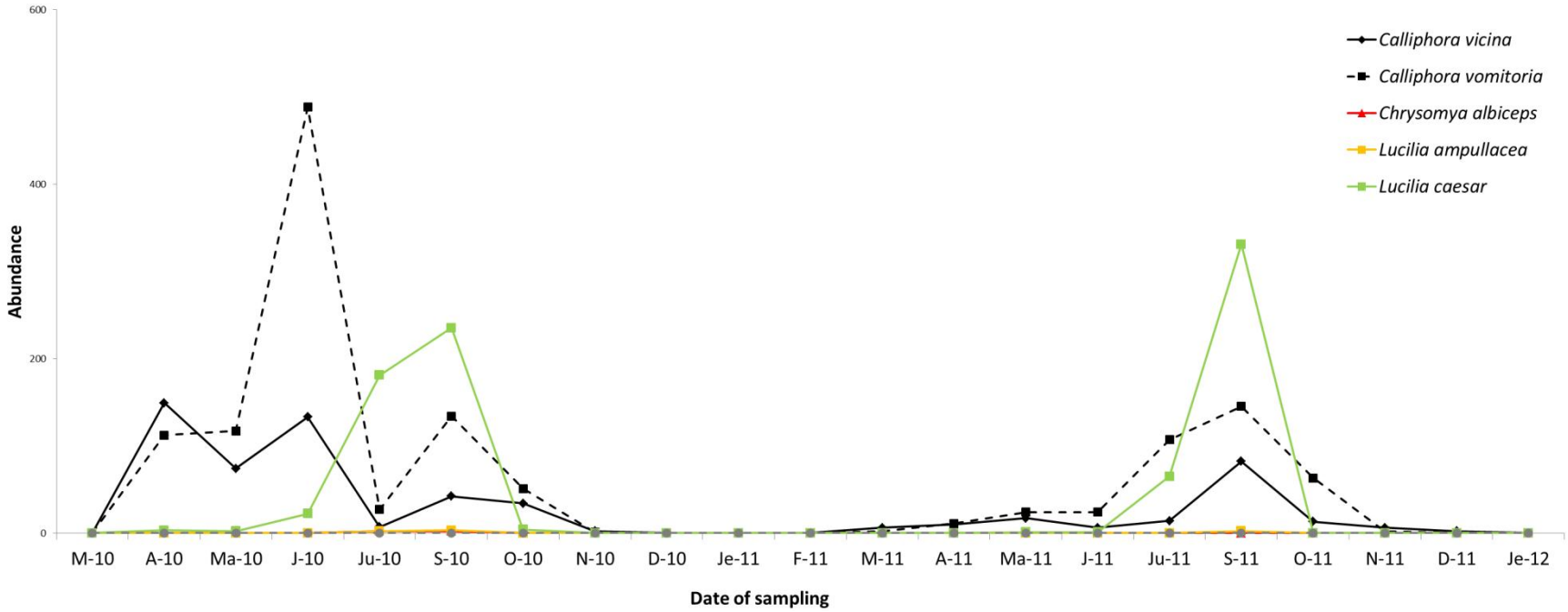
Table 13. Pearson Chi-square for abundance values of Calliphoridae during the sampling date in *urban area*.

URBAN AREA	Valore	df	Sig. asint. (2 vie)
Pearson's Chi-square	9821,463	140	,000
Likelihood ratio	8479,910	140	,000
Linear-linear association	573,414	1	,000
N. of valid cases	9936		

Tables 14, 15 and 16 report the abundance data of each species in each study area, shown in graphs 9, 10 and 11.

Table 14. Abundance of Calliphoridae species sampled in *wild area*.

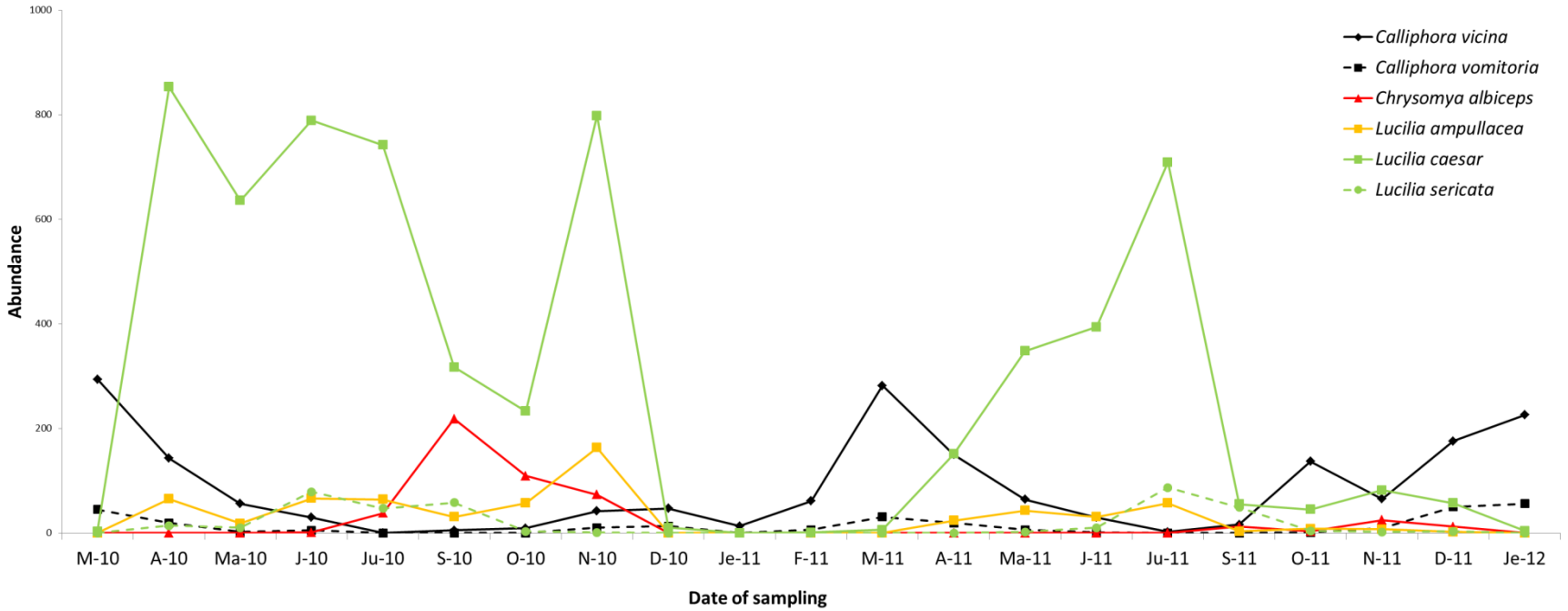
WILD	M-10	A-10	Ma-10	J-10	Ju-10	S-10	O-10	N-10	D-10	Je-11	F-11	M-11	A-11	Ma-11	J-11	Ju-11	S-11	O-11	N-11	D-11	Je-12
Calliphora vicina	0	149	74	133	7	42	34	2	0	0	0	6	10	17	6	14	82	13	6	2	0
Calliphora vomitoria	0	112	117	488	27	134	51	1	0	0	0	2	11	24	24	107	145	63	2	0	0
Chrysomya albiceps	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lucilia ampullacea	0	0	0	0	2	3	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
Lucilia caesar	0	3	2	22	181	235	4	0	0	0	0	0	0	1	1	65	331	0	0	0	0
Lucilia sericata	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0



Graph 9. Phenology of Calliphoridae species sampled in *wild area.*

Table 15. Abundance of Calliphoridae species sampled in *rural area*.

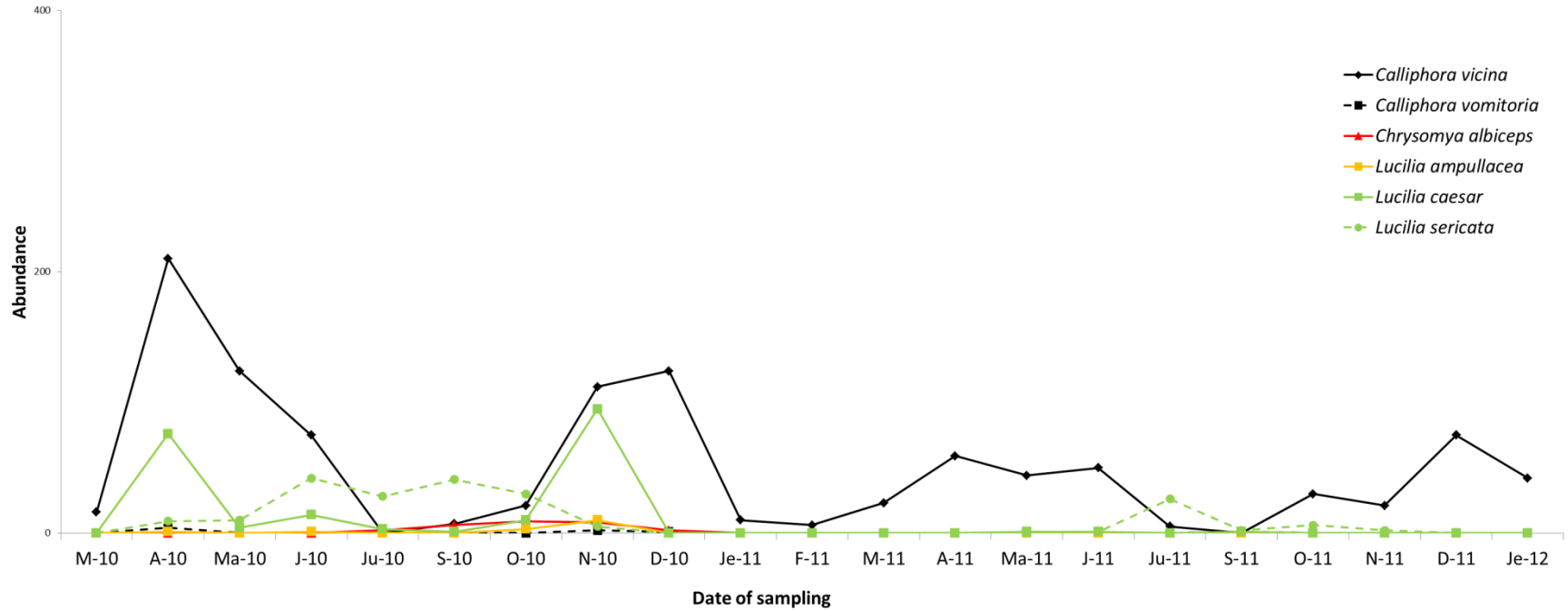
RURAL	M-10	A-10	Ma-10	J-10	Ju-10	S-10	O-10	N-10	D-10	Je-11	F-11	M-11	A-11	Ma-11	J-11	Ju-11	S-11	O-11	N-11	D-11	Je-12
Calliphora vicina	294	143	56	30	0	5	9	42	47	13	61	282	150	64	30	2	16	137	65	176	226
Calliphora vomitoria	45	19	2	5	0	0	0	10	13	0	6	31	19	6	1	0	0	1	9	50	56
Chrysomya albiceps	0	0	0	1	38	218	109	73	0	0	0	0	0	0	0	0	12	3	25	12	0
Lucilia ampullacea	0	65	18	66	64	31	57	163	0	0	0	0	24	43	31	57	3	8	7	2	0
Lucilia caesar	3	853	636	789	742	317	233	798	10	0	1	6	151	348	394	709	55	45	82	57	4
Lucilia sericata	0	14	10	79	47	58	3	1	0	0	0	0	0	2	10	87	49	4	2	3	0



Graph 10. Phenology of Calliphoridae species sampled in *rural area.*

Table 16. Abundance of Calliphoridae species sampled in *urban area*.

URBAN	M-10	A-10	Ma-10	J-10	Ju-10	S-10	O-10	N-10	D-10	Je-11	F-11	M-11	A-11	Ma-11	J-11	Ju-11	S-11	O-11	N-11	D-11	Je-12
Calliphora vicina	16	210	124	75	0	7	21	112	124	10	6	23	59	44	50	5	0	30	21	75	42
Calliphora vomitoria	0	4	0	0	0	0	0	2	1	0	0	0	0	0	0	0	1	0	0	0	0
Chrysomya albiceps	0	0	0	0	2	6	9	8	2	0	0	0	0	0	0	0	0	0	0	0	0
Lucilia ampullacea	0	1	0	1	0	0	3	10	0	0	0	0	0	0	0	0	0	0	0	0	0
Lucilia caesar	0	76	4	14	3	1	10	95	0	0	0	0	0	1	1	0	1	0	0	0	0
Lucilia sericata	0	9	10	42	28	41	30	5	0	0	0	0	0	0	1	26	2	6	2	0	0



Graph 11. Phenology of Calliphoridae species sampled in *urban area*.

As reported in the previous tables, in the *wild area* the most abundant species are *C. vomitoria*, *C. vicina*, mainly active during spring and autumn monts, and *L.caesar* dominant in summer months. *Ch. albiceps* and *L. ampullacea* are quite rare, in fact they have a low number of individuals in this area, instead *L. sericata* is totally absent, never trapped in the *wild area* for the entire investigation period. During the winter months, all the species are absent in this study area.

In the *rural* area, the dominant species is *L. Caesar*, which prevails over the other species from the spring to the autumn months. The winter period is instead dominated by *C. vicina*. In the same area *C.vomitoria* is related to winter months and *L. ampullacea* and *L. sericata* show almost the same phenology of *L. caesar* but their abundance is less. *Ch. albiceps* is active from spring to late autumn.

In the *urban area* the dominant species is *C. vicina*, which is the most abundant for almost the entire sampling period except for summer months when *L. sericata is* the most representative. *L. caesar* is active in spring and autumn instead the remaining species are very rare in this area.

Seasonal activity of sampled species

In relation to obtained data, the activity of the species related to the meteorological seasons was also analyzed, summing the sampling data collected over two years and grouping them together based on the season. The following tables (from 17 to 22) report the abundance data summed for each species for each area in the meteorological seasons and the significance of Pearson Chi-square.

Table 17. Abundance of Calliphoridae related to meteorological season in the *wild area*.

WILD	WINTER	SPRING	SUMMER	AUTUMN
Calliphora vicina	2	256	160	179
Calliphora vomitoria	0	266	634	469
Chrysomya albiceps	0	0	2	0
Lucilia ampullacea	0	0	2	3
Lucilia caesar	0	6	365	408
Lucilia sericata	0	0	0	0

Table 18. Pearson's Chi-square for the abundance values of Calliphoridae related to meteorological season in the *wild area*.

WILD	Value	df	Sig.
Pearson's Chi-square	912,138	12	,000
Likelihood ratio	1023,485	12	,000
Linear-linear association	120,215	1	,000
N. of valid cases	2752		

Table 19. Abundance of Calliphoridae related to meteorological season in the *rural area*.

RURAL	WINTER	SPRING	SUMMER	AUTUMN
Calliphora vicina	523	989	62	274
Calliphora vomitoria	125	122	6	20
Chrysomya albiceps	12	0	39	440
Lucilia ampullacea	2	150	218	269
Lucilia caesar	72	1997	2633	1530
Lucilia sericata	3	27	223	117

Table 20. Pearson's Chi-square for the abundance values of Calliphoridae related to meteorological season in the *rural area*.

RURAL	Value	df	Sig.
Pearson's Chi-square	5644,069	15	,000
Likelihood ratio	5428,735	15	,000
Linear-linear association	570,668	1	,000
N. of valid cases	9936		

Table 21. Abundance of Calliphoridae related to meteorological season in the *urban area*.

URBAN	WINTER	SPRING	SUMMER	AUTUMN
Calliphora vicina	257	476	130	191
Calliphora vomitoria	1	4	0	3
Chrysomya albiceps	2	0	2	23
Lucilia ampullacea	0	1	1	13
Lucilia caesar	0	81	14	107
Lucilia sericata	0	19	108	86

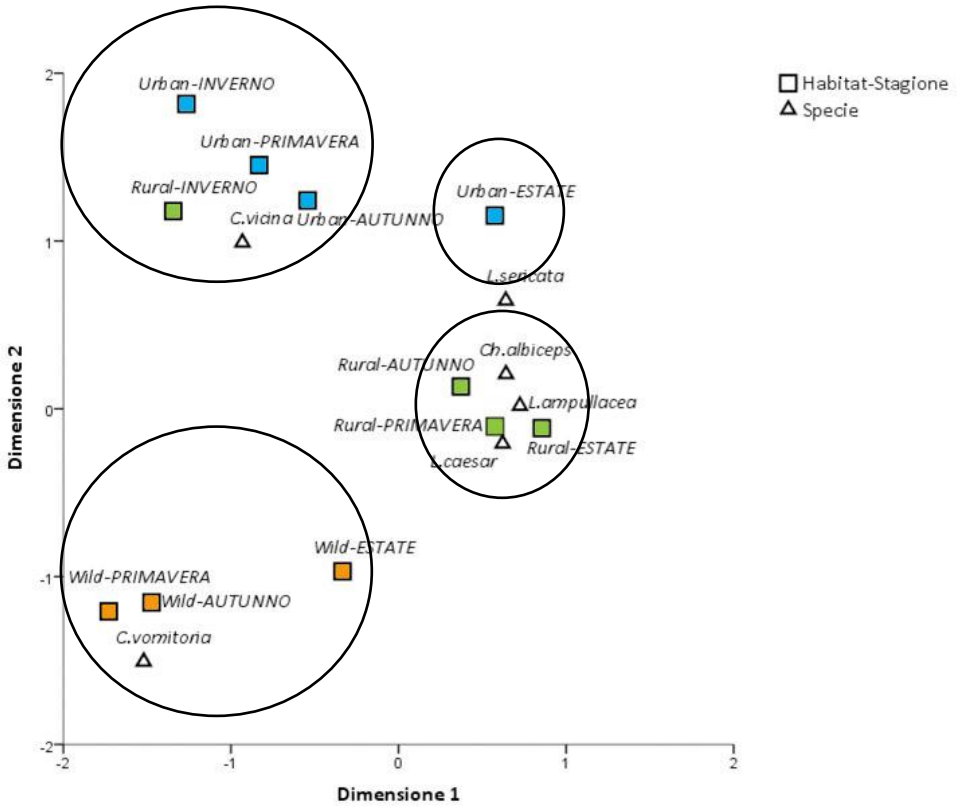
Table 22. Pearson's Chi-square for the abundance values of Calliphoridae related to meteorological season in the *urban area*.

URBAN	Vale	df	Sig.
Pearson's Chi-square	584,644	15	,000
Likelihood ratio	458,509	15	,000
Linear-linear association	50,307	1	,000
N. of valid cases	1519		

Data relative to seasonal activity of the species were subjected to Correspondence Analysis (CA) (table 23; graph 12).

Table 23. Result of *Correspondence Analysis*. SPSS Software

Dimension	Singular value	Inertia	Chi-square	Sig.	Proportion of inertia		Confidence of singular value	
					Explained	Cumulated	Standard deviation	Correlation 2
1	0,736	0,541			0,514	0,514	0,005	0,222
2	0,546	0,298			0,283	0,796	0,009	
3	0,377	0,142			0,135	0,931		
4	0,266	0,071			0,067	0,998		
5	0,041	0,002			0,002	1		
		1,054	14971,96	0,000	1	1		



Graph 12. The *Correspondence Analysis* shows the species distribution related to the habitat and the meteorological season. SPSS Software

As shown in graph 12, the seasonal activity of the species is mainly related to their habitat preferences.

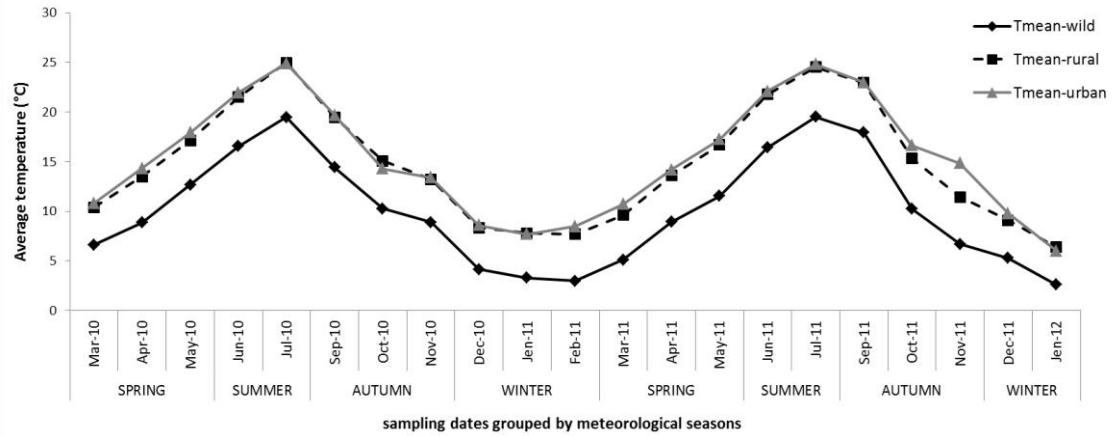
Moreover, as reported in graphs 9, 10 and 11, the species present in all the three study areas show a different phenology, probably because of the different trend of environmental temperatures. The seasonal average temperatures are lower in the *wild area* than those of the *rural* and *urban* areas (table 24; graph 13).

Table 24. Mean environmental temperatures in the three study areas during the investigated seasons..

WILD	**Winter**	**Spring**	**Summer**	**Autumn**
Tmedia	3,84 ± 1,08	8,96 ± 2,86	17,99 ± 1,73	11,42 ± 4,07
Tmax	7,42 ± 1,24	14,59 ± 3,20	24,01 ± 1,66	16,22 ± 4,56
Tmin	1,88 ± 1,22	5,78 ± 2,51	14,26 ± 2,16	9,41 ± 3,74

RURAL	**Winter**	**Spring**	**Summer**	**Autumn**
Tmedia	7,91 ± 0,99	13,49 ± 3,10	23,20 ± 1,82	16,25 ± 4,25
Tmax	11,07 ± 0,76	17,69 ± 3,38	27,74 ± 1,84	20,12 ± 4,83
Tmin	5,05 ± 1,19	9,68 ± 2,77	18,67 ± 1,77	12,88 ± 3,79

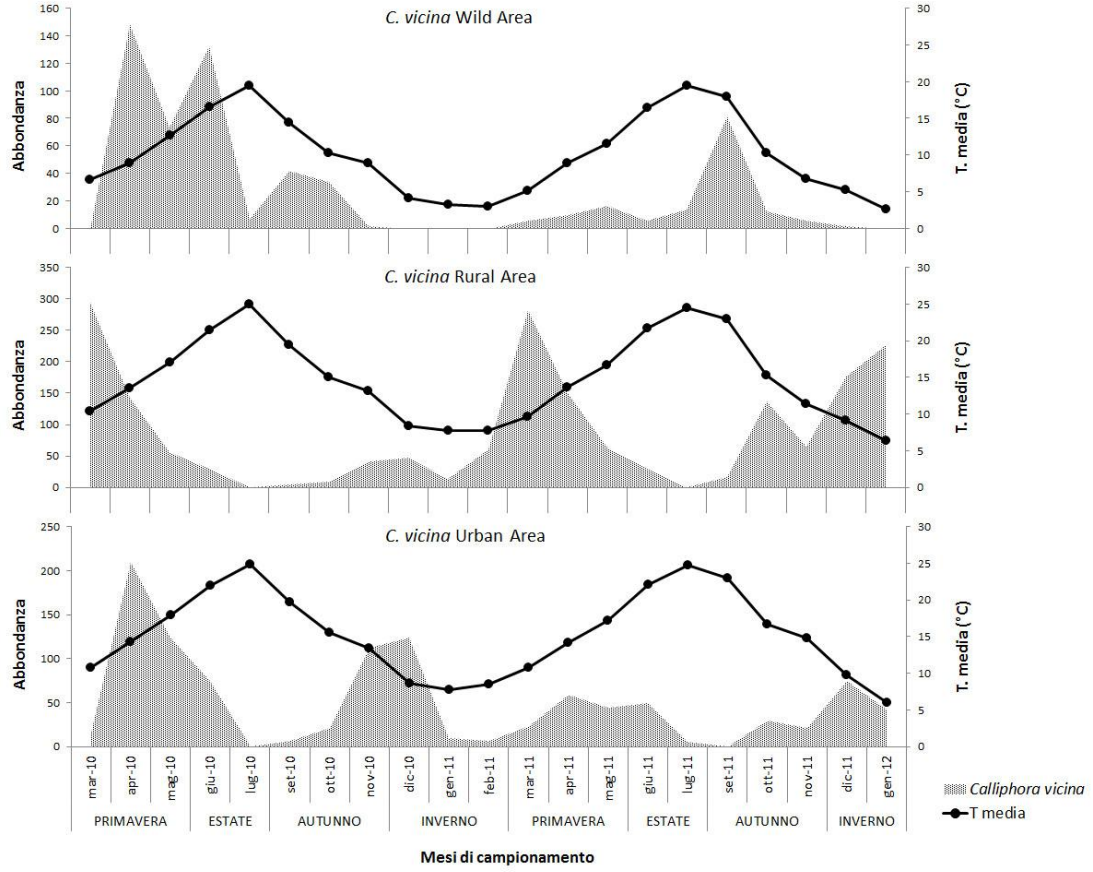
URBAN	**Winter**	**Spring**	**Summer**	**Autumn**
Tmedia	8,12 ± 1,39	14,21 ± 3,05	23,43 ± 1,64	16,98 ± 3,69
Tmax	13,52 ± 1,39	19,80 ± 3,49	29,88 ± 2,09	22,90 ± 4,26
Tmin	3,87 ± 1,71	8,88 ± 2,70	17,15 ± 1,29	12,30 ± 2,98



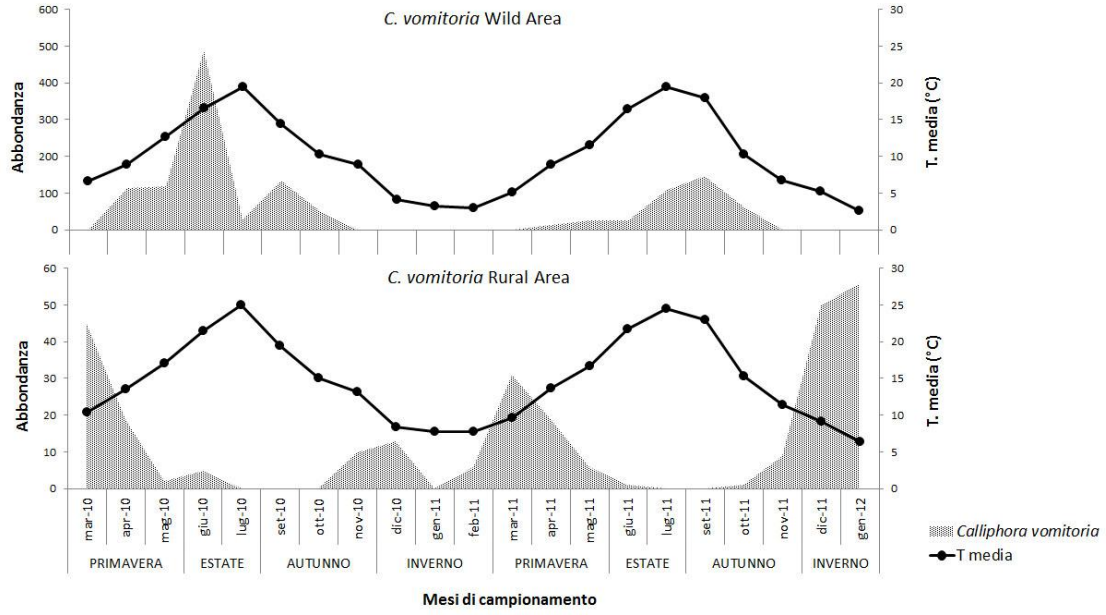
Graph 13. Trend of mean seasonal temperatures in the three study areas during the sampling period.

During the sampling months, the species were trapped with different abundance according to the period and above all depending on the area, strongly influenced by the environmental temperature.

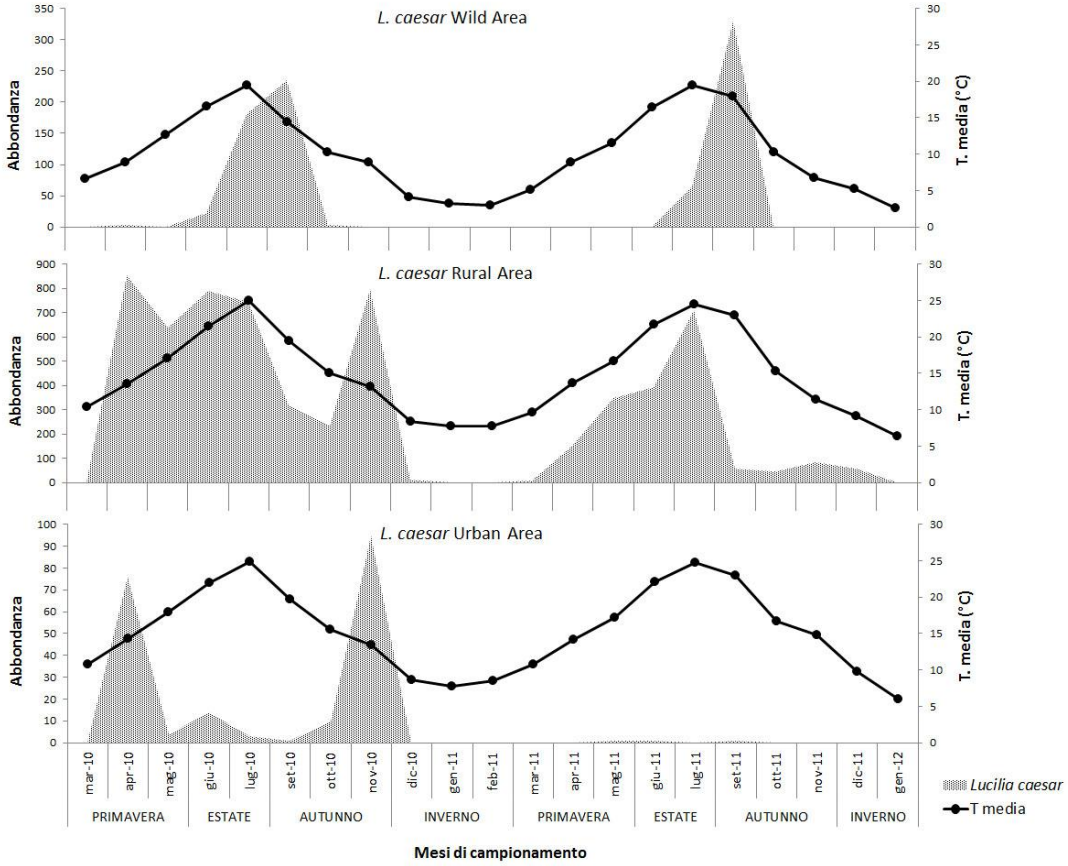
The following graphs (from 14 to 17) show the periods of activity of the most representative species depending on the study area and the environmental temperature. *Ch. albiceps* and *L. ampullacea* are excluded because both are related only to the *rural area* and they are rare in the *wild* and *urban areas*.



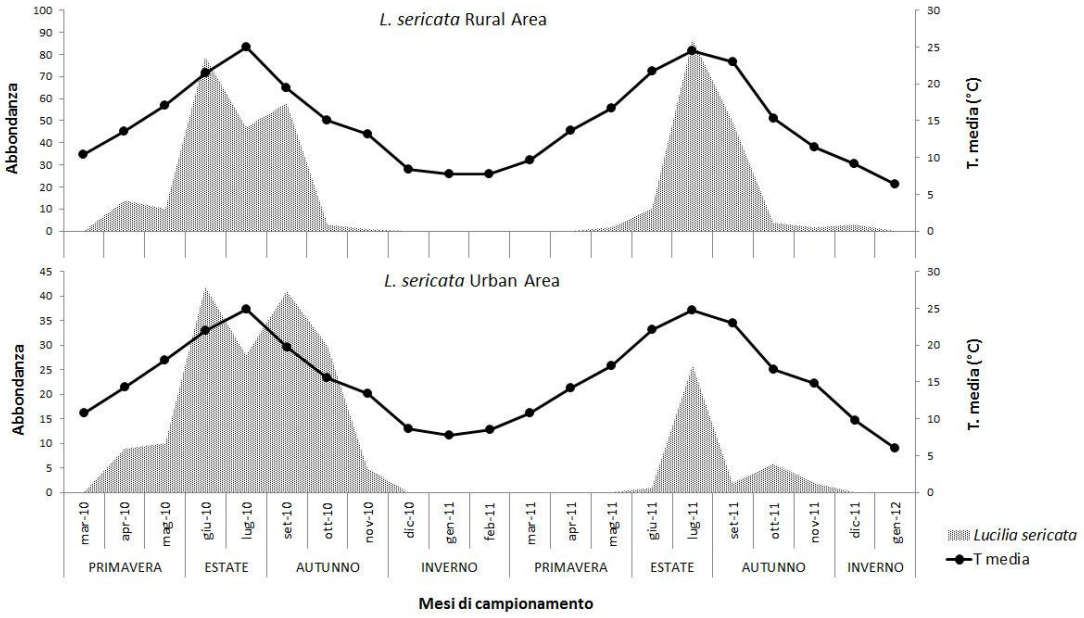
Graph 14. Activity period of *C. vicina* in the three study areas.



Graph 15. Activity period of *C. vomitoria* in the *wild* and *rural* areas.



Graph 16. Activity period of *L. caesar* in the three study areas.



Graph 17. Activity period of *L. sericata* in the *rural* and *urban* areas.

Influence of environmental temperature on the species activity

In order to evaluate the relations between the abundances of the species and the environmental temperatures, both the Pearson and Spearman correlation were performed, giving identical results (table 25).

Table 25. Pearson Correlation between temperatures and abundance of Calliphoridae.

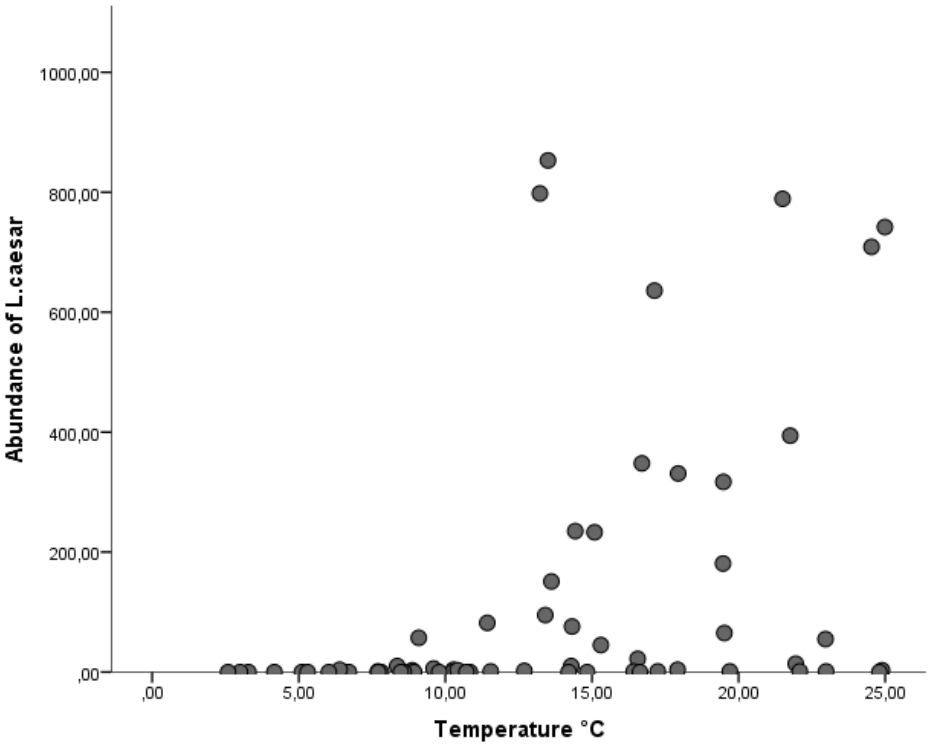
Correlazioni		Tmedia	Tmax	Tmin	*C.vicina*	*C.vomitoria*	*Ch.albiceps*	*L.ampullacea*	*L.caesar*	*L.sericata*
Tmedia	Correlazione di Pearson	1	,989**	,984**	-0,106	0,048	0,168	,268*	,407**	,608**
	Sig. (2-code)		0,00	0,00	0,408	0,708	0,188	0,034	0,001	0,00
	N	63	63	63	63	63	63	63	63	63
Tmax	Correlazione di Pearson	,989**	1	,959**	-0,127	0,068	0,116	0,197	,335**	,567**
	Sig. (2-code)	0,00		0,00	0,319	0,596	0,367	0,121	0,007	0,00
	N	63	63	63	63	63	63	63	63	63
Tmin	Correlazione di Pearson	,984**	,959**	1	-0,131	0,09	0,206	,301*	,453**	,617**
	Sig. (2-code)	0,00	0,00		0,307	0,481	0,104	0,016	0,00	0,00
	N	63	63	63	63	63	63	63	63	63
C.vicina	Correlazione di Pearson	-0,106	-0,127	-0,131	1	,259*	-0,13	-0,05	-0,03	-0,187
	Sig. (2-code)	0,408	0,319	0,307		0,04	0,309	0,696	0,814	0,142
	N	63	63	63	63	63	63	63	63	63
C.vomitoria	Correlazione di Pearson	0,048	0,068	0,09	,259*	1	-0,083	-0,104	-0,042	-0,164
	Sig. (2-code)	0,708	0,596	0,481	0,04		0,518	0,418	0,744	0,2
	N	63	63	63	63	63	63	63	63	63
Ch.albiceps	Correlazione di Pearson	0,168	0,116	0,206	-0,13	-0,083	1	,424**	,278*	,301*
	Sig. (2-code)	0,188	0,367	0,104	0,309	0,518		0,001	0,027	0,017
	N	63	63	63	63	63	63	63	63	63
L.ampullacea	Correlazione di Pearson	,268*	0,197	,301*	-0,05	-0,104	,424**	1	,840**	,352**
	Sig. (2-code)	0,034	0,121	0,016	0,696	0,418	0,001		0,00	0,005
	N	63	63	63	63	63	63	63	63	63
L.caesar	Correlazione di Pearson	,407**	,335**	,453**	-0,03	-0,042	,278*	,840**	1	,512**
	Sig. (2-code)	0,001	0,007	0,00	0,814	0,744	0,027	0,00		0,00
	N	63	63	63	63	63	63	63	63	63
L.sericata	Correlazione di Pearson	,608**	,567**	,617**	-0,187	-0,164	,301*	,352**	,512**	1
	Sig. (2-code)	0,00	0,00	0,00	0,142	0,2	0,017	0,005	0,00	
	N	63	63	63	63	63	63	63	63	63

** La correlazione è significativa al livello 0,01 (2-code).

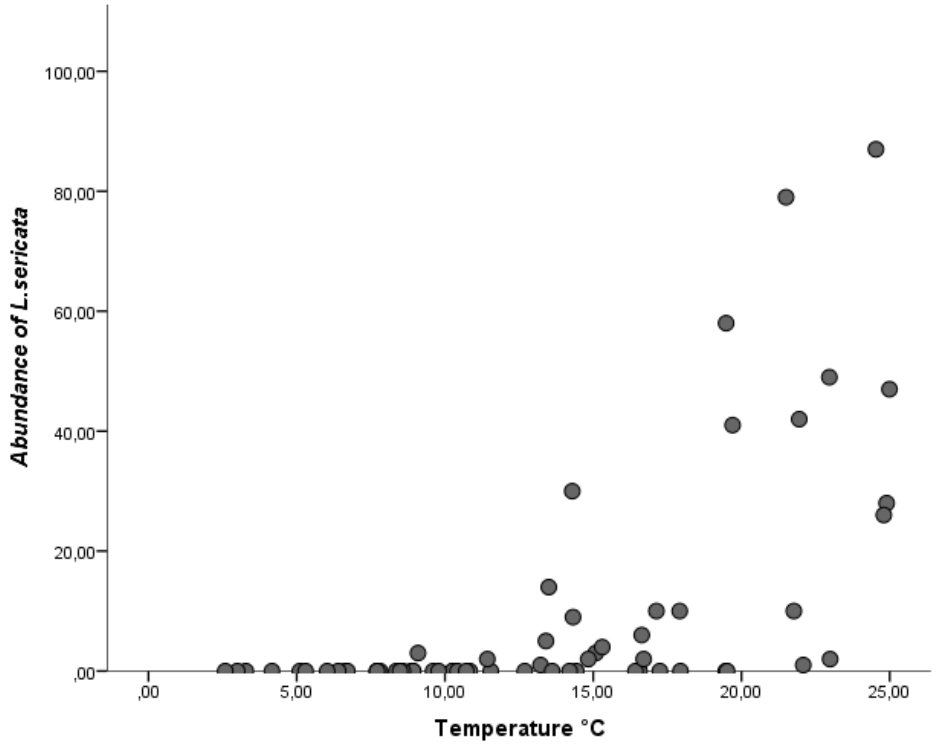
* La correlazione è significativa al livello 0,05 (2-code).

From the report, it seems that *Calliphora* spp and *Ch. albiceps* are not influenced by temperature, instead *Lucilia* spp show a positive correlation, stronger in *L. caesar* and *L. sericata*. In the following graphs (18, 19, 20, 21) the abundance distribution of the interesting species in relation with the mean environmental temperature recorded for each area are reported (*L. ampullacea* is excluded due to being related to only one area and *Ch.*

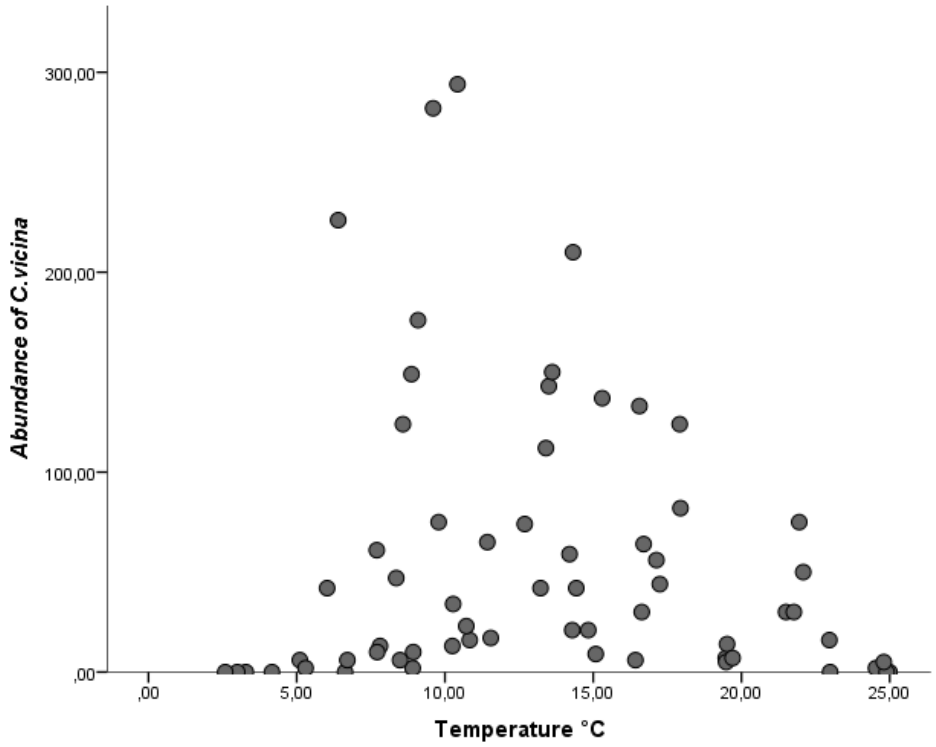
albiceps is excluded because of the low number of individuals). The scatterplots show as *Lucilia* spp have a temperature-dependent activity unlike *Calliphora* spp.



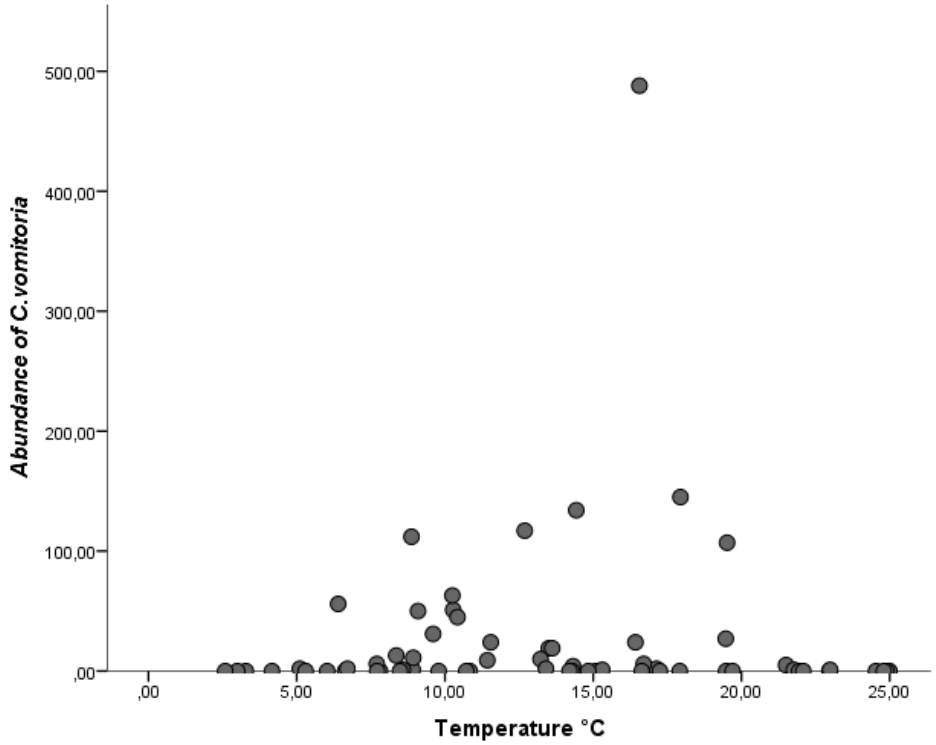
Graph 18. Abundance of *Lucilia caesar* in relation with the environmental temperature.



Graph 19. Abundance of *Lucilia sericata* in relation with the environmental temperature.



Graph 20. Abundance of *Calliphora vicina* in relation with environmental temperature.



Graph 21. Abundance of *Calliphora vomitoria* in relation with the environmental temperature.

Chapter 4

CASEWORK

Thanks to the collaboration of Dr. Vannio Vercillo (from A.S.P. 4 of Cosenza – Legal Medicine section) it has been possible, using the insect evidence, to evaluate the PMI in two human corpses found in outdoor environments and to identify *post mortem* artifacts caused by Hymenoptera Formicidae in a human corpse.

Thanks to the collaboration with veterinaries Dr. Ugo Curcio (Montalto Uffugo, Cosenza) and Dr. Gianni Marinacci (Rende, Cosenza), we were able to collect and identify the species that caused myasis and pseudomyiasis in domestic and stray animals.

Thanks to Dr Claudio Tersaruolo we collected diptera larvae in a pseudomyiasis mase in a child.

PMI ESTIMATION

Case 1

The first case dates back to February 2007. The body of a 37aged man was found in an underground passage in the city of Cosenza. The discovery site of the body, appeared neglected, with surrounding garbage spread on the floor. The walls were very humid and partially covered with moss. There weren't windows; the entrance was partially closed with wooden boards which prevented entry of any light

The corpse was found kneeling, with the face resting on a garbage container; the left arm was folded against the wall, the right one outstretched on the garbage container (figure 43).

Figure 43. The corpse founded in February 2007 (from Bonacci *et al*., 2009).

Eggs and larvae first stage, identified as *Calliphora vicina*, were collected *in situ* at the moment of discovery of the body and also during the autopsy. During the on-the-spot investigation the eggs were collected from the right mastoid region, while larvae at the first stage, were collected from the right ear, lips and lower right eyelid. During the autopsy other eggs were found in the mouth, inside the right nostril, on the right cheek and on the scalp.

The mean environmental temperature recorded on the day of discovery was about 10,8°C. The mean diurnal temperature of February 2007 was 14°C, instead the mean night temperature was 5-6°C.

According to the older sample found on the body (L1) and to the environmental temperatures (which affect the larval age duration) a PMI of 68-72 hours was estimated, in agreement with the medico-legal estimation.

Case 2

The second case dates back to November 2010. The body of an elderly man was found outside the Civil Hospital of Cosenza, under a wall of a passageway that connects the ward of patients with the outside the hospital, in a surrounding garden.

at the moment of discovery, no collections of entomological finds were made, but the body was transported to the morgue and placed inside of the refrigerator. During the autopsy performed the next day, larvae at the second stage of *Calliphora vicina* were found inside the nasal cavity (figure 44).

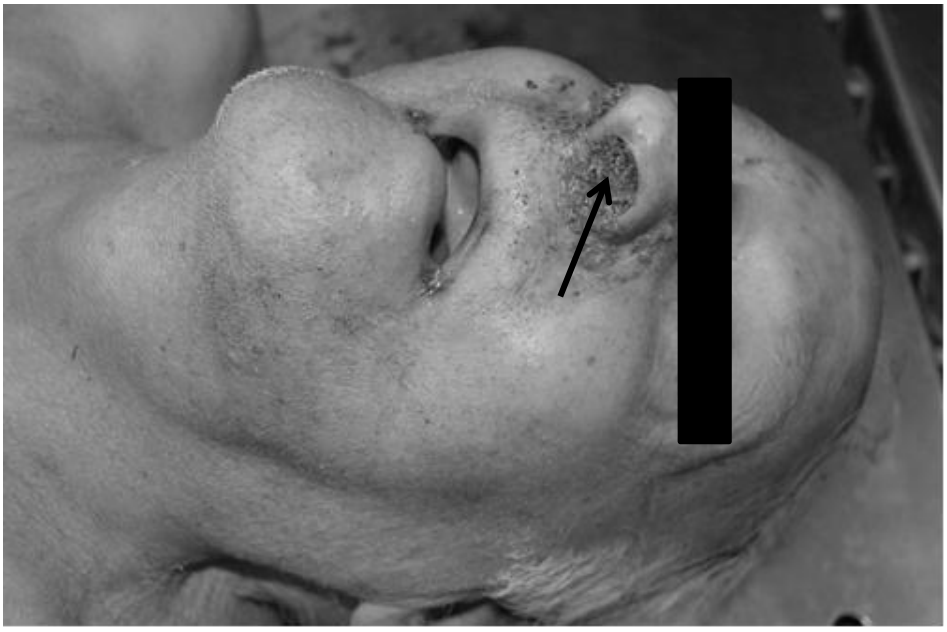


Figure 44. Larvae at the II stage of development found in the nasal cavity of the deceased (from Vercillo V.).

The mean environmental temperature recorded on the day of the discovery was 15,9 ° C. The second stage larvae were found after 2 days of discovery. The PMI estimation has been calculated at about 32 hours before the discovery of the body. No information was provided about the cause of death. The body has been recognized and identified as a patient of the

hospital, hospitalized a few days earlier, probably escaped from the hospital controls and lost outside the hospital where the death occurred.

POST MORTEM ARTIFACTS

Case 3

The body of a 53 year old man was found in a rural area in the province of Cosenza. At the moment of discovery, the corpse was dressed, lying in a supine position and there were no blood stains. From the autopsy, it was estimated a PMI of 72 hours before the discovery of the body. The cause of death was attributed to acute cerebral ischemia associated with cocaine intoxication. Traces of cocaine and alcohol were found in the blood and urine. The body was infested by Hymenoptera Formicidae *Tapinoma nigerrimum* (Nylander 1856), found mainly in the orifices respiratory and at the level of the chest and back (figure 45).

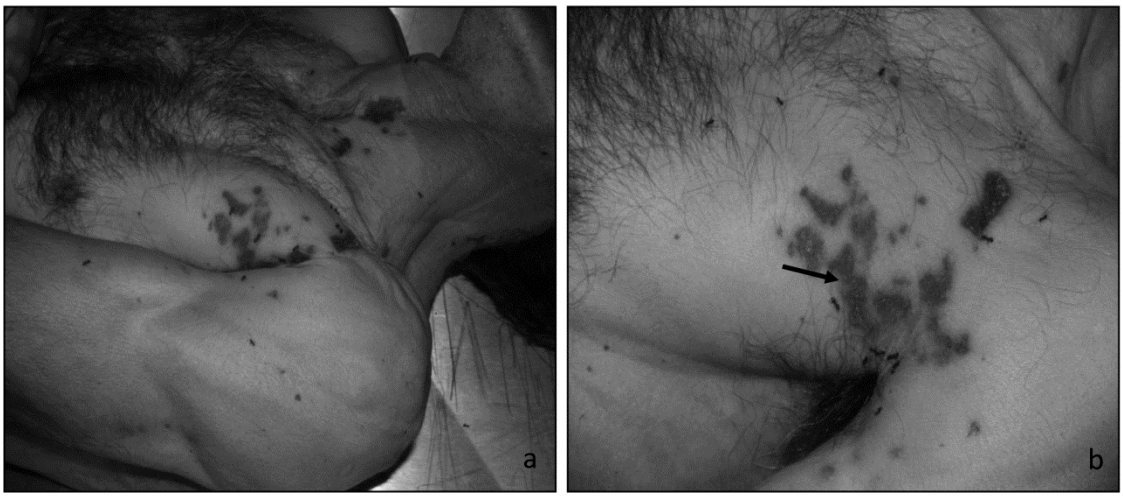


Figure 45. *Post mortem* artifacts caused by *Tapinoma nigerrimum* (from Vercillo V.).

On the body, at the level of the armpits, neck and arms, there were multiple irregular lesions (0,5 to 5,5 cm), excoriation types, dark red, identified as *postmortem* artifacts, caused by Formicidae. The attribution of the injuries to the activity of the ants was confirmed by microscopic examination of injured tissue (figure 46). A histological examination of the epidermis injured party has shown that the erosion caused by the action of Formicidae was caused by the secretion of chemicals rather than by the mechanical action of the jaws.

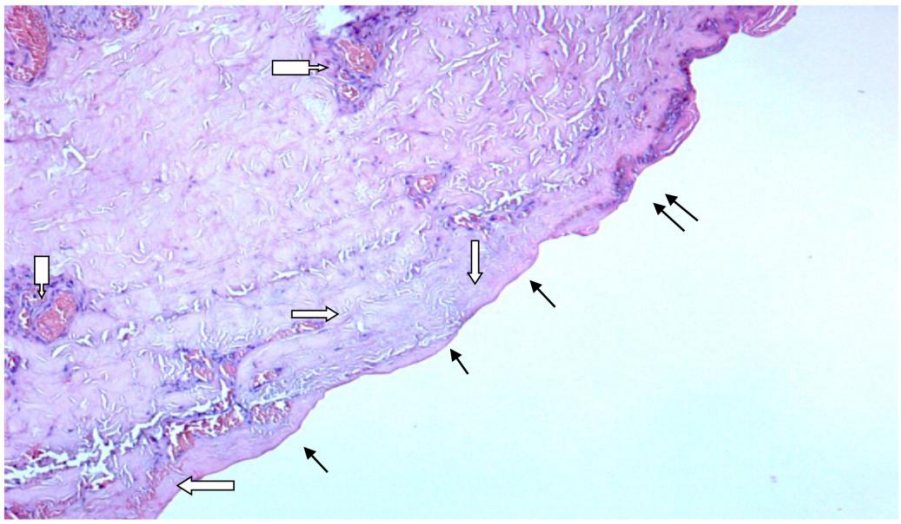


Figure 46. Histology of the injured tissues by *Tapinoma nigerrimum*. The arrows indicate the erosions caused by chemical agents secreted by Formicidae (from Vercillo V.).

MYIASIS

Case 4

A stray dog, presumed wounded due to a car accident, was found near Fago del Soldato, a woodland area of the Cosenza province situated at about 1400 meters a.s.l.. The dog showed Diptera larvae infestations (at different development stages) in the anal region and on the back (figure 47).

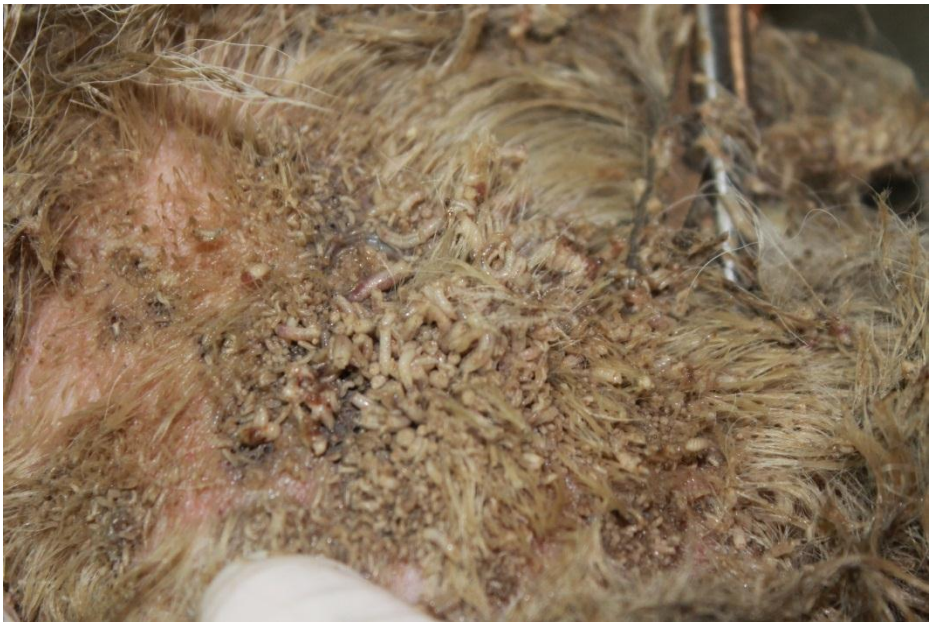


Figure 47. Myiasis on the back of the dog found near Fago del Soldato (from Cordoano S.).

Eggs and larvae samples were collected and reared in the Laboratory of General and Forensic Entomology of University of Calabria. The emerged adults, belonging to several families, were identified as *Lucilia sericata* (Calliphoridae), *Muscina stabulans* (Fallen 1817) (Muscidae) and *Sarcophaga (Liosarcophaga*) *portschinskyi* (Rohdendorf 1937) (Sarcophagidae).

This case is interesting due to the presence of *L. sericata*, a species which usually colonizes dead bodies and occasionally can cause secondary myiasis. Moreover, the woodland habitat where the dog was discovered is not a favoured place for this species, which is strongly synanthropic. This

data suggests that the dog was moved (or maybe abandoned) in the place of discovery after the infestation.

Case 5

A domestic dog was subjected to a surgery for removing a tumoral cyst from abdominal area. The entire cyst was infested by diptera larvae at the third stage of development (figure 48). The collected larvae have been reared in the Laboratory of General and Forensic Entomology (University of Calabria) and the emerged adults were identified as *Wohlfartia* sp Brauer & Bergenstamm 1889.

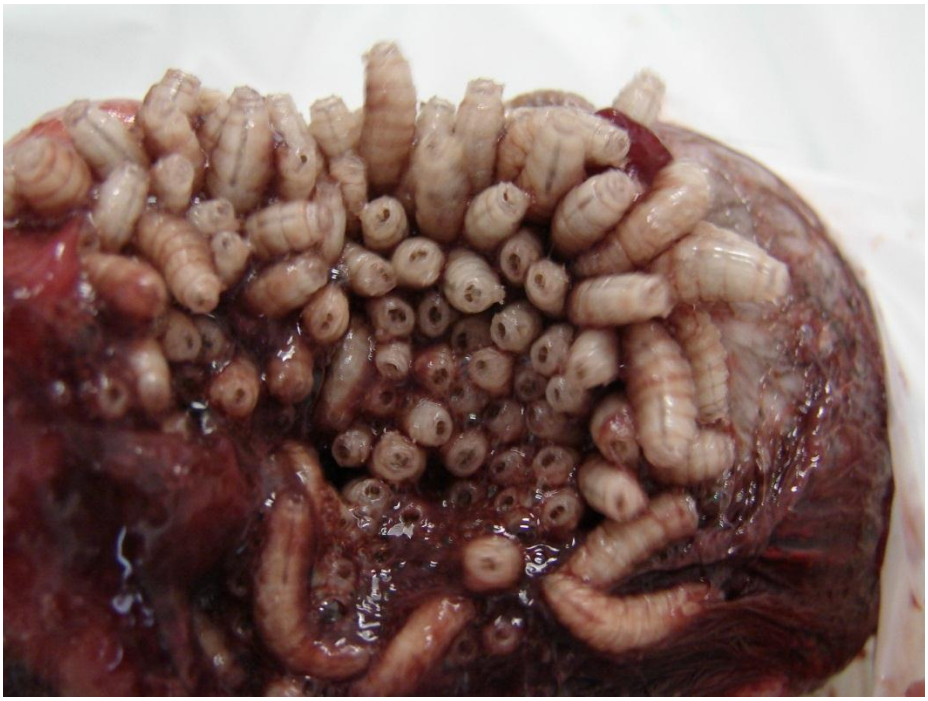


Figure 48. Larvae of *Wohlfahrtia* sp found in the tumor cyst (from Bonacci T.).

Case 6

This case concerns a pseudomyiasis case caused by diptera larvae found living in child feces. From the few information collected, it could be assumed that larvae were accidentally ingested by the child while he played in a riding stable. The collected larvae have been reared in the laboratory of General and Forensic Entomology (University of Calabria). The emerged adults were identified as *Sarcophaga* (*Bercaea*) *africa* (Wiedemann 1824) (Sarcophagidae).

Chapter 5

CONCLUSIONS AND DISCUSSIONS

The investigation strongly contributes to the knowledge of forensic interest species living in Calabria, in particular, in the Cosenza province[127].

Thanks to the obtained results, it has been possible to understand which species are able to colonize the decomposing corpses and the modalities of successions along with the duration of stay of the ecological categories which occur in the outdoor environment over several season. From these studies we observed the environmental factors that affect both the thanatological changes of exposed corpses and necrofauna activity as well as the way in which the ecological categories occur on the cadaveric resource and replace them as the decomposition process proceeds.

First colonizers, main PMI indicators, are Diptera Calliphoridae which occur before the other categories during the succession process, except when the environmental conditions are unsuitable.

Calliphoridae which colonize the bodies are different according to the season, as each species is related to its own phenology.

[127] Bonacci *et al*., 2010

Calliphora spp are active during the winter months, whereas *Lucilia* spp prefer the warm seasons. *Chrysomya albiceps* was the dominant species in the summer experiment, when mean environmental temperatures were very high (27,65 ± 5,58°C).

Diptera Sarcophagidae, Muscidae and Fanniidae were observed occurring always after Calliphoridae colonization; Piophilidae are instead related to advanced stages of decomposition. The role of the Coleoptera predators is also important (Staphylinidae, Histeridae, Nitidulidae, Silphidae) which occur in the majority of cases when a great amount of prey, represented by immature of Diptera, are available.

Saprophagous Coleoptera (Cleridae and Dermestidae) occur during the late stages of decomposition, or when dry remains become available.

Time of arrival of the ecological categories was different for each seasons.

In agreement with the international literature[128], the species activity is strongly influenced by environmental factors (above all by environmental temperature) and the usual colonizer can be replaced by other species when climatic conditions impede their dominance on the resource.

The behavior of Coleoptera Silphidae *Thanatophilus rugosus* and *T. sinuatus* during winter experiment was interesting. Low temperatures recorded during the first months (10,90± 4,46°C) impeded the normal

[128] Campobasso *et al*, 2001

development of the *Calliphora* spp larvae on the carcass, dead during the first feeding stages (I-II instars). In these limiting conditions, the absence of the usual necrophagous agents promoted the arrival of Coleoptera Silphidae, that occurred conspicuously on the carcass as main colonizers. In nature, Silphidae are used to colonize little mammalian carcasses, showing a strong territoriality. In this case, the abundant resource avoided the interspecific competition betweeen *Thanatophilus* species which shared the body as breeding site and trophic resource for their offspring[129].

Investigations on entomological successions also allowed obtaining interesting data about the trophic activity of Hymenoptera Formicidae on the exposed carcasses. For the medico-legal point, the activity of Formicidae on the pigs skin caused *post mortem* artifacts, which indicate not only the thanatological changes of the body, but often they give more information about PMI estimation, as reported in the results for the summer experiment when *Crematogaster scutellaris* delayed the Calliphoridae colonization of 24 hours[130].

In general, the information obtained from carcass experiments give a valid contribution to the knowledge of the forensic importance species living in

[129] Bonacci *et al*., 2010
[130] Bonacci *et al*., 2011

Calabria, their time of arrival during the decomposition process and their daily and seasonal activity.

The investigation on the necrophagous species sampling provides more information about the ecology of scavenger Diptera, with particular reference on Calliphoridae species.

From data analysis it emerges that the interesting species show different ecological and phenological preferences. In particular *Calliphora vomitoria* is the characteristic species for natural environments, instead *Lucilia sericata* is the most synanthopic, as confirmed by the results of Correspondence Analysis (CA) and the Sinanthropy Index (see results at pages 83 and 84), in agreement with the data already present in international literature[131,132,133]. *Calliphora vicina* is the species with wide distribution, as well as being the most active, although it shows different phenology in the three areas, maybe because of the interference with the other species. *Lucilia caesar* is the dominant species for the *rural area*, except for the winter months. *Lucilia ampullacea* is related to *rural area*, in fact it is almost absent in the other habitats. This species is an amphibian parasite, it therefore prefers humid environments where their hosts are present. *Chrysomya albiceps* was the rarer species among Calliphoridae, in

[131] Figueroa-Roa&Linhares, 2002
[132] Fischer O. A., 2000
[133] Vianna *et al*., 1998

fact it was sampled in a very little number of individuals, despite being the dominant species in the summer carcass experiment. The activity of this species could be affected by the size of the trophic resource; in fact the traps bait used in the sampling investigation was not particularly attractive for this species.

Moreover, the investigation pointed out how the species show a different seasonal activity in relation with the area, due to the differences in the environmental temperature trends. The low temperatures affect the presence and the activity of Calliphoridae, as confirmed by the total lack of samples during the winter months in *wild area*, the cooler habitat situated at about 950 meters a.s.l.

The presence of Diptera Platystomatidae, *Platystoma seminationis*, *P. gemmationis* and *P. lugubre* in the traps was very interesting, in fact they dominated on Calliphoridae in the *rural area* only during spring months. The *sex ratio* confirms that females were more abundant than males, resulting in the possible use of the bait for their offspring. No data is present in literature concerning their implication in body colonization, but this could be an interesting data to bear in mind for further investigations[134].

[134] Greco *et al*., 2012, in press

The data obtained from successional experiments and ecology study of necrophagous species, represent the first official *data base* for forensic species living in Calabria which can be applied in real cases.

The investigation focused on the study of Calliphoridae (main indicators of PMI) allowing the identification of the species of forensic importance that occur on the bodies (*Calliphora* spp, *Lucilia* spp, *Chrysomya albiceps*). But further information about other Diptera families was collected, some of them already known as corpse invaders (Sarcophagidae, Muscidae, Fanniidae, Phoridae), others not yet classified as forensic indicators (Platystomatidae).

It could also be interesting to continue with this research investigating in other kinds of habitats in order to obtain further information about the factor that affect the species composition of necrophagous communities in Calabria.

Another point to be developed in future investigations concerns the biology of interesting species, in particular their life cycle, studying in detail the time of development for each larval stage in controlled temperatures in laboratories. Thereby, it will possible to evaluate with precision the duration of life cycle of a species at a particular temperature and obtain growth tables to use as comparison data in real situations.

In conclusion, the entomological investigations, subject of this study, provided useful elements in the following applicative aspects: i) determining the season in which the body was placed in the discovery place; ii) establishing the time of death or the period of permanence of the body in the discovery place; iii) establishing if the discovery place coincides with the place where the death occurred.

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