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# Study on Diptera Hippoboscidae of the genus *Lipoptena*, parasites of ungulates, and morphological and bioecological investigations on *L. fortisetosa*, a new species for Italy

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# Declaration

I guarantee that this dissertation contains the PhD results I personally obtained during the doctoral period (2018-2021) at the University of Florence. Everyone who collaborated on aspects concerning my work (experimental designs, data collections, analyses, elaborations, or paper writing) was included as co-author, acknowledged, or cited. I also declare that this thesis has not been submitted for the award of any other degree or diploma to another University or Institution.

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# Abstract

This research explored different aspects of two hippoboscid species: *Lipoptena cervi* (Linnaeus, 1758) and *L. fortisetosa* Maa, 1965, which are hematophagous ectoparasites infesting mainly cervids and occasionally biting humans. Investigations aimed at enlarging the general knowledge on these species particularly renowned for their possible role in human and animal health implications. To achieve the research goal several studies groupable into five sections have been conducted. In the first section an overview on the Hippoboscoidea superfamily has been presented in order to show the scenario in which the studied hippoboscids are included. The second section regards morphological investigations that allowed to underline the peculiar body characters discriminating *L. cervi* and *L. fortisetosa* which are commonly mistaken. Additionally, several morphological adaptations that some hippoboscid species differently evolved to efficiently live together with their hosts have been shown. The process by which ectoparasites locate the victims has been carefully discussed in the third section. This topic has been assessed by examining *L. fortisetosa* colour attraction trough an experiment conducted in field. This trial allowed to prove that the fly is able to discriminate colours and uses visual stimuli to find hosts. Besides, observations on the antennae of different hippoboscid species have been performed using scanning and transmission electron microscopes approaches. Morphological studies provided a detailed description of the conformation of these structures with a particular focus on the sensory pattern. Sensilla have been mapping and typing, and, thanks to ultrastructure performed on *L. fortisetosa,* the involvement of chemoreception played by basiconic and coeloconic sensilla, as well as by other antennal features, has been

hypothesized. The four section includes the core data of the PhD program. It deals with the diffusion of the two studied *Lipoptena* spp. in central Italy and is focused on the relation these insects established with their main cervid hosts. Parasitism dynamics and infestation preference, in terms of species, sex, and age classes of hosts, have been investigated comparing the results of the two hippoboscid species. Further, phylogenetic analyses on *L. fortisetosa* have been performed with the purpose of giving information useful to trace the route this ectoparasite travelled from the native country Japan to Europe, probably carried around by its original host, *Cervus nippon*. Finally, the last section reports the results obtained by the characterization of the bacterial community of *L. fortisetosa*. This research proved that the fly harbours several pathogens of medical interest confirming that it represents a possible risk for human health as carriers of microorganisms potentially responsible of diseases.

# Introduction

Parasites are considered as organisms evolved to be dependent on other individuals of different species with which they are linked at diverse association levels (Barnard, 1990). Among the numerous arthropods, a relatively small group of species (ticks, mites, some flies, myiasis, fleas, and lice) developed the ability to live directly at the expense of other animals, entailing a relationship potentially detrimental for these latter. If the relationship is harmful to the host, it is named as parasitism (Wall and Shearer, 2001). The degree of damage can vary considerably and generally it does not directly entail lethal consequences for the host, allowing in this way the parasite to survive at host' expense (Paakkonen, 2012). Organisms that live externally to their victims, on the epidermis or burrow into the coats, are called ectoparasites. The parasitism can be facultative if the ectoparasite exploits the victim occasionally not being totally dependent on it, otherwise it is described as obligatory. In addition, a parasitic relationship varies according to the intimacy of the association between insects and hosts. In fact, the ectoparasites can be defined as permanent if they complete the life cycle entirely on the host, semipermanent if they just spend several days continuously on the subject, or occasional ectoparasites if they parasitize a host only sporadically (Mullens, 2003).

Ectoparasites are exploitative animals that obtain from the host everything they need to survive. Firstly, the host provides the food source (blood, epidermis, or skin secretions), but, in case of permanent ectoparasites, the host's body also represents the environment in which the parasites persistently live and offers them the proper degree of heat and moisture, the protection from the external habitat and the

site for the reproduction (Wall and Shearer, 2001). Additionally, the ectoparasites can unintentionally use their hosts as transports capable to carry them worldwide, with the possible risk of an arthropod colonisation in new geographical areas. The number of species (plants, animals, and microorganisms) invading new territories has greatly increased in the last centuries; for example in Europe the introduced species expanded 76% in less than 40 years (Mazza *et al*., 2014). This growth is mainly a consequence of changes in the social organisation and the world population growth, which lead to increase human movements, transports, and commerce, raising the probability of accidental introductions of alien species (Mack *et al*., 2000; Pimentel *et al*., 2001). Relocations of plants and animals have been carried out by humans both accidentally and intentionally, but the majority of invasive insects has been likely dispersed inadvertently (Mack *et al*., 2000). Regardless of the causes, invasions of biotic species may likely entail ecosystem damage, although with great variations in the adverse consequences. A particularly high concern regards the spread of insects potentially harmful to animal and human health (Mazza *et al*., 2014).

Large vertebrates spreading throughout the world can be dispersing-agents for the ectoparasites they carry (Boulinier *et al*., 2001). According to the "enemy release hypothesis", in fact, the hosts can lose the ectoparasites during the invasion, increasing consequently their survival and gaining advantages over native animals to which insects are likely to adapt (Prenter *et al*., 2004). Among large vertebrate groups, also deer have frequently been agents of accidental or deliberate introductions all over the world due to several different reasons. These animals were traded for restocking farms or to be kept in captivity for recreational or conservational

purposes, as well as for hunting activities. Additionally, natural wanderings or migratory movements may have contributed to alien deer spread, with consequent hybridization with native species in new colonized areas. Deer naturalization has important direct and indirect impact on invaded ecosystems and on autochthonous animal balance. In fact, food competition, spatial distribution, population dynamics and hybridization are just few examples of animal invasion consequences (Dolman & Wäber, 2008). The success of alien parasite colonisation is strongly influenced by the adaptability level of the species. However, it can be further ascribed to many biotic and abiotic factors (Kaunisto, 2012), including the scarcity of natural enemies in the introduced territory compared with the native region. Parasite dispersion can be favoured also by climate change. Many invertebrates such as ticks are known to be affected by the weather, especially by temperature and rainfall, both at a wide and a small scale (Allen *et al*., 2002; Cumming & Van Vuuren, 2006). Moreover, environmental temperature influences the flight capacity of ectotherms (Mellanby, 1939). For this reason, it is plausible that the global warming currently affecting the planet may promote the dispersion of ectoparasites, making other geographical areas suitable for insect survival and settlement.

Diverse ectoparasite species infest a different array of host species. Some are characterized as monoxenous and parasitize only a single host species, while others are classified as generalists and can infest a large range of species. In between these two extremes, some parasites are defined as stenoxenous and can attack a group of phylogenetically related species, though others are named as oligoxenous infesting hosts restricted by ecological factors only (Maa & Peterson, 1987; Hutson, 1984; Lourenço & Palmeirim, 2008).

Undoubtedly the presence of parasites is strongly affected by the availability of suitable host species. For this reason, generalist parasites have a greater potential of successfully invading a new territory, since their settlement will not be limited by the availability of a particular host species. In Europe, the distribution and diffusion of ectoparasites infesting ungulates is currently favoured by the substantial expansion of free-living species that has occurred in last years (Apollonio *et al*., 2010). The increase in wildlife abundance is worrying also for the health of other animals and humans since they are known as the principal reservoir of infectious agents. Deer indeed carry a huge number of parasites such as insects and ticks able to vector pathogenic microorganisms which are potentially responsible of diseases, especially zoonoses whose cases are continuously growing (Bengis *et al*., 2004).

Considering the scenario above depicted (naturalization of alien animals and spread of related parasites, global warming, noticeable increase in ungulate abundance, emerging and re-emerging vector-borne zoonoses), the study of ectoparasites is extremely important and requires great attention.

Among the known ectoparasites of mammals or birds, the family Hippoboscidae encompasses three subfamilies, 21 genera, and 213 species of obligate, nearly permanent, hematophagous ectoparasites (Bequaert, 1953; Dick, 2006). They belong to the superfamily Hippoboscoidea, together with Nycteribiidae and Streblidae (bat flies), and Glossinidae (tsetse flies). Actually, the taxonomic classification of Hippoboscidae is contentious (Reeves & Llyod, 2019). On the based of similarity of morphological features and feeding mechanism between these families, a monophyly of the Hippoboscoidea has been proposed. However, recent interpretations,

supported by molecular techniques, propose a likely paraphyly of the Streblidae or suggest nycteribiids and some streblids as a single group (Dittmar *et al*. 2006).

Except for Glossinidae, whose members are free-living parasites of various species, the other three families comprise flies that establish a closer relationship with a variable array of host species (Hutson, 1984). The degree of association determines several aspects of the physiology, behaviour, and morphology of the ectoparasites (Guerin *et al*., 2000). The closer the association is, the deeper the level of adaptation developed by the insects; however, this specificity consequently limits the ability to exploit other species (Lehane, 2005). Hippoboscid species take advantage of different features to functionally live together with the victims: they have a flattened body and a sclerotized exoskeleton useful for withstanding mechanical stresses caused by host activities; their tough legs are equipped with specific organs designed to adhere to the host's fur, and the piercing mouthparts are adapted to hematophagous behaviour (Maa & Peterson, 1987). Additionally, these parasites synchronize their life cycle with that of the host species (Bequaert, 1953).

In general, hippoboscids are not considered as generalist ectoparasites since they target a few host species. Among the genera, host specificity is more marked in flies attacking mammals, while bird parasites live at the expense of a higher range of hosts (Hutson, 1984). However, different species of Hippoboscidae display a diverse parasitic biology. In fact, some of them (Lipopteninae subfamily) live almost all their life cycle on the skin of the same subject, being apterous or becoming wingless (Bequaert, 1942), while others (Ornithomyinae and Hippoboscinae subfamilies) are able to fly and

frequently change individual, although they infest just one or a few different host species (Bequaert, 1930; 1953).

Since the degree of association with the hosts may have influenced the evolution of some parasite body features, the study of the morphology of hippoboscids with different parasitic behaviour and host range is particularly interesting from an evolutionary point of view, and it is a topic worthy of investigation. Similarly, a parasite that has a higher degree of specialization displays a deeper level of specificity in the host choice process (Lehane, 2005). The host location is crucial for an obligate parasite since its survival depends on the quick success of this activity. Host signals are perceived by these ectoparasites throughout sensory organs (sensilla) located in several regions of their bodies (e. g. antennae, mouthparts, wings, legs, genitalia, cerci). Sensilla are deputed to perceive several kinds of cues (Zacharuk, 1980). Different morphological features of sensilla can be reasonably linked to different functions, although in order to prove their role it is necessary to provide electrophysiological evidences corroborated by behavioural trials (Zacharuk, 1985). Generally, olfactory sensilla are mainly present on antennae (especially on the third segment, the flagellum) together with maxillary palps (Schneider, 1964; Stocker, 1994; de Freitas Fernandes *et al*., 2005; Liscia *et al*., 2013). Like the other body features, antennae are subjected to the selection pressure and evolved accordingly to the different needs of the insect. The host searching usually involves different kinds of stimuli, both visuals and chemicals, that may act in combination, as demonstrated for example for nycteribiids (Lourenço & Palmeirim, 2008) and other hematophagous insects (Gibson & Torr, 1999; Hariyama & Saini, 2001; Kortet *et al*., 2010).

As already underlined, some hippoboscids do not need to continuously search for a host, since they establish a close association with one subject; however, almost all species spend the post-emergence period to find an appropriate host. Investigating how hippoboscids locate their hosts is fundamental to understand the relationship among these ectoparasites and their victims and allows to determine which kind of stimuli are involved in this process. Furthermore, this study can be useful for the development of strategies to monitor and control hippoboscid populations, for example by setting appropriate and effective traps or sampling tools. Although hippoboscids target a rather limited array of species, they can accidentally feed on animals not suitable as definitive hosts, since they do not have all the requisites the parasites need to survive (Bequaert, 1953). It cannot be ruled out that an occasional host could become appropriate as permanent host after an adaptation process. Often hippoboscids have been spread worldwide by unintentional artificial dispersal. Especially infesting-deer hippoboscids have been established in other territories following the importation of related hosts (Bequaert, 1954). In new areas these adventive ectoparasites may adapt to different species, enlarging their host range. For this reason, it is important to monitor ectoparasite populations, especially those that are known for their medical and economic importance. Thanks to regular sampling, it should be possible to promptly underline a massive expansion or an adaptation to other host species, that in turn could lead to a further ectoparasite diffusion with negative consequences both in ecological and sanitary perspectives. Furthermore, the acquisition of information on the presence of insects in different countries and on the host species they target should be

useful to piece together the route travelled by alien ectoparasites probably spread with their hosts.

Livestock and wildlife are attacked by hippoboscid flies with health implications and consequent economic losses. Through their bites hippoboscids can produce direct negative effects on the hosts causing severe bleedings, skin damage with potential onset of secondary infections, dermatitis, and anaemia (Kaunisto *et al*., 2009; Madslien *et al*., 2011; Reeves & Llyod, 2019). Additionally, in case of heavy infestations they are bothersome to the hosts mainly due to the swarming inside the fur. This stress can lead to behavioural alterations reducing time spent grazing with a consequent decrease in body weight, welfare, and productive performance (Kynkäänniemi *et al*., 2014; Mullens, 2003). *Melophagus ovinus* is the most renowned hippoboscid for the economic losses caused by the effects it produces on sheep (Small, 2005). Infestations of this fly result in wool loss and in "cockle", a vertical ridging of the skin, which determines a devaluation of sheepskins. In US the overall losses caused by keds is estimated to be about 40 million $ every year (Wall & Shearer, 2001). Besides, hippoboscids can be responsible for the maintenance and transmission of several pathogenic microorganisms harmful to humans (Baker, 1967; Bezerra-Santos & Otranto, 2020). This aspect is especially worrying for people that routinely handle sheep or domestic pigeons, which are commonly infested by two ectoparasites possibly vectors of pathogens, *M. ovinus* and *Pseudolynchia canariensis*, respectively (Reeves & Lloyd, 2019). Also deer keds have been suggested as potential carriers of microorganisms (Böse & Petersen, 1991; Dehio *et al*., 2004; Reeves *et al*., 2006; Duodu *et al*., 2013; De Bruin *et al*., 2015; Korhonen *et al*., 2015; Buss *et al*., 2016; Lee *et al*., 2016; Szewczyk *et al*., 2017; Regier *et al*., 2018; Werszko *et al*., 2020; Bartosik *et al*., 2021;

Gałęcki *et al*., 2021; Sato *et al*., 2021) increasing concern for people that work in or visit natural habitats for recreational purposes or hunting activities (Härkönen *et al*., 2009).

Due to their biological and behavioural traits, hippoboscids are particularly suitable candidates for the maintenance and transmission of pathogens (Bezerra-Santos and Otranto, 2020). In fact, they parasitize animals (wildlife and birds) that are known as important reservoir of microorganisms (Baker, 1967; Bengis *et al*., 2004), but they can occasionally bite other hosts raising the possibility of transferring etiological agents among different species. Additionally, both sexes are hematophagous and feed repeatedly up to 20 times in a day (Ivanov, 1974) increasing the chance of acquiring infected blood. Besides, they reproduce through an obligate pseudo-placental unilarviparity (Meier *et al*., 1999), meaning that just a single fully-grown larva is hold in the mother's uterus and is nourished by secretions produced from milk glands. This reproductive strategy could allow a vertical transgenerational transmission of pathogens that is necessary for an efficient biological spread between vertebrate hosts (de Bruin *et al*., 2015). Finally, ectoparasites that are strictly associated with their hosts, on which feed frequently and intermittently, have a higher probability of transmitting parasites mechanically, usually through infected mouthparts (Barker & Reisen, 2019). Unfortunately humans can be accidentally attacked by hippoboscid flies with possible risk of pathogen transmission. Moreover, their bites lead to different symptoms and reactions on humans (Reeves & Lloyd, 2019). Usually, the bites are painful and result in a variable number of itching papules that can persist for several weeks or up to a year. Several deer ked attacks have been documented, and it has been observed that intense pruritus can lead to scratching with subsequent irritation, secondary

infections, allergic rhinoconjunctivitis, or dermatitis (Rantanen *et al*., 1982; Laukkanen *et al*., 2005; Härkönen *et al*., 2009; Buczek *et al*., 2020; Maślanko *et al*., 2020).

The possibility that deer ked species could be dangerous for public health makes them worthy of accurate investigations from a sanitary point of view in a One Health perspective.

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# Objectives

The present research is focused on the study of the superfamily Hippoboscoidea (Diptera) with particular reference to the deer keds (family Hippoboscidae, subfamily Lipopteninae) currently present in Italy, *Lipoptena cervi* (Linnaeus, 1758) and *L. fortisetosa* Maa, 1965.

Since both these species seem to be involved in the possible transmission of pathogens harmful to animals and humans, the general aim of the PhD was to expand the knowledge related to these two ectoparasites. More in-depth investigations were focused on *L. fortisetosa* which has never been deeply studied, especially in Italy where it has been recorded just in the last years.

Specifically, the research deals with different topics, grouped into the following sections:

- Section 1. The Hippoboscoidea superfamily
  - *review of the superfamily Hippoboscoidea, with particular emphasis on species with medical and veterinary importance*

- Section 2. Morphological traits and evolutionary adaptations
  - *observation of morphological features of hippoboscid species, with detailed description of peculiar body differences between* L. cervi *and* L. fortisetosa*, often confused*
  - *investigation on morphological adaptations differently evolved by four hippoboscid species belonging to the three subfamilies (Ornithominae, Hippoboscinae, and Lipopteninae), which have a diverse parasitic behaviour and infest different host species*

- Section 3. Hippoboscid host location
    - *study on the host location played by* L. fortisetosa *during the emerging period of the winged adults, with special reference to the use of visual stimuli and to the role of colour attraction during the host-seeking process*
    - *scanning and transmission microscopy observation of the antennal structure of the four hippoboscid species previously studied for their evolutionary morphological adaptations (section 2), with more in-depth investigation on the sensory pattern and sensillar ultrastructure of* L. fortisetosa *antennae*

- Section 4. Relationship between deer keds and hosts
    - *evaluation of deer ked distribution in the Tuscan-Emilian Apennines (central Italy), and of the parasitism level these two ectoparasites reached on the most infested deer species*
    - *development of a morpho-molecular approach to investigate the phylogenetic interrelationship of Italian and Asian individuals of* L. fortisetosa *to hypothesise the way of introduction and the route travelled by this ectoparasite from the original area (Japan) to Europe*

- Section 5. Health implications associated with *L. fortisetosa*
    - *characterization of the bacterial community of* L. fortisetosa *considering potential implications for human health*

# 1. Keds and bat flies (Hippoboscidae, Nycteribiidae and Streblidae)

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## Abstract

Ked and bat flies (superfamily Hippoboscoidea) are ectoparasites of primary veterinary importance, for both livestock and wildlife. Besides, they may be relevant also for the public health since can attack accidentally also humans producing in some cases severe pathologies. Their peculiar morphological structures, evolved during the adaptation process, allowed them to live together with the hosts during their whole life, thanks to the presence of flattened and sclerotized body, as well as of legs provided with strongly developed claws and adhesion organs. All these species are hematophagous and both sexes feed on the victim blood with a typical trophic behavior present in all the members of this superfamily. Due to this characteristic, these flies are involved potentially in the transmission of pathogens responsible of diseases and/or zoonoses.

## Introduction

The order Diptera, more than the other orders, has many families characterized by an evolutionary adaptation in the trophic behavior: the hematophagy (Petersen *et al*., 2007). Representatives of these groups have modified the mouthparts in piercing-sucking

appendages to feed on the blood of many vertebrate species. Thus, the mouth apparatus has evolved in different types influenced by the feeding behavioral mechanisms: species that insert their thin appendages into the skin of the host reaching the capillary net, injecting saliva and taping the blood, termed solenophages, or species that by the external teeth-like structures scratch the host skin and feed on the spilled blood termed pool feeders (Afonso *et al*., 2012; Mehlhorn, 2018).

Mosquitoes, which belong to the suborder Nematocera, are important vectors that carry the most severe diseases plaguing humans. A major example is represented by malaria, caused by *Plasmodium* parasites vectored by *Anopheles* spp. (Benelli and Beier, 2017), a plague yearly affecting more than two million of people and has produced 405,000 deaths worldwide in 2018 (WHO, 2019) Of note, *Aedes* and *Culex* vectors are crucial in spreading arboviruses. For example, *Aedes aegypti* and *Aedes albopictus* act as vectors of dengue, chikungunya, and Zika in humans (Powell, 2018; Benelli *et al*., 2020).

In the suborder Brachycera there are important flies acting as mechanical transmitters of pathogens (e.g. bacteria) and parasites (e.g. protozoan cysts and helminth eggs), and some species play an important role also as intermediate host or biological transmitters. Furthermore, some are blood-sucking and attack both humans and animals (Iwasa, 1983; Onmaz *et al*., 2013). Acalyptrate flies of the family Psychodidae, Simuliidae Ceratopogonidae are important vectors of diseases such as haemosporidian parasites or bacteria of genus *Bartonella* as well as filarial nematodes (Durden and Mullen, 2009; Santiago-Alarcon *et al*., 2012; Afonso *et al*., 2012). In high Calyptrate Diptera, the family Muscidae has some important hematophagous

species such as *Stomoxys calcitrans*, a worldwide distributed fly responsible of considerable economic losses in dairy farms. Flies generate direct nuisance such annoyance and blood loss in cattle, and some representatives are implicated as mechanical vectors of important viruses like West Nile Fever Virus (WNFV) or the African Swine Fever Virus (ASFV) (Baldacchino *et al*., 2013). In the superfamily Hippoboscoidea, the family Glossinidae includes representatives responsible for the transmission of some *Trypanosoma* spp. that cause the Human african trypanosomiasis or the African animal trypanosomiasis, which cause the "sleeping disease" and the "nagana" respectively in humans and in livestock (Vreysen *et al*., 2012). Finally, Hippoboscidae (ked and louse flies), Streblidae and Nycteribiidae (bat flies) are implicated in the horizontal and vertical transmission of protozoa, bacteria as well viruses and nematodes to wildlife, and often responsible of direct damage to humans causing allergic reactions by their bite (Reeves and Lloyd, 2019). The present chapter reviews current knowledge about these interesting families, describing the biology, ecology, morphology and behavior of some species which are important from veterinary and medical point of view, and providing insights on current control tools available in the Integrated Pest/Vector Management scenario.

## The superfamily Hippoboscoidea

Hippoboscoidea is a superfamily of hematophagous ectoparasites belonging to the order Diptera and includes four recognized family level-taxa: Glossinidae, Hippoboscidae, Nycteribiidae and Streblidae. All of these families were grouped under the name Pupipara because they are viviparous and deposit larvae that

quickly pupate. Currently, the name Pupipara is recognized as being improper because females lay fully developed larvae instead of pupae. For this reason, it is incorrect to consider this superfamily as pupiparous but larviparous (Reeves and Lloyd, 2019).

Hippoboscidae commonly are referred to as keds or deer keds, or as feather, bird, or louse flies, while Nycteribiidae and Streblidae are known as bat, flat or spider flies (Reeves and Lloyd, 2019). The family Hippoboscidae includes several parasites of mammals and birds, while the other two families are exclusively limited to bats (Hutson, 1984).

Members of Hippoboscoidea are characterized by several peculiar morphological and biological traits. Their reproductive strategy is adenotrophic viviparity: larvae are contained singly in the mother's uterus and are nourished from structures named "milk-glands," well-described by Benoit, *et al*., 2015. When a third-instar larva is fully developed, it is deposited by the female and shortly thereafter pupates. Females larviposit in different substrates depending on the species. Some flies deposit larvae in nests, others on the host animal fur, but some attack larvae to roost walls or glue them to the host wool. Fully-grown larva is legless with a barrel-like soft body (Maa and Peterson, 1987), while the puparium is similar to the mature larva with more sclerotized cuticle <Figure 1.1>. In many hippoboscids, the emergence of a new generation occurs simultaneously in a determined range with the year, with pupae able to overwinter in diapause, depending on the larviposition period (Bequaert, 1953; Hutson, 1984).

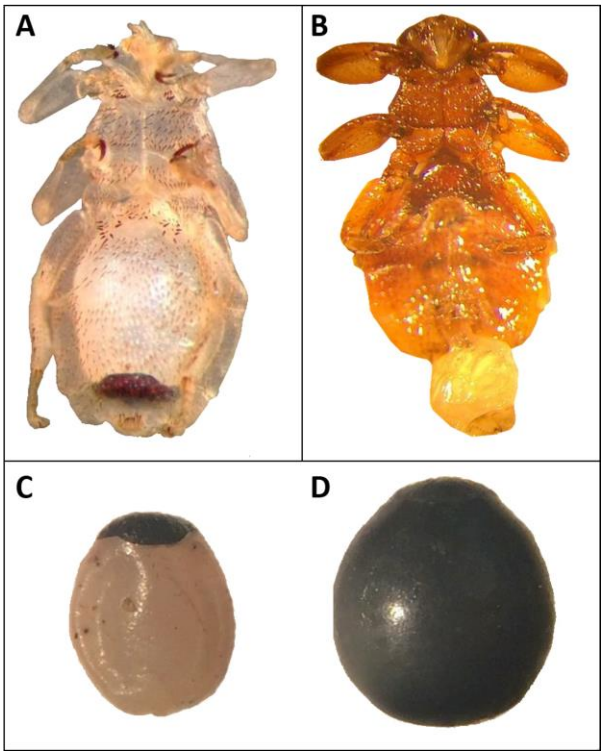



Figure 1.1. *Lipoptena cervi*. Gravid female (artificially depigmented) showing a mature larva inside the uterus (A); female larvipositing (B); barrel-like larva just out of the mother's body (C); typical dark sclerotized puparium (D).

In the adult stages, all members of Hippoboscoidea are obligate and permanent blood-sucking ectoparasites, feeding on various host species. Generally, a fly is specialized in attacking a specific host or a few related groups, but occasionally can feed on other species, called "accidental hosts," which are used as food source but on which reproduction does not occur (Maa and Peterson, 1987). Within the superfamily, species exhibit varying parasitism levels. Some species are monoxenous, meaning they are associated exclusively with a single species; others are stenoxenous, and can infest a phylogenetically related group, or oligoxenous if they parasite a limited number of hosts restricted by ecological factors only; others instead are polyxenous

and are able to live on a wide range of species (Hutson, 1984; Lourenço and Palmeirim, 2008).

To live in close association with hosts, these ectoparasites have co-evolved with them adapting morphologically and physiologically (Guerin et al., 2000). For instance, parasites of birds synchronize their life cycle with host seasonality: adults die when birds migrate, and the emergence of a new generation coincides with host return (Bequaert, 1953). Similarly, aggregations of bat flies live in caves together with their gregarious hosts. When a bat exits the cave to search for food, female parasites leave the host to deposit pupae on the walls (Peterson and Wenzel, 1987). Finally, deer keds are strictly dependent on a single suitable host specimen and spend the entire life on the fur of the host (Bequaert, 1942).

Representatives of Hippoboscoidea are adapted morphologically for ectoparasitic life and display numerous structures that allow them to efficiently live together with their hosts (Petersen et al., 2007). Their body is dorsoventrally flattened with several reduced and fused regions and a sclerotized exoskeleton that is able to withstand mechanical stresses caused by host movements. Females show a strong reduction or disappearance of abdominal sternites with large intersegmental membranous areas allowing the develop of the larva in the mother's uterus <Figure 1.2>. Additionally, parasites have peculiar adhesion organs that permit the adherence to animals (Maa and Peterson, 1987; Petersen et al., 2018; Reeves and Lloyd, 2019).

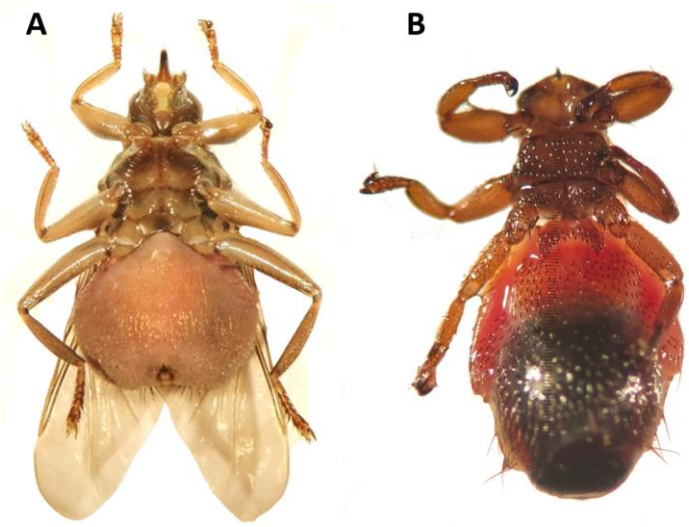



Figure 1.2. Gravid females of *Pseudolynchia canariensis* (A) and *Lipoptena mazame* (B). Note the disappearance of abdominal sternites in both species allowing the development of the larva.

Among the three families, Nycteribiidae are modified morphologically to the extent that they no longer resemble most Diptera, while Streblidae are unusually variable in features and structures between their species (Hutson, 1984; Petersen et al., 2007). The head is prognathous in Hippoboscidae and Streblidae, while in Nycteribiidae it is protruding from the dorsal thoracic area (Maa and Peterson, 1987; Dick and Patterson, 2006). Both sexes of Hippoboscoidea are hematophagous and feed using highly adapted mouthparts consisting of a slender proboscis embraced in two concave, bristled, and sclerotized palpi. The alimentary canal is formed by the union of labrum, hypopharynx, and labium (theca sensu Snodgrass, 1943). Labella end the proboscis and bear at the tip different kinds of teeth and sensilla <Figure 1.3> (Snodgrass, 1943; Peterson and Wenzel, 1987; Wenzel and Peterson, 1987; Andreani et al., 2019).

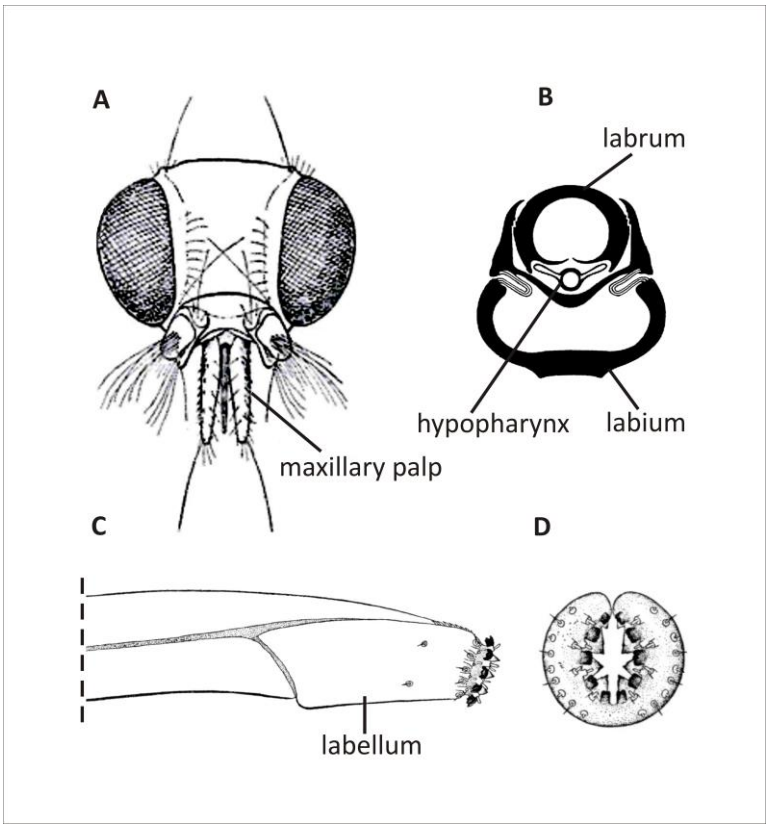



Figure 1.3. Head and mouthparts of Hippoboscidae (modified from Jobling, 1926 and Snodgrass, 1943). (A) Head; (B) Cross-section of mouth appendages; (C) proboscis; (D) details of the proboscis tip.

Some species are wingless in the adult stage, e. g. nycteribiids or some hippoboscids such the sheep ked, *Melophagus ovinus* (Linnaeus, 1758), while others are caducous-winged and shed wings once settled on a suitable host, as in the subfamily Lipopteninae of Hippoboscidae. Finally, others, instead, maintain either reduced or fully developed wings, like in Streblidae and some species of the Hippoboscidae family, such as *Hippobosca equina* (Linnaeus, 1758) or the louse fly *Pseudolynchia canariensis* (Macquart, 1840) (Hutson, 1984; Liu *et al*., 2019).

Life-cycle of some Hippoboscoidea have been drawn and presented below <Figures 1.4, 1.5, 1.6, 1.7>.

Figure 1.4. Life-cycle of *Lipoptena* spp. on red deer, *Cervus elaphus*.

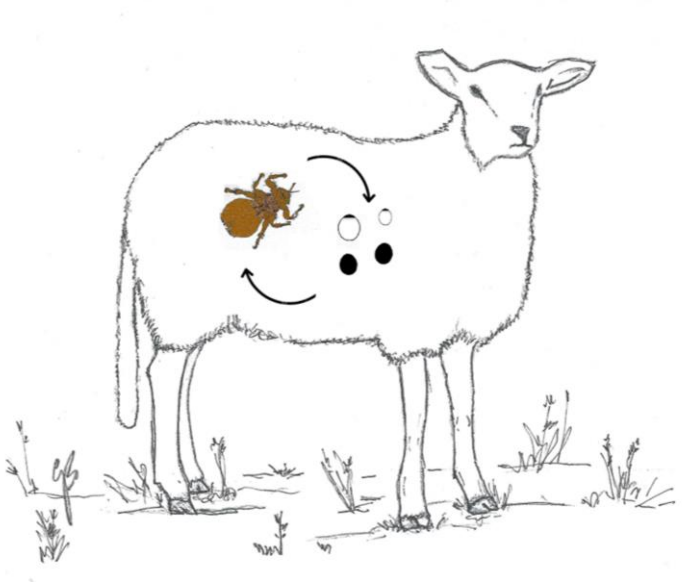


Figure 1.5. Life-cycle of the sheep fly, *Melophagus ovinus*.

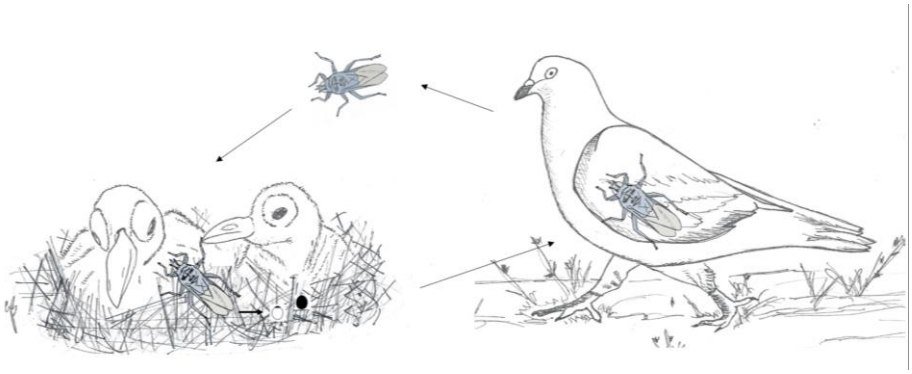


Figure 1.6. Life-cycle of the pigeon louse fly, *Pseudolynchia canariensis*.

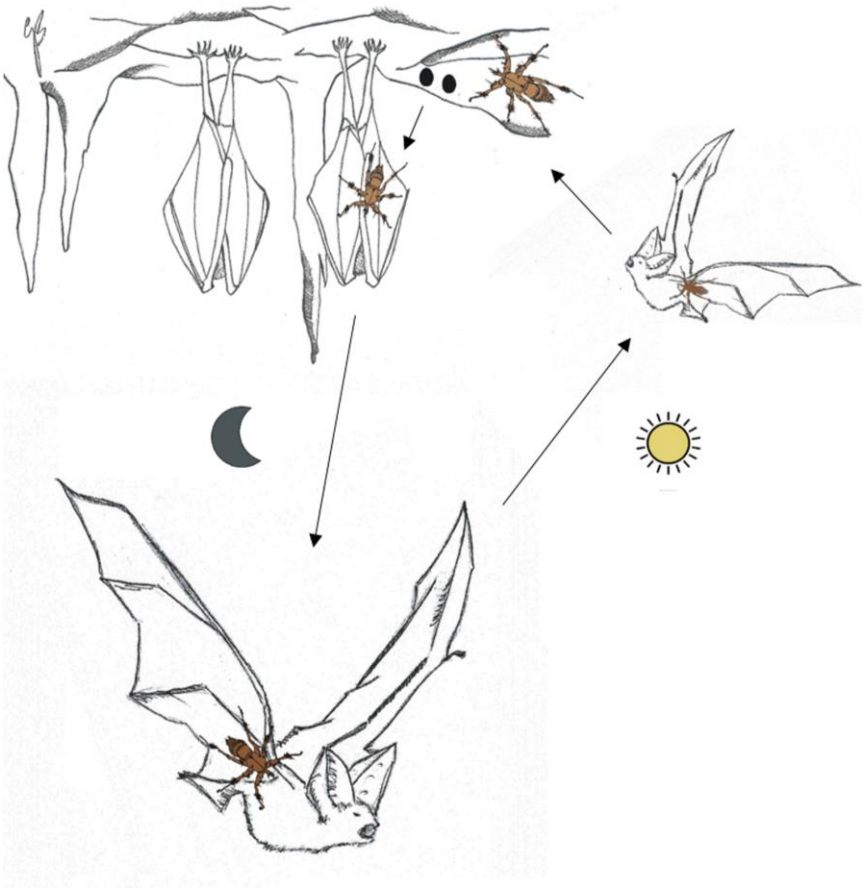


Figure 1.7. Life cycle of a bat fly of the family Nycteribiidae.

# Hippoboscidae

## Bio-taxonomy, morphological adaptations and behavior

The family Hippoboscidae is composed by three subfamilies and includes a total of 213 described species divided in 21 genera (Dick, 2006). The subfamily Ornithomyinae is the most numerous with 16 genera and 171 species; the subfamily Hippoboscinae, instead, consists of two genera with eight species, while three genera and 34 species form the Lipopteninae subfamily (Maa and Peterson, 1987; Dick, 2006).

Among the genera, host specificity is more marked in flies attacking mammals, while bird parasites live at the expense of a higher range of hosts (Reeves and Lloyd, 2019). Compared to the other two families belonging to the Hippoboscoidea, Hippoboscidae includes highly variable species in terms of morphology, biology, and behavior.

The head of hippoboscids is prognathous with mouthparts consisting of two well-sclerotized palpi embracing the piercing apparatus, called also proboscis (haustellum *sensu* Snodgrass, 1943). It ends with two labella bearing a series of different types of sensilla and teeth-like structures which allow to scratch the skin of the host. The fly thus can feed on the blood spilled from the injury. Other interesting features strongly adapted in these flies are the legs. They are provided with an acropod (pretarsus) equipped with modified adhesion organs such as claws, pulvilli, and empodium. These structures are armed differently among species, but they allow the parasite to live together with the host during most of the life-cycle <Figure 1.8>.

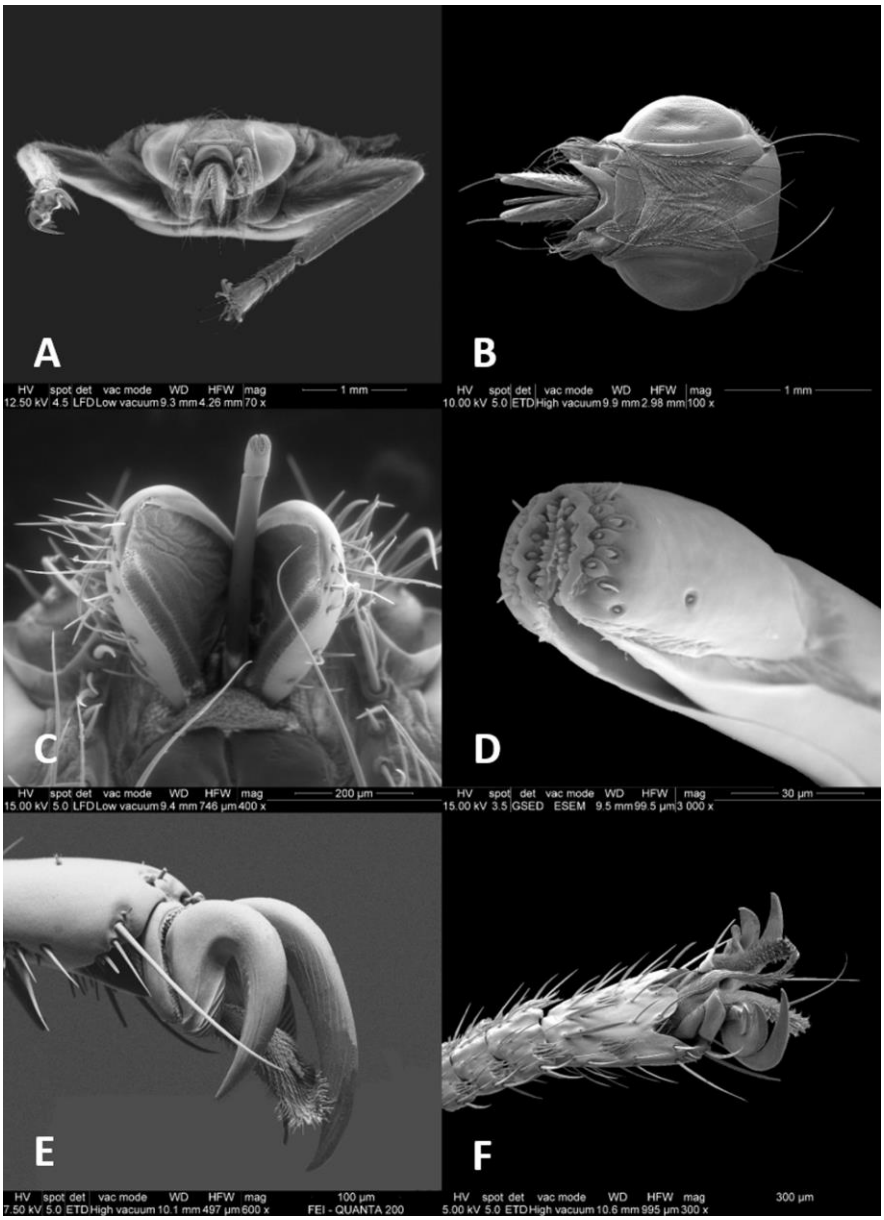



Figure 1.8. *Pseudolynchia canariensis*. Frontal view of the head and anterior legs (A); dorsal view of the prognathous head (B). *Hippobosca equina*. Mouthparts with sclerotized palps embracing the proboscis (C); detail of the proboscis tip bearing teeth-like structures and sensilla (D). *Lipoptena* spp. Acropod with asymmetric claws (E). *P. canariensis*. Acropod with three differently sized claws (F).

Lipopteninae subfamily is known to infest ruminant Artiodactyl mammals, especially Cervidae and Bovidae (Bequaert, 1942; Hutson, 1984). This group is divided into three genera: *Lipoptena*, *Melophagus* and *Neolipoptena*. Except *Melophagus* genus, which is totally wingless, the others have caducous wings that show a predetermined horizontal breaking line. Flies lose these structures during the passage between the fur of the host (Haarløv, 1964). Lipopteninae species need a single suitable specimen to survive. Due to their loss of wings once on-host, it is difficult for these species to switch host. Pupae are laid on the hairs of the fur but fall to the ground as a result of the mammal movements; the emergence of a new generation occurs in a specific range of time. New adults are winged and search for a specific host, they are not able to move for long distances and remain near the emergence site (Bequaert, 1942).

Ornithomyinae is the largest subfamily of Hippoboscidae. About 75% of hippoboscid species exclusively parasite birds and all of them belong to this subfamily (Hutson, 1984). Eighteen bird orders are infested by Hippoboscidae (Santos *et al*., 2014). The genera within this group are related more strictly than those of the other two subfamilies. Parasites live in close association with their victims and display different level of host specialization, some are limited to one or few species, while others affect a wider range of hosts. Pupae are deposited in bird nests and the life cycle is synchronized with those of the hosts (Bequaert, 1953). These species have peculiar morphological structures that assist them in remaining on the host during its flight.

Subfamily of Hippoboscinae is indigenous throughout the continental areas of the Old World. All species of this subfamily are ectoparasites of mammals, with the exception of *Struthibosca struthionis* (Janson, 1889) which solely parasite ostriches. Flies of this

subfamily infest mainly ungulates and carnivores showing less host specificity compared to Lipopteninae subfamily. They are winged during all their adult life and are good fliers able to switch host frequently (Bequaert, 1930).

Species of Hippoboscidae family can establish phoretic associations with mites, fleas and lice (Maa, 1966; Maa and Peterson, 1987; Megat Abd Rani *et al*., 2011; Amaral *et al*., 2013). This aspect can be relevant for the public health, because of the possible transmission of zoonotic microorganisms carried by flies.

Several species of the three subfamilies have been confirmed as potential and/or vectors of various pathogenic agents from veterinary and medical point of view. Consequently, studies on the biology of these parasites are increasing thanks to the modern molecular technologies in order to understand their sanitary role as well as their economic importance.

## Relevant Hippoboscidae species for animal and human health

### Subfamily Lipopteninae

#### *Lipoptena cervi* (Linnaeus, 1758)

The fly is a Palearctic species, nowadays distributed worldwide <Figure 1.9>. Originally it has been recorded in Europe, Siberia and North China, but subsequently the species has spread into Asia, North Africa and North America, mainly as a result of both intentional and accidental introductions (Bequaert, 1942). Currently, this fly is the most widespread species in Europe (Salvetti *et al.*, 2019).

It is usually named "deer ked" as it infests several species of deer. In fact, many species belonging to the Cervidae family are suitable hosts for the parasite (Haarløv, 1964).

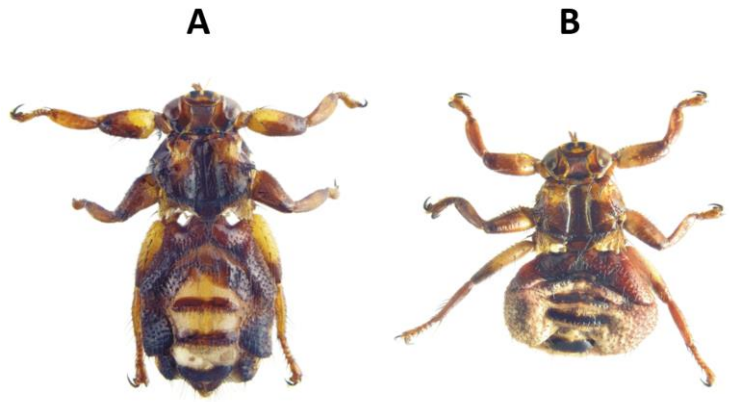



Figure 1.9. *Lipoptena cervi* adults. Female (A) and male (B).

In Europe, the fly predominantly parasites moose (*Alces alces*), red deer (*Cervus elaphus*) <Figure 1.10>, and to a lesser extent reindeer (*Rangifer tarandus fennicus*) roe deer (*Capreolus capreolus*) and fallow deer (*Dama dama*). Recently it adapted to few Bovidae species; in fact, some flies have been recorded on chamois (*Rupicapra rupicapra*) and mouflon (*Ovis musimon*) (Ferron, 2008; Bianchi *et al*., 2016). Moreover, it can occasionally parasite other species used as a food source, such as horses, cows, dogs, cats, badgers, boars, and humans (Bequaert, 1942; Hermosilla *et al*., 2006). *Lipoptena cervi* has become a pest for animals and humans. In the past this parasite has reached a very high density in Finland, causing such nuisance that people strongly reduced recreational and professional activities in woodland (Härkönen *et al*., 2009). The infestation can be surprisingly high, in fact up to 17,500 flies have been counted on a single moose, with severe consequences for the host, as skin injuries, dermatitis and alopecia (Kaunisto *et al*., 2008; Madslien *et al*., 2011). Parasitism affects

also the behaviour of the hosts, increasing defensive or restless actions (scratching, grooming, shaking) with a decrease in the general animal welfare (Kynkäänniemi *et al*., 2014).

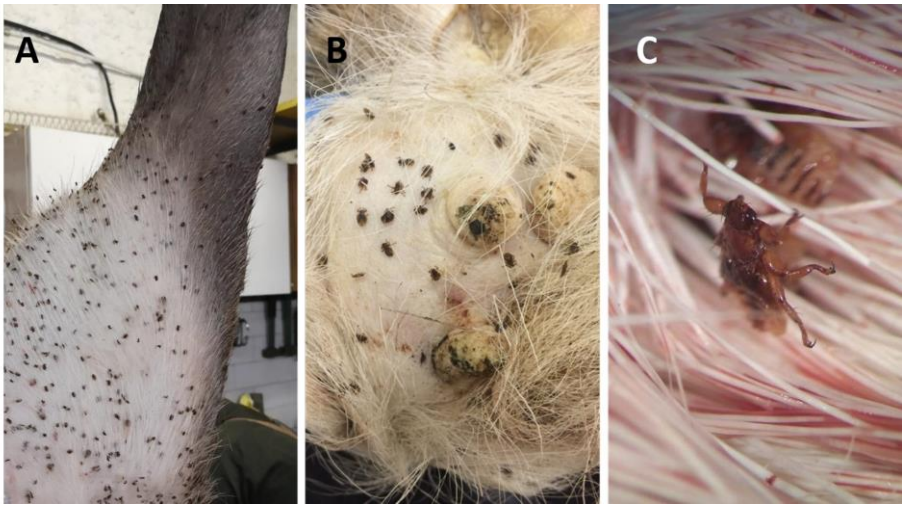



Figure 1.10. Red deer thigh and breast with a high infestation of *Lipoptena cervi* and *L. fortisetosa* (A-B); detail of an adult of *Lipoptena cervi* moving through the guard hairs of the host (C)

Besides mechanical harms, recent studies have identified *L. cervi* as potential vector for several pathogens. *Borrelia burgdorferi*, etiologic agent of Lyme disease, has been detected and identified in flies, as well as *Anaplasma phagocytophlyum*, responsible for human and animal granulocytic anaplasmosis; in some case the specimens were infected with both pathogens (Buss *et al*., 2016). It is possible that the parasite is a mechanical vector for the transmission of these agents, acquiring them from the blood of an infected deer. This hypothesis is confirmed also by lack of these pathogens in the winged flies (Víchová *et al*., 2011). Moreover, *L. cervi* is considered a potential carrier for *Trypanosoma* spp. (Böse and Petersen, 1991) and *Bartonella* spp (Dehio *et al*., 2004; Halos *et al*., 2004; Regier *et al*., 2018), responsible

of human dermatitis. Presumably, *Bartonella schoenbuchensis* is vertically transmitted from the infected females to the new generations, in fact the pathogen has been detected in larvae and pupae, winged and wingless adults, and colonize the midgut of flies (Dehio *et al*., 2004; de Bruin *et al*., 2015). In Norway *L. cervi* seems to contribute to infect moose with *Bartonella* spp (Duodu *et al*., 2013). In addition, *Acinetobacter baumannii* DNA has recently been detected in one specimen of *L. cervi*, proving that the parasite can contribute to the spread of this pathogen, affecting people with compromised immune systems, in human and animals (Regier *et al*., 2018). Although the presence of pathogen DNA in the flies does not imply their competence as biological vector, it suggests that *L. cervi* can increase the spread of the agent to humans and animals. However, the possible mechanical transmission of the pathogen entails the risk to infect other subjects via the bite of the parasite.

### *Lipoptena depressa* (Say, 1823)

*Lipoptena depressa* has been divided taxonomically in two different subspecies: *L. depressa depressa* (Say, 1823) and *L. depressa pacifica* Maa, 1969 (Dick, 2006). These two subspecies are differently distributed: the first occurs in several central states of the USA, while the other occupies the eastern part of USA and Canada (Reeves and Lloyd, 2019). *Lipoptena depressa* attacks several subspecies of the ungulate *Odocoileus hemionus* (i.e *O. h. hemionus, O. h. columbianus, O. h. californicus, O. h. fulginatus*) (Bequaert, 1953). Moreover, it is a likely parasite of *Odocoileus virginianus leucurus*, the Western white-tailed deer, and has been found on other accidental hosts, such as horses and pigeons. Although in USA the infestation areas occupied by *Lipoptena* species seem to be divided geographically, in western

North America *L. depressa* and *Neolipoptena ferrisi* frequently occur together on the same host, as well as *L. cervi* and *Lipoptena fortisetosa* in Italy (Andreani *et al*., 2019; Skvarla and Machtinger, 2019). *Lipoptena depressa* is considered a potential vector for *Anaplasma* (Skvarla and Machtinger, 2019).

### *Lipoptena fortisetosa* Maa, 1965

The species <Figure 1.11> is native to Japan but currently has been identified in few other countries in Asia and Europe (Choi *et al*., 2013).

Its original host is the Japanese deer, named Sika deer (*Cervus nippon*) (Maa, 1965; 1967), although it has been recorded also on the other hosts, such as deer, cattle, goat, sheep, dog, passerine birds and humans (Schumann and Messner, 1993; Yamauchi *et al*., 2009; Sokół and Gałęcki, 2017; Kurina *et al*., 2019). In all the countries in which *L. fortisetosa* has been recorded, its presence is considered to be related to the Sika deer, frequently introduced and considered one of the major naturalized alien ungulates in Europe (Mogi, 1975; Sonobe, 1979; Yamauchi and Nakayama, 2006; Choi *et al*., 2013; Raganella Pelliccioni *et al*., 2013). On the contrary, Kurina *et al*., 2019 report that just few Sika deer recently have been counted in Estonia, suggesting that this host cannot be the mean by which *L. fortisetosa* arrived in this area. However, it is undoubted that this hippoboscid is continuously expanding its range, in fact in the last years several occurrences have been recorded in further European countries (Sokół and Gałęcki, 2017; Andreani *et al*., 2019; Kurina *et al*., 2019; Mihalca *et al*., 2019).

*Lipoptena fortisetosa* is poorly investigated for possible implications to animal and human health, but, as other hippoboscid species, mechanical damages on the host skin have been attributed to

this species, as well as anemia and hair loss (Kurina *et al*., 2019). Besides, this fly is considered a potential vector for several pathogens. In fact, the presence of *Coxiella* spp., *Theileria luwenshuni* and *T. ovis* have been detected in some fly specimens, though its role in the transmission of other agents (*Babesia* spp., *Hepatozoon* spp., *Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp., *Bartonella* spp., *Borrelia* spp.) has not been clarified (Lee *et al*., 2016). For this reason, further investigations are needed to verify if the parasite acts as biological vector for pathogens, as well as which effect it can produce on the host (Reeves and Lloyd, 2019).


Figure 1.11. *Lipoptena fortisetosa* adults. Female (A) and male (B).

### *Lipoptena mazame* Rondani, 1878

The species has been recorded in the southeastern United States and several central and south America countries (Reeves and Lloyd, 2019; Skvarla and Machtinger, 2019). This parasite infests mainly Mazama deer in central and south America and white-tailed deer in the United States, but the fly may accidentally parasite pampas deer, domestic cattle, pumas and humans (Reeves *et al*., 2006; Graciolli *et al*., 2011). Recent studies have proved that *L. mazame* acts as vector for

*Bartonella* spp. Every year *B. henselae* is responsible for over 20,000 human infestations in the United States; its main vectors are domestic cats, but the presence of this pathogen has been detected in *L. mazame* as well (Reeves *et al*., 2006). Moreover, the fly may transmit *B. schoenbuchensis,* which is well-known to cause deer ked dermatitis in humans, and it is implied also in the transmission of *Anaplasma* spp. in cattle and *Trypanosoma cervi* in cervids (Reeves *et al*., 2006; Trout *et al*., 2010).

### *Melophagus ovinus* (Linnaeus, 1758)

This parasite is an important economic species of sheep. Adults are wingless <Figure 1.12>, 4-7 mm long brown colored (Yevstafyeva *et al*., 2017). *Melophagus ovinus* is distributed in the major part of temperate and subtropical areas where sheep are bred.

Figure 1.12. *Melophagus ovinus* adult.

The life cycle of the parasite is completely carried out on the host: gravid female generates a creamy fully grown larva which is attached to the host wool by means a glue-like secretion and in few hours it

molts in a dark puparium. A single female can generate up to five-six larvae and the pupal stage lasts about 19-30 days in relation to the temperature. Even if the species is wingless it can switch from one host to another very easily, especially from the mother to lambs (Small, 2005). A study conducted on Wyoming unshorn lambs to investigate the distribution of keds over the bodies, showed that the most colonized area of the host was the rib for both sexes of the ked, while the next most heavily populated area for the males was the thigh. Ked populations increased in winter and spring, with an average of 400 keds/host approximately, decreased in summer and increased again in the autumn (Legg *et al*., 1991).

The number of infesting keds on the sheep can vary a lot: in Ukraine the mean number of the keds/host was 92.72 (Yevstafyeva *et al*., 2017), while in Canada the mean number was higher. In fact, the peak populations for barren ewes ranged from 61 to 659 keds and for pregnant ewes from 110 to 1348 keds; anyway a general reduction of keds in all animal categories was noticed (Nelson and Qually, 1958). Reduction of the populations seems to be due to several factors such climatic conditions, physiological status as well the intrinsic resistance of the hosts. This latter has been fully investigated in some experimental trials and it has been proved that sheep during the time develop a resistance to the insect trophic activity in terms of inflammatory response to keds. This resulted in changes to the skin that reduced the keds' ability to feed successfully. In other words, the development of resistance was at the beginning, determined by the frequency of attempts for the feeding activity as well the ability and time taken to engorge (Nelson and Kozub, 1980). Later, investigations carried out on artificially infested lambs showed elevated antibody titers within five months after the infestation which reached the

maximum peak. Further experiments highlighted that the resistance was temporary and mainly due to an inflammatory response to the skin lesions produced by the keds activity as the direct result of the localized arteriolar vasoconstriction that makes blood unavailable to the keds (Baron and Nelson, 1985; Nelson and Kozub, 1980; Small, 2005).

Presence of keds on the host causes a skin reaction such as pruritis, aggravated by the rubbing and scratching in response to the irritation, with consequent reduction of growth rates and production. Besides, losses of the leather quality due to the nodules produced by the keds feeding activity have been observed (Legg *et al*., 1991; Small, 2005).

*Melophagus ovinus* is a species of veterinary and medical importance since it is a vector of some important diseases. A recent study conducted in China during the years 2013-2017 aimed at investigating the presence of pathogens inside the sheep flies, showed as primary result, the detection of *Anaplasma ovis* DNA in pupae, confirming the potential vertical transmission. *Anaplasma ovis* is an obligate pathogen infecting sheep, goats, and some wild ruminants and its presence in animals causes the anaplasmosis which is an important disease for public and animal health producing economic losses to sheep breeding (Zhao *et al*., 2018). Moreover, *M. ovinus* is capable to transmit the Blue Tongue Virus (Luedke *et al*., 1965) as well as the Border Disease Virus, which is an important infection in sheep and goats (Liu *et al*., 2019). In a survey carried out in Ethiopia, on different domestic animals, it has been shown that about 86% of keds collected from sheep were infected by *Acinetobacter lowfii*. *Acinetobacter* spp. are ubiquitous bacteria implicated in different types of human infections especially in immunocompromised

individuals (Kumsa *et al*., 2013). Halos *et al*., 2004 demonstrated the vertical transmission of *Bartonella* in *M. ovinus* due to the presence of *Bartonella* DNA in all sampled pupae of the sheep ked, suggesting a symbiotic association between the bacterium and the vector. Further researches allowed the identification of this bacterium as *B. melophagi* (Kumsa *et al*., 2013; Liu *et al*., 2018). Finally, some authors demonstrated the presence of *Borrelia burgdorferi* and *Rickettsia* in different sheep ked samples (Chu *et al*., 2011; Liu *et al*., 2016).

## Management strategies for the sheep ked

Shearing practice can reduce about 75% of the ked population; ewes have to be shorn prior to lambing, otherwise keds can move from them and infest later their lambs. Unshorn lambs, until the following spring, could became a reservoir of infestation for the flock (Johnson, 2011).

Topic application of chemical insecticides is a spread practice and spray, or dust are usually applied in ked control programs. Best results have been obtained treating sheep after shearing; the replacement of animals should take into consideration to treat them before introducing into the flock. It is important to keep treated animals away for about seven - ten days in order to allow the insecticide to kill all the external parasites. Best results in the treatment procedures have been obtained by the application pour-on of permethrin plus piperonyl butoxide (Johnson, 2011) or diazinon and cypermethrin that showed, in addition to a remarkable effectiveness, a long-lasting preventive action (Small, 2005).

### *Neolipoptena ferresi* Bequaert, 1935

This is the only species of *Neolipoptena* genus (Dick, 2006). It is a volant fly infesting predominantly Cervidae, to a lesser extent Bovidae, and occasionally humans (Hutson, 1984). It is well-represented in western America and occurs in the same areas of *L. depressa*. The species is considered a potential vector for *Anaplasma* spp. (Skvarla and Machtinger, 2019).

## Subfamily Ornithomyinae

### *Crataerina pallida* (Latreille, 1812)

The "swift louse fly" is a monoxenous ectoparasite, so named because commonly collected on the European swift (*Apus apus*) and, to a lesser extent, on martin (*Delichon urbicum*) (Hutson, 1984). This hippoboscid is widespread in the Palaearctic region, although its populations are decreasing due to the decrement of host species. (Oboňa *et al*., 2019). An interesting trait of this hippoboscid is its scarce fly ability caused by reduced forewings unsuitable for this activity (Liu *et al*., 2019). However, it is agile inside the plumage and establishes a permanent association with a single host, adapting its life cycle with the bird seasonality. Since the swift is a migratory bird usually returning to the same places every year, *C. pallida* lays larvae directly in or around the nest and dies when birds leave the nesting site to migrate back to Africa. The new adult emergence, occurring after a dormant period until the next breeding season, coincides with host return (Bequaert, 1953; Hutson, 1984). Currently, this hippoboscid has been studied for its interesting morphological and physiological adaptations, mainly for its capability to remain adhere to the victim which is able to reach altitudes exceeding 3500 m and velocities faster

than 40 km/h (Petersen *et al*., 2018; Liu *et al*., 2019). Differently to *Pseudolynchia canariensis*, *C. pallida* attacks mainly adult instead of young swifts; it has been recorded with a density of maximum 31 parasites on a single host subject (Hutson, 1984). Nevertheless, damaging effects have never been detected on hosts, although the parasite can remove up to 5% of the host's blood volume (Liu *et al*., 2019). Recently, this species has been proved as a potential vector of *Rickettsia bellii* and *R. monacensis,* suggesting its role in the transmission or in the spread of these pathogens (Cerutti *et al*., 2018).

### *Icosta americana* (Leach, 1817)

The species belongs to the Nearctic genus *Icosta*, which is the largest of the Hippoboscidae family (Hutson, 1984). This fly is a parasite of owls, which can be infested from a single individual to more than 12 specimens. *Icosta americana* has been identified as a possible vector of West Nile Virus (Gancz *et al*., 2004). Positive specimens, both unengorged or with blood, have been collected from positive owls in Pennsylvania and in Canada in Ontario (Farajollahi *et al*., 2005). Although the competence of the vector has not been confirmed yet, it is particularly worthy of attention that some positive parasites were unengorged. It suggests that further investigations are needed to understand the role of *I. americana* as a carrier for the virus.

### *Pseudolynchia canariensis* (Macquart, 1840)

This parasite is known as the "pigeon louse fly" because it is generally associated with tame or wild pigeons, and doves; it is the only hippoboscid attacking domestic birds (Maa, 1966). This species shows the highest affinity for its host, *Columbia livia*, <Figure 1.13>

although it has been found on other birds, such as Falconiformes (Hutson, 1984; Yamauchi *et al*., 2011; Santos *et al*., 2014).

Figure 1.13. *Pseudolynchia canariensis* on the external plumage of a feral pigeon, *Columba livia*.

The fly is present worldwide, in tropical, subtropical and temperate areas, where its host occurs. *Pseudolynchia canariensis* can attack occasionally humans, but it happens rarely, in case the parasite has lost the pigeon and encounters humans nearby. The bite is a painful annoyance for humans, but the fly seems to not transmit any diseases to them (Kern, 2013); however, *C. livia* is a reservoir for zoonotic pathogens, which means that the parasite could play a significant role in the transmission (Amaral *et al*., 2013). This hippoboscid is known to cause several health problems to the hosts, such as skin irritation or dermatitis. Moreover, it is a potential carrier for

*Haemoproteus columbae* an avian malaria parasite highly dangerous for young birds and able to make hosts more susceptible to predation (Earle *et al*., 1993; Pirali-Kheirabaldi *et al*., 2016). It is well-known that the fly infests more frequently young subjects instead of adults, probably due to the immunity acquired by adults, which additionally use claws and beak as defense weapons against parasites (Amaral *et al*., 2013). Besides, *P. canariensis* can potentially transmit *Trypanosoma hannae* and DNA of *Bacillus burgdorferi* has been detected in this species as well (Nartshuk *et al*., 2018). The pigeon fly establishes phoretic association with mites <Figure 1.14> (particularly genus *Myialges*), chewing lice and mallophaga as reported by Maa, 1966; Macchioni *et al*., 2005; Amaral *et al*., 2013 and Kern, 2013.

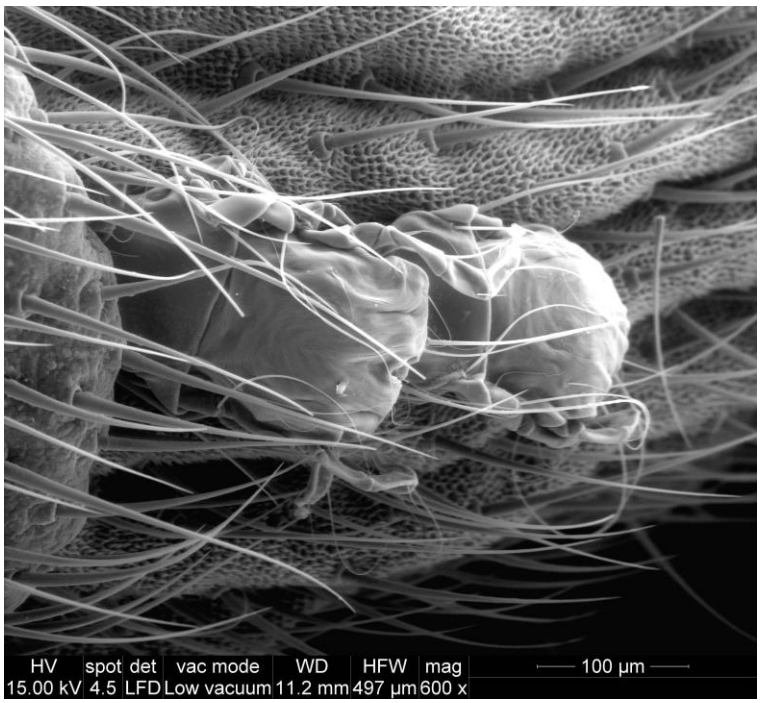



Figure 1.14. Abdomen of *Pseudolynchia canariensis* with phoretic mites.

## Subfamily Hippoboscinae

### *Hippobosca equina* Linnaeus, 1758

*Hippobosca equina* is a medium-sized hippoboscid species, spread in several temperate areas of the Palaearctic and West Oriental Regions (Sóos and Hurka, 1986). The fly is an obligate parasite that feeds on several mammal host species, primarily on domestic horses. The species can reproduce also on cattle (Maa, 1969; Hutson, 1984) or on different secondary hosts such as red deer (Kadulski, 1996), camel and rabbit (Maa, 1969). Further, also birds such the grey heron (Olafsson, 1985) and northern goshawk (Kristofik and Stefan, 1980) have been recorded as occasional hosts. The species is commonly termed also "forest fly". Wings <Figure 1.15> display a vein reduction typical of nearly all representatives of the family, but primary veins remain hardened allowing the adults to fly very fast and also for a long time (Turner and Mann, 2004).

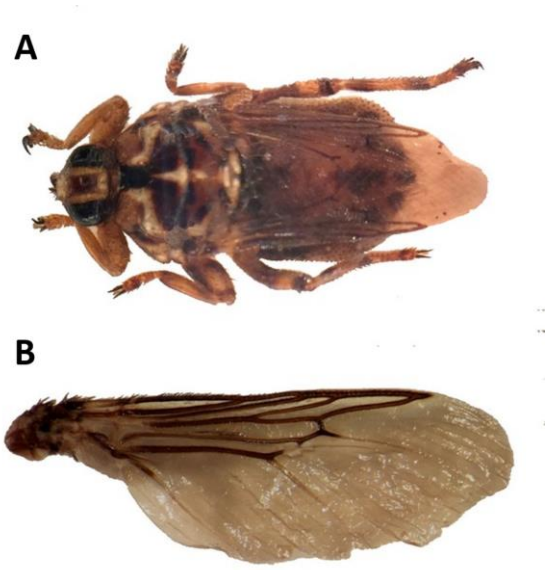



Figure 1.15. *Hippobosca equina*. Adult (A) and typical wing pattern (B).

Gravid females larviposit a full-grown larva in suitable sites, mostly in organic litter (Roberts, 1925). Main biological information for this species have been given by Hafez *et al*., 1977, which report that *H. equina* was reared artificially in Egypt on guinea pigs. Main results relied on some physiological features of lab-reared adults who survived up to 40-44 days when fed on the blood of factitious hosts and only one-two days when starved. The female fecundity ranged from seven and nine larvae during the entire life and the duration of intrauterine life of the larvae lasts from three to eight days according to different climatic conditions. A two-year study carried out in Poland on primitive horses showed that *H. equina* was present in this area mainly with highest presence from mid-June to the end of July and the most attacked horses were working geldings, leading stallions, and 1.5-year-old colts (Sokół and Michalski, 2015).

*Hippobosca equina* is a potential vector of some important animal diseases such as the haemopathogen *Anaplasma* spp. on horses in Tunisia (Selmi *et al*., 2019) or the Buffaloe Oedematous Skin Disease (OSD) produced by *Corynebacterium pseudotuberculosis equi* which cause closed skin lesions either edematous or nodular or open ulcerative lesion in buffaloes in Egypt (Arafa *et al*., 2019). In a research carried out in France in several specimens of *H. equina* the intracellular Gram-negative bacterium *Bartonella comelii* has been detected and identified (Halos *et al*., 2004). *Bartonella* spp. are considered to be emerging pathogens in humans and animals and are present in a wide range of wild and domestic mammals, some of which have been associated with zoonoses (Breitschwerdt and Kordick, 2000; Chang *et al*., 2000; Halos *et al*., 2004).

Some anaphylaxis cases due to the bite of *H. equina* to humans have been described. In South America, a man 54 year-old showed

severe reactions developing generalized pruritus, followed shortly by generalized urticaria, palpebral and labial edema, dyspnea, and hypotension after a bite of *H. equina* (Vidal *et al*., 2007). In Italy a 48-year-old female showed after a bite a generalized pruritus and then extensive urticaria, abdominal pain, nausea, angioedema on the face, and dyspnea (Quercia *et al*., 2005). Finally, in Hungary a 46-year-old female patient showed different symptoms such as hard swelling at the border of the forehead, with oedema. After, erythema and itching developed locally and all over the body, with oedema in the hands, face and lips, later accompanied by shivering, nausea and vomiting (Decastello and Farkas, 2010).

### *Hippobosca longipennis* Fabricius, 1805

*Hippobosca longipennis* is a common hippoboscid species spread in several countries of southern Europe, Africa and Asia, particularly China and India, associated especially to arid and semi-arid areas. This parasite is frequently called "dog fly", because wild and domestic dogs are its principal victims, and it has been found also in mummified dogs in Egypt (Sokół and Gałęcki, 2017). However, it has been collected from different other species, such as fox, cat, hyena, cheetah, lion mongoose or civet (Megat Abd Rani *et al*., 2011; Reeves and Lloyd, 2019) and occasionally from humans (Bequaert, 1942). *Hippobosca longipennis* is considered the main vector of the filarial nematode *Acanthocheilonema dracunculoides*, found in several European countries in different host species. Moreover, the involvement of this fly as vector of the filarian nematode *Acanthocheilonema* spp. has been proved in northern India (Megat Abd Rani *et al*., 2011; Mihalca *et al*., 2019). The health importance of this nematode seems to be little, with just one case in Australia: a

female larva was detected in a human eye (Megat Abd Rani *et al*., 2011), but further investigations are needed to better know its implications. The transmission occurs trough the parasite bite, since the infective larvae migrate from the fat-body cells to the mouthparts of the fly. In addition, *H. longipennis* can have a phoretic association with the mite *Cheyletiella yasguri*, zoonotic agent harmful for dogs and humans since it can cause itching, erythema and exfoliative dermatitis (Megat Abd Rani *et al*., 2011; Sokół and Gałęcki, 2017).

## Bat flies (Nycteribiidae and Streblidae)

### Bio-taxonomy, morphological adaptations and behavior

Bat flies are a highly specialized group that includes two families, Streblidae and Nycteribiidae, both strictly limited to bats (Dick and Patterson, 2006). A total of 520 recognized species belong to the bat flies, making this group the richest of species among the Calyptrate Diptera related to mammals (Dittmar *et al*., 2006). Although they are spread worldwide, no species, genus or even subfamily are present in both hemispheres; in fact, streblids are well-represented in the Western Hemisphere, while nycteribiids are common in the Eastern (Dick and Patterson, 2006). Two subfamilies among the Streblidae (Nycteriboscinae, Ascodipterinae) are well-represented in the Old World, while the other three (Trichobiinae, Nycterophilinae and Streblinae) have been recorded mainly in the New World (Petersen *et al*., 2007). Bat fly families occur in different climate areas as well: Nycteribiidae occupy temperate regions, while Streblidae have been found in tropical and subtropical climates (Dittmar *et al*., 2006).

Life cycle of bat flies seems to be quite similar to that of hippoboscids, and it is likely the same among species (Peterson and

Wenzel, 1987). All of them live in close association with their hosts inside caves together with gregarious bats and show a high level of host specificity although different bat species occupy the same sites (Lourenço and Palmeirim, 2008). About every ten days, females leave the infested subject to stick on roost walls a single larva, and then actively search for a new host. Since males do not lay larvae, it could be thought that they always stay on the victim, but, actually, several male flies have been collected from cave walls, proving that they leave the host at least for brief periods (Dick and Patterson, 2006). Several factors affect host specificity in bat flies, for example climate conditions, isolation, competition or predation, and morphological or physiological adaptations (Autino *et al*., 2011). Bat flies are monoxenous or stenoxenous, and, although they are able to infest alternative bat species that live in the same cave, their preference in parasitizing one species have been demonstrated recently (ter Hofstede *et al*., 2004; Lourenço and Palmeirim, 2008; de Vasconcelos *et al*., 2015). Host specificity presumably is affected also by fly ability, but, despite of nycteribiids are totally wingless, they are as specific as the winged streblids (ter Hofstede *et al*., 2004). Different parasite species can infest the same subject, and the presence of several flies at the same time on a single host has been proved positively associated (Dick and Patterson, 2006).

Bats are the first cause of parasite death. In order to avoid the predation during host auto-grooming behavior, flies adapted to occupy specific micro-niches, in fact, they generally are located on the pelage or under the membranous wings of bats (ter Hofstede *et al*., 2004). Moreover, they move very fast in all directions and have structures, e.g. claws, that allow them to adhere to the host (Dick and Patterson, 2006; Kim *et al.*, 2012). Bat flies have a peculiar general

morphology, and, like the other Hippoboscoidea, they display several adaptations that make them able to efficiently live together with the hosts (Peterson and Wenzel, 1987; Wenzel and Peterson, 1987). Nycteribiidae evolved the same structures, while, among Streblidae species, morphological adaptations are more variable. Differently from Hippoboscidae except for *Melophagus* spp., bat flies have reduced or absent compound eyes due to an evolutive response towards the dark environment in which they live (Mayberry, 2014). Despite the two families show several common characteristics in both morphological and bio-ecological traits, they have been divided in two groups because of differences in some body structures and in their geographical distribution <Table 1.1>.

## Subfamily Ascodipterinae

### Genus *Ascodipteron* Adensamer 1896

Members of this subfamily are distributed in tropical and subtropical areas especially in Africa, Middle East, Asia and Australia (Wenzel and Peterson, 1987) *Ascodipteron* spp. represent the only exception to the ectoparasitic nature of bat flies. After mating, an eyeless female embeds herself in the tissue of the host thanks to a strongly modified mouth apparatus that has series of cheliceral blades located at the tip of the labial thecum (Hastriter *et al*., 2006), and becomes an endoparasite (Wenzel and Peterson, 1987; Dick and Patterson, 2006). When she settles on a host, immediately sheds wings, halters and all legs except for coxae; moreover, thorax and mouthparts invaginate within the abdomen forming a cyst-like body. This feature is a rare example of neosomy in the adult stage; in fact, there is an additional cuticular secretion that allows the enlargement of the whole

abdomen. This latter protruding from the host skin displays three pairs of respiratory spiracles close to the anal opening (Hastriter *et al*., 2006).

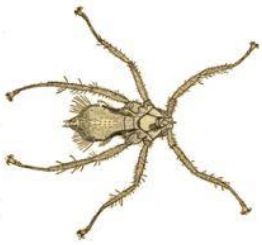
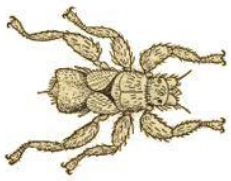
| | | | | | | |
|---|---|---|---|---|---|---|
| **Nycteribiidae** |  | *dorsoventrally flattened; spider-like* | *backwardly folded at rest* | *absent* | *dorsally inserted* | *Eastern Hemisphere* | *temperate regions* |
| | | | | | | *Western Hemisphere* | *tropical and subtropical regions* |
| **Streblidae** |  | *varying from generalized convex, laterally compressed to dorsoventrally flattened; flea-like* | *prognathous* | *fully developed, brachypterous, stenopterous, or apterous.* | *laterally inserted* | | |

Table 1.1. Main differences between Nycteribiidae and Streblidae families.

## Health importance of bats and bat flies

Bats are a great diverse mammalian group, second only to rodents. They play an essential role in ecosystems and have also public health significance (Szentiványi *et al*., 2019). In fact, bats are known to be reservoirs of several etiological agents, such as Rabies, Marburg, Ebola, measles, mumps, parainfluenza, canine distemper and hepatitis C viruses (Han *et al*., 2015; Reeves *et al*., 2016). For many reasons these animals are likely carriers of pathogens, they are the second largest order of mammals with a high diversity of species and a long evolutionary history allowing the co-evolution with viruses. Moreover, bats are social animals that create aggregation of millions of subjects, they are able to fly long distances facilitating the spread of agents and have a relatively long-life span (Han *et al*., 2015). Bats are hosts for many different ectoparasites, among which Nycteribiidae and Streblidae flies are the most common. These parasites have been implicated as vectors of several pathogens, like protozoa (*Nycteria* spp.), arboviruses and trypanosomes (*Trypanosoma vespertilionis*). In addition, nycteribiids are able to transmit *Polychromophilus* spp., while filarial nematode DNA have been detected in streblids, although it is not clear if it is only present in the last blood meal (Reeves *et al*., 2016; Szentiványi *et al*., 2019). DNA of *Bartonella* spp. was detected in *Trichobius major* (Diptera: Streblidae), demonstrating that it may play a role in the transmission of the agents but not explaining the competence of the vector (Reeves *et al*., 2005). Bat flies more host specific display a lower diversity of *Bartonella* spp., but a general high prevalence of these pathogens, suggesting they have co-evolved with bats. Viviparity could cause vertical transmission of agents from the mother to the offspring through the "milk glands" (Szentiványi *et al*., 2019). Finally, parasites can affect host behavior, inducing bats to

change roost in case of a high pupal density on the walls. Moreover, a high infestation level can reduce the survival and the reproduction success of the host (Szentiványi *et al*., 2020).

## Concluding remarks and challenges for further research

After this literature analysis and critical discussion on keds, louse and bat flies, it is clear that much work has been done in these last years especially for the detection and identification of pathogens carried by these parasites, proving in some cases that they could play an important role as vectors of etiological agents responsible of diseases in both animals and humans. Investigations have been carried out thanks to new molecular tools allowing the identification of the species as well of the different strains of pathogens. Nevertheless, we have to stress the importance of future basic studies on these flies mainly concerning their morphology, biology and behavior that may contribute to better understand the real role of these parasitic flies, particularly in relation to both wild and domestic hosts as well to the environment. Finally, it will be particularly worthy of attention the studies dealing with the possible zoonoses transmitted to humans.

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# 2. Comparative morphology of the deer ked *Lipoptena fortisetosa* first recorded from Italy

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## Abstract

Hippoboscidae flies parasitize various animal species. Knowledge about these insects remains sparse, although they are known to cause stress and damage to their hosts, and can also accidentally infest humans, causing different sanitary risks. Research conducted in Tuscany assessing the biology and distribution of *Lipoptena cervi* (Linnaeus, 1758) (Diptera: Hippoboscidae), the most common ectoparasite of ungulates in Italy, revealed the presence of *Lipoptena fortisetosa* Maa, 1965 in Italy for the first time. This study includes a morphological comparative description of *L. cervi* and *L. fortisetosa*, emphasizing the peculiar differences between the two species to facilitate their accurate identification. The most pertinent morphological differences between the two species are highlighted, such as the external features of the antennae, distribution of bristles, and different features in the external genitalia. In both species, scanning electron microscopy of mouthparts revealed strong adaptive convergence in the feeding apparatus. Modified palps and a very thin proboscis are described in relation to feeding behaviour.

## Introduction

*Lipoptena fortisetosa* Maa, 1965 is a haematophagous ectoparasite belonging to the family Hippoboscidae, subfamily Lipopteninae (Maa, 1965). The fly parasitizes mammals, particularly cervids. This species is native to Japan, but has spread into Korea and Russia. It has been recorded in a few European countries, such as Germany, Lithuania, Moldova, Poland, the Czech Republic, Romania, Slovakia and Switzerland (Choi *et al*., 2013). The main host of *L. fortisetosa* is the Japanese deer [*Cervus nippon* Temminck, 1838 (Artiodactyla: Cervidae)], but other mammal hosts have been reported, such as the Siberian roe deer [*Capreolus pygargus* Pallas, 1771 (Artiodactyla: Cervidae)] (Choi *et al*., 2013). *Lipoptena fortisetosa* can also infest humans (Schumann & Messner, 1993). *Lipoptena cervi* (Linnaeus, 1758) is another common hippoboscid species that attacks ungulates. *Lipoptena cervi* was originally recorded in Europe, Siberia and northern China, but it has spread into northern Africa, North America and other parts of Asia (Bequaert, 1942). This fly lives on various species of ungulate and can accidentally infest other species, including humans (Härkönen *et al*., 2009a; Kaitala *et al*., 2009; Kaunisto *et al*., 2010). In Italy, *L. cervi* predominantly parasitizes red deer [*Cervus elaphus* Linnaeus, 1758 (Artiodactyla: Cervidae)], roe deer [*Capreolus capreolus* (Linnaeus, 1758) (Artiodactyla: Cervidae)] and, to a lesser extent, fallow deer [*Dama dama* (Linnaeus, 1758) (Artiodactyla: Cervidae)] (Haarløv, 1964). When a ked finds a suitable host, it settles on the mammal for the rest of its life and gradually loses its wings as a result of its passage between the hairs of the host. Both *L. fortisetosa* and *L. cervi* are viviparous species that generate full-grown larvae that fall to the ground and pupate. Both species occur year-round, but the emergence of winged adults occurs from summer to early autumn

(Haarløv, 1964). Both species have been poorly investigated, although, in recent years, the spread of *L. cervi* over northern European countries has stimulated research into its population dynamics and invasiveness (Härkönen *et al*., 2009b; Kaitala *et al*., 2009). These species are known to cause sickness and stress in their hosts, and they facilitate the transmission of pathogens and zoonoses such as borreliosis, anaplasmosis and trypanosomiasis (Härkönen *et al*., 2009a; Víchová *et al*., 2011; De Bruin *et al*., 2015; Buss *et al*., 2016; Lee *et al*., 2016). The present paper reports the first record of *L. fortisetosa* in Italy, along with a comparative morphological assessment of *L. fortisetosa* and *L. cervi* to facilitate the accurate identification of the two species. Moreover, morphological observations on some features found to be common to both species, such as in the legs and mouthparts, are described in relation to their parasitic lifecycles and feeding activity.

## Materials and methods

### Sampling procedures and taxonomic identification of Hippoboscidae

Observations were made in Tuscany, central Italy. Hippoboscids were collected from five ungulates. Three *C. elaphus* specimens were examined: a male fawn (44°4′ 23.82″ N, 11°5′ 46.74″ E); a female fawn (44°4′ 49.28″ N, 11°6′ 58.83″ E), and a male yearling (44°6′ 19.08″ N, 11°7′ 17.10″ E). Flies were collected from hunter-harvested deer in 2017 in the province of Prato, on 29 January, 11 February and 11 March, respectively. The other two parasite-yielding specimens were *C. capreolus* and included a male fawn harvested by hunters in the province of Grosseto (42°52′ 55.66″ N, 11°15′ 10.45″ E) on 26 August 2017, and a tame adult female kept at the experimental farm of the Department of Agrifood Production and Environmental Sciences

(DISPAA), University of Florence in Florence (43°47′ 3.30″ N, 11°13′ 20.02″ E), examined on 29 June 2017 (Figure 2.1). The hippoboscid specimens were identified using taxonomic keys proposed by Bequaert (1942), Mogi (1975) and Maa & Peterson (1987).

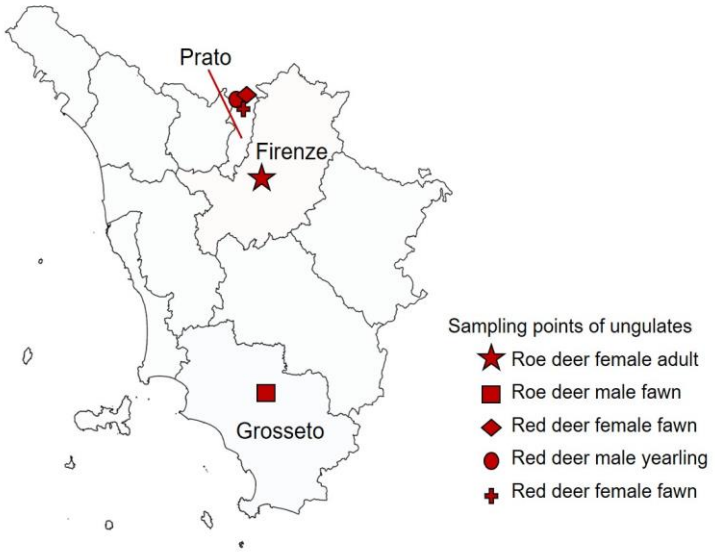


Figure 2.16. Locations of sampling of hippoboscids in Tuscany, Italy.

## Morphological investigations

A morphological study was conducted using optical and scanning electron microscopes housed at the Department of Agricultural, Food and Agro-Environmental Sciences, University of Pisa, Italy. Several specimens were prepared for the observations. Adults of both species were anaesthetized and killed at −20°C and then immersed in hexane and sonicated for 10 min to clean them and to remove impurities and secretions from their bodies. Subsequently, flies were sonicated again for 10 min in water with two drops of soap (Ausilab™; Carlo Erba Reagents Srl, Cornaredo, Milano, Italy) to rehydrate the previously

cleaned samples. After this procedure, the specimens were air-dried, quickly pinned, and prepared for optical observations. At least fifty specimens of each sex and species were observed using an optical microscope. Two dimensions were measured: the total length of the body and the largest width of the abdomen. For scanning electron microscopy, specimens in toto or some excised parts were placed in hexane, sonicated for 10 min, and then dehydrated in a graded ethanol series (70%, 80%, 90%, 95% and 100% ethanol). Subsequently, samples were air-dried, mounted on stubs and gold-coated in a sputter coater device (S150B; BOC Edwards, Burgess Hill, U.K.). Observations were made using an FEI Quanta 200 high-vacuum scanning electron microscope (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.).

## Results

The examined ungulates hosted a large number of ectoparasites. Among these, the presence of the hippoboscid *L. fortisetosa* was detected for the first time in Italy. A total of 802 parasites were collected from the five ungulates. These included 622 *L. cervi* and 180 *L. fortisetosa* (Table 2.1). However, it was not possible to remove all the flies from the two roe deer and hence the number of infesting insects is assumed to have been greater.

Table 2.1. Number of hippoboscid flies, divided by species and sex, collected from ungulate hosts

| Hosts | *L. cervi* ♂ | *L. cervi* ♀ | *L. fortisetosa* ♂ | *L. fortisetosa* ♀ |
|---|---|---|---|---|
| Red deer male fawn | 12 | 25 | 34 | 40 |
| Red deer female fawn | 12 | 30 | 17 | 19 |
| Red deer yearling male | 220 | 323 | 0 | 1 |
| Roe deer female* | - | - | 7 | 10 |
| Roe deer male fawn* | - | - | 31 | 21 |

*only few specimens sampled from the animal

## Morphological and taxonomic differences between the species

The morphological features of both ectoparasites show an extreme level of adaptation to parasitic life. This adaptation includes the flattening of the body to enable the insect to remain on the host, the thickening of the integument to withstand the mechanical pressures caused by host movements, and the development of many bristles for protection (Figures 2.2 and 2.3). The largest difference between the two species refers to body size: *L. fortisetosa* is smaller than *L. cervi* and females are larger than males in both species (Table 2.2).

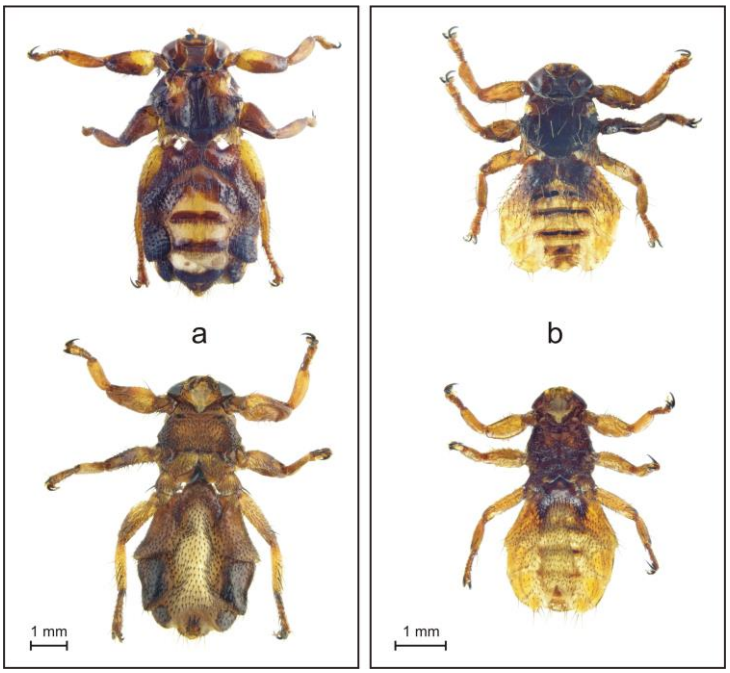


Figure 2.2. Dorsal and ventral views of females of (A) *Lipoptena cervi* and (B) *Lipoptena fortisetosa*.

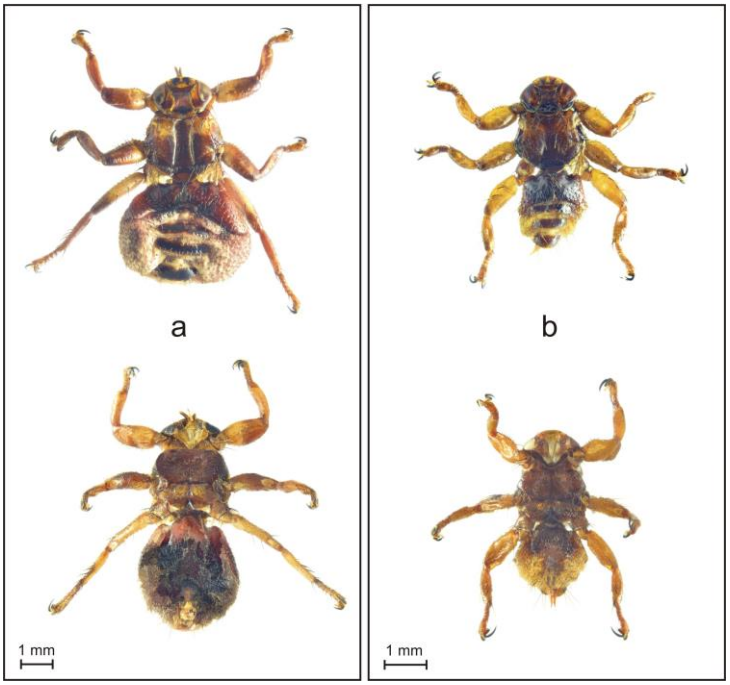


Figure 2.3. Dorsal and ventral views of males of (A) *Lipoptena cervi* and (B) *Lipoptena fortisetosa*.

Table 2.2. Body dimensions (mean ± SD) measured in both sexes of *L. cervi* and *L. fortisetosa*

| Hippoboscid flies | Number of measured specimens | Body length (mm) | Abdomen width (mm) |
|---|---|---|---|
| *L. cervi* female | 55 | 7.40 ± 0.527 | 3.84 ± 0.285 |
| *L. cervi* male | 54 | 6.64 ± 0.790 | 3.83 ± 0.454 |
| *L. fortisetosa* female | 59 | 4.74 ± 0.230 | 2.62 ± 0.222 |
| *L. fortisetosa* male | 51 | 3.89 ± 0.472 | 2.03 ± 0.302 |

Closer observation shows that the head of *L. cervi* is ovoid, whereas the head of *L. fortisetosa* has a characteristic rhomboidal shape (Figure 2.4). Other noticeable differences concern the frontoclypeus area, which is trapezoid in *L. cervi* and elliptical in *L. fortisetosa*.

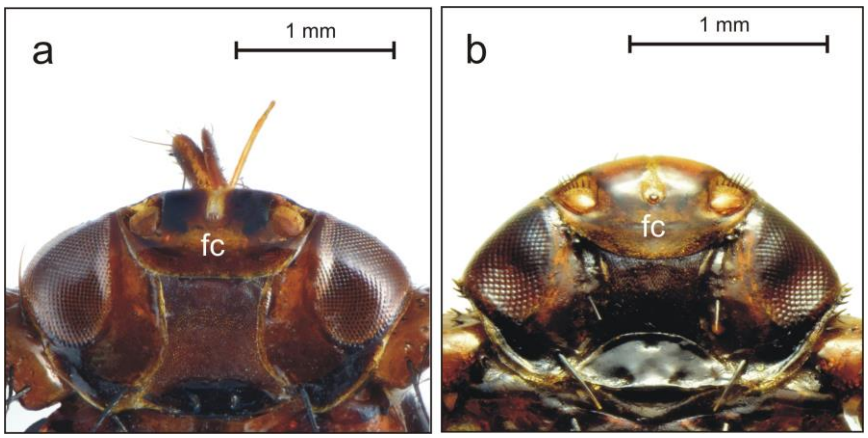


Figure 2.4. Features on the head (dorsal view) of (A) *Lipoptena cervi* and (B) *Lipoptena fortisetosa*. Differences between the species are detectable on the frontoclypeus area (fc) and in the numbers of mechanosensory bristles and their arrangement on the external side of the antennae.

Additionally, the sensillar pattern present on the external surface of the antennal segment differs between the two species: *L. cervi* antennae bear two trichoid, one basiconic and seven coeloconic sensilla (Figure 2.5), whereas there are nine strongly socketed sensillar bristles in *L. fortisetosa* (Figure 2.6).

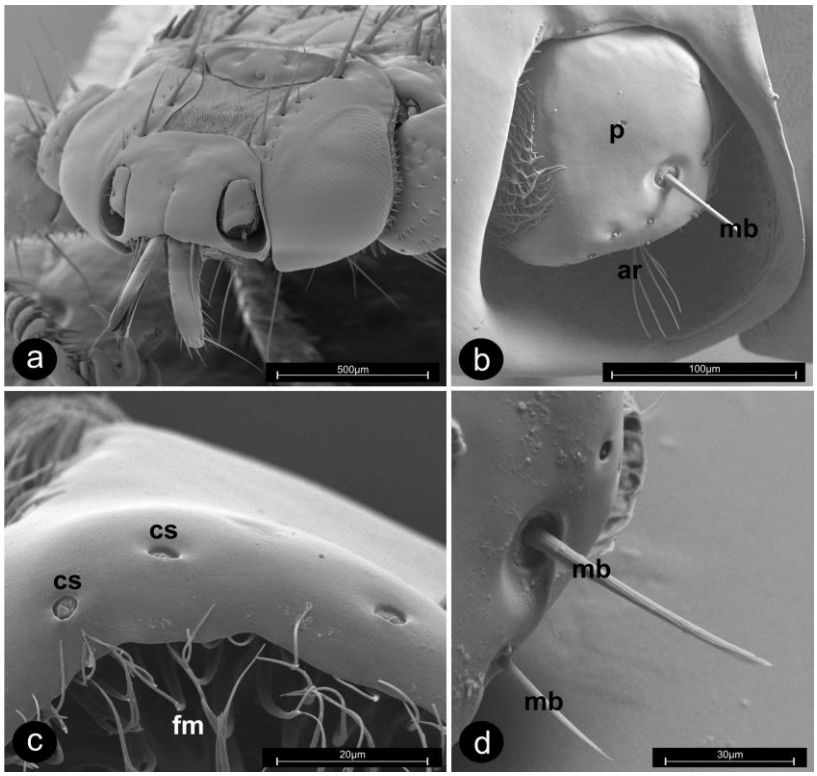


Figure 2.5. *Lipoptena cervi*. (A) Head with antennae and mouthparts. (B) Typical arrangement of the antennae inside the frontal pit; note the branched arista (ar) protruding from the antennal pedicellum (p). (C) Magnification of the antenna edge with coeloconic sensilla (cs) and furcate microtrichia (fm) from the internal hollow. (D) Socketed mechanosensory bristles (mb) on the external part of the antenna.

The thoracic region differs between the two species in both the sutural pattern and the distribution of bristles. *Lipoptena cervi* displays two protruding post-scutellar sutures that border a prominent central area (acrostichal area) of the generally flattened thorax. Conversely, in

*L. fortisetosa* these sutures are not present and the medionotal suture is well marked and crosses the whole thorax longitudinally (Figure 2.7).

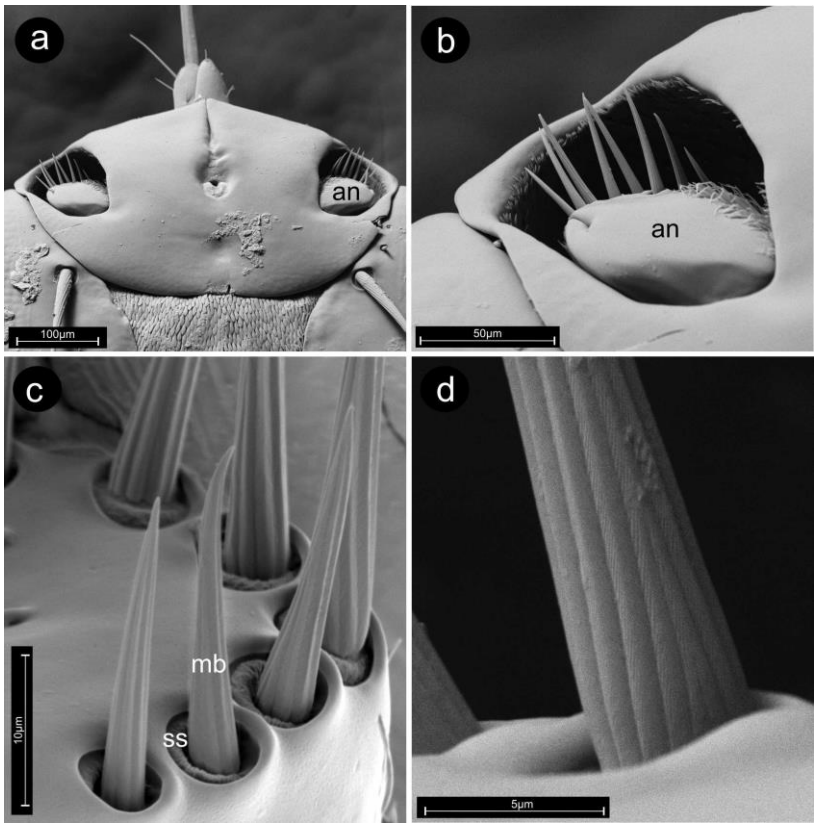


Figure 2.6. *Lipoptena fortisetosa*. (A) Head with antennae (an) and base of the mouthparts. (B) Antenna inside the frontal pit with elongated bristles. (C) Detail of the mechanosensory bristles (mb) with noticeable sensillar sockets (ss). (D) High magnification of the grooved wall of one mechanosensory bristle.

A very important taxonomic feature of this region is the chaetotaxy, which can help differentiate the two species. *Lipoptena cervi* is hairier than *L. fortisetosa* and the dimensions of its bristles vary, whereas all bristles in *L. fortisetosa* are of equal dimensions. The distribution of bristles is very different. *Lipoptena cervi* exhibits some groups of bristles that are not observed in *L. fortisetosa* and *L. cervi* has a peculiar feature that is absent in the other species: the presence of three bristles above the thoracic spiracle (Figure 2.7 C, D).

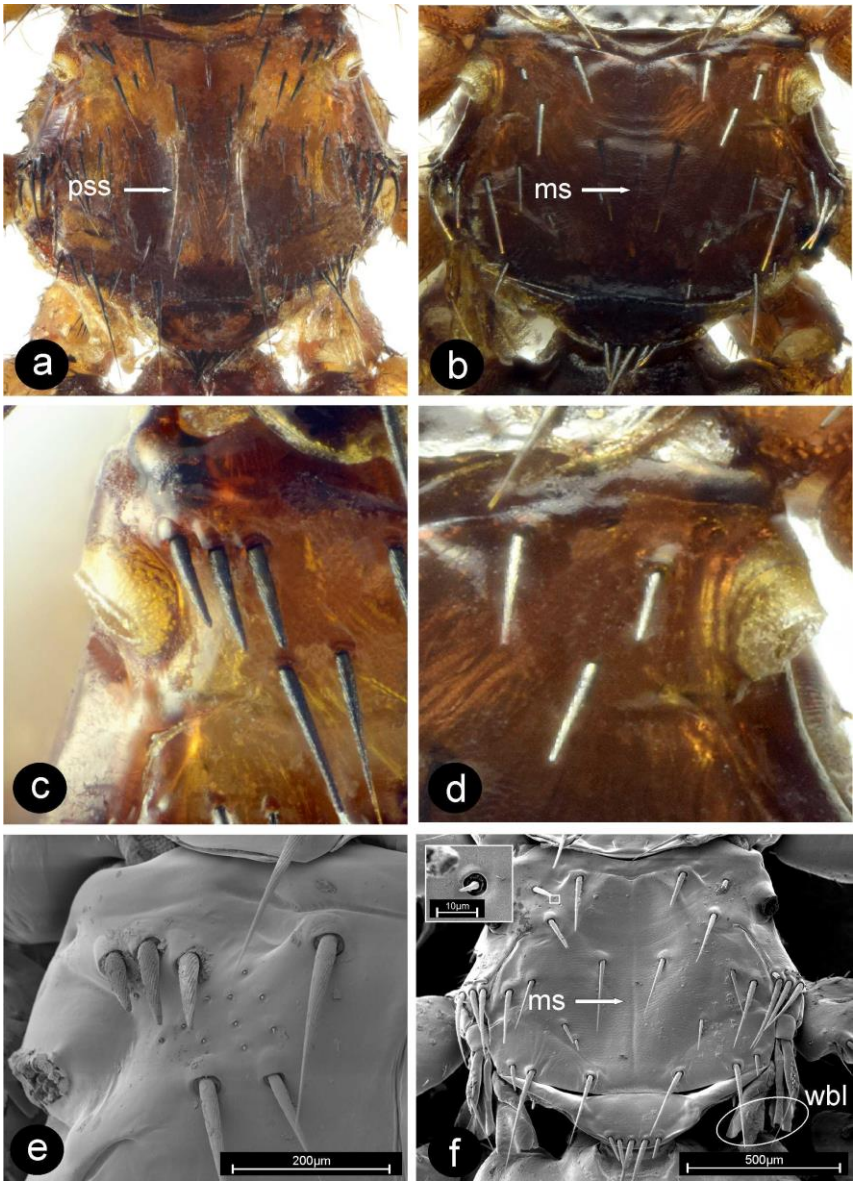


Figure 2.7. Features on the thorax (dorsal view) of females of (A, C, E) *Lipoptena cervi* and (B, D, F) *Lipoptena fortisetosa*. Morphological differences between the two species are easily detectable on the prescutellar (pss) and medionotal sutures (ms), in the number of spiracular bristles and in the number of coeloconic sensilla on the prescutellar area (E, F). (F, inset) Coeloconic sensilla belong to the uniporous type. (F) Note the wing-breaking line (wbl) on the *L. fortisetosa* thorax.

Interestingly, both species display a sensory area consisting of a group of coeloconic sensilla on the prescutellar region, close to the spiracular bristles; there are approximately 10 sensilla in *L. cervi* but fewer in *L. fortisetosa* (Figure 2.7 E, F). At high magnification, these sensilla appear to be uniporous (Figure 2.7 F, inset). These insects lose their wings after settling in a suitable host and the breaking line is notable (Figure 2.7 F).

The abdomen of *L. fortisetosa* is less sclerotized and consequently of a lighter colour, and in males is smaller than that of *L. cervi*. It is notable that in both species the membranous tegument of the abdomen of females is wider than the sclerotized areas, which allows the extension of the body for progeny development. Female terminalia differ between the species in features and in the number of bristles on the genital opening (Figure 2.8). *Lipoptena cervi* shows three pregenital aligned sclerites; each external sclerite bears two or three bristles and the central sclerite has four bristles. Furthermore, the pregenital plate is bilobate, whereas the hypoproct is semi-circular and bare, with two nearly hairless cerci. *Lipoptena fortisetosa* has only a central pregenital sclerite bearing two long and strong bristles, and the pregenital plate is composed of two distinct narrow urotergites. The hypoproct is semi-circular and, by contrast with the other species, is hairy with well-developed bristles. The male terminalia are characterized by a short aedeagus and two external gonopods that protect it and guide it during mating (Figure 2.9). In *L. cervi*, the aedeagus is cone-shaped and ends with a ridge-shaped process, and the surstyli are well developed and bear strong bristles (Figure 2.9 A, C, E). In *L. fortisetosa*, the aedeagus is membranous with a bilobate tip, and each lobe bears spines on its edge (Figure 2.9 D, F). The gonopods are elongated with tiny spines and cuticolar depressions on

the surface, homogeneously distributed but different in size. Some of these cuticolar depressions are presumably coeloconic sensilla (Figure 2.9 F). The surstyli are not as evident, but they exhibit some long bristles.

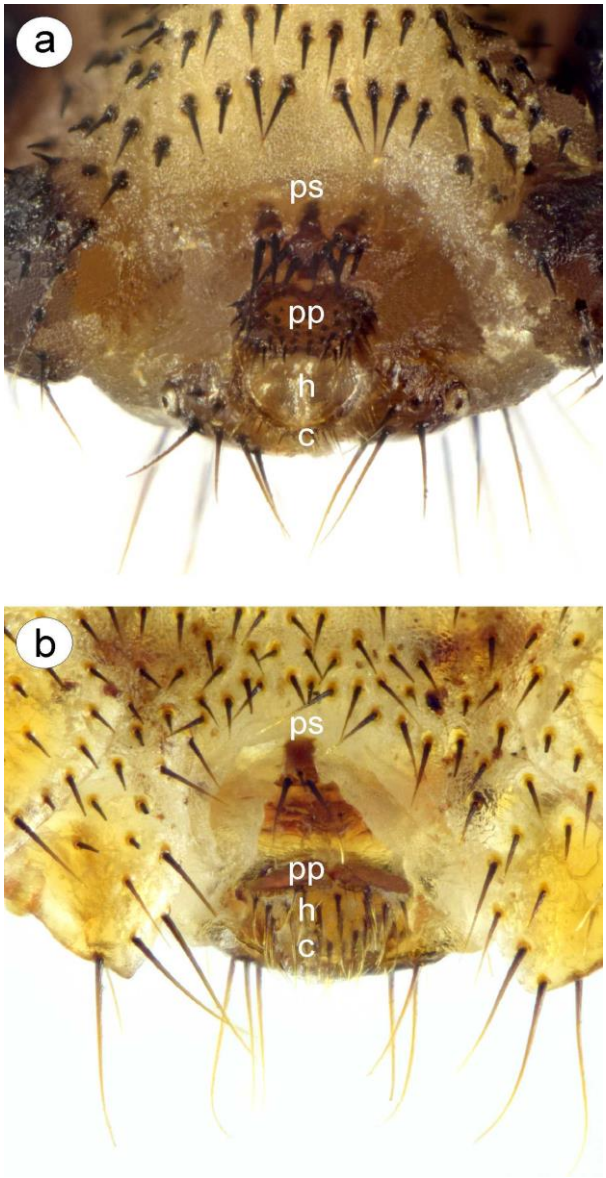


Figure 2.8. Female terminalia in (A) *Lipoptena cervi* and (B) *Lipoptena fortisetosa* showing main differences on pregenital sclerites (ps) and pregenital plate (pp) features. h, hypoproct; c, cerci.

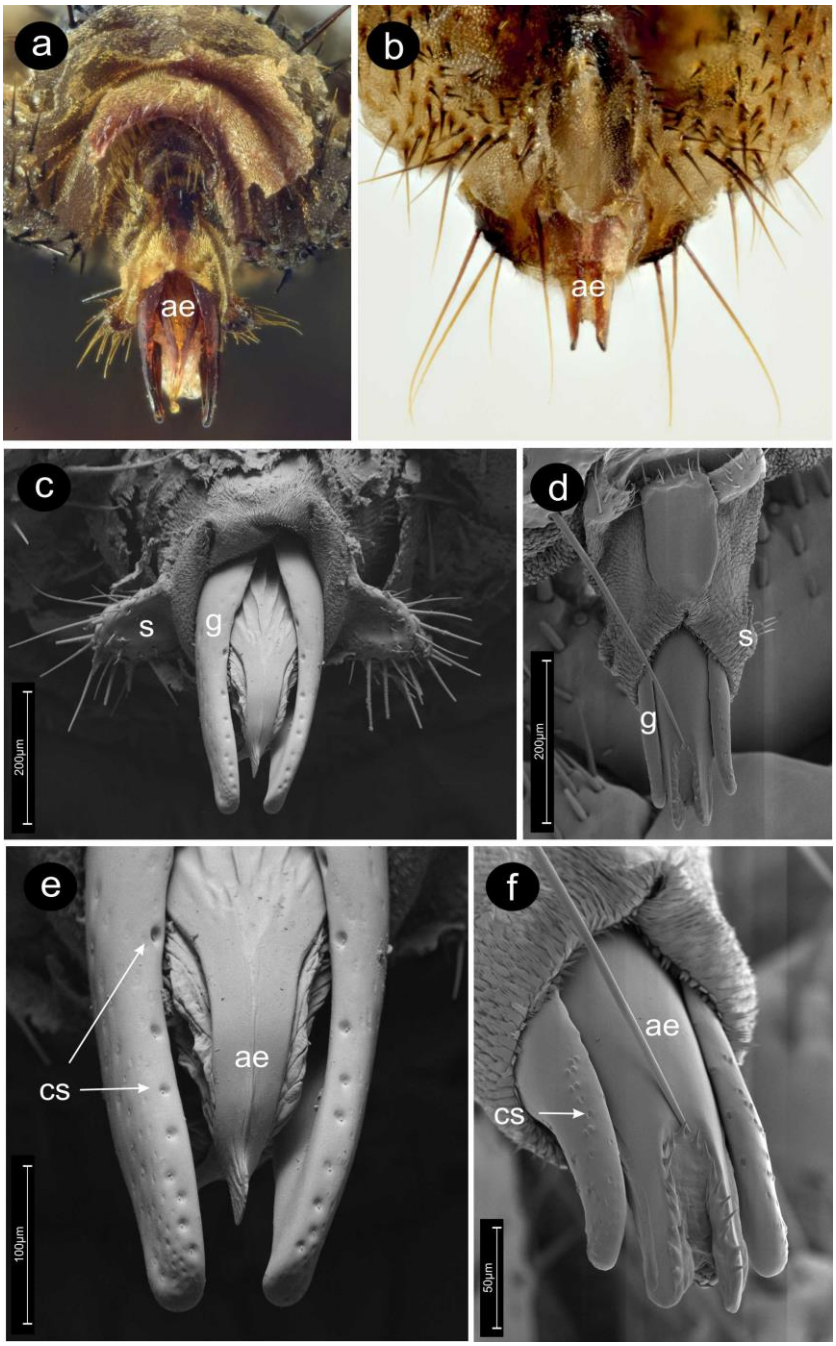


Figure 2.9. Male terminalia in (A, C, E) *Lipoptena cervi* and (B, D, F) *Lipoptena fortisetosa*. Note the morphological differences between the two species in the surstyli (s), gonopods (g) and aedeagi (ae). Coeloconic sensilla (cs) are spread on the gonopods in both species.

### External features common to both species

The legs and feeding apparatus in the two species are identical. These structures are efficiently adapted to parasitic life. The legs are robust and bear strong bristles that probably serve as mechanoreceptors (Figure 2.10). Moreover, these bristles are also useful for clasping on to the host and may help claws to hook firmly to the fur of the ungulate host. Claws are the most important tools of adhesion and are stout, asymmetrical and widely grooved to better hold the hairs of the mammal. Two additional adhesion organs are also present: the empodium and pulvilli (Figure 2.10 B).

The feeding apparatus is completely adapted for blood sucking. It consists of a retracted proboscis embraced in two sclerotized, single-segment, bristled maxillary palps (Figure 2.11). The proboscis comprises three segments: the labella, labrum and labium [the latter two, respectively, represent the labial gutter and thecal section *sensu* Snodgrass (1943)]. The apical portion is formed by the labella, whereas the main part of the proboscis is divided lengthwise into a thecal section and a labial gutter that includes the hypopharynx (Figure 2.12 A). Numerous sensilla are arranged in a circle on the tip of the labella. There are two types of sensilla: four basiconic sensilla symmetrically arranged at the four corners, and various differently sized coeloconic sensilla. Furthermore, coeloconic sensilla are also present along the surface of the thecal section of the proboscis (Figure 2.12 A, inset). Finally, on the tip of the labella, the biting apparatus consists of a group of prestomal teeth that scrape the host's skin (Figure 2.12 B, C).

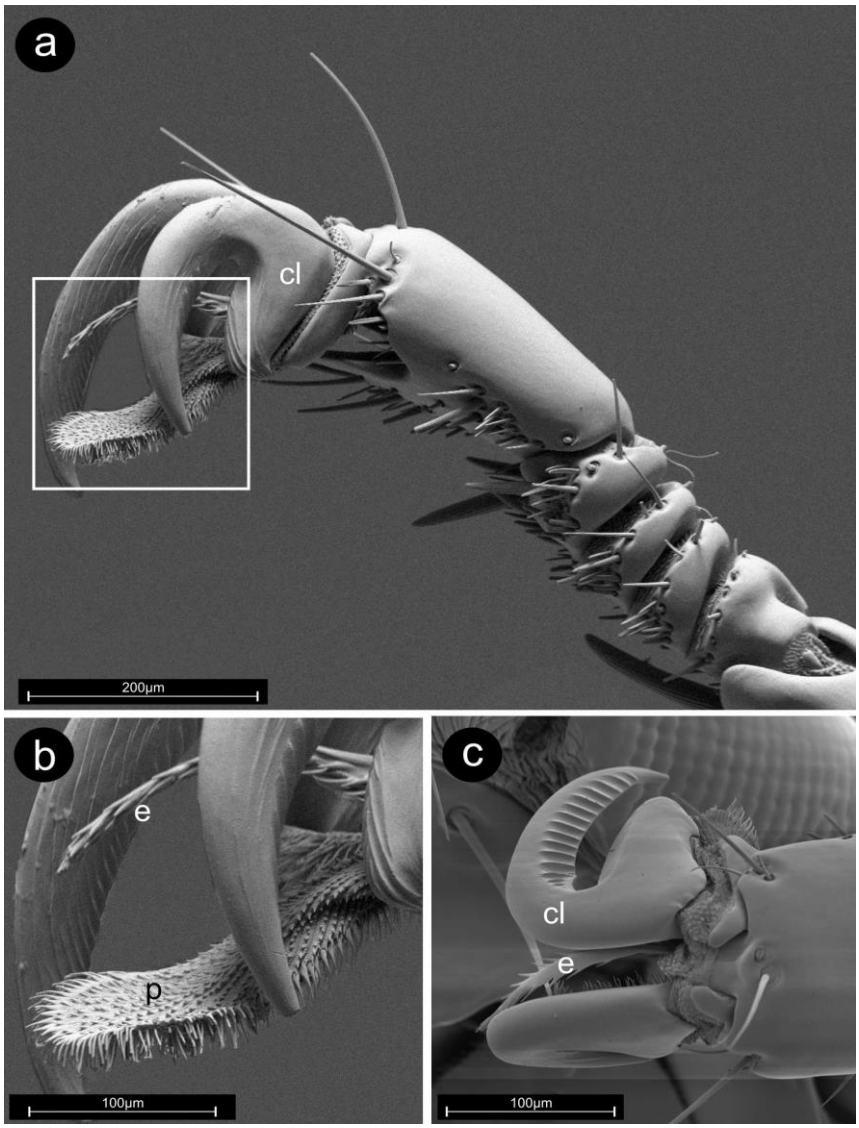


Figure 2.1017. Common features of the distal segments of the leg in (A, B) *Lipoptena cervi* and (C) *Lipoptena fortisetosa*. cl, claw; e, empodium; p, pulvilli. Note numerous long mechanosensory bristles on each tarsal segment.

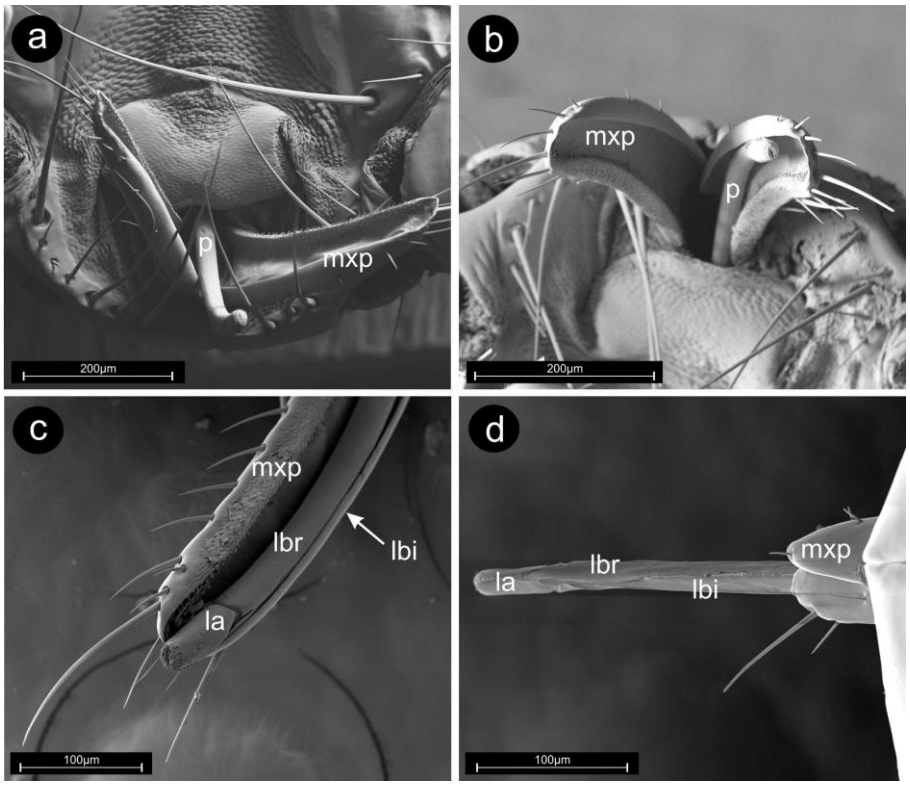


Figure 2.11. Common features of the mouthparts in (A, C) female and (B) male *Lipoptena cervi* and (D) the *Lipoptena fortisetosa* female. Single-segment, bristled, maxillary palps (mxp) serve as a sheath for the proboscis (p). The proboscis, formed by the junction of labrum (lbr) and labium (lbi), ends with the labella (la). The proboscis is embraced by the maxillary palps in (A–C) *L. cervi*, but is everted in (D) *L. fortisetosa*.

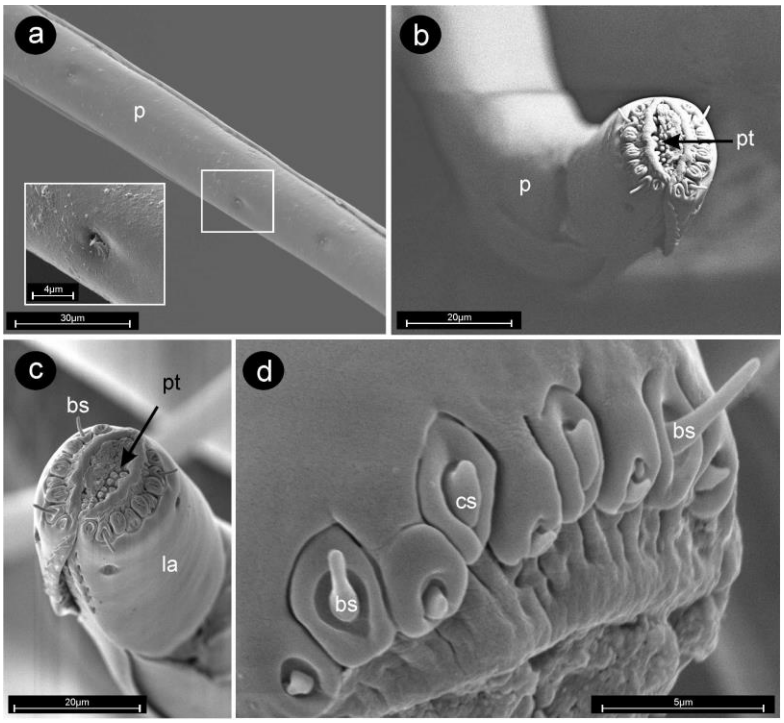


Figure 2.12. Common features of the proboscis (p) in (A) the *Lipoptena fortisetosa* male and (B–D) the *Lipoptena cervi* female. (A) Sensillar arrangement on the medial proboscis with magnification of a coeloconicum sensillum (inset). (B–D) Labella (la) with a sensory area at the tip formed by a circle of coeloconic sensilla (cs) and four basiconic sensilla (bs) symmetrically placed. Prestomal teeth (pt) are embraced by the rim of the labella.

## Discussion

In the present study, 802 hippoboscid specimens were collected from five ungulates. These investigations showed that substantial numbers of *L. fortisetosa* were present on each mammal, except for the yearling male. This ungulate carried a high number of *L. cervi* and only one *L. fortisetosa* specimen. This may reflect the predilection of *L. cervi* for parasitizing yearlings. The fly attacks yearlings over fawns because it uses visual stimuli during host seeking and hence tends to parasitize hosts with larger body sizes (Kortet *et al.*, 2009). However, *L. cervi* prefers yearlings to adults because the latter are less active and

movement has been shown to be one of the most relevant factors in host selection in this species. In fact, the parasite rests in vegetation and waits for a host to pass by and hence is more likely to encounter a host that moves more (Madslien *et al*., 2012).

The other two sampled red deer had equivalent numbers of parasites of the two species. Although it is not possible to determine when *L. fortisetosa* colonized Italy, it can be assumed that this parasite is currently spreading into a new area and is strongly competing with the native *L. cervi*, as well as adapting to a new environment. This competition is related to the host, which represents the refuge, reproduction site and food source for both species. In fact, although *L. fortisetosa* has not been well studied, it shows a similar lifecycle to *L. cervi* (Sonobe, 1979). Initially, both the autochthonous parasite and the ungulate hosts may withstand the invasion, but later there may be a reaction and an adaptation process that may lead to the establishment of a new balance among *L. fortisetosa* and other competing parasites (such as ticks) and hosts. However, the coexistence of two *Lipoptena* species on the same host in the same geographical region is reportedly unusual and leads to several ecological problems that should be more deeply investigated (Mogi, 1975).

The first important issue worthy of attention is how *L. fortisetosa* has spread into Italy. The present authors hypothesize that this species may have arrived via *C. nippon*, its original host, because the fly is native to Japan (Maa, 1965, 1967). Indeed, in all countries in which the parasite has been discovered, its presence is considered to be related to this ungulate (Mogi, 1975; Sonobe, 1979; Yamauchi & Nakayama, 2006; Choi *et al*., 2013). The Japanese deer is originally from the Far East, but it is now distributed worldwide as a result of both intentional and accidental introductions, and is one of the major naturalized alien

ungulates in Europe (Raganella Pelliccioni *et al*., 2013). In Italy, this mammal was recently discovered in the provinces of Modena and Trento (approximately 100 km and 300 km, respectively, north of Florence), and its presence in the country is undisputed (Ferri *et al*., 2016). It is interesting to note that a recent study conducted in the province of Sondrio in northern Italy revealed the absence of *L. fortisetosa* among the ectoparasites of ungulates (Bianchi *et al*., 2016). Because the parasite has been recorded in Switzerland, the country bordering Sondrio, it would be interesting to study the reasons why it is absent from this area of Italy although, based on the present results, it is clearly established in central Italy. As other authors (Mogi, 1975; Choi *et al*., 2013) have assumed, *C. nippon* may have spread through Europe carrying its ectoparasites, which later may have switched to other cervid hosts. The current findings clearly demonstrate that *L. fortisetosa* has adapted to other hosts. It should be noted that the present study represents the first record of this species infesting roe deer. Moreover, the fact that one of the roe deer from which *L. fortisetosa* was collected was born and raised in Florence at the DISPAA experimental farm shows the adaptability of the parasite to heavily urbanized areas.

With respect to the morphological differences between the two parasites, this study highlights the peculiar characteristics that facilitate their identification. Among the numerous traits described, three major differences should be emphasized. Firstly, body size provides important information at a glance because *L. cervi* is visibly larger than *L. fortisetosa*, and males of both species are smaller than females. Secondly, the distribution of thoracic bristles is a fundamental taxonomic feature. Overall, *L. fortisetosa* has fewer bristles, whereas *L. cervi* is hairier and presents some groups of bristles that are not

observed in the other species. In particular, it is possible to identify three strong bristles above the spiracles, present only in the native species, in agreement with the taxonomic key of Maa (1965). The third marked difference refers to the sensillar pattern on the external surface of the antennal segment. In *L. cervi*, two trichoid, one basiconic and seven coeloconic sensilla are present, whereas *L. fortisetosa* shows only nine strongly socketed sensillar bristles. This feature is important for differentiating the two species and allows for further considerations with respect to the host. The nine bristles of *L. fortisetosa* have grooved surfaces and are probably mechanoreceptors, as has been shown in horse stomach both flies (Zhang *et al*., 2016). However, *L. cervi* presents three types of sensilla, which means that this species is likely to use different stimuli to perceive the environment and locate a host. In fact, the trichoid sensilla probably have a unique mechanoreceptive function, whereas the basiconic and the coeloconic sensilla are chemoreceptors and may allow the parasite to perceive changes in temperature and humidity that help it to locate a host, as demonstrated by Kortet *et al*. (2009). The presence of different receptors on the antennae shows more developed sensory perception and indicates a major opportunity for signal transduction in this species. Nonetheless, additional information should be obtained by further studies on the sensory area of the antenna, as recently shown in three different hippoboscid species (Zhang *et al*., 2015).

The bodies of both *L. cervi* and *L. fortisetosa* are covered with a number of bristles, which are useful for protection but also help in clasping the host. Moreover, some of them may serve as mechanoreceptors that increase sensory perception.

The legs and feeding apparatus are very similar in both species and represent examples of adaptive evolution common to both

species. The adaptation process has led to the development of all the regions of the body for parasitic life. As previously noted, the legs are strongly adapted to hook the fly to its host's fur and show asymmetrical claws together with additional tools of adhesion, such as strong bristles and spurs. Other flies that parasitize bats, such as those of the dipteran families Nycteribiidae and Streblidae, also show modified legs (Peterson & Wenzel, 1987; Wenzel & Peterson, 1987).

The feeding apparatus of these hippoboscid flies displays some interesting characteristics, such as the sclerotized palps and the presence of several sensilla arranged in a circle at the tip of the labella. Although different groups of flies have evolved mouthparts according to specific needs, maxillary palps are generally devoted to monitoring the environment for both gustative and olfactive purposes, together with other sensory structures present in the antennae, labial palps, tarsi and ovipositor. In blood-sucking species, such as tabanid flies, palps may play an important role in host location, as well as in the environmental monitoring usually carried out by different types of sensilla (Krenn & Aspöck, 2012).

In other ectoparasitic dipterans, such as mosquitoes, maxillary palps present specialized sensilla that respond to specific stimuli involved in host-seeking behaviours. For example, sensilla on the maxillary palps of *Aedes aegypti* (Linnaeus) (Diptera: Culicidae) detect carbon dioxide. The same organs in *Aedes albopictus* (Skuse) show four different sensilla, such as capitate pegs, and campaniform, basiconica and chaetica sensilla, whereas the labial palps are covered with three types of smooth chaetica sensilla at the tip of the labellum. Similar structures have been reported in other mosquitoes and biting insects (Seenivasagan *et al*., 2009). The presence of differently specialized sensilla indicates a well-developed sensory perception

system capable of detecting various stimuli. Other blood-sucking flies, such as the Ceratopogonidae, present maxillary palps bearing a relevant number of sensory structures mostly within a well-defined sensory pit (Alexandre-Pires *et al*., 2010). In tabanids, the palps are short and two-segmented, and bear different kinds of bristles, but have not yet been investigated in terms of sensory structures (Stoffolano & Yin, 1983).

In phytophagous dipterans such as tephritids, maxillary palps represent a specified sensory area. In the olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), the internal side of the palps is covered by microtrichi, and the external side shows mainly mechanosensory bristles with basiconica sensilla interspersed among them. The palps are equipped with olfactory sensilla involved in semiochemical perception and respond to various volatile compounds (Liscia *et al*., 2013).

In *L. cervi* and *L. fortisetosa*, the palps evolved differently from those in other haematophagous flies. These parasites show two strongly sclerotized, elongated and concave (channelled) palps that enclose and protect the proboscis. These species live among the hairs of the fur of their ungulate hosts in a hostile and cumbersome environment. Moreover, in these species, the palps maintain sensory function to a lesser extent as they show only bristles that are probably mechanoreceptor sensilla and no other types of sensorial structures.

Although there are no detailed descriptions of the feeding mechanisms of hippoboscid flies, it can be assumed that they have followed an evolutionary path similar to that of the Glossinidae (Diptera). In these parasitic flies, the palps are paired to form a sheath that embraces the proboscis and, during feeding, they separate from the proboscis, which penetrates the host skin with repeated

movements to allow the fly to feed on the pool of blood that accumulates under the skin (Krenn & Aspöck, 2012).

With respect to the sensorial area located on the tip of the proboscis, the present authors observed two types of sensilla that differed in size: basiconic and coeloconic. The flies probably need all these sensilla to test the skin of the host in order to find the most appropriate feeding point, while the sclerotized palps lead the proboscis. Snodgrass (1943) described the mouthparts of hippoboscids, but not in great detail. After finding a suitable feeding location, the parasite damages the skin of the ungulate using two specific fixed rows of teeth and some specific eversible teeth near the sensillar area on the labella. When blood emerges, the fly feeds through the food canal.

Haarløv (1964) concluded that *L. cervi* is a pool feeder and not a capillary feeder like some mosquitoes because it needs to injure the skin of the host in order to suck blood from a haemorrhage made by its teeth. *Lipoptena fortisetosa* shows mouthpart structures that are the same as those in *L. cervi* and both are very similar to the feeding apparatus of the Glossinidae (Snodgrass, 1943; Haarløv, 1964; Krenn & Aspöck, 2012; Gibson *et al*., 2017). The mouthparts of tsetse flies include a proboscis that is equipped with arrays of teeth and rasps. The proboscis is formed by labella at the top of the organ and is divided lengthwise into a labrum and a labium that includes the hypopharynx (Gibson *et al*., 2017).

Several species of biting insect are provided with sharp elements that are able to tear the skin of the attacked animal; some tabanids exhibit developed mandibles armed with marginal teeth, suggesting haematophagous feeding. Conversely, smaller mandibles with vestigial or absent marginal teeth, or those that are covered with

micropilosity, may indicate different feeding behaviours in other tabanid species (González & Flores, 2004). Indeed, some muscids within the Stomoxyinae also have similar structures to *L. cervi* and *L. fortisetosa*. *Stomoxys calcitrans* (Linnaeus) (Diptera: Muscidae) has a particularly hard and sclerotized haustellum with rows of teeth and spines and two short palps, whereas *Haematobia irritans* (Linnaeus) (Diptera: Muscidae) and *Haematobia titillans* (Bezzi) present two aligned rows of bristles (Giangaspero *et al*., 1996).

In summary, *L. fortisetosa* and *L. cervi* are two parasitic hippoboscids that were detected during a survey carried out on ungulates in Tuscany. This finding of *L. fortisetosa* represents the first record of this species in Italy. The present report highlights some of the most relevant differences in gross morphology between the two species, such as those in the external parts of the antennae, the distribution of bristles, and different features in the external genitalia. Scanning electron microscopy of the mouthparts revealed a strong adaptive convergence developed for feeding on the skin of the host in both species, such as modified palps and a very thin proboscis with teeth at the apex and a characteristic sensory area that suggests a specialized feeding behaviour. The presence of an exotic species may represent a new challenge to the health of its hosts in Italy, particularly as *L. cervi* can transmit several disease-causing pathogens to animals, as well as to humans. Further investigations into the importance of *Lipoptena* species, especially *L. fortisetosa*, are worthy of attention.

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# 3. Evolutionary adaptations in four hippoboscid fly species belonging to three different subfamilies

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## Abstract

*Lipoptena cervi* (Linnaeus, 1758), *Lipoptena fortisetosa* Maa, 1965, *Hippobosca equina* Linnaeus, 1758, and *Pseudolynchia canariensis* (Macquart, 1840) (Diptera: Hippoboscidae) are haematophagous ectoparasites that infest different mammal and bird species and occasionally attack humans. They are known for the health implications they have as vectors of pathogens to humans and animals, and for the injuries they inflict on their host's skin. This study focused on the morphological structures evolved by parasites in terms of their biology and the different environment types that they inhabit. To this aim, we examined four hippoboscid species, as well as their hosts' fur (ungulate and horse), and feather (pigeon) through light and Scanning Electron Microscopy (SEM) observations in order to highlight the main morphological features that evolved differently in these flies and to explain the effect of hosts' fur/feather microhabitats on the morphological specializations observed in the investigated ectoparasites. The studied species showed main convergent characters in mouthparts while remarkable differences have been detected on the antennal sensillar pattern as well as on the leg acropod that displayed divergent characters evolved in relation to the host.

## Introduction

Members of the Hippoboscidae family belong to the Diptera order and are haematophagous flies that parasitize birds and mammals (Hutson, 1984; Reeves & Lloyd, 2019). The Hippoboscidae family has three subfamilies, Ornithomyinae, Hippoboscinae and Lipopteninae (Maa & Peterson, 1987), of which 213 species have been described (Dick, 2006; Obonă *et al*., 2019).

Hippoboscid adults are obligatory blood-feeding insects; some species remain on the host for the duration of their life cycles, while others usually have multiple hosts during their life span (Maa & Peterson, 1987). These flies are "pool feeders", meaning that they receive nourishment by the haemorrhage they produce with their prestomal teeth on the host's skin (Haarløv, 1964). In order to secure the blood, these parasites have a heavily adapted mouthpart (Snodgrass, 1943). Their feeding apparatus is similar to the mouthparts of members of the Glossinidae family (Diptera), with which they are very closely related (Popham & Abdillahi, 1979; Gibson *et al*., 2017). Members of both groups are equipped with a piercing proboscis, which has a sensory area at the tip that allows these parasites to detect the most appropriate feeding spot. The proboscis is embraced by two sclerotized maxillary palps and is equipped with sharp elements that allow it to tear the host's skin (Andreani *et al*., 2019). Although several biting insect species use their teeth and rasps to injure the skin (Krenn & Aspöck, 2012), only members of the Glossinidae and Hippoboscidae families have been classified as pool feeders (Haarløv, 1964); however, hippoboscids have recently been considered solenophages or vessel feeders (Reeves & Lloyd, 2019) such as other blood feeding flies (e.g. mosquitoes). Thus, further studies are needed to better clarify the feeding behaviour of these parasites.

Members of the *Lipoptena* genus (subfamily Lipopteninae) infest ruminant artiodactyl mammals, especially cervids (Bequaert, 1942; Hutson, 1984). Among these keds, *Lipoptena cervi* (Linnaeus, 1758) is the most widespread species in Europe (Salvetti *et al*., 2020). It predominantly parasitizes red deer, *Cervus elaphus* Linnaeus, 1758, roe deer, *Capreolus capreolus* (Linnaeus, 1758), and to a lesser extent, fallow deer, *Dama dama* (Linnaeus, 1758) (Haarløv, 1964). Until 2017, this ectoparasite was considered to be the only species of the *Lipoptena* genus present in Italy (Pape *et al*., 1995); however, during a research conducted in Tuscany, *Lipoptena fortisetosa* Maa, 1965 was detected for the first time in Italy (Andreani *et al*., 2019). This fly is native to Japan but has spread into Korea and Russia and, in addition to being detected in Italy, it was identified only in a few other European countries (Choi *et al*., 2013). Its original host is the Japanese deer (*Cervus nippon* Temminck, 1838), but currently it has switched to other host species as demonstrated by new records on different cervids (Choi *et al*., 2013). All representatives of the *Lipoptena* genus are obligate ectoparasites and remain on a single, suitable host for the duration of their lives, feeding and reproducing into its fur. While they pass through the hairs of their hosts, these keds lose their wings and become wingless (Haarløv, 1964). This is a typical phenomenon in the genus *Lipoptena*; in fact, other species of different genera have functional wings, depending on their host's coat and environment (Maa & Peterson, 1987). *Lipoptena cervi* and *L. fortisetosa* are able to co-exist on the same host subject (Andreani *et al*., 2019).

Little information is available on the role of these species as disease vectors; however, several authors have claimed that when the flies reach very high infestation intensities, they can impair the health condition of their hosts, causing alopecia, skin lesions, sickness and

stress (Víchová *et al*., 2011; Madslien *et al*., 2012; Paakkonen *et al*., 2012; Kynkäänniemi *et al*., 2014).

*Hippobosca equina* Linnaeus, 1758 (subfamily Hippoboscinae) is spread worldwide and is a common parasite of horses, donkeys and cattle (Obonă *et al*., 2019). This is a pest species of veterinary importance as it causes direct skin damage and general health problems; in addition, it is a possible carrier of different pathogens (Hafez *et al*., 1977; Reeves & Lloyd, 2019).

Approximately 75% of Hippoboscidae infest birds (Hutson, 1984). Among them, *Pseudolynchia canariensis* (Macquart, 1840) (subfamily Ornithomyinae), better known as the pigeon fly, is a nearly cosmopolitan obligate parasite of birds. This species attacks predominantly Columbiformes, especially feral and domestic *Columba livia* Gmelin, 1789 individuals, and Falconiformes; nevertheless, this fly has been recorded on a wide range of other birds (Maa, 1966; Hutson, 1984; Yamauchi *et al*., 2011). It can cause several health problems to its hosts, such as irritation or dermatitis; moreover, it is a potential vector of *Haemoproteus columbae* Kruse, 1890 (Pirali-Kheirabadi *et al*., 2016).

All the aforementioned parasites need a suitable and permanent host to survive; however, they can also accidentally infest other species, including humans, in order to feed (Maa & Peterson, 1987; Reeves & Lloyd, 2019). This characteristic makes these species important for public health since they serve as pathogen vectors that are known as causal agents of zoonoses. Nevertheless, further research on the medical and veterinary importance of hippoboscid flies is required, as their role as pathogen vectors is probably much greater than what we currently perceive it to be.

Ectoparasites infest an array of host species; some of them are characterized as monoxenous and parasitize on a single host, while others are classified as generalists and can infest a large range of species (Maa & Peterson, 1987). Hippoboscids are restricted to only a few host species; in fact, although they can accidentally infest different hosts on which they feed, only some species are suitable hosts and can guarantee the parasites' survival. Parasites that are limited to a few hosts are highly adapted physiologically to an ectoparasitic lifestyle and have specific morphological features (e.g. blood-feeding apparatus, legs, and adhesion organs) that evolved to enable them to survive efficiently on their host species (Kaunisto, 2012; Andreani *et al*., 2019). This adaptation process has led to a differential development of certain body parts, depending on the distinct environments in which the parasites live.

The aim of this study was to highlight the main morphological characters that *L. cervi*, *L. fortisetosa*, *H. equina* and *P. canariensis* have evolved in their parasitic lives. In fact, these four species attack different hosts that live in very peculiar environments and exhibit distinctive behaviours; thus, the parasites require specialized morphological characters. On the other hand, other features have evolved similarly demonstrating a strong structural convergence related with the type of parasitic life. A description of divergent and convergent morphological features is provided with a focus on the external part of the insect antennae.

## Materials and methods

### Sampling procedures

During a wildlife ectoparasite sampling session conducted in the Central Apennines, several Hippoboscidae specimens were collected. *Lipoptena cervi* and *L. fortisetosa* were collected from hunter-harvested cervids in 2018–2019 from some Tuscany and Emilia-Romagna provinces, while *H. equina* specimens were collected from horses in Marradi (Firenze, Italy). Finally, several *P. canariensis* specimens were collected from pigeons thanks to the collaboration of the Provincial Wildlife Police of Pisa, in the context of an official wildlife surveillance program performed in San Miniato (Pisa).

### Taxonomic identification of Hippoboscidae

Hippoboscid species were identified morphologically using a stereomicroscope (Leica/Wild MZ16, equipped with an L2 illuminator; Leica Microsystems, Wetzlar, Germany) and the taxonomic keys proposed by Bequaert (1942), Hutson (1984), Maa (1966, 1967), Mogi (1975), Maa & Peterson (1987), and Graciolli & Carvalho (2003).

### Investigations on fly morphology

From the collected ectoparasites, several specimens belonging to the four hippoboscid species were morphologically studied using optical and Scanning Electron Microscopes (SEM). Adults were anaesthetised by being kept at −20°C for 20 min, being subsequently killed. Then, both the males and females were subjected to a specific preparation in order to be cleaned. For the SEM observations, the specimens were immersed in 10% potassium hydroxide and sonicated for 15 min to remove impurities and secretions from their bodies. After this procedure, the insects were rinsed in distilled water, dehydrated

in a series of graded ethanol concentrations (70–99% ethanol, for 10 min in each concentration), and, subsequently, air-dried. Specimens in toto or some excised parts were mounted on stubs with a double-sided adhesive tape and gold-coated with a sputter coater device (S150B; BOC Edwards, Burgess Hill, U.K.). Observations were made using a FEI Quanta 200 high vacuum, low vacuum and ESEM environmental scanning electron microscope (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.).

The morphology and the external sensillar pattern of the antennae were examined and described using the terminology and nomenclatures reported by Maa & Peterson (1987) and Zhang *et al*. (2015).

### Ungulate and bird body hair observations

Skin samples of hunter-harvested ungulates belonging to red deer, roe deer and fallow deer were prepared for the light and SEM microscopes. Several neck and groin region pieces (approximately 0.5 × 0.5 $cm^2$) as well as hairs were cut from different specimens and their morphological composition was observed. Winter coat pieces were obtained in October 2019; parasites were present in the fur of some of these skin samples. Additionally, some feathers were collected from a pigeon nest and their morphology was examined by light and SEM microscopes. None of the samples were subjected to a specific preparation either for the SEM or for the optical investigations.

Observations were made using a FEI Quanta 200 low-vacuum scanning electron microscope (Thermo Fisher Scientific, Inc., Waltham, MA, U.S.A.) and a Leica Z16 Apo microscope equipped with the Helicon Remote software (Helicon Soft Ltd., Kharkov, Ukraine) for

capturing single images and the Helicon Focus software (Helicon Soft Ltd.) for the staking process.

## Results

### Main morphological adaptive features

*Antennae*. In hippoboscid flies, the antennal structure is deeply modified compared with other Muscomorpha, as their main sensory area (flagellum) is introflexed in the other segments that originate from a fusion of the first and second segments (scape and pedicel) (Figure 3.1).

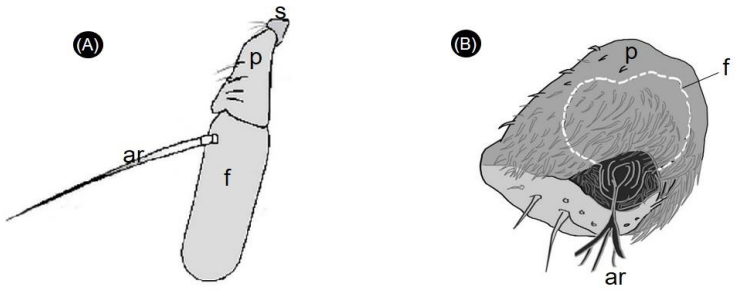


Figure 3.1. Typical antennal morphology of higher dipterans (A); antennal adaptation in *L. cervi* (B). Note the external part (scape and pedicel fused) and the introflexed flagellum (dashed line). s, scape; p, pedicel; f, flagellum; ar, arista

In addition, hippoboscid antennae are housed in two hollows, termed antennal sockets, on the face and close to the compound eyes (Figure 3.2).

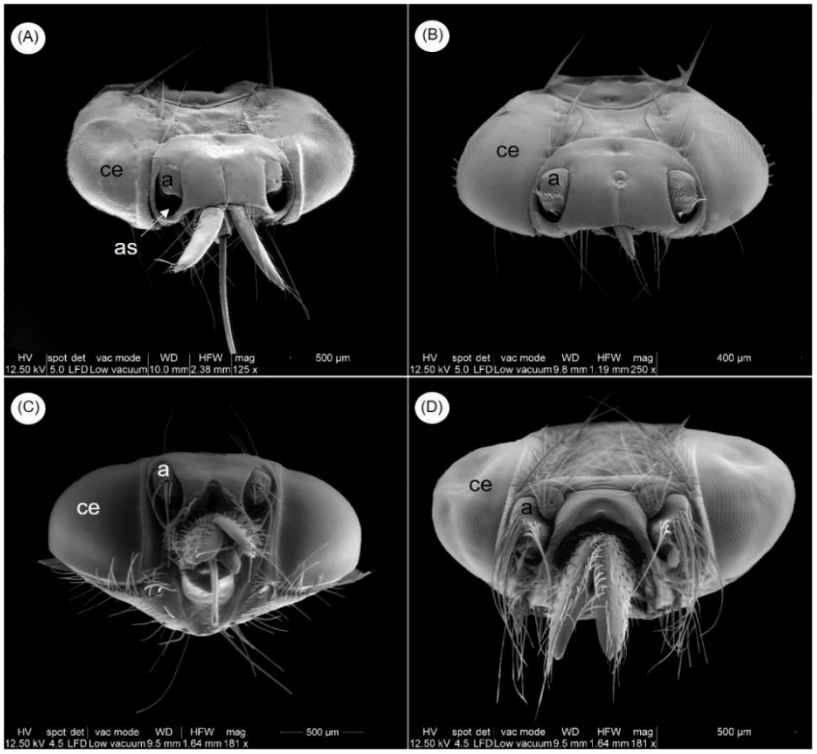


Figure 3.18. Frontal view of the heads of four hippoboscid species. (A) *L. cervi*; (B) *L. fortisetosa*; (C) *H. equina*; (D) *P. canariensis*. a, antenna; ce, compound eye; as, antennal socket.

In *L. cervi* and *L. fortisetosa*, the antennae originate from a complete fusion of the scape and pedicel, which protrudes externally (Figure 3.3 A and B). In the former, the bell-shaped antenna has different kinds of sensilla; seven coeloconic sensilla close to the segment rim, two trichoid socketed sensilla, and one basiconic sensillum, all located in the medium distal part of the pedicel (Figures 3.3 A and 3.4 A). The arista is slender, thin and branched (Figures 3.3 A, 3.4 A, and 3.5 A). *Lipoptena fortisetosa* has a different sensillar pattern consisting of nine characteristic long sensory bristles arranged along the pedicel edge, while the arista has a jagged fan structure at the edges (Figures 3.3B, 3.4B, and 3.5B).

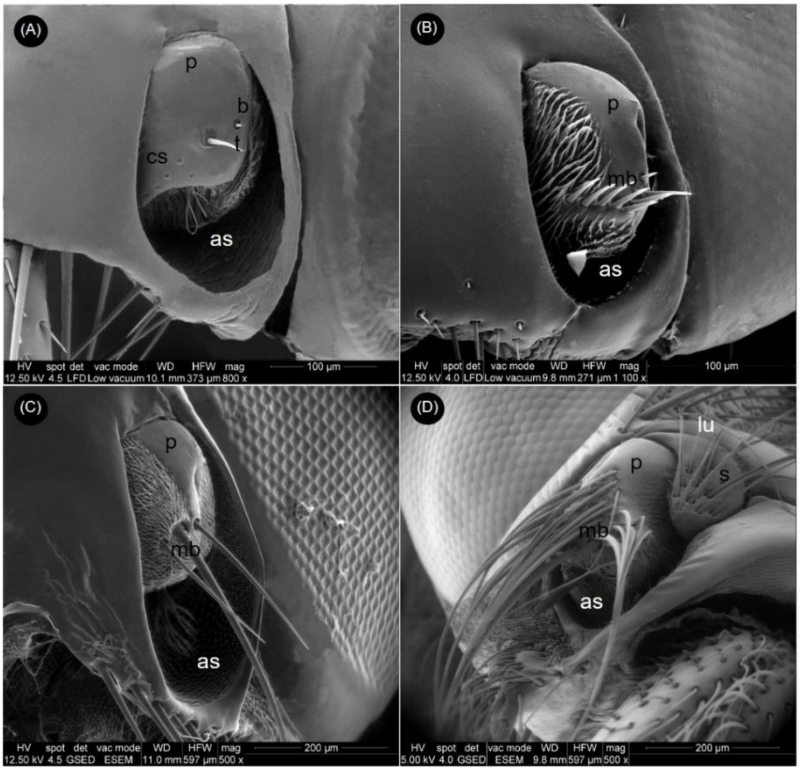


Figure 3.3. Antennae of *L. cervi* (A); *L. fortisetosa* (B); *H. equina* (C); *P. canariensis* (D). Note the presence of the scape bearing several mechanosensory bristles in the latter species. p, pedicel; s, scape; as, antennal socket; lu, lunula; b, basiconic sensillum; cs, coeloconic sensillum; t, trichoid sensillum; mb, mechanosensory bristle.

The antenna of *H. equina* is inserted inside a deeper socket as compared with that of the other three species and occupies about a half of the hollow (Figure 3.3 C). The inner surface of the antennal pit is covered by microtrichia while in the other species it is bare. Furcate microtrichia occupy the entire external surface of the pedicel except for a little portion adjacent to the compound eye that is uncovered. Three mechanosensory bristles, with the central longer and more developed than the others, constitutes the external sensillar pattern; in addition, seven stout setae are present on the frontoclypeal rim bordering the antennal socket. The arista is stocky and has numerous ramifications (Figures 3.3 C, 3.4 C and 3.5 C).

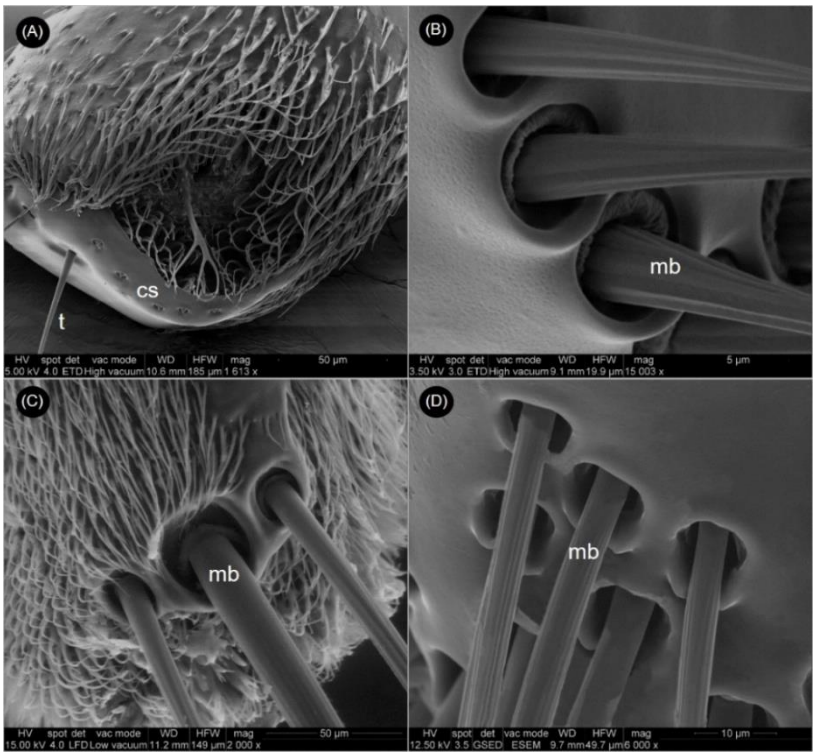


Figure 3.4. External part of the antennal pedicel with different sensillar arrangements. (A) *L. cervi*; (B) *L. fortisetosa*; (C) *H. equina*; (D) *P. canariensis*. Note the well-developed sensory bristles present on *H. equina* and *P. canariensis*. cs, coeloconic sensillum; t, trichoid sensillum; mb, mechanosensory bristle.

The antenna of *P. canariensis* has different adaptive features compared with those of the other three species. The scape articulates with the pedicel, it is visible and partially fused with the lunula (Figure 3.3 D); moreover, although the antenna is inserted in the socket as in the other hippoboscid species, the pit is less hollow and the antenna protrudes externally. The scape is smaller than the pedicel, drop-shaped and bears several bristles that vary in size. The pedicel displays numerous long, socketed mechanosensory bristles and the arista is spatulate (Figures 3.3 D, 3.4 D and 3.5 D). A common feature among the four examined species is the presence of furcate microtrichia

originating from the inner pedicel surface and protruding from the opening (Figure 3.4).

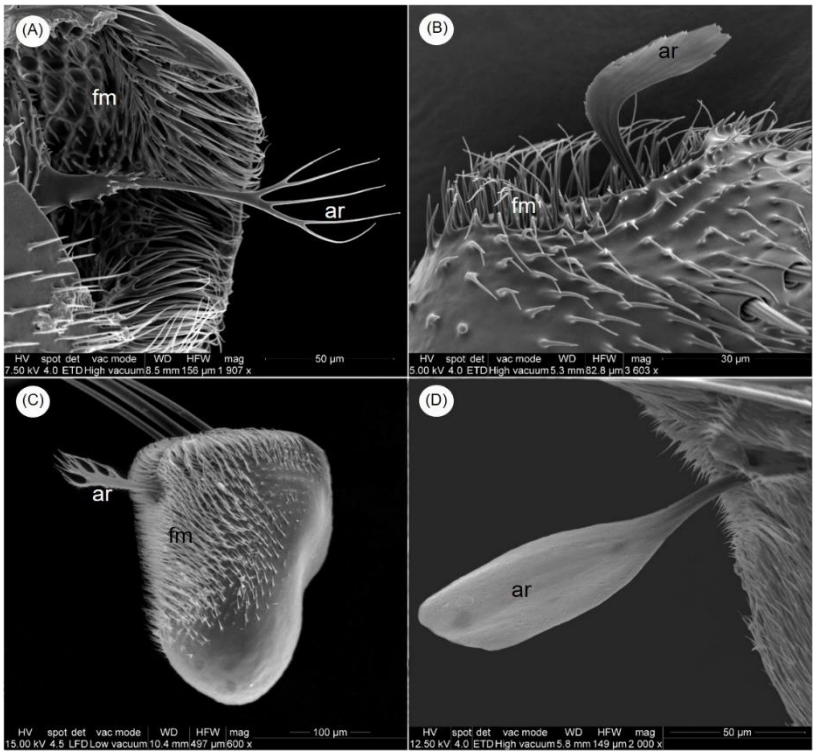


Figure 3.5. Different shape of the arista in *L. cervi* (A); *L. fortisetosa* (B); *H. equina* (C); *P. canariensis* (D). ar, arista; fm, furcate microtrichia.

*Feeding apparatus*. The feeding apparatus is a convergent feature and evolved in a similar manner in all four described hippoboscids. The hippoboscid mouthparts consist of a long proboscis enclosed into a pair of sclerotized maxillary palps, which have a concave inner surface, allowing them to embrace the proboscis (haustellum sensu Snodgrass, 1943) and bear numerous mechanosensitive bristles. The proboscis originates from the union of the labrum (upper part), hypopharynx (internal part) and labium (lower part) with the distal part formed by the fused labella.

The feeding apparatus has several sensilla and their arrangement is the only feature that differs between *P. canariensis* and the other three species (Figures 3.6 and 3.7). In fact, the tip shows a circular crown that consists of different types of sensilla; *L. cervi*, *L. fortisetosa* and *H. equina* have four long basiconic sensilla arranged at the corners and a circular row of coeloconic sensilla. Moreover, smaller coeloconic sensilla are present on the medium-basal portion of the labella and are differently arranged in these three species. (Figure 3.6).

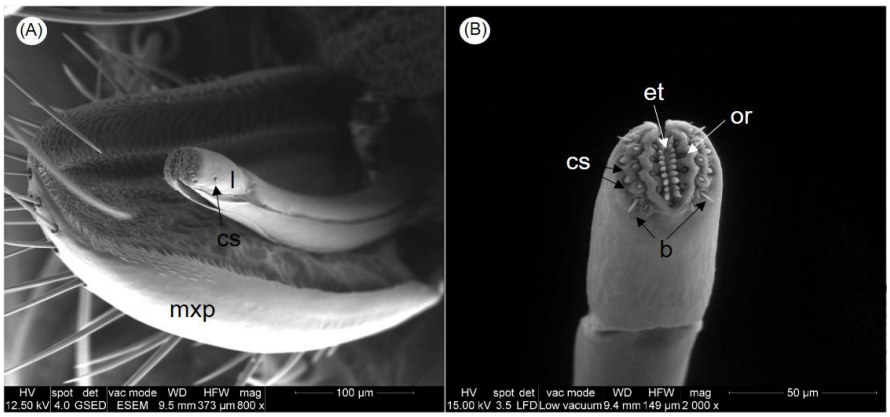


Figure 3.6. Feeding apparatus of *H. equina*. (A) Maxillary palp embracing the proboscis; (B) proboscis tip with characteristic sensory pattern. Note the four basiconic sensilla located at the corners and several coeloconic sensilla bordering the oral rim. l, labellum; cs, coeloconic sensillum; mxp, maxillary palp; et, eversible teeth; or, oral rim; b, basiconic sensillum.

*Pseudolynchia canariensis*, on the contrary, displays two circular crown rows of sensilla; the external row has long basiconic sensilla of different sizes and the inner row is composed of coeloconic sensilla. The internal part of the labellar tip shows differently shaped teeth; some are furcate and others two-segmented or acuminated (Figure 3.7). Finally, the presence of two typical rows of sharp teeth located along the labellar edges is a common characteristic observed in the species evaluated.

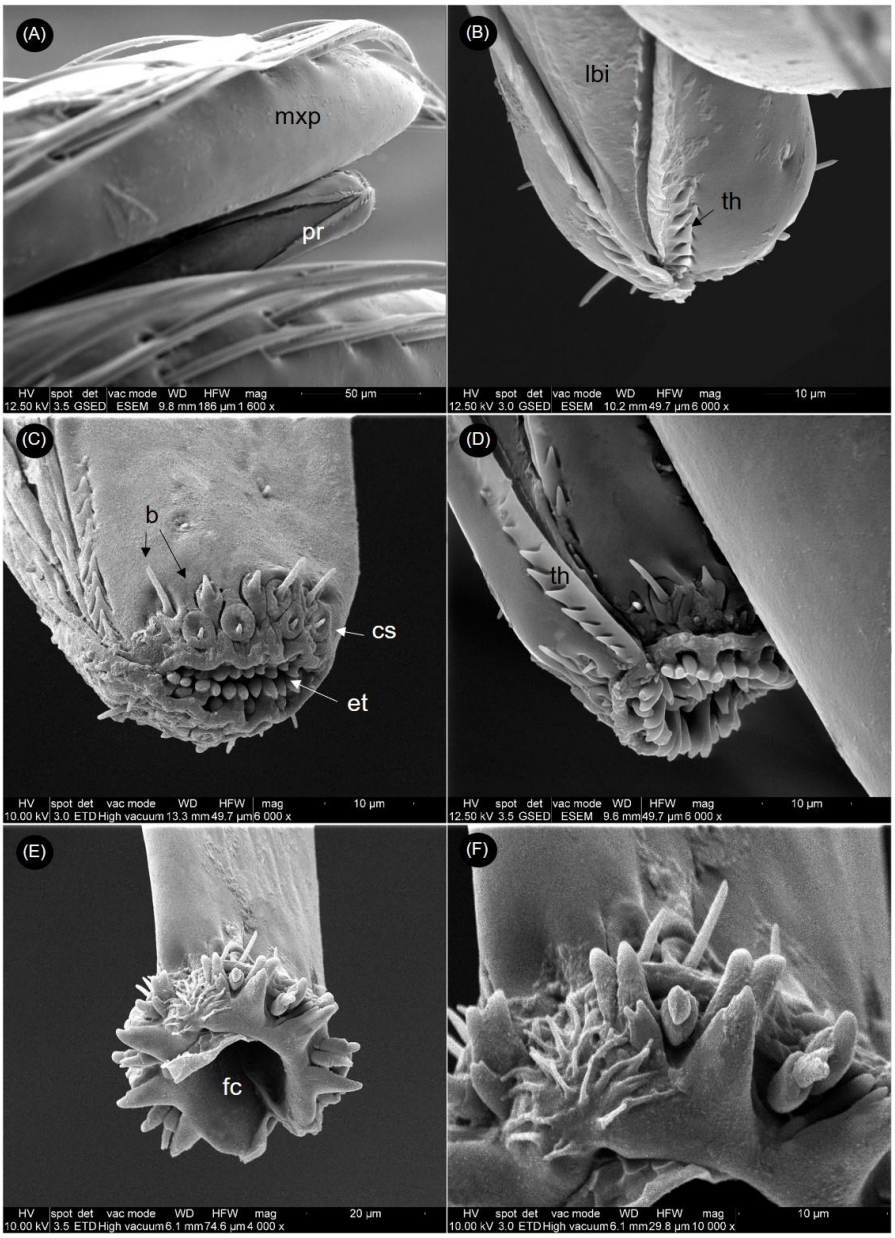


Figure 3.7. Feeding apparatus of *P. canariensis*. (A) Ventral view of the apical part of the mouthpart; (B) Magnification of the labellar tip; (C) Sensillar and tooth arrangement on the proboscis tip; (D) Ventral view of the labellum with the feeding canal slightly open. Note the rows of teeth useful to scrape the host skin; (E) Open proboscis showing the food canal; (F) Magnification of the sensillar pattern. mxp, maxillary palp; pr, proboscis; lbi, labium; th, teeth; b, basiconic sensillum; cs, coeloconic sensillum; et, eversible teeth; fc, food canal.

*Wings*. There are clearly noticeable morphological differences among the wings of the evaluated species (Figure 3.8). A general reduction and a fusion of some structures are observable features compared with other Muscomorpha. The wings of *L. fortisetosa* and *L. cervi* have a similar conformation and are characterized by a well-developed membranous surface with a scarcely sclerotized costa. Some veins disappeared, except for radial 1 (R1) and radial 4 that are fused with radial 5 (R4+5) veins, which form just two cells. The wing lacks the radial 2 fused with radial 3 (R2+3), as well as the posterior branches 1 and 2 of the media veins (M1+2). In particular, the costa ends together with the fusion of the radial 4 and 5 veins (R4+5) forming a pointed tip. The basal portion of the costa has some spines, one of which is long (Figure 3.8 A).

The anterior part of the wing in *H. equina* has remarkable veins and a well-developed costa resulting in a less membranous area than the corresponding section of *Lipoptena* wings. The costa is robust and covered with strong hairs along its length; moreover, its proximal portion has numerous short spines. Some structures disappeared, but subcostal veins, radial 1 (R1) and radial 2 fused with radial 3 (R2+3), and radial 4 fused with radial 5 (R4+5) are present and form the respective cells. In addition, branch 1 of the anal vein (A1), the anterior branch of the cubital vein (CuA1), and the posterior branches 1 and 2 of the media veins (M1+2) are slightly visible and do not join the distal margin of the wing. The posterior part of the wing is membranous and wrinkled. The alula is well developed and clearly recognizable (Figure 3.8 B).

The wings of *P. canariensis* are more membranous compared with those of *H. equina* and better equipped with veins compared with those of *Lipoptena*. The subcostal and radial cells are slender and

flattened. Branch 1 of the anal veins (A1), the anterior branch of the cubital veins (CuA1), and the posterior branches 1 and 2 of the media veins (M1+2) are barely marked and almost reaching the wing margin. This species displays lower and upper calypteres, while the alula is almost fused with the anal lobe (Figure 3.8 C).

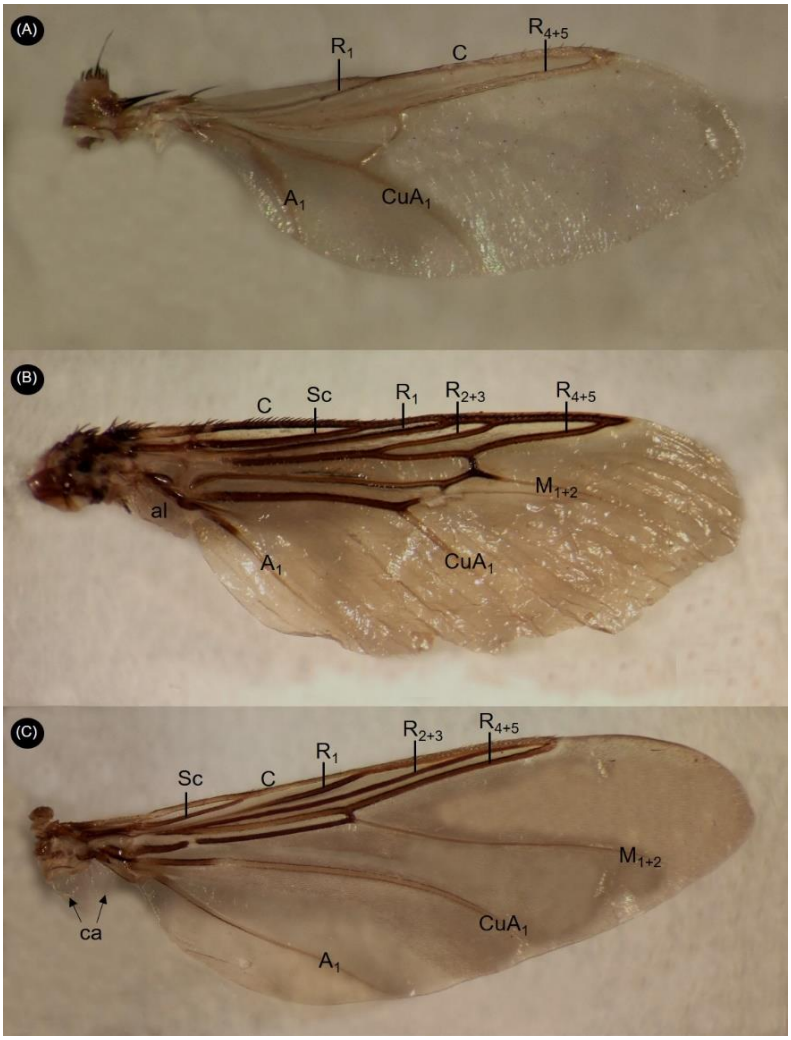


Figure 3.19. *Lipoptena fortisetosa* (A), *H. equina* (B), and *P. canariensis* (C) wings. Note the visible reduction of wing veins in *L. fortisetosa* and the heavily sclerotized veins in *H. equina*. C, costa; Sc, subcostal vein; R1, radial 1 vein; R2+3, radial 2 vein fused with radial 3 vein; R4+5, radial 4 vein fused with radial 5 vein; A1, branch 1 of anal vein; CuA1, anterior branch of cubitus vein; M1+2, posterior branches 1 and 2 of media veins; al, alula; ca, calyptra.

*Legs*. The legs are perfectly developed to guarantee a firm attachment to the host's fur. As the studied species infest different hosts, their legs are equipped with specific elements that allow them to achieve a firm grip.

*Lipoptena cervi*, *L. fortisetosa* and *H. equina* show the same adaptive features, probably owing to the similarity of their hosts' hairs. The acropod (pretarsus) is stout and armed with asymmetric and grooved claws that allow flies to clasp the host. In addition to these structures, the parasites are equipped with other adhesion organs, such as pulvilli and the empodium. In these species, one of the two pulvilli is pad-like and more developed than the other that is reduced (Figure 3.9 A and B). The empodium is elongated and presents little spurs that are useful in grasping the host's hairs (Figure 3.9 A–D).

The claws of *P. canariensis* are less grooved, symmetric and consist of three hooks per side; moreover, the two pulvilli are similar in size and shape and the empodium is hairier than in the other species (Figure 3.9 E and F).

In all the studied hippoboscids, the tarsi are covered with setae and bristles that probably serve as mechanoreceptors (Figure 3.9 A–C).

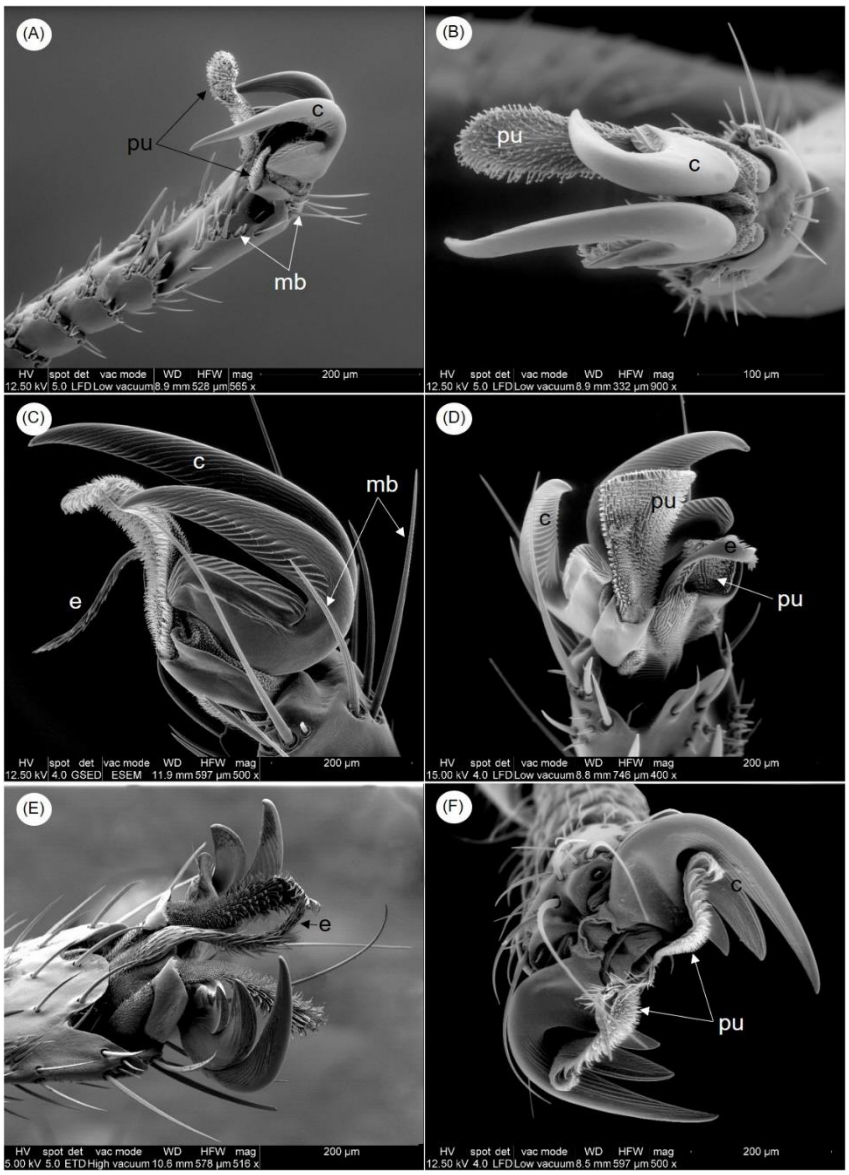


Figure 3.9. *Lipoptena fortisetosa* (A and B), *H. equina* (C and D), and *P. canariensis* (E and F) acropods, equipped with adhesion organs. Note the asymmetrical claws and pulvilla present in *L. fortisetosa* and *H. equina* and the three, differently sized hooks in *P. canariensis*. c, claw; pu, pulvillum; e, empodium; mb, mechanosensory bristle.

*Terminalia*. Terminalia are stable features, which are typically evolved in each observed hippoboscid species. Although hippoboscids generally have a similar genital structure, the differences among the species are represented by the shape of the aedeagus in

males and by the genital opening conformation and number of bristles in females. In addition, all of the studied parasites have reduced cerci bearing several bristles along the lower rim.

In all of the four studied species, the male terminalia are composed by a differentially shaped and sized aedeagus, two slender gonopods that guide it and two bristled surstyli at the base. In *L. cervi* the aedeagus is cone-shaped with an apical ridge at the tip and a membranous area in the ventral part; the surstyli display differently sized bristles, many of which are strong and very long (Figure 3.10 A). *Lipoptena fortisetosa* is equipped with a bifurcate aedeagus with a dentate curved rim in the distal portion and, similarly to *L. cervi*, displays a marked ventral membranous area (Figure 3.10 C and D); the surstyli are reduced and a sclerotized area bearing a bristled basal edge is identifiable (detail not shown). *Hippobosca equina* has a bilobate aedeagus with a smooth curved rim in the distal portion; additionally, its inner surface is covered by tiny spines that probably facilitate the adherence to the female terminalia. This species has two well-developed and strongly bristled surstyli and a bean-like pregenital plate with an underlying circular sclerotized area bearing several differentially sized bristles (Figure 3.10 B). *Pseudolynchia canariensis* instead, is equipped with a slender aedeagus, ending in an axe-shaped tip, and with two bristled surstyli. The aedeagus has a marked genital opening located in the dorsal part close to the apex (Figure 3.10 E and F). These four species have some convergent genital features. Firstly, in all of them the gonopods are thin, blunted at the tip and covered lengthwise with cuticular depressions, most of which seem to be coeloconic sensilla (Figure 3.10). The terminalia of hippoboscid males have a high number of setae, many of which are

well-developed and long with a presumable mechanosensitive role during mating.

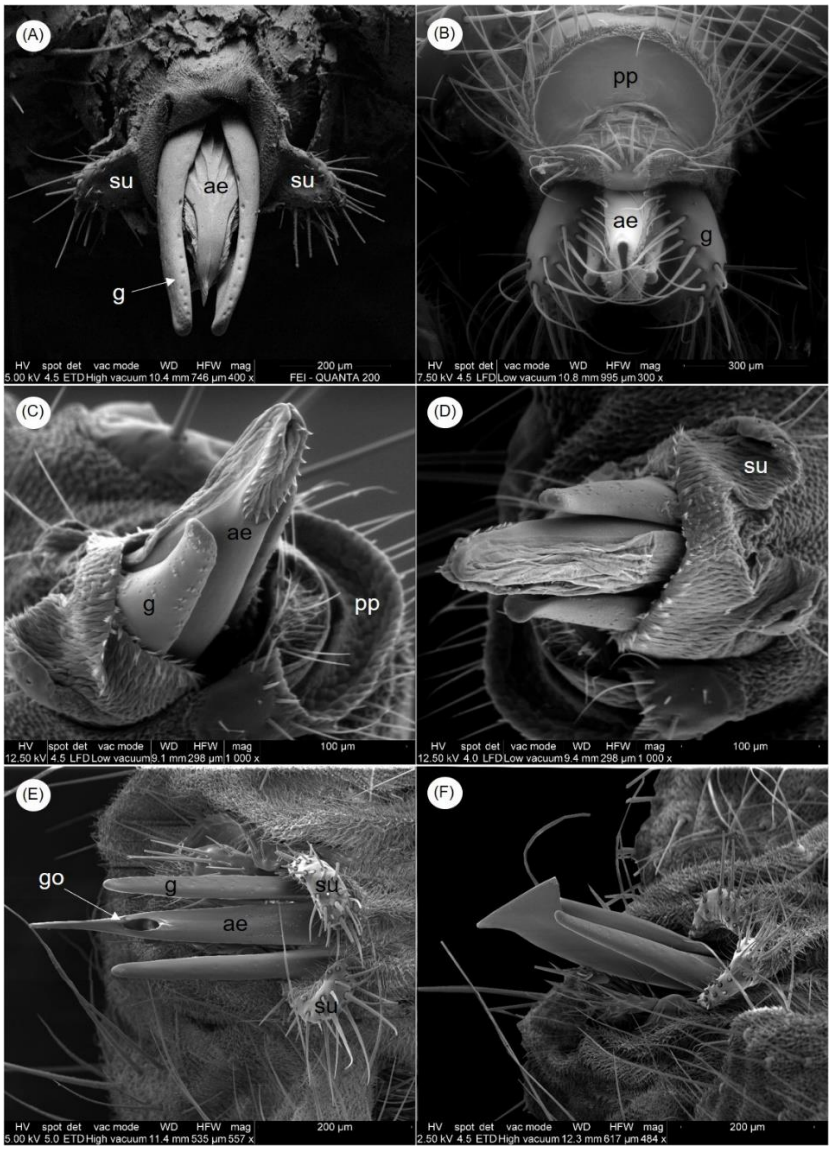


Figure 3.10. Male terminalia of *L. cervi* (A), dorsal view; *H. equina* (B), ventral view; *L. fortisetosa* (C and D), ventro-lateral view and dorsal view, respectively; P. *canariensis* (E and F), ventral and lateral view, respectively. Note the membranous area in the ventral part of the aedeagus in *L. fortisetosa* and a typical presence of coeloconic sensilla along the surstyli in the four species. P. *canariensis* displays a large genital opening and an axe-shaped aedeagus tip. ae, aedeagus; su, surstylus; g, gonopod; pp., pregenital plate; go, genital opening.

Female terminalia consist of a differentially shaped genital plate, named hypoproct, which is placed between a pregenital plate (ventral) and two fused flap-like cerci (dorsal); in addition, these species show a rudimental pregenital sternite located above the pregenital plate. In *L. cervi* this latter structure is slightly bilobate, while the hypoptroct is semicircular; the pregenital sternite consists of three segments each bearing two or three bristles (Figure 3.11 A). The main differences between the terminalia of this parasite and those of *L. fortisetosa* are detected in a differentially shaped pregenital plate; in *L. fortisetosa*, this plate consists of two sclerotized urotergites and a single pregenital sternite with two long external bristles and a smaller one in the middle (Figure 3.11 B). In *H. equina*, the hypoproct is strongly bilobate at the top and has several setae arranged along its lower edge, while the pregenital plate is slightly curved. In this species, it is possible to identify two well-developed circular surstyli with numerous long bristles in the distal part (Figure 3.11 C). *Pseudolynchia canariensis* differs from the other species in terms of its hypoproct that is drop-like, the pregenital plate that consists of two distinct areas fused externally with the abdomen but separated from each other in a central line, and the pregenital sclerite that is triangle-shaped and bare. It is interesting to notice that *P. canariensis* shows a marked genital opening located in the internal part of the hypoproct (Figure 3.11 D), compared with the other species.

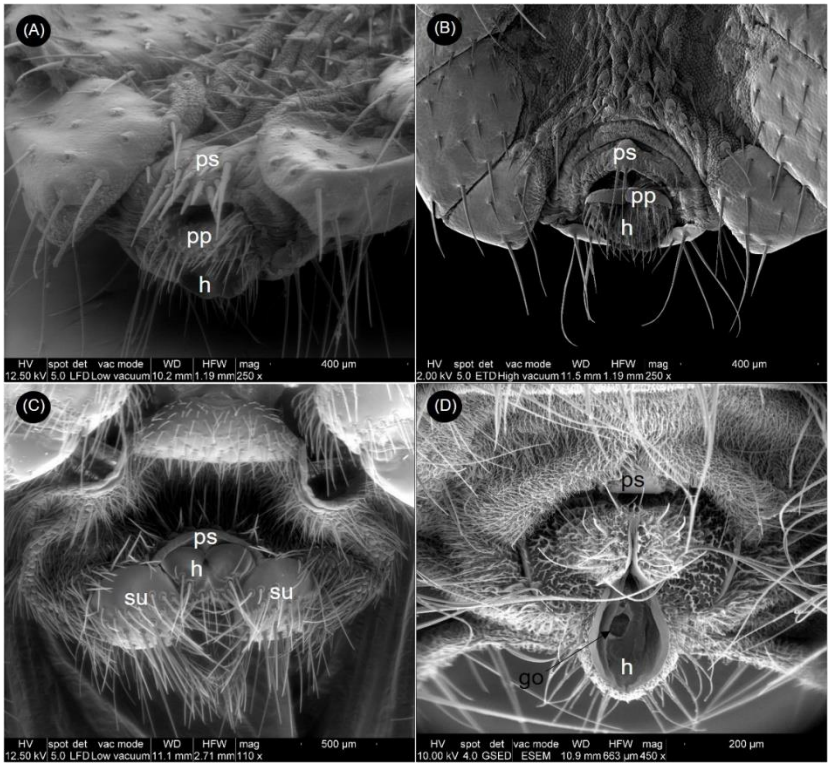


Figure 3.11. Female terminalia of *L. cervi* (A); *L. fortisetosa* (B); *H. equina* (C); *P. canariensis* (D). In *P. canariensis* it is possible to observe the genital opening. ps, pregenital sternite; pp., pregenital plate; h, hypoproct; su, surstylus; go, genital opening.

## Host coat conformation

*Ungulate fur*. In all of the ungulate fur samples, we identified two predominant hair types, which were classified according to Woods *et al*. (2011) (Figure 3.12). Guard hairs are long straight fibres, stocky and varied in diameter (Figure 3.12 A and D). Each hair has a bulb-shaped root, starts out thin and gets gradually thicker, and becomes thinner at the tip. These hairs are different in color along their length; they are white at the basis and darker at the tip. Guard fibres are bigger in diameter than underhairs, although their width varies. Their surface pattern is an irregular mosaic of plates with different dimensions. The underhairs constitute a knotted mass lying at the base of the fur (Figure

3.12 B–D). These fibres are generally thin, very long, wavy, and white along their length. The underhairs have a distinct surface pattern consisting of a regular, coronal mosaic with raised margins between adjacent scales.

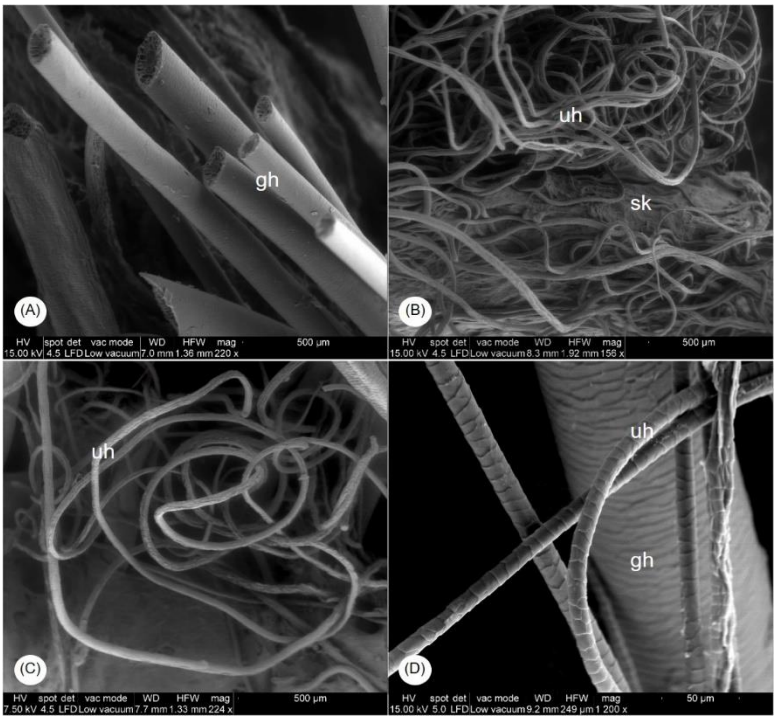


Figure 3.12. Different hair types present in *C. elaphus* fur. (A) Cut guard hairs; (B and C) Underhairs; (D) High magnification of guard hairs and underhairs. Note the pattern surface of both hair types. gh, guard hairs; uh, underhairs; sk, skin.

The red deer has different hair arrangements in the neck and the groin regions (Figure 3.13). The neck is covered by packed underhairs that form a superficial dense layer on the skin. Among them, long and strong guard hairs stand out (Figure 3.13 A and B). In the groin, both underhairs and guard hairs are less numerous, with the former being even scarcer (Figure 3.13 C and D).

In the roe deer, guard hairs on the neck are shorter in length, but more abundant, forming a thicker fur than what is observed on the red

deer's neck. Conversely, the underhairs are sparser in this area (Figure 3.14 A). The groin has a lower density of both fibre types and the guard hairs are generally thinner (Figure 3.14 B). Typically, roe deer's fur is softer than that of the red deer, which is bristly. The neck's fur of fallow deer is thicker than the fur of the other two species in both of the observed body areas and the hairs covering the skin form a dense layer.

*Horse fur*. The horse coat consists of thick and short hairs, especially in the groin area, which is almost bare. In other body regions, such as the neck, the fur is longer but has rather soft fibres (Figure 3.15).

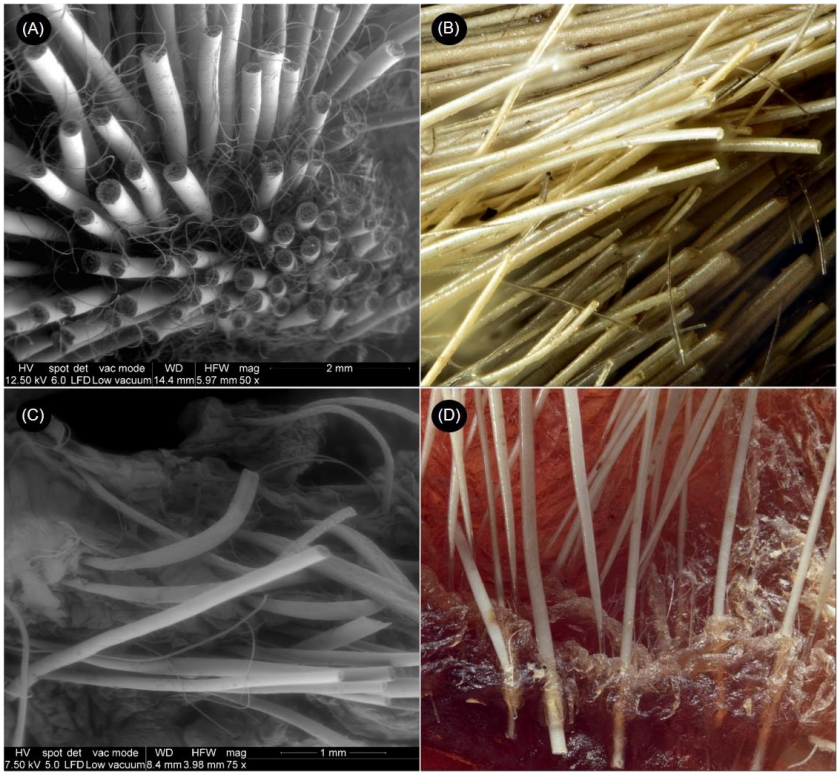


Figure 3.13. SEM and light microscope pictures of *C. elaphus* fur. (A and B) Neck hair arrangement; (C and D) Groin hair arrangement. Note the different underhair density in the two body regions.

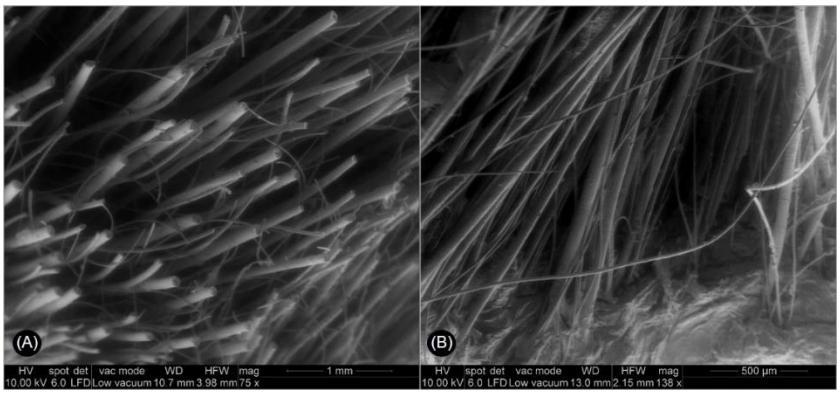


Figure 3.14. *C. capreolus* fur. Hair composition in the neck (A) and in the groin (B) areas.

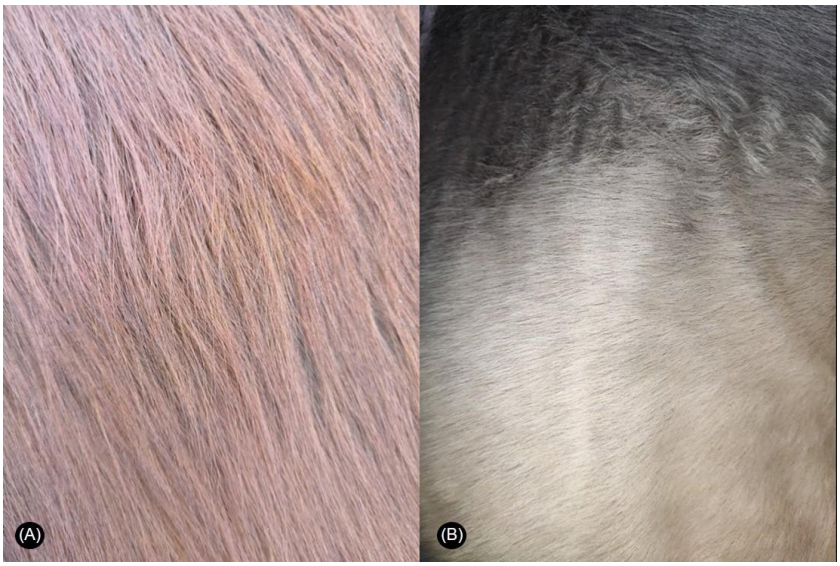


Figure 3.15. Horse fur. Hair composition in the neck (A) and in the groin (B) areas.

*Pigeon plumage*. The pigeon plumage consists of a variety of morphologically different pens (Figure 3.16). Near the hollow shaft there is a lower part of soft dowry barbs and after feathers (Figure 3.16 A). An inner surface of soft feathers is present under a coat of better-developed quills. A single pen consists of a thick rachis and a set of variably thin barbs, formed by several barbules composed by slim

hooklets (Figure 3.16 B–D). The pen layers are superimposed and form a tangled environment.

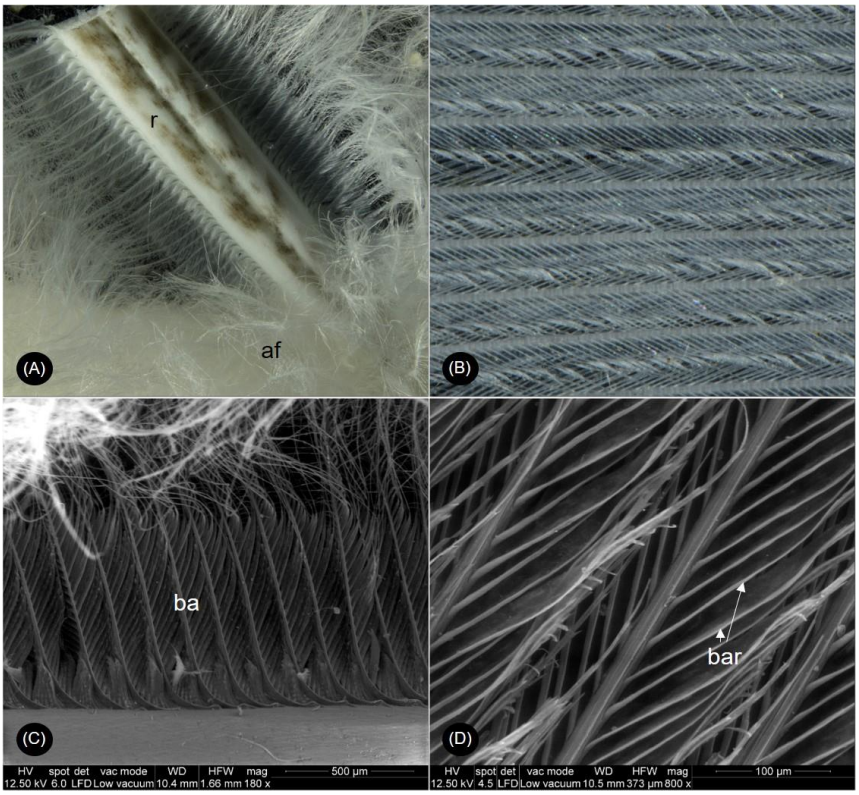


Figure 3.16. Light and SEM microscope pictures of pigeon feathers. r, rachis; af, after feather; ba, barb; bar, barbula.

## Discussion

The need to find and survive on a suitable host, able to satisfy a parasite's requirements, is an integral part of what it means to be a parasite. The adaptation level depends on the type of parasitism and on the relationship established between the parasite and the host species (Paakkonen, 2012). The studied hippoboscid flies are obligate ectoparasites and have a rather close association with few species,

which are considered as definitive hosts. Making a distinction between a permanent and an accidental host is crucial. The former is not only essential for the parasite's survival but it also provides refuge, protection and nourishment; the latter, on the other hand, only satisfies the trophic requirements of the parasite (Bequaert, 1942). The accidental host range accepted by parasites can be wide, while that of suitable definitive hosts is generally restricted (Bequaert, 1953). Parasite specialization permits flies to successfully exploit the victims, but at the same time, it limits them to specific conditions (Reeves & Lloyd, 2019). Several variables such as temperature, humidity and the environment influence the survival of a parasite; however, the host's presence and its characteristics are obviously the most important factors (Kaunisto, 2012; Paakkonen, 2012). Living at the expense of another species entails a combination of physiological, behavioural and morphological adaptations depending on the parasite's needs (Guerin *et al*., 2000). For example, some parasites have coevolved with their hosts by synchronizing their life cycles with those of the latter or are physiologically adapted to an extreme parasitic lifestyle. The most important evolved features include the host's location and the involved sensory apparatus, mouthpart structures to feed on, adapted legs and their adhesion organ and appropriate reproductive strategies (Guerin *et al*., 2000; Lehane, 2003).

The interactions between hosts and ectoparasites lead to the development of similar morphological and behavioural traits in insects belonging to the same family; however, it is also possible for taxonomically related insects to evolve a few different features depending on their life cycles (Guerin *et al*., 2000). Hippoboscid species of the genus *Lipoptena* have a so-called direct life cycle; this means that they reside permanently on a single definitive host. These

flies need to live in a particularly close association with their hosts and must be able to settle quickly on a suitable victim (Hutson, 1984). Members of the *Hippobosca* and *Pseudolynchia* genera, instead, do not completely depend on a single subject, but they can change hosts frequently. The reasons why these parasites evolved their wings differently in the first place, could be related to their different behaviour and life cycles. Although *L. cervi* and *L. fortisetosa* have a direct life cycle, they are not good flyers and are not able to cover long distances by flying probably due to the scarce numbers of veins in these structures as compared to other species (Bequaert, 1953). It is well known, that the more wings are equipped with veins the better their resistance is during flight (Wootton, 1990; Gullan & Cranston, 1994). A typical characteristic of the *Lipoptena* genus is wing loss when the parasites land on a suitable host; this is a result of their passage among the hairs of the ungulate fur, with a specific horizontal breaking line along which the gradual wing detachment occurs (Haarløv, 1964). These species live in an unfavourable environment consisting of a high density of strong and long guard hairs and dense underhairs that facilitate the parasites' attachment to their hosts while simultaneously obstructing their mobility. Becoming wingless facilitates the movement of ked flies through the host's coat and increases the probability of finding skin substrate for feeding as well as a partner for reproduction (Haarløv, 1964). *Hippobosca equina* and *P. canariensis*, on the other hand, have well-developed wings. This is presumably due to their life cycle as they often need to fly in order to switch hosts. Moreover, the environment in which they live is less harsh; horses and cattle have shorter hairs and less dense fur, especially in the groin region that is usually infested by parasites, while birds have a soft plumage composed of different pen and feather types. The number of

ectoparasites infesting an individual may be one of the explanations why some species may switch hosts during their life cycles. For example, hundreds of *Lipoptena* individuals can infest a host simultaneously (Andreani *et al*., 2019), while only few *P. canariensis* and *H. equina* individuals have been observed on a single host. Therefore, these two species need to switch hosts frequently in order to encounter partners and facilitate a wider genetic exchange.

In addition to a different wing evolution, Hippoboscidae have peculiarly adapted legs and accompanying adhesion organs. These structures are designed to provide a strong adherence of the parasite to the hairs or pens of the host's coat; thus, they play a significant role in the survival of haematophagous ectoparasites, especially members of the *Lipoptena* genus, which can have many problems if they accidentally lose contact with the host (Haarløv, 1964). At a glance, the legs of the four studied hippoboscids appear to be developed in a similar manner and have the same general gross structure. What differs among these species is their claw shape and the adhesion organ arrangement. *Lipoptena cervi*, *L. fortisetosa* and *H. equina* have similar pretarsal equipment compared with *P. canariensis*, which infests birds. The first three species have two well-developed, asymmetric and strongly grooved claws, while their adhesion organs consist of an asymmetrically developed pair of pulvilli and a spiny empodium. Surprisingly, the bigger claw is associated with the reduced pulvillum and vice versa. Considering the fur features of their host species, we can hypothesize that two kinds of differentially developed claws could be useful to efficiently hook the coat, which is predominantly formed by two types of hairs with different dimensions. Based on our laboratory observations during sampling, we assume that claws do not play a primary role in the attachment, as they do not clasp hairs laterally

(Figure 3.17 C and D). In fact, in *L. cervi* and *L. fortisetosa* the role of the adhesion organs is to firmly hold the hairs of the fur and keep the insect grasped to the host.

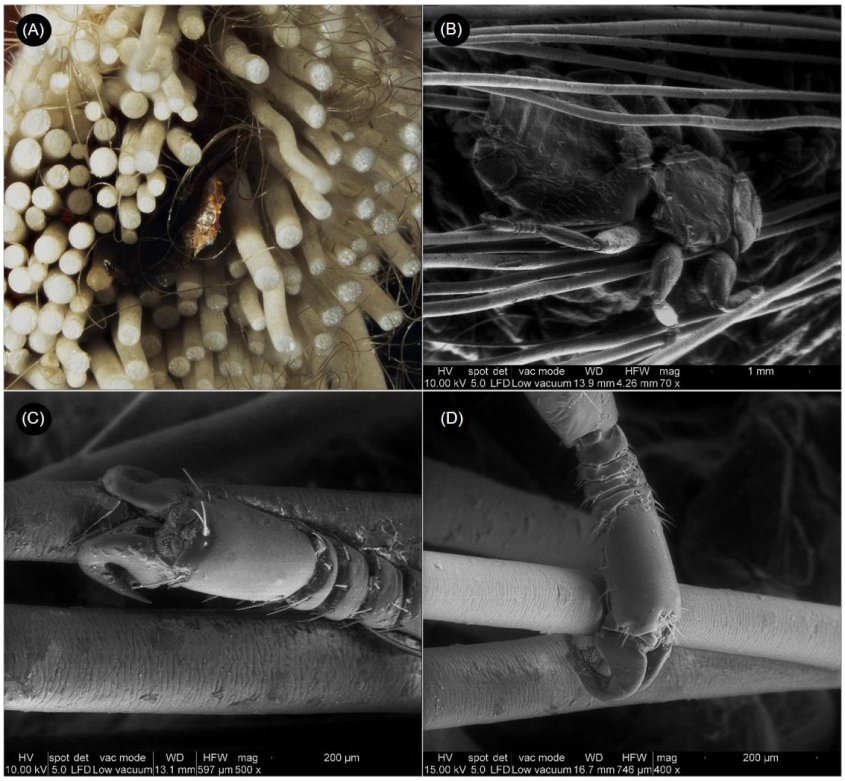


Figure 3.17. Light and SEM microscope pictures of *L. fortisetosa* inside the host fur (A and B); acropod organs allowing adhesion to the host's hair (C and D).

*Hippobosca equina* potentially uses these pretarsal appendages in a similar manner. On the contrary, bird plumage is completely different from ungulate or equid coats, therefore *P. canariensis* has a different leg equipment. First, their claws are symmetric and show three hooks with various dimensions. As pigeon feathers consist of three differently sized fibres (barbs, barbules and hooklets), we can speculate that these structures are useful for holding on to the different pen types, as was observed in the other hippoboscids. We do not have

actual evidence of which organ, the claws or the pulvilli, is used by *P. canariensis* to adhere to its hosts. However, in the louse fly *Craterina pallida* (Latreille 1812) pulvilli were proved to have an important role in adhesion to smooth surfaces in laboratory trials (Petersen *et al*., 2018). Moreover, in ornithophilic black flies, the claws of members of the *Nevermannia* subgenus are morphologically similar to those of *P. canariensis* and are considered useful for clamping one or two barbules of the feather therefore allowing an efficient adhesion to the bird's plumage (Sukhomlin *et al*., 2018).

The surface on which ectoparasites live may have affected the conformation of the external part of the antennae. As previously described, these structures are inserted in two deep antennal sockets located between the compound eyes and the lunula. In the *Lipoptena* and *Hippobosca* genera, these features originated from the complete fusion of the first two antennal segments, while *P. canariensis* shows a partial articulation between the scape and the pedicel. Another marked distinction among *P. canariensis* and the other three species is that in the former the antenna protrudes externally, while in the latter it is completely inserted into the hollow. This arrangement could potentially be attributed to the softer plumage of pigeons compared with the bristly hairs of large mammals' fur. Furthermore, long sensillar bristles are present in all the members of the Ornithomyinae subfamily, which are specialized in infesting birds (Maa & Peterson, 1987). Antennae are the most important olfactory organs and it is fundamental that ectoparasites protect them from the external environment (Zhang *et al*., 2015); otherwise, the passage through the strong hairs of the ungulate fur could affect these organs and damage their external sensory structures. The sensilla of *L. fortisetosa* and *L. cervi* do not exceed the bounds of the antennal sockets, owing to the

unsuitable environment these parasites live in, while those of *P. canariensis*, which live among the softer pens of birds, have numerous and very long sensory bristles. The antennae of *H. equina*, on the other hand, are completely enclosed in the cavity; however, several long setae are present on the external surface of the pedicel and along the frontoclypeal rim; therefore, this species combines antennal elements from the other species. In fact, horse, donkey or cattle coats are not as soft as those of birds; however, they are shorter and less thin than ungulate fur.

The scape of *P. canariensis* is partially fused with the lunula and articulated with the pedicel; this structure is different from those of the other observed species in which the lunula is completely fused with the scape. Insect bodies are generally divided in several segments, but different groups have different division levels; species with greater segmentation are more ancestral than species with fused body elements (Zrzavy & Stys, 1997). Similarly, we can confirm that *L. cervi*, *L. fortisetosa* and *H. equina* show a higher adaptation level in terms of their antennae. This characteristic could be attributed to the unpleasant environment in which these species live that could have exerted major evolutionary pressure on them compared with the environment of *P. canariensis*.

Regarding the external sensillar pattern observed in these species, we can confirm that there are strong differences both within the same genus (i. e. *Lipoptena*) and among the different species. *Lipoptena cervi* and *L. fortisetosa* display a variable number of sensory bristles that are arranged variably on the pedicel surface. In many phytophagous and haematophagous dipterans, sensory bristles on the pedicel edge are supposed to have a tactile function (Schneider, 1964; Zacharuk, 1985; Seenivasagan *et al*., 2009). In hippoboscid flies,

the pedicel is the exposed part of the antennae, but the main sensory area is located on the flagellum, which is inside the pedicel (Zhang *et al*., 2015). Nevertheless, we could suggest that the presence of different sensory bristles in *L. fortisetosa*, *P. canariensis* and *H. equina*, may also play an important role while they move through the host's coat, especially for feeding and mating purposes. In addition to having fewer mechanotactile bristles than the other species, *L. cervi* has several coeloconic sensilla on the pedicel rim, which could have a specific function on the host's location the parasites were positioned at. Although we do not have any supporting data or electrophysiological evidence on the fine structure of these receptors, coeloconic sensilla have been shown to have chemo-, thermo- and hygro-receptive functions in insects (Altner & Loftus, 1985; Zacharuk, 1985; Zacharuk, 2003); therefore, we can state that *L. cervi* is more equipped for environmental monitoring compared with the other hippoboscids. Moreover, we have to highlight that the sensory bristles present on the *P. canariensis* pedicel are quite numerous and very long compared with those of the other three species. The presence of such long bristles could be attributed to the lifestyle of this species that moves easier throughout the pigeon plumage than the other three species do throughout the mammalian fur. It is noteworthy that *H. equina* has three very long, protruding and straight bristles, which differ morphologically from those of *P. canariensis*; this could probably be attributed to the short and less dense hair coat of their hosts. This kind of sensillar pattern suggests an external sensory perception of the environment, rather than a proprioceptive function. Not only was the sensillar pattern different among the four parasites, but the aristae were also differently shaped in all the species. Finally, further research, especially on the habitat and location on the host, is required in order

to understand the different antennal sensillar arrangement between the two *Lipoptena* species, which parasitize the same ungulates.

One of the few convergent features, that is common in all of the studied species, is the feeding apparatus. In fact, the mouthpart conformation is similar in all the described species and consists of an alimentary canal formed by the union of the labrum and the labium with the internal hypopharynx. The proboscis has a very equipped tip bearing both basiconic and coeloconic sensilla, which are likely specialized in assessing the host's skin thanks to their chemoreceptive functions, especially the gustative ones. Furthermore, we also have to take into consideration the prestomal teeth present along the labellar rim that play a primary role in scratching the skin and allowing the blood to spill. These structures are similar to those observed in the haematophagous muscid fly *Haematobosca stimulans* Meigen (Giangaspero *et al*., 1996) and more evidently, in members of the Glossinidae family (Gibson *et al*., 2017), which belong to the same Hippoboscoidea superfamily (McAlpine, 1989).

Although the thickness of the skin tissue differs among the host species, the conformation of the parasites' mouthparts is similar. This could probably be attributed to the feeding mechanism employed by these species, which are defined as pool feeders, as previously stated. Flies use the sensory structures present on the tip to locate the most appropriate feeding point; when they find it, the parasites presumably align the proboscis horizontally and scrape the host's skin with the teeth of the labellar rows. Then, they may slightly insert the labella in the injured area, which is assisted by the eversible teeth. These structures could adhere to the host's skin and raise the laceration margins, thereby allowing the food canal to intercept the blood, as is suggested in Figure 3.7 D and E.

Hippoboscids feed on the blood haemorrhage caused by teeth placed on different rows, so they do not need to insert their proboscis deep into the host's skin. It follows that the morphology of the host's pelt has probably not affected the evolution of the feeding apparatus of these ectoparasites, which share many similar features with haematophagous tsetse flies (Snodgrass, 1943; Gibson *et al*., 2017).

The aedeagus in the terminalia of *Lipoptena* species and *H. equina* consists partly of membranous and sclerotized areas; this is very similar to the terminalia arrangement of the Nearctic species *Hippobosca longipennis* (Maa & Peterson, 1987). This well-developed membranous surface of the aedeagus could play a mechanic role and/or acts as a temporary storage area for spermatic fluid during mating. On the contrary, the aedeagus of *P. canariensis* is completely sclerotized with a very remarkable genital opening on the ventral part, which is clearly visible in the female hypoproct. These openings presumably correspond during mating and allow the passage of spermatozoa and male secretions.

The morphological investigations on the four studied hippoboscid species suggest that the development or the regression of important insect body structures have allowed or induced adaptive strategies. Concerning the external part of the antennae, we highlighted marked differences among these taxa that are mainly due to their parasitic lifestyles, such as the reduction in the number and size of pedicellar sensilla in *Lipoptena* species and the development of long mechanosensory bristles in *H. equina* and *P. canariensis*. Of course, more studies are required to examine the third antennal segment (flagellum), which is the main sensory area, as well as the role played by its different chemoreceptors. To achieve this goal, behavioural and electrophysiological trials are necessary in order to

understand how these flies locate their hosts and move in the environment. In terms of the mouthparts, we have to stress a noticeable adaptive convergence observed in all the studied species, which display a similar sensillar arrangement on the tip of the proboscis, in addition to the mouthparts forming the apparatus. The study of the characterization of these sensilla from a physiological point of view could clarify the feeding behaviour of these haematophagous flies and their possible role as vectors of pathogens to animals and humans. The morphological comparisons of the wings and legs showed that these structures have been highly influenced by the adaptive selection pressure that occurred during the evolutionary process. Thus, our observations raise some important questions about the biology, behaviour and evolution of the Hippoboscidae family. Genetic studies coupled with physiological and ultrastructural investigations could provide a deeper understanding of these poorly studied ectoparasitic dipterans.

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# 4. Colour preference of the deer ked *Lipoptena fortisetosa* (Diptera: Hippoboscidae)

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## Simple Summary

Insects use visual stimuli to find habitats, food, or a mate while moving around. This trait might be exploited to intercept flying insects to monitor their populations and reduce their presence. Among the various visual stimuli, colours are commonly used to attract insects. *Lipoptena fortisetosa* is a hematophagous deer ectoparasite native to Japan that has spread to several central European countries and was recently recorded in Italy. Measures to monitor and control *L. fortisetosa* would be helpful given its potential threat as a pathogen vector for animals and humans. The objective of this research was to assess the potential use of colour to attract and trap *L. fortisetosa*. The response of the winged adults was evaluated through an experimental trial carried out in a wooded area of Tuscany using differently coloured sticky panels as traps. Blue panels attracted the highest number while yellow panels showed the lowest performance. This preference for blue could be useful in the design of traps to reduce the population of this parasitic fly which, at certain times, can reach a very high density, causing annoyance to wildlife and humans visiting natural areas.

## Abstract

*Lipoptena fortisetosa*, a deer ked native to Japan, has established itself in several European countries and was recently recorded in Italy. This hippoboscid ectoparasite can develop high density populations, causing annoyance to animals and concern regarding the potential risk of trans-mitting pathogens to humans. No monitoring or control methods for *L. fortisetosa* have been applied or tested up to now. This research evaluated the possible response of *L. fortisetosa* winged adults to different colours as the basis for a monitoring and control strategy. In the summer of 2020, a series of six differently coloured sticky panels were randomly set as traps in a wooded area used by deer for resting. The results indicated a clear preference of the deer ked for the blue panels that caught the highest number of flies during the experimental period. Lower numbers of flies were trapped on the red, green, black, and white panels, with the yellow panels recording the fewest captures. The response clearly demonstrates that this species displays a colour preference, and that coloured traps might be useful for monitoring and limiting this biting ectoparasite in natural areas harbouring wildlife and visited by people.

## Introduction

*Lipoptena fortisetosa* is a small blood-sucking ectoparasite whose primary hosts are various species of ruminant artiodactyl mammals, especially cervids and bovids [1], although it is also known to bite humans. Phylogenetically, it belongs to the subfamily Lipopteninae, but unlike other hippoboscids, it is not able to frequently switch host and attaches itself to a single animal for life. Newly emerged *Lipoptena* flies are fully winged adults that immediately search for a suitable host.

Once found, the fly settles down to live in the host's fur and gradually loses its wings, which separate at a predetermined break line on the proximal part of the wing [2–4].

*Lipoptena fortisetosa* is native to Japan but has spread to many European countries [5], including Italy, where it was recorded for the first time in 2019 in wooded areas in Tuscany [4]. *Lipoptena fortisetosa* is an obligate, permanent ectoparasite which, apart from its original host, sika deer, thrives on a limited range of closely related mammalian species, especially cervids [6–9]. Before settling on the so called "definitive host", this fly may occasionally bite other species for food, including humans [10,11].

In general, parasites are either dependent on a single species or have adapted to a wider range of hosts. The level of host selectivity varies greatly among parasites and affects the degree of specificity of the host choice process, that in some cases needs to be highly precise since any host may not provide all the requirements necessary for a parasite's survival. The closer the parasite's association with a few species, the deeper its level of adaptation; consequently, exploiting other species becomes more difficult [12]. The host location is determined by many factors that may act in concert, such as insect morphology, physiology, behaviour, ecology, genetics, and circadian cycle. These are distinct, evolved responses that represent adaptations to specific biotic and abiotic constraints [12,13]. This process is affected by many issues, including habitat, movement, odour, and moisture, and is crucial for ensuring the parasite's survival [14]. For blood-sucking insects, the search for a host can be divided into three phases that are not strictly consecutive: a) appetitive searching; b) activation and orientation; and c) attraction [12,14]. In general, the location of the host involves a variety of chemical, physical, and visual

signals, such as specific odours emitted by animals, carbon dioxide, movements, and the shape and colour of the host [13]. Usually, visual and olfactory stimuli act over a long distance, while humidity and heat are more significant at closer range [12].

In the superfamily Hippoboscoidea, some species belonging to Nycteriibidae and Streblidae were studied in terms of host location [15,16], but a substantial amount of information is available especially on other Hippoboscoidea, such as tsetse flies (Glossinidae) due to their great economic, medical, and veterinary importance [17,18]. Host finding by Glossinidae was found to entail two kinds of behavioural responses: long-range olfactory responses and short-range responses, determined by olfactory and visual factors [13]. Visual stimuli are of primary importance at short distances: past experiments on tsetse flies demonstrated that, in a series of tested colours, phthalogen blue traps had a significantly higher capture rate than those of other colours, such as yellow [19].

Host location behaviour has been poorly investigated in members of the Hippoboscidae family; in fact, except for earlier observations reported by Bequaert [20], the only experimental research has been carried out in Finland, where the preferences of *L. cervi* for host body parts, colour, and temperature were investigated using people as dummies. Among other findings, the winged adults displayed a clear attraction toward people wearing dark and red clothing [21].

*Lipoptena fortisetosa* has never been studied for its host location process although it is currently receiving renewed attention, especially given its medical and veterinary importance. In fact, as ascertained in other hippoboscid species, *L. fortisetosa* may be a potential vector of pathogens that are harmful for animals and humans [22–26]. Since this parasite lives on just a few host species, completes its life cycle while

dwelling permanently on a single subject, and is not able to frequently switch victims, we believe it has developed efficient mechanisms to locate a host after emergence.

Investigations into two hippoboscid flies, *Hippobosca equina* and *L. cervi*, demonstrated that visual signals are involved in host location and that mainly colour stimuli are used [21,27]. Thus, evaluating the colour preferences of *L. fortisetosa* might be useful for disclosing behavioural traits of this allochthonous ectoparasite which is spreading through Europe, causing concern for its medical and veterinary importance. Moreover, a possible response to colours could be exploited to define monitoring and control strategies. In fact, coloured traps coated with odourless glue are frequently used to sample different blood-feeding insects since they are inexpensive and easy to assemble [28].

The objective of this paper is to provide a fresh account on the response of *L. fortisetosa* to visual stimuli through an experiment carried out in a wooded area of Tuscany using differently coloured sticky panels as traps.

## Materials and methods

The field trial was conducted in a wooded area in Schignano (Prato, Tuscany, central Italy) at about 550 m a.s.l. (43.967432 N; 11.101761 E), where many warnings about the abundance of ked flies have been reported by people visiting this area. The study area consisted of a sloped clearing enclosed on three sides by a forest of mainly oak, holm oak, and chestnut that is frequently used by deer as a passageway or rest area.

## Experimental Design

To evaluate the possible response of ked flies to colour, three series of differently coloured sticky traps were arranged in three different locations within the experimental area (Figure 4.1). The first series bordered the forest to the southwest of the clearing and had an east–west orientation (trap sides with exposure north–south); the second was placed inside the woods and was oriented north–south (trap sides with east–west exposure); and the third series was placed to the north of the glade with the same orientation and trap exposure as the second series.


Figure 4.1. Schignano (43.967432 N; 11.101761 E) (Prato, Italy), 2020. Experimental area with the three series of chromotropic sticky traps arranged for the field trial.

Each series consisted of three repetitions of six solid colours (black, transparent, blue, green, yellow, and red) in a randomized sequence for a total of 54 traps (18 traps per series) (Figure 4.2).


Figure 4.2. Schignano (Prato, Italy), 2020. Sticky traps of Series 1, formed by three repetitions of six differently coloured plastic panels in a randomized sequence.

The colours were chosen on the basis of studies conducted on tsetse flies and other hematophagous insects [18,21,29–31]. The transparent colour was used as a control. Coloured traps consisted of plastic alveolar polypropylene "plastonda" panels while transparent traps were made of Poliver (artificial glass polystyrene); all of them measured 20 cm × 30 cm × 2.5 mm (width, height, and thickness, respectively) and were purchased at the home improvement retailer OBI Italia. The spectral reflectance of these panels in the visible and UV regions, between 250 and 800 nm, was measured using a PerkinElmer Lambda 1050 spectrophotometer coupled with a specific accessory

for re-flectance measurements (150 mm InGaAs Integrating Sphere) (Perkin Elmer Inc., Waltham, MA, USA). The black and transparent traps showed nearly constant reflectance over the wavelength range from 250 to 800 nm. The black colour exhibited a mean reflectance of about 6%, while for the transparent sample it was 17%. The blue trap displayed a maximum reflectance of 420–470 nm (~60% of reflectance) while the green displayed 500–550 nm (~20% of reflectance). The yellow panel showed a maximum reflectance of 520–550 nm (~75% of reflectance), and the red, 620–650 nm (~55% of reflectance) (Figure 4.3).

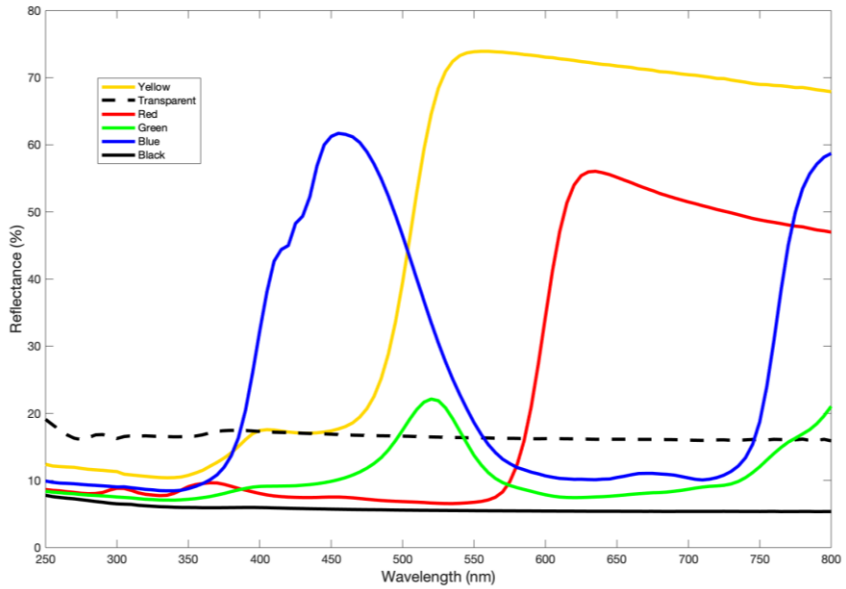


Figure 4.3. Reflectance spectra of the black (black line), transparent (dotted line), blue (blue line), green (green line), yellow (yellow line), and red (red line) sticky traps exposed to *Lipoptena fortisetosa* winged adults in Schignano (Prato, Italy) between July and October 2020.

Both sides of the panels were coated with a glue applied by brush (Planatol VP 1854 PSA, Ivog biotechnical systems GmbH, Neüsaß,

Germany). In each series, traps were arranged 1.5 m above the ground and hung from two cords in a stationary position; the 18 panels within each series were placed close to each other. To avoid any interference from the juxtaposition of colours, the order was changed every two weeks according to a randomized experimental design. The traps were set on 6 July 2020 and checked weekly: flies caught on each side were counted separately for every panel and then removed to estimate their response to colours. The traps remained continuously exposed until 29 October 2020, and this time period was chosen based on *L. fortisetosa* adults in Europe being reported as present from June to October [5]. On the same day as the trap control, sweeping paths were performed in different environments near the experimental area (woodland, forest edge, open field, track).

### Statistical analyses

Analyses were carried out considering the mean number of flies caught by each trap per day as a dependent variable and the following as independent variables: the position of the series (position 1, position 2, and position 3), trap colour, sampling date, and trap activity (dichotomised as traps that captured *L. fortisetosa* or did not). From 1 October onwards, most of the traps were inactive and an extremely low number of flies (9) was caught. For this reason, only data from 15 July to 1 October were analysed.

To highlight potential differences in trap attractiveness, the data structure was checked through factor analysis of mixed data (FAMD), and then inferential statistics were applied.

The goal of the FAMD analysis was to explore the association between all the variables and highlight which factors determined the variability of the average number of flies caught by the traps. FAMD

was performed with the open-source software RStudio (RStudio Version 1.3.1093, 2009–2020, PBC, Boston, MA; http://www.rstudio.com/) using the packages FactoMineR and FactoExtra. All variables were considered active, and missing data were managed using the package missMDA [32,33].

After the FAMD observation, inferential analysis was carried out to highlight differences among active traps compared to inactive traps as well as the average number of caught flies.

The proportion of active with respect to inactive traps was analysed using a Chisquare test (6 × 3 contingency tables) for the null hypothesis that all proportions were equal. When necessary, multiple pairwise comparisons were applied to the number of active traps according to the variable series position (H0: p1 = p2; H0: p1 = p3; and H0: p2 = p3) and to the variable trap colour (H0: p1 = p2 = . . . = p6). The Type I error rate was adjusted, thereby reducing the maximum error rate of 0.05 by the total number of comparisons [34]. Chi-square tests were carried out using Excel software (Microsoft 365, 2016, Microsoft Italia, Milano, Italy).

Differences in the average number of *L. fortisetosa* captured on traps of different colours were analysed using univariate analysis of variance with the sampling dates from 15 July to 1 October and panel colours as factors, while the dependent variable was the average number of *L. fortisetosa* captured per day. Data were log(x + 1) transformed [34], and a pairwise comparison was performed using the Bonferroni test if a main effect was highlighted. All statistical analyses were performed using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp, Armonk, NY, USA) software.

## Results

### Overall evaluation of the experimental set on *Lipoptena fortisetosa* captures

The eigenvalue to explain the total variance of data was selected according to the criteria defined by Karlis *et al*., [35]. Dimension 1 (Dim. 1) satisfied the criterion (eigenvalue >1.31) and summarized most information; however, to interpret the FAMD results, the second dimension (Dim. 2) was also considered. Both dimensions explained 17.04% of the overall variability. Although it was less than 50%, the representation was valid for describing the variance distribution within the dataset. The results of the FAMD analysis are reported in Table 4.1.

Figure 4.4a shows that the two variables of number of captures and trap activity were linked to the first dimension. Total variability was mainly explained by the number of captured *L. fortisetosa* (Contr. = 33.93%), and these observations were positively correlated with the first dimension (Corr. = 85.70%). The variable trap activity also contributed to the construction of Dim. 1 (Contr. = 27.91%) and was positive correlated to active traps (Corr. = 1.39) as opposed to the inactive traps (Corr. = –1.05) (Figure 4.4b). Observations that positively correlated to the Dim. 1 may be characterized by a higher average number of captures per trap.

In addition, the variable position of a series was related to Dim. 1 (Figure 4.4a), with position 1 contributing to the first component structure (Contr. = 16.07%, Corr. = 1.52). Position 1 was opposed to position 3 as evidenced by its high contribution and significant positive ratio compared to Dim. 2 (Contr. = 15.65, Corr. = 0.73) (Figure 4.4b and Table 4.1).

Table 4.1. Contribution and correlation of the active variables and factors of the categorical variables for the first two principal dimensions of the FAMD.

| Variables and Factors | Dim. 1 | | Dim. 2 | |
|---|---|---|---|---|
| | Contribution | Correlation * | Contribution | Correlation * |
| **Flies caught/day** [a] | **33.93** | **0.86** | **4.02** | **0.19** |
| **Trap activity** [b] | **27.91** | **0.65** | **5.32** | **0.51** |
| Active traps | 12.75 | 1.39 | 2.43 | 0.24 |
| Inactive traps | 1.16 | −1.05 | 2.89 | −1.02 |
| **Series position** [b] | **25.07** | **0.54** | **23.48** | **0.25** |
| Position 1 | 16.07 | 1.51 | 3.68 | −0.35 |
| Position 2 | 1.72 | −0.49 | 4.15 | −0.37 |
| Position 3 | 7.28 | −1.01 | 15.65 | 0.73 |
| **Sampling date** [b] | **10.53** | **0.23** | **47.88** | **0.19** |
| 15 Jul | 0.30 | −0.39 | 9.89 | −1.10 |
| 22 Jul | 0.06 | | 9.42 | −1.08 |
| 27 Jul | 0.66 | −0.58 | 1.03 | 0.36 |
| 6 Aug | 0.66 | 0.41 | 1.09 | 0.37 |
| 12 Aug | 0.33 | 0.96 | 5.46 | 0.82 |
| 20 Aug | 4.09 | 1.45 | 15.33 | 1.37 |
| 26 Aug | 0.34 | 0.42 | 1.61 | −0.45 |
| 3 Sept | 0.59 | −0.55 | 3.55 | −0.66 |
| 9 Sept | 0.00 | | 0.00 | |
| 17 Sept | 0.60 | −0.56 | 0.23 | |
| 1 Oct | 1.79 | −0.96 | 0.28 | |
| **Trap colours** [b] | **2.56** | **0.06** | **19.30** | **0.20** |
| Black | | | 7.17 | −0.69 |
| Transparent | 0.19 | | 0.31 | |
| Blue | 1.50 | 0.65 | 5.17 | 0.59 |
| Green | 0.03 | | 0.50 | |
| Yellow | 0.81 | −0.48 | 2.12 | −0.38 |
| Red | 0.02 | | 4.03 | 0.52 |

* Correlation for the variables. Flies caught/day refers to the correlation coefficient, while for the other variables, it refers to the square of the correlation ratio. Correlation was reported when the value was significantly different from 0 (p = 0.05). a continuous variable; b categorical variable and factors.

The variable sampling date was less correlated with Dim. 1 than with Dim. 2 (Figure 4.4a); only three dates correlated with Dim. 1, with 12 and 20 August being positive (Corr. = 0.96 and 1.45, respectively), and 1 October being negatively correlated (Corr. = –0.96).

Regarding the variable colour, Dim. 1 and Dim. 2 contrasted blue traps with yellow ones (Figure 4.4b). The blue traps were correlated with both dimensions (Dim. 1: Corr. = 0.65, Dim. 2: Corr. = 0.59) while the yellow ones were negatively correlated (Dim. 1: Corr. = –0.48, Dim. 2: Corr. = –0.38).

The FAMD results emphasized that position 1 was more appropriate for insect monitoring since it explained most of the variability and was linked to active traps. Moreover, 12 and 20 August seemed to be the most favourable days for *L. fortisetosa* capture. The outcomes suggest that there were optimal capturing periods that should be taken into account. The traps with the most divergent results were the blue and the yellow ones, with the blue traps being the most active.

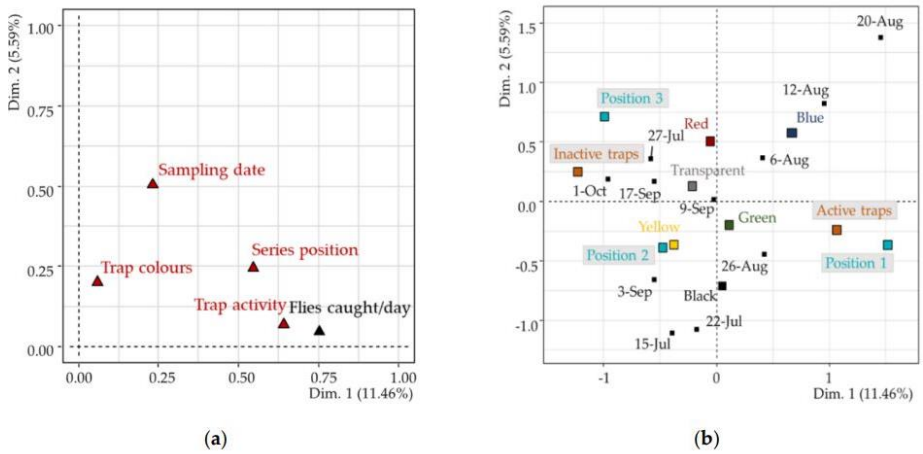


Figure 4.20. FAMD results: (a) Graph of the variables showing the correlation between both quantitative (black triangle) and qualitative variables (red triangles) to Dim. 1 and Dim. 2; (b) Graph of the categories: trap activity (active traps and inactive traps), series position (position 1, position 2, and position 3), sampling date (data from 15 July to 1 October), and trap colours (black, transparent, blue, green, yellow, and red). The point for each of the categories indicates the barycentre of the observations.

## Trap activity

A significantly different number of active traps was highlighted between the three positions of the series ($\chi$ 2 = 19.93, df = 10, p = 0.03). Pairwise comparisons showed a significantly higher number of active traps in position 1 (71.48%) compared to position 2 (37.50%) ($\chi$

2 = 20.88, df=5, p < 0.001) and position 3 (20.37%) ($\chi$ 2 = 15.85, df = 5, p = 0.007). The number of active traps in position 3, where the lowest values were recorded, was comparable to the number of active traps in position 2 ($\chi$ 2 = 9.74, df = 5, p = 0.08). Considering the variable colour, there was a similar proportion of active compared to inactive traps ($\chi$ 2 = 11.23, df = 5, p = 0.34) (Figure 4.5).

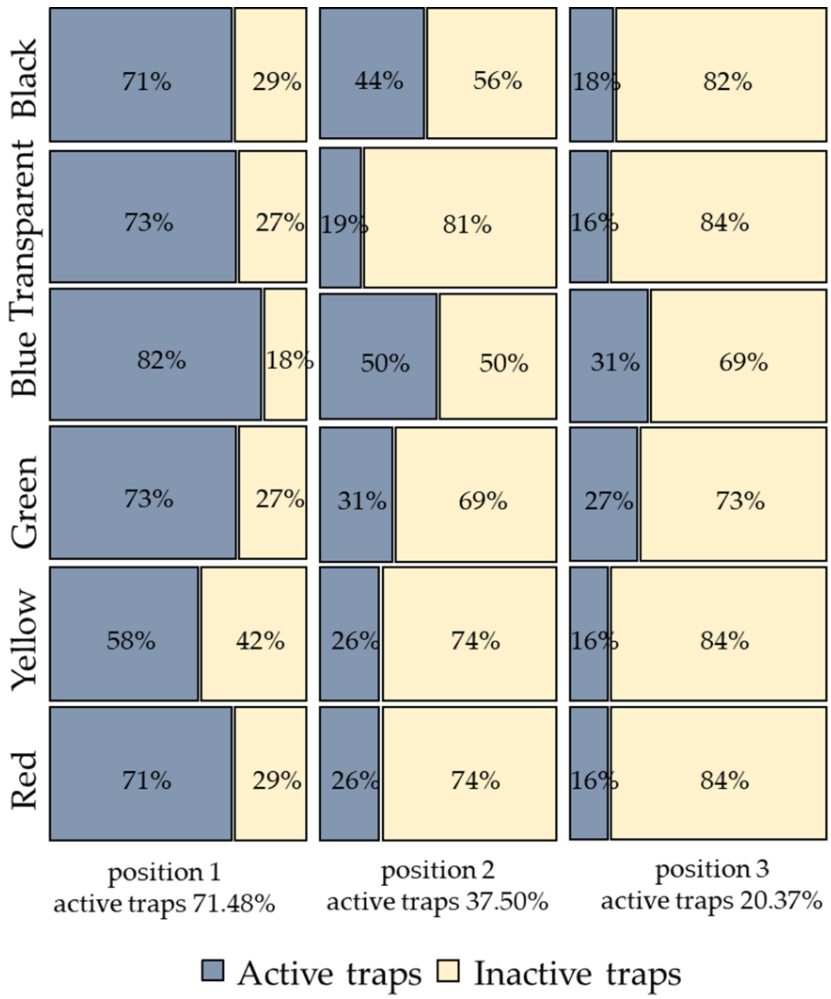


Figure 4.5. Mosaic plot showing the percentage of active and inactive traps observed in the three series positions. Percentages in the boxes refer to the total number for the colours, while the percentages reported outside the boxes refer to the total number of active traps for each series position.

### Colour preference of *Lipoptena fortisetosa* winged adults

The FAMD and trap activity results show low effectiveness for series positions 2 and 3; thus, only *L. fortisetosa* captured by traps in position 1 were analysed. The log-transformed data of the captured *L. fortisetosa* highlighted significant differences among trap colours (main effect colours $F = 15.82$, $p < 0.001$) and sampling dates (main effect of the sampling dates $F = 43.54$, $p < 0.001$). Differences among the average number of flies caught daily by the panels were consistent across all sampling periods (interaction effect $F = 1.42$, $p = 0.06$). The blue panels recorded the highest number of individuals with an average daily catch of 1.28 ± 1.08 (mean ± SE). On the other hand, the yellow traps showed the lowest average values (0.33 ± 0.29 mean ± SE). The blue and yellow colours were significantly different from all the other panel colours (black, green, red, and transparent), which showed a similar average number of insects (respectively: 0.63 ± 0.58, 0.77 ± 0.72, 0.80 ± 0.88, and 0.64 ± 0.10 mean SE) (Figure 4.6).

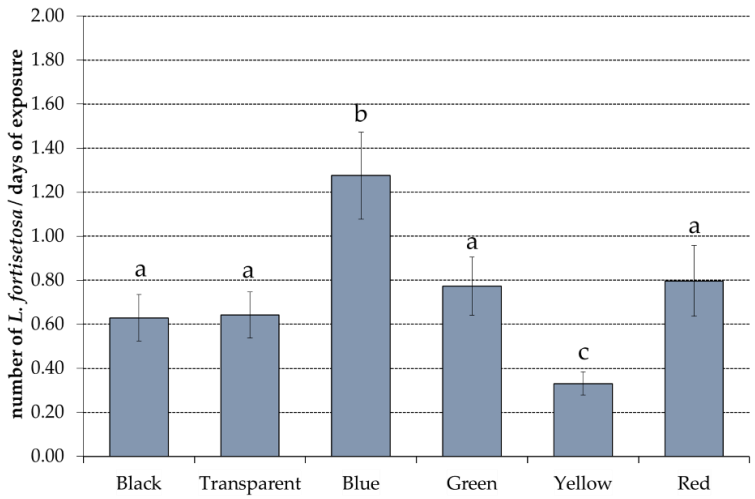


Figure 4.6. *Lipoptena fortisetosa* captured by panels of six different colours (average calculated on the number of insects of each panel per day of exposure). Different letters above the columns indicate significant differences among colours (main effect

colours F = 15.10, p < 0.001, followed by multiple comparisons: Bonferroni test, p < 0.05).

Regarding the variable sampling date, the most significant numbers of *L. fortisetosa* were recorded in August (mean ± SE of 1.09 ± 0.16, 1.49 ± 0.21, and 1.90 ± 0.21, 0.82±0.14 respectively for 6, 12, 20, and 26 August). The averages recorded on these days were significantly higher than the values obtained on earlier dates (15–27 July: avg ± SE 0.19 ± 0.03, 0.33 ± 0.06 and 0.34 ± 0.06) and after 26 August. On other days, the averages were statistically similar; however, 9 September (0.68 ± 0.12) was an exception, with a slight increase in the daily catch. This value was similar to those recorded on 6 and 26 August (Figure 4.7).

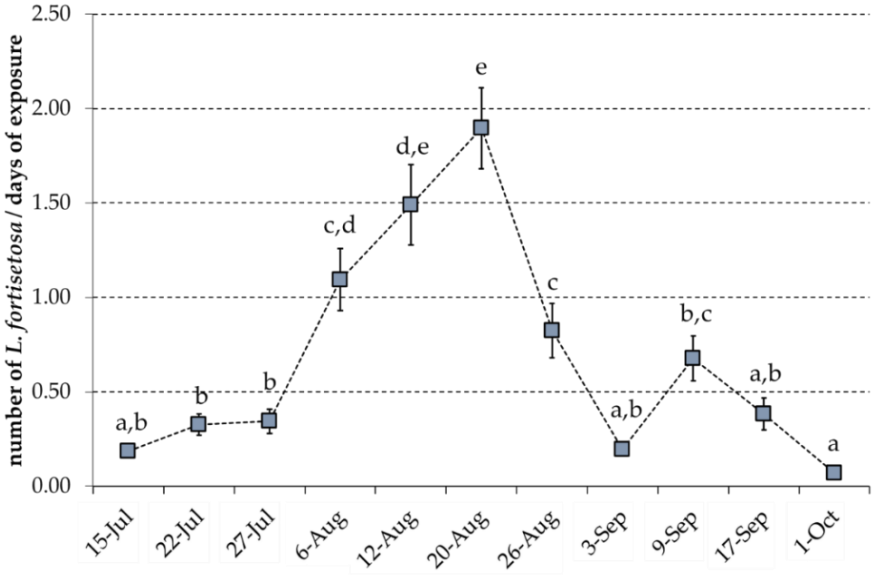


Figure 4.7. Average number of *Lipoptena fortisetosa* captured by panels on different sampling dates (average calculated from the number of insects caught by each panel per day) in the series position 1. Different letters above the columns indicate significant differences (main effect sampling dates; F = 37.21, p < 0.001, followed by multiple comparisons: Bonferroni test, p < 0.05).

In Figure 4.8, which shows the trend of flies trapped by the blue panels over the whole period, the average number of *L. fortisetosa* was considerably relevant, reaching the highest value of 22 specimens per trap. Moreover, after the August peak, the blue traps also caught flies effectively in September although that peak was less than half (8 insects/trap).

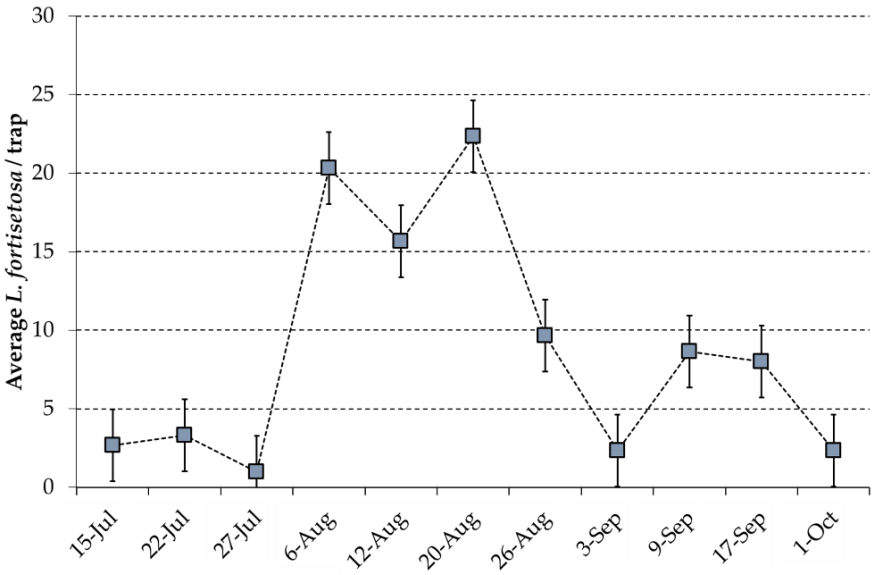


Figure 4.8. Average number of *Lipoptena fortisetosa* per trap as caught by blue panels from mid-July to 1 October.

## Discussion

### Colour preference

Colours tested in this study can be qualified or distinguished based on their spectral characteristics: yellow and red fit into the so-called "cut-off" colours, with a steeply sloped spectrum; blue and

green relate to the "band reflecting" colours with discrete reflectance peaks; transparent and black, having constant reflectance at any wavelength, can be considered neutral [19,36].

As far as we know, most dipterans possess five types of photoreceptors, which can be sensitive in a wide band of wavelengths from UV up to the green wavelength range [37,38], so we speculated that *L. fortisetosa* followed this general model, although the spectral sensitivity of the deer keds has never been measured.

The capture of *L. fortisetosa* in Schignano (Prato, Italy) using differently coloured traps clearly demonstrates that this parasitic species displays a colour preference, since the winged adults selected colours according to a preference scale. Blue traps caught a significantly higher number of adults, compared to black, red, green, and transparent traps, while yellow traps seemed to be the least attractive. This colour ranking was consistent throughout the experimental period. Colour attractiveness has, to date, been poorly investigated in hippoboscid flies, with the only reported observations on *L. cervi* in Finland [21] where black-coloured clothing worn by moving people was compared to white, blue, and red clothing by evaluating the number of flies that landed on them or were caught by transparent flypaper. In the field trials, deer keds preferred black to white clothing, but showed no significant preferences for blue or red.

Our field trial results on *L. fortisetosa* differ from those of Kortet *et al*. [21] in that a blue colour preference was determined, though there are differences in the experimental design (coloured plastic panels in a stationary position instead of coloured clothing worn by moving people) and *Lipoptena* species. In the experiment carried out in Finland, other factors influencing host location may have affected deer ked choice, such as movement, chemical cues, and host size. Usually,

moving objects are detected by an achromatic photoreceptor channel [39]; however, many insects are attracted by moving objects with particular colours showing the so-called "wavelength-specific behaviour" [40]. Different long-distance stimuli could also have influenced these flies, such as carbon dioxide, or odour cues emitted by the host skin as was demonstrated for different groups of hematophagous dipterans [13]. However, it is difficult to separate the effect of different stimuli and to ascribe, with certainty, the location of a host to movement per se, since a moving subject may also release more carbon dioxide or may be warmer than one that is stationary [41].

In other hematophagous Diptera, such as Glossinidae (tsetse flies), blue proved to be the most attractive colour over a short distance [36]. Tsetse and ked flies show quite similar mouthparts and feeding activity [4,42], but once a suitable host is found they display different parasitic behaviour. Unlike deer keds, *Glossina* species frequently change host.

Additionally, the morphological affinity between these families could also pertain to eye structure and colour preference, which have been thoroughly investigated in tsetse flies [43,44]. In a field trial in Zimbabwe, *Glossina morsitans morsitans* and *G. pallidipes* showed a marked preference for traps covered by royal blue cotton, strongly reflecting blue–green wavelength bands, but poorly reflecting ultraviolet or green–yellow–orange bands [19]. A similar behaviour was described for another important tsetse fly, *Glossina fuscipes fuscipes*, which was particularly attracted by blue cloth panels, especially phthalogen blue panels, while yellow was the least attractive [36]. However, olfactory stimuli can affect the attraction of tsetse flies to different colours [17].

Other flies of economic importance, such as *Stomoxys calcitrans*, were investigated to highlight possible colour preferences that could be used to improve monitoring and control interventions. Experiments with blue and black targets with different patterns demonstrated the importance of blue in attracting flies of this species [45]. In addition, the attraction of *S. calcitrans* to polyethylene blue screens was not affected when different hues of blue were used [31]. Phthalogen blue with spectral sensitivity at 350, 450, and 625 nm markedly influenced *Stomoxys* spp. capture [31,46].

Other blood-sucking dipterans have shown a response to colours partly comparable to that exhibited by *L. fortisetosa*. For example, in Canada, Tabanidae and Simuliidae were tested with differently shaped and coloured traps and generally responded more to colours than to shapes [29]. Remarkably, all the investigated species belonging to both these families were consistently not attracted by yellow silhouettes. Moreover, when subjected to solid blue and solid yellow traps, tabanids were more attracted by the blue ones, and when subjected to blue/yellow striped traps, they always chose the blue part. Similar outcomes were highlighted in other studies [30] all of these tabanid data were quite consistent with our observations on *L. fortisetosa*, especially for the poor attractiveness of yellow. Nevertheless, for some herbivorous dipterans, such as Tephritidae and especially the Mexican fruit fly, *Anastrepha ludens*, laboratory trials showed a particular preference for yellow together with green, over black, red, blue, and white [47]. Similarly, yellow traps are commonly used in monitoring and control strategies in different olive growing areas for the olive fruit fly, *Bactrocera oleae* [48,49]. The recognition of green fruit by fruit flies, as well as the detection of green leaves by other herbivorous insects, assumes an interaction between two receptor

types: green-sensitive, which contributes positively, and blue-sensitive, which contributes negatively. The preference displayed by many phytophagous insects for yellow over green stimuli can be explained by this model [50].

A similar mechanism based on receptor opposition was recently proposed for tsetse flies. In these hematophagous insects, the attraction to blue visual bait might be due to an interaction with a positive contribution by a blue-sensitive photoreceptor against photoreceptors sensitive to green–yellow–UV, which contribute negatively [18,36]. Moreover, based on the four sensitivity peaks of the tsetse fly, the number of specimens attracted by different colour panels correlated positively with the blue colour band and with reflectance at 460 nm (blue wavelengths), whereas the correlation was negative in the green colour range and for reflectance at 520 nm (green wavelengths) [36]. Glossinidae and Hippoboscidae share phylogenetic, morphological, and some behavioural features [51,52]; thus, as already supposed, deer keds are thought to display the same visual ecology as seen in the *Glossina* species. As a consequence, catches of *L. fortisetosa* by different coloured panels might have been determined by different reflectance in relation to the sensitivity of fly photoreceptors. The blue panel had a distinct reflectance peak at about 450 nm, which could be in the proximity of the sensitivity peak of *L. fortisetosa*. The green traps showed a reflectance maximum at about 500 and 550 nm, while the yellow ones displayed a "cut off" spectrum between 450 and 520 nm. The lower attractiveness of the yellow panels might be explained by an opposition mechanism of the photoreceptor sensitive to the yellow–green band as proposed for tsetse flies.

In a natural environment, green leaves have a green reflectance peak at 555 nm due to chlorophyll. They contrast with groups of objects that do not reflect the same wavelength (for instance, fruit and flowers). Moreover, grey–red surfaces (such as bark, soil, and animal) are also present, and these have a reflectance that increases gradually as a function of wavelength [53]. For glossinids, it has been suggested that the contrast of blue against the green–yellow reflectance of vegetation could represent a "non-vegetation" stimulus that induces flies to move towards a more feasible stimulus likely coming from a host. Moreover, the stronger attraction for blue could be explained by hypothesizing an important role of the shadow in creating contrasts: the shaded areas where flies rest, or darker areas on the bodies of potential hosts can appear as bluish patches that contrast with the background [18,36].

In different species of Glossinidae, a negative contribution of the green–red and ultraviolet wavelength in attracting tsetse flies was highlighted [18]. In *L. fortisetosa*, the number of individuals caught by green and red traps was higher than for yellow traps but was lower than for blue traps. In the red and green colour panels, however, the number of flies was similar to that found in black and transparent traps. Spectrophotometric analysis of the green panels showed a nearly constant reflectance at different spectrum bands with a maximum 20% at about 500 and 550 nm. On the other hand, the red panels showed a cut-off transition that varied from a minimum value of 535 to a maximum of 635 nm; the lower wavelength chromatic bands were therefore excluded (blue–green region, 410–520 nm).

Because deer have crepuscular and nocturnal activity, they move and find refuge inside the woodland during the day but feed and rest in open areas in the evening and during the night [54,55]. As proposed

for the genus Glossina, we hypothesized that *L. fortisetosa* flies also show a colour response depending on the perception of the host against the background of green foliage or other background material. Alternatively, winged adults might prefer blue targets because they resemble the shaded areas where hosts rest or the shaded zones of the host body where insects can more easily carry out their parasitic action.

### Trap activity at different positions

As reported in Figure 4.6, the blue traps caught a higher percentage of insects than those of other colours for all positions. Regarding the three tested series positions, we found that hippoboscids were not caught in the same numbers in the different locations. In fact, in position 1, located at the border between the forest and the open area, captures were significantly higher than those in the other two, both of which were positioned among the trees. The more abundant captures of *L. fortisetosa* at the boundary between the woods and clearing were also supported by observations conducted in Japan [56]. These outcomes showed that the environment seemed to affect parasitic attraction, which was higher in position 1 probably due to the ecology of both fly and host. Since *L. fortisetosa* adults spend their entire lives in the fur of their host, reproducing and laying larvae, the pupae are more likely to fall from the host body to the ground in areas where the deer stay and repose. Because newly emerged winged adults are unlikely to be able to fly long distances, as reported for *L. cervi* [2], they are probably more abundant at emerging sites. Moreover, as already shown, the series were differently orientated with the first east–west oriented (trap sides with north–south exposure) and the second and the third north–south oriented (trap

sides with east–west exposure). In Glossinidae, as already mentioned, ultraviolet radiation was negatively associated with fly catches [19,36]. Surface UV reflection varies with solar zenith angles and surface type and orientation [57,58]. The different orientation and exposure of the rows in the three positions may have determined a different reflectance that probably induced a lower presence of *L. fortisetosa* in areas with higher ultraviolet reflectivity. This marked difference in captures in the three positions with a total number of 1013 flies in the first compared with the 162 and 77 insects in positions 2 and 3, respectively, may have also been influenced by wind direction and speed. In the end, the flies showed a similar preference to colours in all three series, with blue being the most attractive and yellow the least.

### Pattern of *Lipoptena fortisetosa* captures in the sampling period

Concerning the number of *L. fortisetosa* adults caught during the sampling experiment (from 15 July to 1 October), it is important to highlight the remarkable peak in August, which might be due to adult emergence from pupae laid during the previous year. The slight increase in the number of flies caught in September might be attributed to a second generation from a small number of specimens originating from earlier emerged adults that quickly found a suitable host. Larvae that pupated in early summer could have completed their development and given rise to another emergence of adults favoured by the high temperature. In the Schignano area, the average temperature from mid-July to mid-September was 23.7 °C [59], while it was 14.1 °C from mid-September to the end of October. Lower temperatures of this latter period probably induced pupae to enter diapause until the following year [60]. Our experiment represents the first field-monitoring survey of *L. fortisetosa* and allows us to conclude

that it seems to be multivoltine, as has been reported for other European countries [5,61] and for the area of origin [62].

## Conclusions

Our research provides insight into the life cycle and basic visual ecology of *L. fortisetosa*, which may be exploited in the development of strategies for its monitoring and control and opening a new path to acquire fundamental knowledge on these ectoparasites.

Our experiment showed a peak presence of *L. fortisetosa* winged adults in mid-August, suggesting the possibility that, in Italy, this species could produce more than one generation per year.

More interestingly, we provide evidence that *L. fortisetosa* exhibits a preference scale for colours, with blue being the most attractive and yellow the least attractive. The colour ranking displayed by *L. fortisetosa* suggests that this species is able to discriminate colours and uses visual stimuli over short distance. These results could help in the design of traps to monitor and reduce the populations of this parasitic, hematophagous fly which, in some periods, can reach a very high density, causing annoyance by biting humans in natural areas. The way these flies locate a host remains a topic of active investigation, and further observations are needed to better define the complex stimuli that govern this behaviour. In particular, the role played by odours at medium and long distance should be clarified. For instance, we can state that *L. fortisetosa*, contrary to what is reported for *L. cervi*, does not passively search for a host by waiting for an animal to pass by; rather, it actively flies in search of one. This is supported by our capture of winged adults by a sweeping net only when they were flying, and not when they were resting on vegetation.

The noticeable consistency in some aspects of host attraction among different groups of hematophagous insects is surprisingly high and may suggest a general similar set of needs and tendencies in blood-feeding ectoparasites. Particularly, the convergence in visual stimuli and colour preference ranking is evident: dark colours, especially blue, red, and black, are often attractive for parasites. The similar colour preference for different groups of ectoparasites made us wonder why some hematophagous dipterans are particularly attracted by blue and seem to be relatively unattracted by yellow. Future observations on different blue wavelengths and reflectance are needed to set appropriate and effective traps for this parasite.

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# 5. Antennal morphology and fine structure of flagellar sensilla in hippoboscid flies with special reference to *Lipoptena fortisetosa* (Diptera: Hippoboscidae)

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## Simple Summary

In insects the host searching usually involves different kinds of stimuli, both visuals and chemicals, that may act in combination. External cues are perceived through specific sensory organs (sensilla), mainly present on the antennae. Understanding how ectoparasites belonging to the Hippoboscidae locate their hosts is crucial, since these flies infest animals and can attack humans, with veterinary and medical implications. The aim of this research was to study the antennae of four hippoboscid species, *Lipoptena cervi* (Linnaeus, 1758), *Lipoptena fortisetosa* Maa, 1965, *Hippobosca equina* Linnaeus, 1758, and *Pseudolynchia canariensis* (Macquart, 1840), investigating the morphology and the sensory structures present on these appendages. A typical conformation of the antennae with the envelopment of the third segment (flagellum) inside the first two have been observed. Moreover, two types of sensilla have been detected and their role in the perception of host odours and $CO_2$ have been hypothesized. Other antennal structures seem to be involved in the detection of temperature and humidity variations. Our findings

confirm that these hippoboscids use chemoreception for host location, giving insights into this complex process in this poorly investigated group.

## Abstract

*Lipoptena cervi* (Linnaeus), *Lipoptena fortisetosa* Maa, *Hippobosca equina* Linnaeus, and *Pseudolynchia canariensis* (Macquart) are hematophagous ectoparasites that infest different animal species and occasionally bite humans. Hosts are located by a complex process involving different kinds of stimuli perceived mainly by specific sensory structures on the antennae, which are the essential olfactory organs. General antennal morphology, together with distribution and ultrastructure of sensilla, have been studied in detail with scanning and transmission electron microscopy approaches. Observations have revealed some common features among the four studied hippoboscids: a) typical concealment of the flagellum inside the other two segments; b) characteristic trabecular surface of the flagellum; c) peculiar external microtrichia; d) presence on the flagellum of basiconic sensilla and grooved peg coeloconic sensilla; e) unarticulated arista. The ultrastructure of *L. fortisetosa* revealed that microtrichia and the flagellar reticulated cuticle are not innervated. Different roles have been hypothesized for the described antennal structures. Microtrichia and the reticulated cuticle could convey volatile compounds towards the flagellar sensory area. Peculiar sensory neurons characterize the unarticulated arista which could be able to detect temperature variations. Coeloconic sensilla could be involved in thermoreception, hygroreception, and carbon dioxide reception at

long distances, while the poorly porous basiconic sensilla could play a role in the host odor perception at medium-short distances.

## Introduction

Hippoboscids are obligate hematophagous ectoparasites of vertebrates. These flies belong to the superfamily Hippoboscoidaea together with other important families such as Glossinidae (tsetse flies), Nycteribiidae and Streblidae (bat flies) [1]. The family Hippoboscidae encompasses three subfamilies - Ornithomyinae, Hippoboscinae, and Lipopteninae - whose members live on birds, various mammal species, and ungulates, respectively [2,3]. Several representatives of these three subfamilies are well known for their veterinary and medical importance, since they can be responsible for diseases harmful to humans and animals [4].

Among members of the subfamily Ornithomyinae, *Pseudolynchia canariensis* Macquart (the pigeon fly) is a medium-sized species living especially on Columbiformes. It may transmit to its hosts the avian malaria parasite, *Haemoproteus columbae* Kruse, and, additionally, it can produce skin dermatitis in case of severe infestations [5]. *Hippobosca equina* L. (the forest fly) belongs to the Hippoboscinae and is an ectoparasite mainly of horses and donkeys, on which it can cause several annoyances and skin injuries. This species can also act as a vector of pathogens dangerous both to animals and humans, such as *Anaplasma* spp. [6], *Corynebacterium pseudotuberculosis equi* [7], and *Bartonella chomelii* Maillard *et al*. 2004 [8]. Within the subfamily Lipopteninae, *Lipoptena cervi* L. and *L. fortisetosa* Maa (the deer keds) predominantly attack cervids, on which they can cause skin diseases and behaviour alterations in case of high parasite population density

[9,10]. Moreover, they can play an important role as carrier of pathogens, mainly *Anaplasma* spp., *Bartonella* spp., *Borrelia* spp., *Coxiella* spp., *Theileria* spp., *Trypanosoma* spp. [11-17]. Recently, the Asian species *L. fortisetosa* has colonized most of the European countries, including Italy, where it is competing with *L. cervi* for territories and host microniches [18].

The four above-mentioned hippoboscid flies live at the expense of a few host species, wherewith they established a strict interaction depending on the host behaviour and morphology. Due to this specialized parasitic life, these flies display extreme specialization of many features, such as a flattened body, robust legs equipped with claws which allows it to firmly adhere to the host's coat, and a prognathous head which allows to firmly adhere to the host [19]. Antennae show a remarkable morphological adaptation with the scape and pedicel fused together in almost all species [20]; moreover, the flagellum is housed within a cavity formed by the first two antennal segments [19,21]. Antennae are almost completely hidden inside two deep hallows, named antennal sockets or fossae. These sensory appendages play a primary role in the host location in hematophagous dipterans, and are responsible for the detection of odour cues. This behavioural aspect has been demonstrated in members of the suborder Nematocera, such as black flies and mosquitoes [22,23], as well as in representatives belonging to the suborder Brachycera, such as muscids, tabanids, and glossinids [22]. Antennal sensory structures of hippoboscid flies have been studied in *Hippobosca equina*, *H. longipennis* Fabricius, and *Melophagous ovinus* L., where the external surface of the flagella have different kinds of sensilla [21]. Given the concealment of the flagellum and the reduction in the other antennal segments, Zhang *et al*. [21] speculated that these modifications may

have caused the lack of the primary sensory function (olfaction), defining these flies as "inactive ectoparasites". Actually, soon after their emergence from puparia, most of hippoboscid species spend the pre-parasitisation period resting on vegetation or flying, using different kinds of stimuli to locate a suitable host. In Finland, *L. cervi* adults are able to survive without feeding over a month [24], so that the host-seeking period may be extended. These insects require remarkable energy expenses to detect external signals, mainly odour cues. Host location in hippoboscid flies needs to be further investigated, especially in those species in which newly emerged adults occur in areas with no availability of hosts, leading to an active host searching mediated by external stimuli, which are especially detected by antennal sensilla. In *P. canariensis*, *H. equina*, *L. cervi*, and *L. fortisetosa,* the antennal sensory structures present on the external segments display peculiar morphological adaptations, since these species have diverse parasitic behaviours and a different association level with their hosts [19].

The present paper deals with a morphological analysis of antennal structures and sensory patterns in these four species, with special reference to the deer ked *L. fortisetosa,* which has been investigated for the sensillar ultrastructure. These observations may contribute to a better understanding of the host location process of these hematophagous ectoparasites.

## Materials and methods

### Insect collection

Hippoboscid flies were collected in several areas of Tuscany (central Italy) for Scanning Electron Microscope (SEM) observations.

Wingless deer keds were manually picked up by cervid skin pieces provided by hunters during the culling season of 2019-2020. Specimens of *P. canariensis* were collected from pigeons during an official wildlife surveillance program performed by Provincial Wildlife Police in San Miniato (Pisa), while *H. equina* adults were picked up from horses in a stable in Marradi (Firenze). For Transmission Electron Microscopy (TEM) winged adults of *L. fortisetosa* were collected in a wooded area in Schignano (Prato) at about 550 m a.s.l. (43.967432 N; 11.101761 E). Winged adults were caught by sweeping, maintained in microvials containing a small piece of cotton soaked with water and sugar, and kept at low temperature (4-6 °C) for a few days, pending TEM analysis.

## SEM procedures

All hippoboscid adults (at least 20 specimens each species, about 60 for *L. fortisetosa*) were anaesthetized at −20°C for 20 min and then maintained in 70% ethanol pending preparation procedures. Specimens were removed from ethanol, rinsed with distilled water several times, and then sonicated for 15 min in 10% potassium hydroxide (KOH) distilled water solution to remove impurities and secretions from their bodies. After that, the samples were rinsed again in distilled water to remove KOH residues. Subsequently, adults were dehydrated in a series of graded ethanol concentrations (from 70% to 90% with 10% increasing concentration each, then 95% and 99%, for 10 min in each concentration). Antennae were excised from the heads and dissected to extract the internal flagella. Then, all samples were air-dried, mounted on aluminium stubs and gold-coated with a sputter coater device (S150B; BOC Edwards, Burgess Hill, U.K.). SEM observations were made using a FEI Quanta 200 high vacuum, low

vacuum and environmental scanning electron microscope (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at the Department of Agriculture, Food and Environment (DAFE), University of Pisa, and a Zeiss Evo 40 at the centre "Centro di Servizi di Microscopia Elettronica e Microanalisi" (MEMA), University of Florence. The morphology and the external sensillar pattern of the antennae were examined and described according to the terminology and nomenclatures reported by Maa and Peterson [20] and Zhang *et al*. [21].

## TEM procedures

Ten live winged adults of *L. fortisetosa* were $CO_2$ anaesthetised and thereafter immersed in a glutaraldehyde/paraformaldehyde solution (2.5% in 0.1 M cacodylate buffer +5% sucrose, pH 7.2–7.3) for 3-4 h. The antennae of some specimens were isolated from the rest of the head capsule to reduce the size of the tissue to be fixed and to facilitate fixative penetration. However, full heads were also processed. After the first fixation step, samples were rinsed twice in 0.1 M cacodylate buffer (15 min each step) and kept at 4°C overnight. Then, samples were post-fixed in a 1% osmium tetroxide solution ($OsO_4$) for about 50 min. After rinsing with the same buffer, specimens were then dehydrated in a graded series of ethanol (from 50 to 90% with 10% increasing concentration each, then 95% and 99%), with each step lasting 15 min. Subsequently, specimens were exposed to pure propylene-oxide, then to a 50/50 blend of propylene oxide and Epon-Araldite resin to improve resin infiltration. Each sample was finally infiltrated with an Epon-Araldite resin and incubated at 65°C for 48 h. Embedded sample were sectioned using a diamond knife (Drukker) using a Bromma ultramicrotome (LKB, Stockholm, Sweden). Ultrathin sections (60-90 nm) were collected using formvar coated, 50 mesh

copper grids, and then stained with uranyl acetate (20 min at room temperature) and lead citrate (5 min at room temperature). Grids were investigated with a Philips EM 208 TEM (Thermo Fischer Scientific, Hillsboro, OR, USA). Digital photographs (1376x1032 pixels, 8 bit, uncompressed greyscale TIFF files) were obtained using a high-resolution digital camera MegaView III (SIS, Muenster, Germany) connected to the TEM. TEM data were obtained at the "Centro Universitario di Microscopia Elettronica e Fluorescenza (CUMEF; Università degli Studi di Perugia, Italy)".

## Results

The four studied species show a similar arrangement of the antennal pattern with antennae inserted inside peculiar head cavities, named fossae or antennal socket. Except for *P. canariensis*, the scape and pedicel are fused and house the third segment, the flagellum.

*Pseudolynchia canariensis*. The outer part of *P. canariensis* antennae, depicted in Figure 5.1 A-C, displays the arrangement of these appendages protruding externally from the socket with the scape articulated with both the lunula and the fronto-clypeus and the proximal part of the pedicel, to some extent, fused with the scape (Figure 5.1 A-B). Several long bristles constitute the external sensillar apparatus of the first two segments, which have been described in a previous paper [19]. Moreover, the pedicel surface adjacent to the fronto-clypeus is partially covered by microtrichia. An unbranched arista, with a shovel-shaped tip (Figure 5.1 C-D), originates from the dorsolateral part of the introflexed flagellum, which is pear-shaped (Figure 5.2 A). The flagellum is marked by an irregular surface of dense

and long microtrichia mixed with cuticular trabeculae, uniformly arranged and forming several pits located on the dorsolateral area (Figure 5.2). Within these hollows, different kinds of receptors, mainly coeloconic grooved and a few basiconic sensilla, are interspersed.

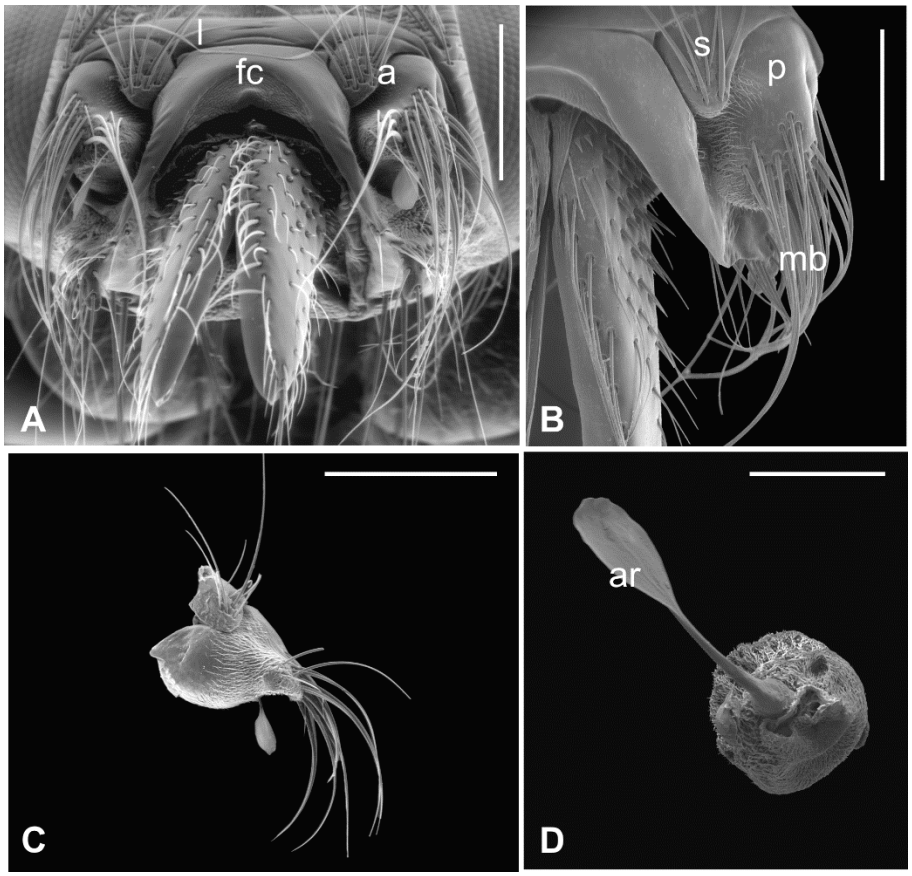


Figure 5.1. *Pseudolynchia canariensis*. (A) Frontal view of the head with antennae (a), which are partially fused with the lunula (l) and the fronto-clypeous (fc); (B) Dorsal view of the antenna with the visible articulation between the scape (s) and the pedicel (p), bearing long bristles (mb) with probable mechanosensory function; (C) Antenna excised from the antennal socket, showing mechanosensory bristles and the protruding arista; (D) Flagellum with the non-articulated, shovel-shaped arista (ar). Bar scale: A 300 µm; B 200 µm, C 400 µm; D 100 µm.

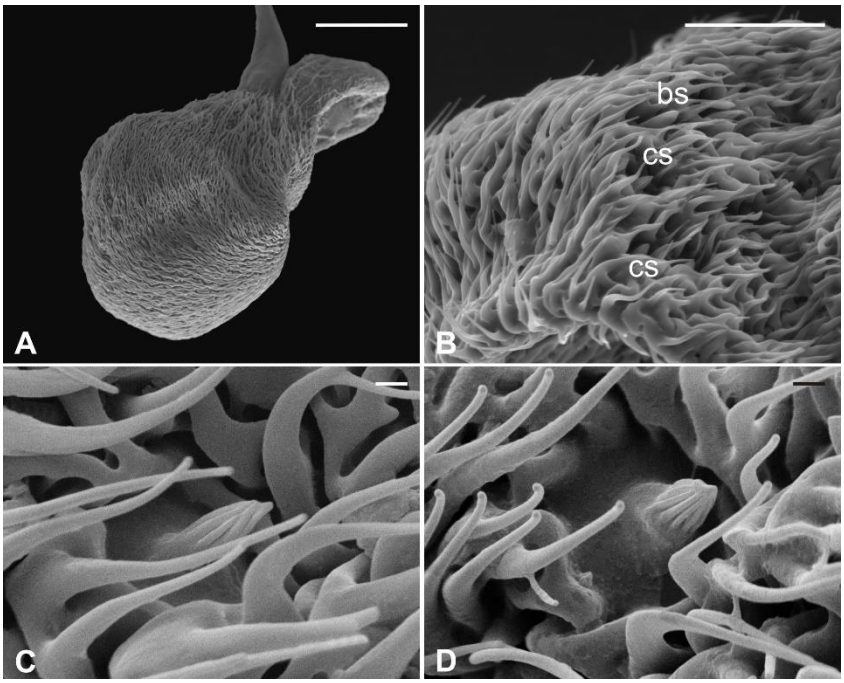


Figure 5.2. *Pseudolynchia canariensis*. (A) Lateral view of flagellum with cuticular pits housing mainly grooved coeloconic sensilla and rare basiconic sensilla. Note the characteristic surface arrangement; (B) Detail of the typical trabecular structure of the flagellum, with coeloconic (cs) and basiconic (bs) sensilla accommodated in pits; (C-D) Magnification of coeloconic grooved sensilla with different features. Bar scale: A 50 µm; B 20 µm, C, D 1 µm.

*Hippobosca equina*. In this species, the antenna lies in the antennal socket with only the dorsal surface externally exposed and entirely covered by microtrichia except for a small area on the top (Figure 5.3 A-B). Three mechanosensory bristles are present on the distal part of the segment [19]. Additionally, a small furcate arista protrudes in the ventral region of the hollow which is wallpapered by a dense microtrichia coverage (Figure 5.3 C-D). The Figure 5.4 A shows the piriform flagellum with a non-articulated arista on the dorsoanterior area. The surface of the flagellum is covered by a reticulated cuticle from which short microtrichia rise up. These microtrichia are shorter and display a wider base than those of *P. canariensis* (Figure 5.4 B).

However, similarly to the cuticular pattern of the pigeon fly, several sensilla are located inside sensory pits on the dorsolateral region. Coeloconic grooved sensilla, almost always sunken in sensory pits, are mainly spread in the proximal part of the flagellum, while multiporous basiconic sensilla occur around the arista (Figure 5.4 A-C-D).

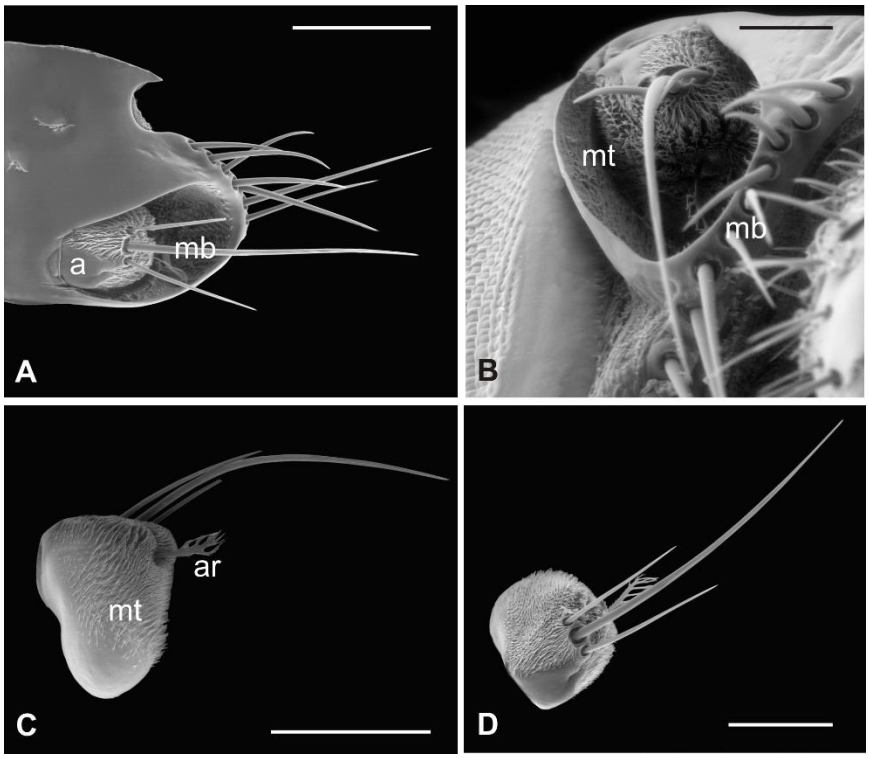


Figure 5.3. *Hippobosca equina*. (A) Dorsal view of antenna housed in the large antennal fossa, bearing three mechanosensory sensilla (mb). Note the long bristles (mb) probably with mechanosensory function at the edge of the structure; (B) Ventral view of the antenna with the pedicel partially covered by a dense layer of microtrichia (mt) present also on the wall of the antennal fossa; (C-D) Lateral and dorsal view of the antenna showing microtrichia (mt) and the typical branched arista (ar). Bar scale: A 300 µm; B 100 µm, C 300 µm, D 200 µm.

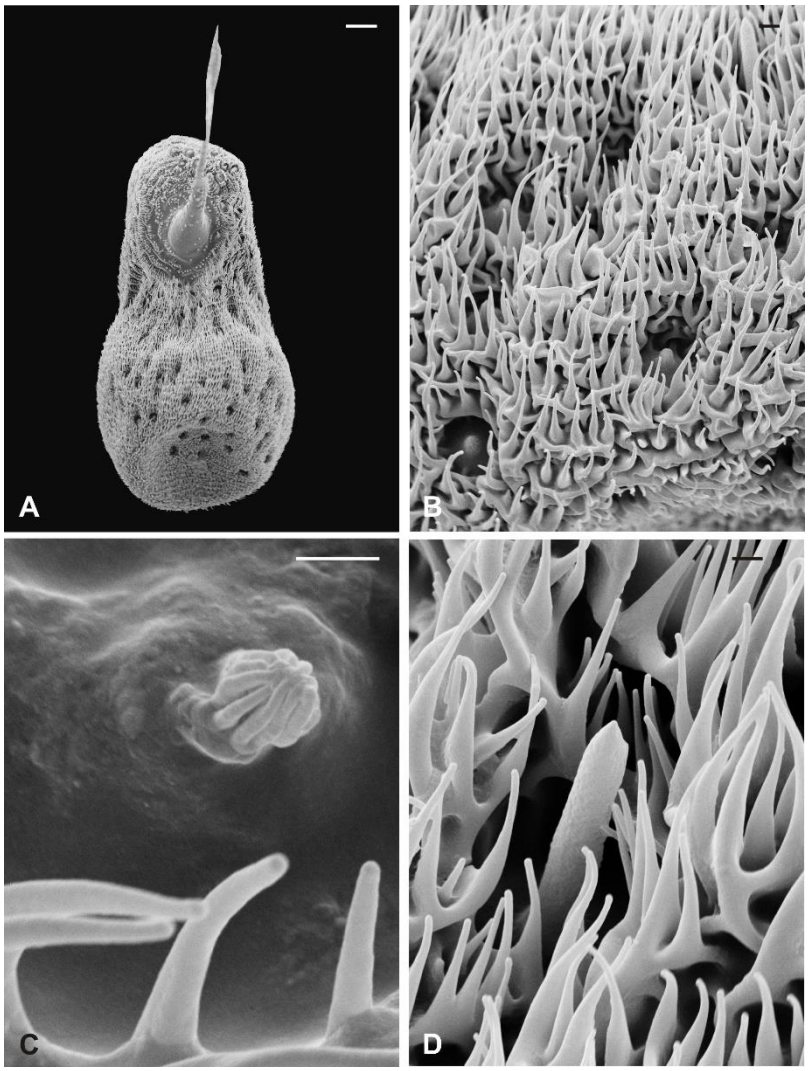


Figure 5.4. *Hippobosca equina*. (A) Dorsal view of the piriform flagellum with numerous sensillar pits located mostly on the dorso-proximal part. Note the non-articulated arista with a large base; (B) Magnification of the surface characterized by differently sized microtrichia with coeloconic grooved sensilla accomo-dated inside cuticular depressions; (C) High magnification of a coeloconic grooved sensillum with evident finger-like projections; (D) High magnification of a basiconic sensillum embedded within microtrichia and cuticular trabeculae. Bar scale: A 20 μm; B 2 μm, C, D 1 μm.

*Lipoptena cervi*. The external edge of *L. cervi* antenna presents different types of sensilla previously described [19]. The external surface shows a typical microtrichia overlay which thickens

approaching the pedicel opening (Figure 5.5), where, interestingly, microtrichia become furcate with two or three prongs (Figure 5.5 D). A non-articulated branched arista protrudes from the pedicel hollow (Figure 5.5 A-C-D). The piriform flagellum displays the typical trabecular surface in the proximal part (Figure 5.6 A). Close to the arista, the cuticle forms shallow depressions in which microspines are present. Similar to the previously described species, *L. cervi* shows several sensory pits on the proximal part of the flagellum, characterized by the presence of basiconic and coeloconic grooved sensilla (Figure 5.6 B-D).

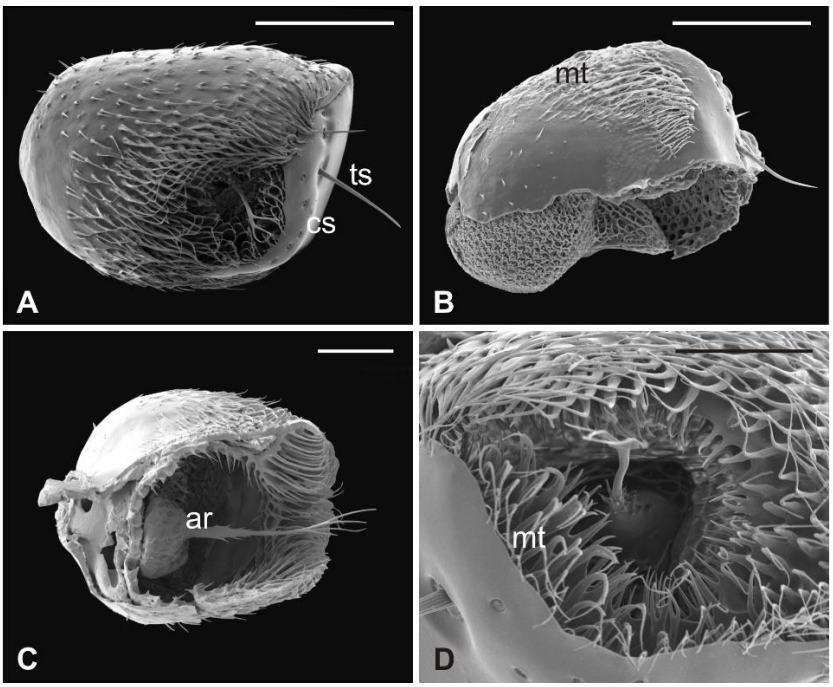


Figure 5.5. *Lipoptena cervi*. (A) Ventral view of the antenna with arista protruding from the hollow densely covered by mi-crotrichia. Note the coeloconic (cs) and trichoid (ts) sensilla present on the pedicel edge; (B) Lateral view of the opened pedicel with microtrichia (mt) showing the introflexed flagellum with the characteristic trabecular surface; (C) Ventro-lateral view of the dissected pedicel displaying the anterior part of the flagellum from which the branched arista (ar) originates; (D) Antennal hollow magnification showing the arista with furcate microtrichia (mt). Bar scale: A, B 100 µm, C 50 µm, D 40 µm.

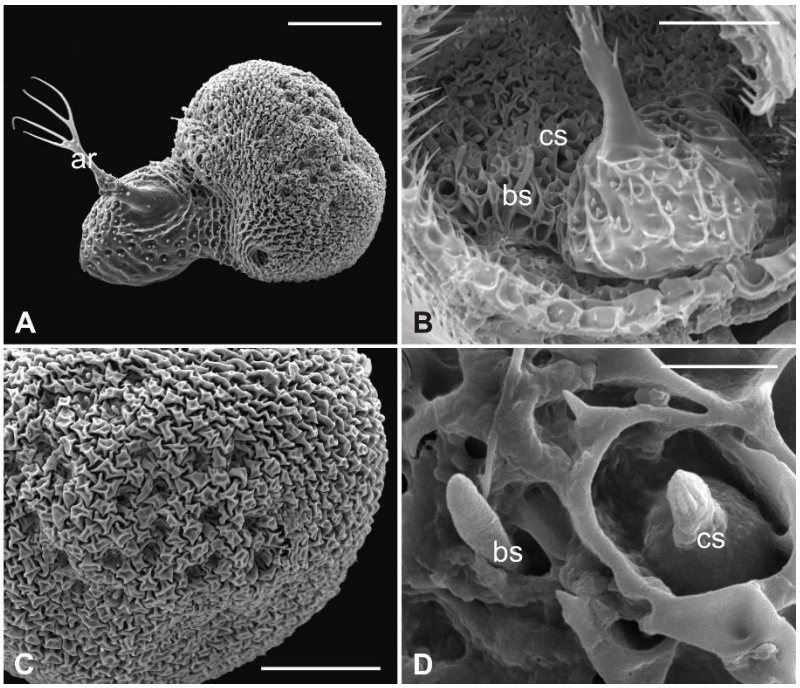


Figure 5.6. *Lipoptena cervi*. (A) Dorso lateral view of the flagellum showing a typical trabecular surface and the non-articulated arista (ar) with the branched tip. Note the cuticular depressions housing sensilla; (B) Magnification of the sensory area close to the arista base with visible coeloconic (cs) and basiconic (bs) sensilla; (C) Magnification of the dorso-distal part of the flagellum showing trabeculae and sensory pits; (D) Magnification of a multiporous basiconic (bs) sensillum and a grooved coeloconic sensillum (cs). Bar scale: A 50 µm, B, C 30 µm, D 5 µm.

*Lipoptena fortisetosa*. In this fly, the antennal apparatus differs externally from those of the other described species by the presence of a series of peculiar aligned robust mechanosensory bristles on the edge of the pedicel (Figure 5.7 A) [19]. The pedicel is bean-like and bears sparse microtrichia on the dorsolateral part, toward the fronto-clypeal area; the fan-shaped tip of the arista emerges from the hollow (Figure 5.7 B, 8 A). The antenna is housed inside a deep antennal fossa which encloses almost all the segment surface (Figure 5.7 C). As in *L. cervi*, the proximal region of the flagellum is covered by a trabeculated surface with sensory cavities in the dorsal area (Figure 5.7 D). This trabecular organization of the surface appears even more evident in

serial cross-sections of the antenna performed at the very distal region of the flagellum, as well as more proximally (Figure 5.8 B, C). TEM cross-sections show shallow cavities occupied by cuticular pegs and sparse basiconic and coeloconic sensilla, while clustered basiconic sensilla are housed in deeper invaginations of the flagellum cuticular wall (Figure 5.8 D, E).

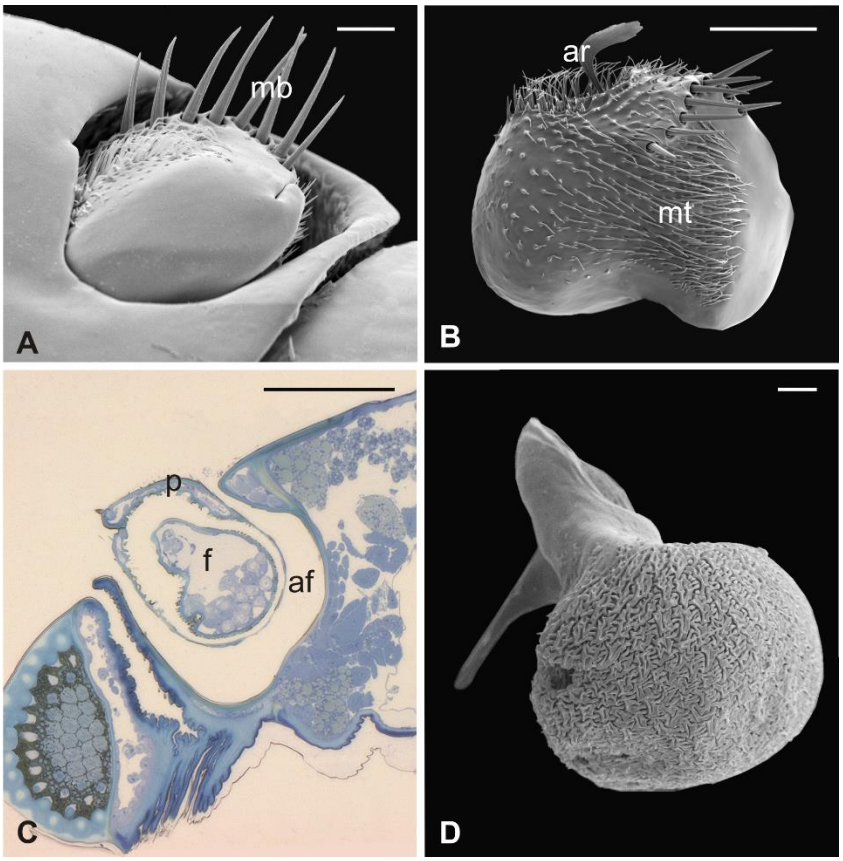


Figure 5.7. *Lipoptena fortisetosa*. (A) Dorsal view of the antenna with the typical aligned mechanosensory bristles; (B) Lateral view of the bean-like pedicel showing sparse microtrichia (mt) thickening close to the hollow from which the arista (ar) protrudes with a spatulate tip; (C) Light microscopy cross section of the head showing the deep and large antennal fossa (af) where the pedicel is inserted. Inside the pedicel (p), it is possible to observe the pear-like flagellum (f) with clustered nuclei; (D) Lateral view of the piriform flagellum with the typical reticulate surface covering the proximal part of the segment. Bar scale: A 20 µm, B 50 µm, C 100 µm, D 10 µm.

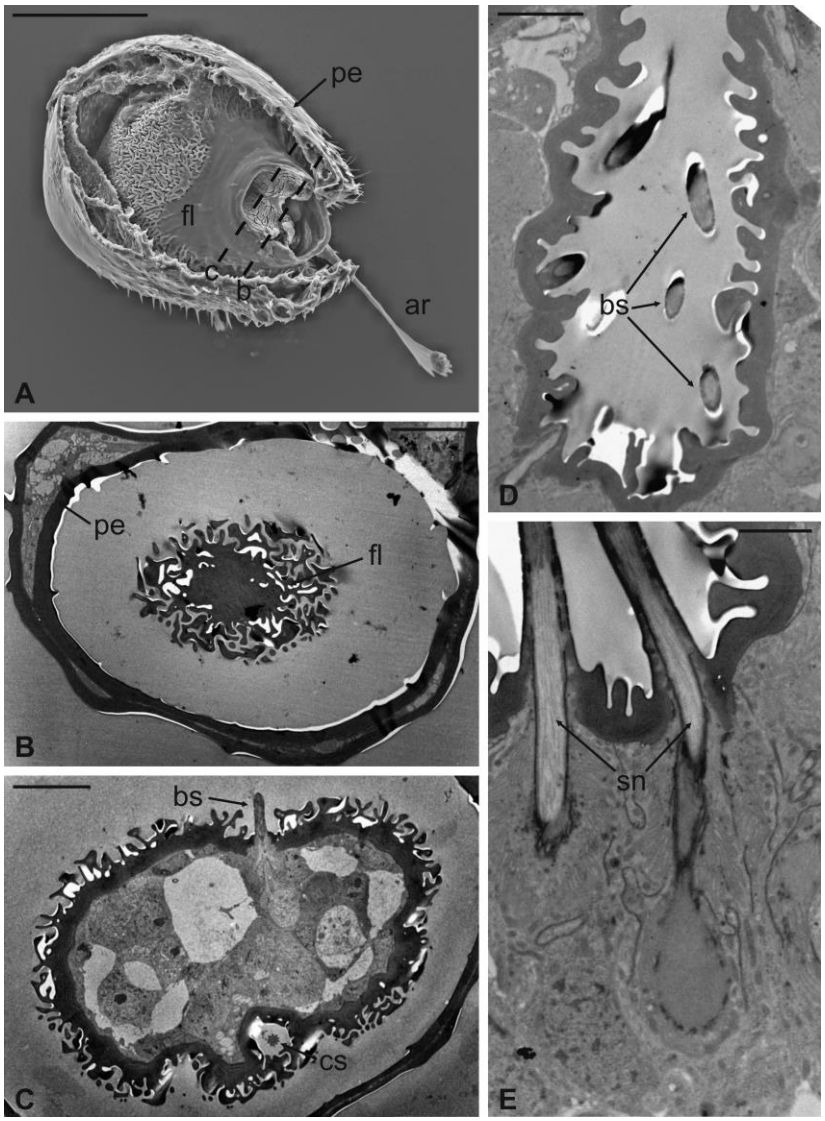


Figure 5.8. *Lipoptena fortisetosa*. (A) SEM ventral view of the antenna isolated from the head and partially opened. The pedicel (pe) surrounds the flagellum (fl). The anterior part of the flagellum bears the arista (ar) that protrudes externally. (B-C) TEM cross section of the whole antenna taken according to the two dotted lines depicted in (A). (B) Antennal section carried out at the distal part of the pedicel (b line in A): the external structure, delineated by two cuticular layers, is the pedicel, housing the flagellum having elaborate and trabeculate cuticle. (C) Flagellum sectioned subapically (c line in A), with some invaginations occupied by coeloconic (cs) and basiconic sensilla (bs), with the typical cuticle. (D) Detail of one of the cuticular cavities (sensory pit) occurring dorsally on the flagellum with several basiconic sensilla. (E) Close up view of the innermost part of a sensory pit: two basiconic sensilla with their innervating sensory neurons (sn). Bar scale: A 50 µm; B, C 10 µm; D 5 µm; E 2 µm.

TEM investigation reveals the following internal organization for the above reported sensory structures.

The long bristles located at the external margin of the pedicel are characterised by long cuticular shafts, straight and sharply pointed (Figure 5.7 A, 5.9 A). The shaft base is housed inside a rounded socket and exhibits sturdy external grooves running from the base to the tip. Serial cross and longitudinal sections reveal the presence of a single sensory neuron with a large cell body containing a prominent nucleus and a relatively short inner dendritic segment (Figure 5.9 D). Right below the socket, from the inner dendritic segment an outer dendritic segment is differentiated (Figure 5.9 C), with a typical ciliary constriction region. The outer dendritic segment ends at the base of the shaft with an electron dense tubular body enveloped by the dendritic sheath (Figure 5.9 B). The cuticular shaft is made of thick cuticle with a small lumen located in the centre, where no dendrites or dendritic branches are found. The thinner and shorter microtrichia present on the dorsalateral side of the pedicel are not innervated (Figure 5.9 E).

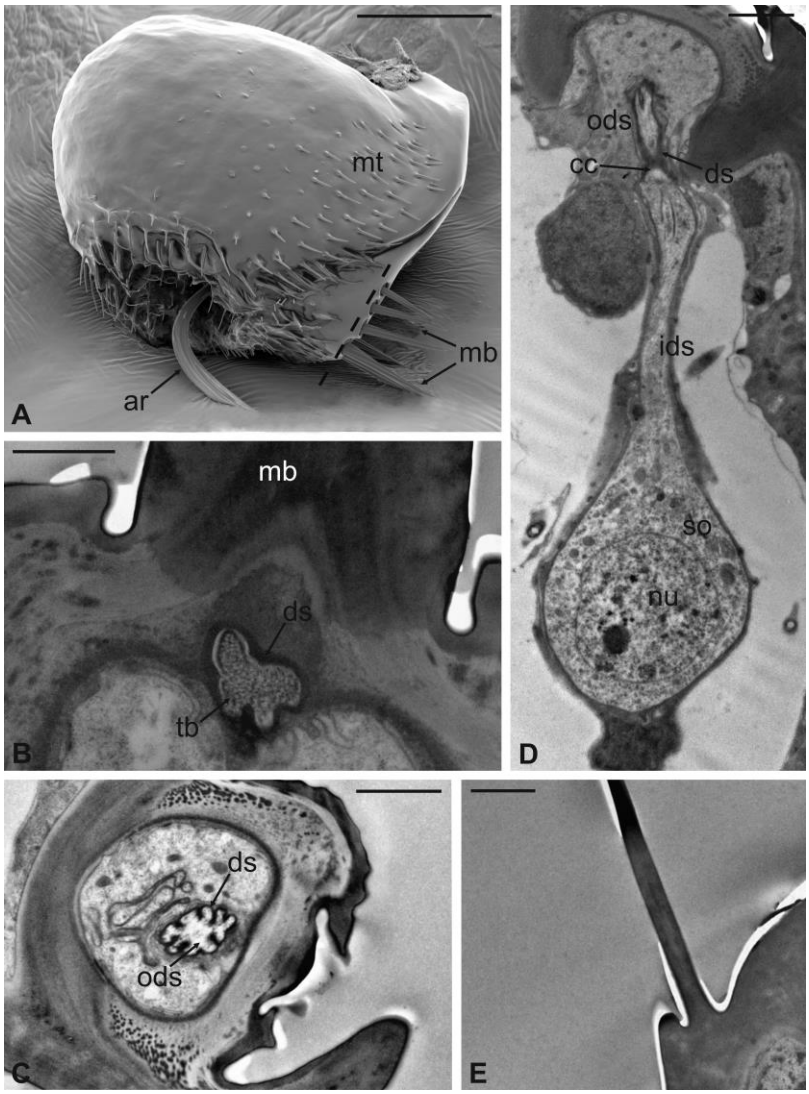


Figure 5.9. *Lipoptena fortisetosa* long bristles. (A) SEM dorso-lateral view of the antenna: arista (ar), long and sharply pointed long bristles (mb), and numerous thin microtrichia (mt) are clearly visible. (B-C) TEM cross sections of the whole antenna taken along the dotted line depicted in (A). The apical part of a single sensory neuron (tubular body, tb) that innervates the associated mechanosensory bristle is depicted. More proximally, the same neuron, encased by a thick dendrite sheath (ds), is visible at the level of the outer dendritic segment (ods). (D) TEM reconstruction, obtained by four different pictures, with the sensory neuron of the mechanosensory bristle. The cell body lies deeper in the antennal lumen. From the somata (so) a relatively short inner dendritic segment (ids) originates and, close to the cuticular wall, evolves in the (ods). The ciliary constriction (cc) region appears as a throttling from which the dendrite sheath starts to be visible. (E) Longitudinal section of a microtrichium with no evidence of associated sensory neurons. Bar scale: A 50 µm; B, 1 µm; C, D, E 2 µm.

The arista is short, slightly curved, and can be divided in two distinct parts of the same length: a proximal portion, stick-like, that is connected to the flagellum; and a distal region that is enlarged and fan-like (Figure 5.10 A). The flattened distal region examined through TEM shows no lumen (Figure 5.10 B, C). The cross-section of the proximal part of the arista displays a lumen filled with extracellular material (Figure 5.10 D). It is noteworthy that externally the arista does not show any specialized cuticular structures that could be related to a sensory function. Cross-sections of the arista's basal region, connected with the pedicel, reveal the absence of a specialised socket, while the arista lumen contains a cluster of cells located eccentrically, close to the wall. In this area (Figure 5.10 E-F), two groups of sensory neurons have been highlighted: the first one formed by two outer dendritic segments and the second one comprising three outer sensory neurons. In both cases, the grouped sensory neurons are embedded by dendrite sheaths.

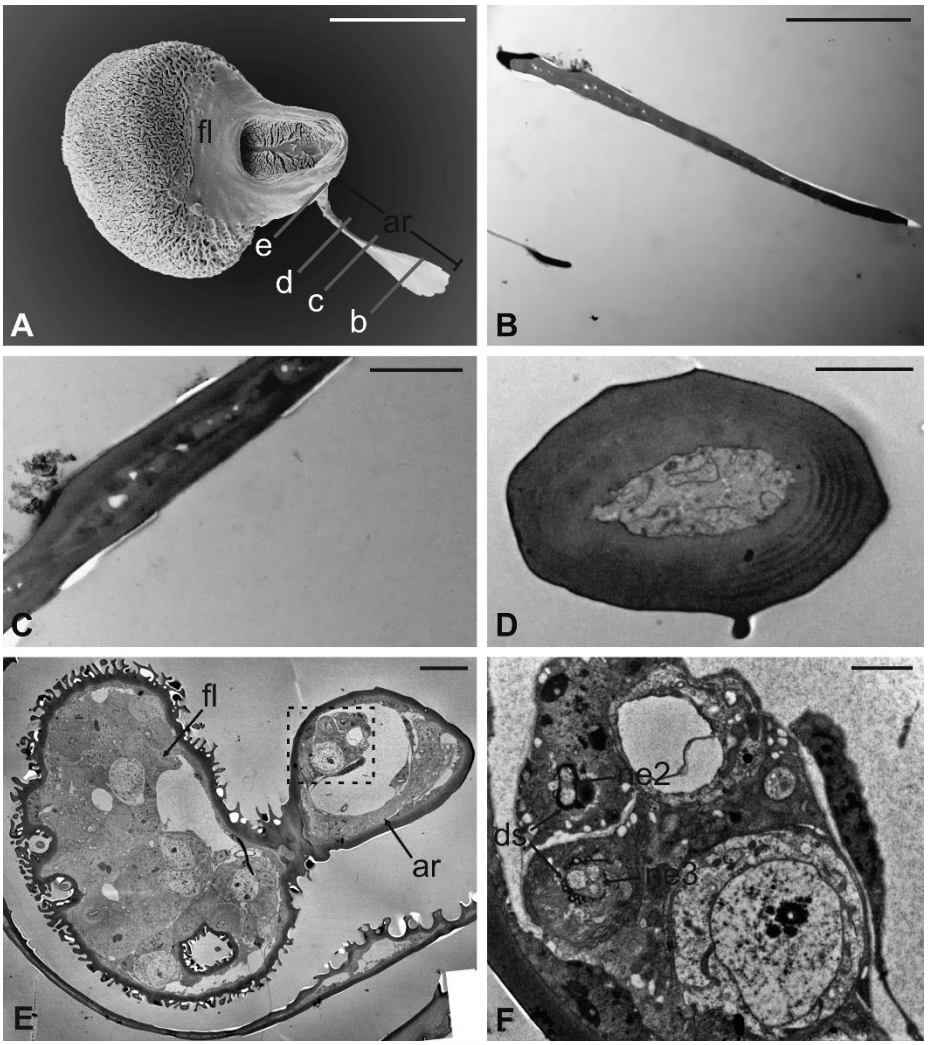


Figure 5.10. *Lipoptena fortisetosa* arista. (A) SEM ventral view of the flagellum (fl) removed from the remaining of the antenna showing the arista (ar). This is a long and slightly bended structure, with a flattened tip fan-shaped, supported by a thin, ellipticalstalk. (B-C-D) TEM pictures showing cross sections of the arista at the section plane levels as reported in (A). The arista appears flattened and without a perceptible internal lumen in (B) and (C), while in (D) the cuticle is thicker, and the small lumen is occupied by extracellular material. (E) TEM reconstruction obtained combining nine different pictures showing a cross section of the region as reported in (A). At this level, the base of the arista is connected with the flagellum. The lumen of the arista displays a region (dotted square in (E) where (F) groups of neurons are visible. Two groups of neurons can be differentiated, one with three (ne3) and another with two neurons (ne2), in both cases enclosed by a dendrite sheath (ds). Bar scale: A 50 µm; B 10 µm; C, D, F 2 µm; E 10 µm.

Basiconic sensilla (BS) can be mainly found inside the deeper cavities that are spread on the dorsal area of the flagellum, as well as on the surface of the flagellum itself. BS are characterized by the presence of an external cuticular peg standing on the antennal wall and surrounded by the trabeculated cuticle that covers most of the flagellum surface (Figure 5.11 A). The representative basiconic sensillum shows a blunt tip and pores on the sensillum wall. Longitudinal sectioning reveals a cuticular shaft made of a thin cuticle perforated by numerous pores distributed on the distal half (Figure 5.11 B-D). The shaft is inserted on the antennal wall without a flexible socket (Figure 5.11 B). Internally, a single sensory neuron innervates each sensillum. The outer dendritic segment enters the proximal part of the sensillum, from where several dendrite branches arise, filling the shaft lumen for all its length (Figure 5.11 B-D). Cross-sections under the sensillum base show the sensory neuron enclosed by a well-defined dendrite sheath and the thecogen cell (Figure 5.11 E-G). The dendrite sheath embeds the outer dendritic segment of the sensory neuron also inside the cuticular shaft, up to the level where the neuron starts to branch (Figure 5.11 B).

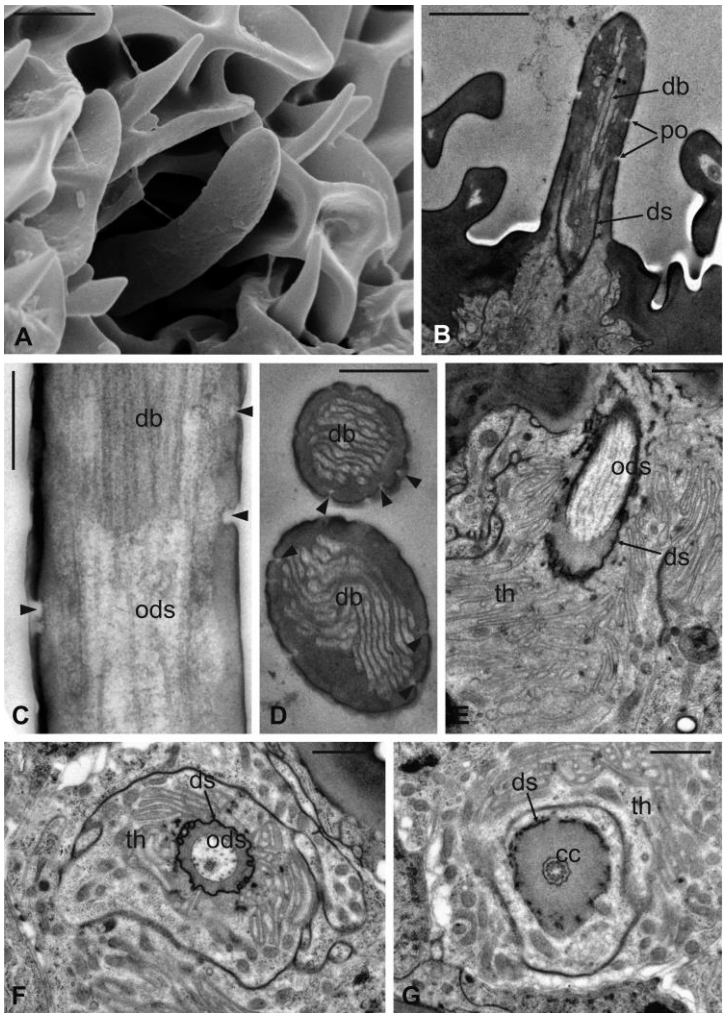


Figure 5.11. *Lipoptena fortisetosa* basiconic sensilla. (A) SEM picture with one basiconic sensillum (bs) with its blunt tip and porous cuticle. It is surrounded by elaborated cuticular sculpture wall. (B-G) TEM images of bs. (B) Longitudinal section of bs with a thin, porous wall and dendritic branches (db) entering the sensillum lumen. The dendrite sheath (ds) enters the sensillum and embeds the sensory neuron until it starts branching. The base of the sensillum is inflexibly inserted into the antennal wall. (C) Detail of a longitudinal section of bs with the unbranched outer dendritic segment (ods) of the sensory neuron that starts forming the db. Pores (po) are visible (arrowheads). (D) Cross section with two bs sectioned at their distal region: clusters of db filling the lumen and several cuticular pores are visible. (E) Longitudinal section of bs taken at the base level unveiling a single sensory neuron embedded by the ds and numerous microvilli belonging to the thecogen cell (th). ( F-G) Cross sections of bs sensory neuron. (F) The outer dendritic segment surrounded by the ds and th. (G) The same sensory neuron depicted more proximally, at the ciliary constrictions (cc) level, where ds originates. Bar scale: A, B 2 µm; C, D 0.5 µm; E, F, G 1 µm.

Coeloconic sensilla (CS) are grooved pegs interspersed on the flagellum surface, often accompanied by basiconic sensilla. Also in this case, the presence of coeloconic sensilla seems to be restricted to the dorsal surface of the flagellum. These sensilla have a typical organization with a short cuticular shaft positioned inside a shallow depression. The shaft displays several grooves on its distal half, due to cuticular ridges that give to the sensillum a unique morphology (Figure 5.12 A-B). TEM cross-sections reveal a distal star-shaped structure (as a result of the external ridges) with nine-ten spikes (Figure 5.12 C). In between the ridges, there are spoke channels that connect the internal lumen with the external environment. The peg lumen shows two-three dendrites (Figure 5.12 C). Proximally, the cuticular shaft exhibits the typical double-walled organization delimiting an innermost lumen, housing two-three dendrites, and an outermost space (Figure 5.12 D). Under the socket, a thick dendrite sheath embeds the associated outer dendritic segments (two-three per sensillum), with a bulk of electron lucid vesicles present in this region (Figure 5.12 E). Sections carried out more proximally show dendrites still embedded by the dendrite sheath, but this appears less thick and tight (Figure 5.12 F).

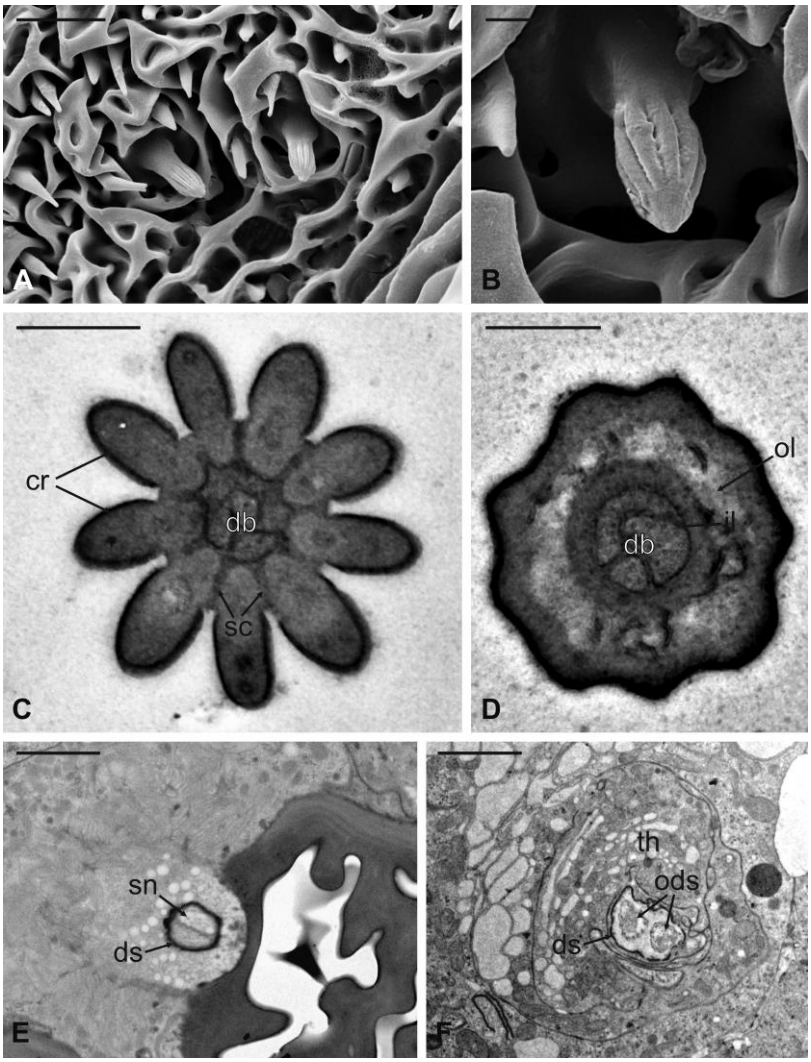


Figure 5.12. *Lipoptena fortisetosa* coeloconic sensilla. (A-B) SEM pictures with two coeloconic sensilla (cs) inserted on the flagellar wall, with distinct longitudinal ridges that define correspondent longitudinal grooves (B) originating at the distal half of the peg and reaching the tip where they converge and merge together. (C-D) Serial TEM sections of cs. (C) Distal section of cs at the groove level. Typical stellate structure related to the presence of cuticular ridges (cr) is visible. Between each cr, a spoke channel (sc), connecting the outside with the sensillum lumen, is visible. At this level, the sensillum lumen shows tree dendritic branches (db) of sensory neurons. (D) Proximal section of the same sensillum where the cr are not present. The internal double-walled organization of the cuticle appears clear, defining an outermost (ol) and an innermost lumen. This latter shows three db. (E) Cross section at the base of cs, where a thick dendrite sheath (ds) embeds two sensory neurons (sn). (F) Cross section of the two sn belonging to cs. At this level the ds is less compact and not so tightly associated with the outer dendritic segments (ods). The thecogen cell (th) envelops both structures. Bar scale: A 5 µm; B 1 µm; C, D 0.5 µm; E, F 2 µm.

## Discussion

The antennal apparatus of the described hippoboscids reveals a general similar arrangement in the structure of the segments in both sexes. In fact, because of their highly specialized parasitic lifestyle the main sensory area, the flagellum, is concealed inside the pedicel in order to protect the sensory area during the movements of the ectoparasites within the host coat. Generally, among hippoboscids the scape is not well recognizable, being partially or completely fused with the lunula [20]. Otherwise, it is also possible that this segment is not completely absent, but fused with the pedicel, as postulated for species of Nycteribiidae [25]. This hypothesis may be acceptable since in several other hippoboscid species, especially belonging to the subfamily Ornithomyinae, the three antennal segments are clearly separate. For example, in *P. canariensis* the scape is distinguishable and partially articulated with the fronto-clypeal region and the lunula, as also seen in the Nearctic louse flies *Olfersia fumipennis* (Sahlberg, 1886), *Ornithoica vicina* (Walker, 1849), *Ornithoctona erythrocephala* Leach, 1817, and *Icosta americana* (Leach, 1817) [20]. The articulation between the first two segments is likely due to the different host selection pressure that louse flies received during their adaptive process in comparison with other hippoboscid species living on mammals. In fact, the partial or total fusion between the scape and the pedicel, together with the introflexion of the flagellum, likely allowed the flies to reduce the antennal surface and consequently to mechanically protect these appendages inside the antennal sockets or fossae. As already mentioned, this morpho-functional organization protects the antennal sensory function of hippoboscids in a dense and harsh environment, such as the animal coat [19].

The morphology and location of antennae in Nycteribiidae and Streblidae (Hippoboscoidea) are quite similar to those of the four species studied in this paper. Representatives of these two families live on bats and, as do other hippoboscid species, establish a strong parasitic association with their hosts [26,27]. In fact, Nycteribiidae and Streblidae species display the reduction or absence of the first antennal segment, the scape, and the complete or partial introflexion of the flagellum inside the previous segment. However, in nycteribiids, the piriform flagellum differs from that of hippoboscids in the surface pattern and for the sensory pits, only in terms of position and number. In fact, the flagellar surface appears reticulose and bears only four sensory pits located on the dorsodistal part [28]. In contrast, in Streblidae the flagellum is different from nycteribiids, since it is not completely encapsulated inside the pedicel and shows a bilobate shape with a surface covered by minute spines [29], which are similar in arrangement to those observed in the described hippoboscid species.

Into the superfamily Hippoboscoidea, representatives of Glossinidae (tsetse flies) show a very different antennal apparatus compared to Hippoboscidae, Nycteribiidae, and Streblidae. In fact, Glossinidae are free-living parasites which do not establish a permanent association with the host, with antennae more similar to Muscomorpha [30]. In these hematophagous flies, antennae are always three-segmented with a well-developed external flagellum bearing a long arista which originates from the dorsoproximal part of the segment [31]. Thus, the arrangement of the antennae in tsetse flies is similar to those of higher dipterans, but these appendages are housed in a deeper antennal socket [32], which presumably protect the flagella during the trophic activity. This arrangement is comparable

to that of Hippoboscoidea and other parasites of veterinary importance, e.g., in the muscid flies *Haematobia irritans* (Linnaeus, 1758) [33] and *Hydrotaea irritans* (Fallén, 1823) [34]. Additionally, in hippoboscid species, the lack of the external flagellum may have led during the evolution process to the development of these sockets which could act as a funnel, directing external volatile compounds towards the sensilla located on the introflexed flagellum.

Noteworthy are the microtrichia which densely cover the antennal hollow of the four hippoboscid flies. These processes have been observed for their ultrastructure on *L. fortisetosa* and are not innervated. For this reason, microtrichia do not play a primary role in the sensory perception but, being furcated in two or three branches and forming a kind of dense carpet of hairs, could be involved in directing odors, conveying them towards the internal sensory area on the flagellum. In particular, in *H. equina* microtrichia are present also on the internal surface of the antennal fossa, lending support to this hypothesized role. Recently, six types of microtrichia, including branched ones, have been detected also on the flagellum of the tabanid fly *Haematopota pandazisi* (Krober, 1936) by Pezzi *et al*. [35], who postulated the role of these structures, together with different kinds of sensilla, in the sensory perception.

The introflexed flagellum is the main sensory area of the antenna, and its surface is covered by trabecular structures which resemble the above discussed microtrichia. Similarly, this reticulate surface could serve to maintain the odor cues, allowing porous chemosensilla, which are mainly present inside the sensory pits, to improve the perception of volatile stimuli. The assortment of flagellar sensilla is very similar in the four studied species since they display only two types of sensory structures: grooved coeloconic and basiconic sensilla. Their

arrangement on the flagellum is diverse, since grooved sensilla are more abundant and more present in the pits on the dorsodistal part of the flagellum, while basiconic sensilla are fewer and are mainly distributed in the anterior part of the segment, around the base of the arista. Additionally, the number and the arrangement of these two types of sensilla is different among the investigated flies. *Hippobosca equina* seems to display a higher number of sensory pits compared with the other three species, with most of these depressions housing coeloconic sensilla. In general, the richness of sensilla is related to the different lifestyle of ectoparasites; in fact, a permanent ectoparasite living in close association with its host does not require many receptors, while a temporary ectoparasite needs a major number of sensilla to frequently locate a new host [36,37]. For instance, *H. equina* has a higher number of sensilla compared with the other three hippoboscid species, and is not strictly associated with a single host specimen. In *Glossina morsitans,* flagellar sensilla show a more complex pattern compared with that of hippoboscid flies, displaying four types of sensilla: basiconic, coeloconic, trichoid, and intermediate sensilla [38]. Trichoid sensilla seem to be involved in sex pheromone transduction as demonstrated in *Drosophila melanogaster* [39,40]. The absence of this type of sensillum in the investigated hippoboscid flies, may be explained by the mating behavior of these dipterans. They mate usually when they have colonized the host microniches, although some species can mate on the wing [37]. In fact, since the wingless adults of both sexes live aggregated on the host body, it is possible to hypothesize that they do not require any pheromone to locate the partner.

In these ectoparasites, the prevalence of coeloconic sensilla compared with basiconic sensilla may be attributed to their function as

chemoreceptors. In *D. melanogaster,* coeloconic grooved sensilla are involved in the detection of ammonia, ketones, and amines [41]; moreover, these kinds of sensilla are able to perceive humidity variations, but not temperature changes [41]. Similarly, in *Anopheles gambiae* these sensilla are involved in ammonia detection [42]. However, coeloconic sensilla have also been classified as thermoreceptors, hygroreceptors, and carbon dioxide receptors in other orders of insects [43]. Further, a thermoreception role has been hypothesized in *Aedes aegypti* L. [44], and an olfactory and hygroreceptive function have been proposed for *Culicoides furens* (Poey) [45], which are hematophagous dipterans of medical and veterinary importance. These supposed functions may also be similar for hippoboscid flies. As a matter of fact, a previous work conducted in field using people with two heated bags to one shoulder and two cold bags to the other shoulder, showed that *L. cervi* winged adults usually landed on the hotter part, demonstrating that they were attracted to and perceived temperature at short distance [46]. As well, in a lab experiment carried out in arenas, two species of Nycteribiidae, *Penicillidia conspicua* Speiser, 1901 and *P. dufourii* (Westwood, 1835), were found to be more active moving more often towards the heat source; although they responded more strongly to the combination of carbon dioxide and heat stimuli [47].

Regarding basiconic sensilla present on the flagellum, our investigations showed that their number is lower compared with that of coeloconic sensilla. This is consistent in all the observed species, and mainly in *H. equina,* where the distribution of these sensilla has been mapped, highlighting the higher abundance of coeloconic compared with basiconic sensilla [21]. Basiconic sensilla occur in higher density on the flagellum of Glossinidae flies [38], as well as of other

hematophagous dipterans, such as *Stomoxys calcitrans* [48]. Similarly, basiconic sensilla are more represented in some saprophagous species [49-52], as well as in phytophagous dipterans like fruit flies [53,54]. Although there are a few electrophysiological studies about the role played by basiconic sensilla, it is known that they are mainly involved in odor detection due to the presence of many pores on the external wall [55]. The multiporous basiconic sensilla occurring on *L. fortisetosa* differ from those described in other dipterans [34,35,54,56], since the ultrastructure shows a reduction in the presence of the wall pores, which occur only in the distal half of the shaft. The limited number of pores on the basiconic walls could be due to the perception of the host odors which is activated just when the parasite is approaching the host at short-medium distance. On the other hand, previous studies conducted on different families of hematophagous dipterans revealed that the main stimuli activating the response at long-distance are carbon dioxide, ammonia, or other volatile substances [22,44,45]. In fact, electrophysiological studies demonstrated that neurons associated with coeloconic sensilla were activated by different kinds of external stimuli, such as $CO_2$, temperature, and humidity, in many species of insects [57,58]. A relevant feature of the basiconic sensilla in *L. fortisetosa* is the presence of a single sensory neuron that innervates each sensillum. The occurrence of a relatively low number of neurons associated with multiporous sensilla is reported for other species belonging to different insect orders. In the planthopper *Hyalestes obsoletus* Signoret, a grooved peg sensillum coeloconicum has been observed at the level of the expanded base of the thread-like flagellum. This sensillum is innervated by a single sensory neuron that is highly branched inside the sensory peg, for which a role in $CO_2$ perception is

hypothesized [59]. Within Diptera, sensilla basiconica are described in detail in *D. melanogaster*, where different types of sensilla are present: small sensilla basiconica (innervated by two sensory neurons) and large sensilla basiconica (innervated by up to four sensory neurons) [60,61]. In *D. melanogaster,* such diversity is related to the high antennal sensitivity to volatile compounds and the gradient distribution pattern of antennal sensilla on the funiculus. In *L. fortisetosa,* we hypothesize that the great reduction in number and size of sensilla basiconica, as well as in the number of associate sensory neurons, could be linked to the reduced range of volatiles exploited during intra- and interspecific interactions.

The arista was unarticulated in all the four investigated hippoboscids. This structure shows a remarkable difference in the morphology of the distal part, being furcate (*L. cervi*), fan-shaped (*L. fortisetosa*), branched (*H. equina*), or spatulate (*P. canariensis*). According to our knowledge, currently, the fusion of the arista with the flagellum is highlighted for the first time in dipterans. This arrangement may be due to the particular adaptation evolved during the introflexion of the flagellum inside the pedicel. Although the arista tips are so diverse, our investigations performed on *L. fortisetosa* revealed that no sensilla on the external surface, nor are cuticular pores present. The lack of external sensory structures differs from that observed on the arista of the human bot fly, *Dermatobia hominis* (Linnaeus Jr. in Pallas, 1781), which showed different kinds of receptors, such as long bristles, coeloconic, and styloconic sensilla [62]. Additionally, on the arista (properly termed as stylus) of the marsh fly *Sepedon fuscipennis* Loew 1859, mechanosensilla arranged differently in females and males may have possible functions related to mating and foraging behaviour [63].

Ultrastructural investigations on *L. fortisetosa* showed that two bundles of sensory neurons are present close to the base of the arista, with an apparent lack of connection with the external cuticle. Similar findings have been reported for *D. melanogaster*, *Calliphora erythrocephala* (Masquart, 1834), and *Musca domestica* Linnaeus, 1758. Here, the arista ultrastructure is much more complex compared with *L. fortisetosa*, and houses a variable number of aberrant sensilla that lie freely in the haemolymph, with a possible thermoreceptive function [64]. It is noteworthy that the number of these unusual sensilla increases with the increase in the arista size, and this could explain the low number of sensory neurons recorded in *L. fortisetosa* (five) compared with the amount found in *D. melanogaster* (six), *M. domestica*, and *C. erythrocephala*, (24 and 36 respectively) [64]. It is conceivable that the aristal sensilla in *L. fortisetosa* could be involved in the perception of temperature variation, a key factor in the detection and discrimination of the warm-blood hosts exploited by these ectoparasites.

Sensillar pattern of the studied hippoboscid flies reveals how the host location process in these ectoparasites is quite complex; in fact, the abundance of coeloconic grooved sensilla supports the long-distance activation of the newly emerged winged adults, which should be mainly stimulated by ammoniacal substances and carbon dioxide. Additionally, we can speculate that, once the principal odour stimulus is detected, other factors may guide the adult towards the host. Heat and colour take part in the host location at medium and short distances, as reported for *L. cervi* [46] and *L. fortisetosa* [65].

## Conclusion

Morphological investigations carried out by SEM and TEM on four hippoboscid species revealed a strong adaptation in the antennal apparatus due to the parasitic lifestyle of these flies. In particular, the main sensory area, the flagellum, is concealed inside the pedicel. This latter is fused with the first antennal segment, the scape, in *L. cervi*, *L. fortisetosa* and *H. equina*, while it is partially articulated in *P. canariensis*. The arista appears differently shaped in the species studied and is not articulated with the flagellum, which is a unique feature in dipterans. The flagellum bears two different types of sensory structures: grooved coeloconic and basiconic sensilla. The number and the arrangement of these sensilla is quite different among the species according to their life cycle and association level with the hosts. However, a prevalence of coeloconic sensilla has been highlighted in all the investigated hippoboscids. These structures are generally involved in volatile detection, such as $CO_2$, ammonia, and other odors, but they can also play a role in perceiving changes in humidity and temperature. Similarly, the arista could be involved in the detection of temperature variations, since, in *L. fortisetosa,* it houses peculiar sensory neurons.

Finally, we hypothesize that locating hosts at medium and long distance in winged adults hippoboscids, occurs mainly due to these sensilla; although it is a complex process that involves visual stimuli as well. Since basiconic multiporous sensilla are present in few numbers and display a reduction in the abundance of wall pores along the shaft, they probably play a role in the host location at medium-short distances. However, further experiments, such as electrophysiological and behavioral bioassays, are needed to confirm these hypotheses.

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# 6. Distribution of deer keds (Diptera: Hippoboscidae) in free-living cervids of the Tuscan-Emilian Apennines, central Italy, and establishment of the allochthonous ectoparasite *Lipoptena fortisetosa*

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## Simple Summary

In recent years, the increased presence of wildlife in habitats close to urban settlements has raised concerns about the risk of pathogen transmission from wild animals to humans due to the spread of different parasites. For this reason, a survey aimed at describing the dispersal and parasitism level of two cervid ectoparasites was carried out in the northern Apennines, in central Italy. The presence of two hippoboscids, the autochthonous *Lipoptena cervi* and allochthonous *L. fortisetosa*, native to Eastern Asia and recently recorded in Italy, were assessed on their main host species (red deer, fallow deer, and roe deer), considering host sex and age. The alien species *L. fortisetosa* was found to be widespread in the study area, most likely competing with *L. cervi*. Moreover, red deer seemed to be the favored host of both flies, with differences in sex and age class preferences. This study demonstrated the importance of regularly monitoring the populations of these parasites, especially the invasive species, due to the risks to human health, as these insects are potential vectors of pathogens.

## Abstract

*Lipoptena fortisetosa* and *L. cervi* are hematophagous ectoparasites belonging to the Hippoboscidae family and preferentially living on cervids. In recent years, they have received specific attention due to the great increase in the abundance of their host species, and to their medical and veterinary importance as possible vectors of pathogens harmful to humans and animals. The aim of this study was to investigate the parasitism level of both of these flies on their main hosts in Italy, which are red deer, fallow deer, and roe deer, and to highlight a possible preference for a species, sex, or age class among the hosts. Deer keds were collected by examining 326 cervids hunted in the Tuscan-Emilian Apennines. Outcomes showed that *L. fortisetosa* has greatly spread throughout the study area, where it competes with the autochthonous *L. cervi*. Moreover, red deer was the favored host species of both ectoparasites, while different preferences for host sex and age classes were observed in the two hippoboscids. The regular monitoring of deer ked populations, especially the allochthonous *L. fortisetosa*, which is continuously spreading in Europe, is recommended to expand the knowledge on these parasitic species that are potentially dangerous to public health.

## Introduction

In recent years, a substantial expansion range of free-living ungulate species has occurred in many European countries [1], including Italy [2,3] and particularly the Tuscany region in the northern Apennines [4], leading to a consequent increase in the abundance of their ectoparasites, which can potentially colonize and adapt to new territories and host species.

Particularly notable is the spread of allochthonous ungulates and their ectoparasites in new countries, which demonstrates the adaptability of some alien species with the consequent risk of competition with native animals and a compromised ecosystem balance. In this respect, both the hippoboscid *Lipoptena fortisetosa* Maa, 1965 and its original host *Cervus nippon* (sika deer) have been recently reported in Italy [5,6].

Members of the genus *Lipoptena* (Diptera: Hippoboscidae) are obligate hematophagous ectoparasites that permanently live on a restricted range of hosts, especially Cervidae [7,8]. These flies attack several species, referred to as "accidental hosts" or "feeding hosts", that are used as food sources only, but they are able to successfully thrive only on a few mammals, referred to as "definitive hosts" or "breeding hosts", which have the requisites to guarantee the reproduction and survival of these flies [9,10].

Parasites establish a close association with their suitable hosts through morphological and physiological adaptations [11,12]. Females are viviparous and give birth to fully grown larvae, one at a time, that thereafter pupate and fall from the host to the ground due to the deers' movements. Reproduction occurs all-year-round, but the emergence of newly winged adults takes place from late spring to autumn. Flies spend the first period as imago searching for a suitable host to settle on for their whole life. Subsequently, they crawl into the fur of the animal and gradually shed wings through a horizontal predetermined breaking line as a result of their passage between the hairs of the host [10]. When the ectoparasites become wingless, they are no longer able to fly, making it quite difficult to switch to other subjects; thus, they strictly depend on the selected host. Nevertheless, moving to other specimens is possible, especially from cervid females

to their fawns and vice versa, or moving can occur during allogrooming behavior among deer [13].

In Italy, the species of the Lipopteninae subfamily infesting deer are *Lipoptena cervi* (Linnaeus, 1758) and *L. fortisetosa* (named deer keds). In this country, this adventive ectoparasite has also been collected from other cervid species, demonstrating its ability to successfully colonize different hosts [6]. *Lipoptena cervi* is widely distributed in more than 20 European countries [14] and has spread across North America since the beginning of the twentieth century [15]. *Lipoptena fortisetosa* is native to Japan but has spread, as far as we know, to at least 12 European countries [16], including Italy. Both of these species show a preference for parasitizing Cervidae: *L. cervi* has been recorded on *Cervus elaphus*, *Dama dama*, *Alces alces*, *Capreolus capreolus*, and *Moschus moschiferus*, while *L. fortisetosa*, although it was considered quite restricted to its original host, *Cervus nippon*, has also been collected from *Cervus elaphus*, *Capreolus pygargus* and *Capreolus capreolus* [15,17,18]. *Lipoptena cervi* and *L. fortisetosa* can concurrently be found on the same deer [6,19], together with ticks. Hippoboscids can heavily infest hosts, compromising them physically and behaviorally [20,21]. Hosts are annoyed by keds, especially because of their movements in the hosts' fur and their recurrent feeding on blood, up to 20 times per day for each fly of both sexes. Severe attacks are mainly documented on moose, on which tens of thousands of *L. cervi* have been collected from a single individual host [20,22]. Such infestations directly harm the hosts, causing issues such as skin inflammation, injuries, and blood loss leading to possible secondary infections. Observing the discoloration caused by fresh blood loss in moose bedding sites, Kaunisto *et al.* [23] showed that a high number of deer keds can cause

bleeding in their hosts, leading to capillary vein and skin damage. In the case of extreme harassment, physiological and behavioral changes can be observed in reindeer, as reported by Kynkäänniemi *et al.* [21]. These authors verified that heavy parasitism induces hosts to react with defense actions, such as shaking their head and body or stamping feet, reducing the time spent grazing and causing a decrease in body weight and welfare.

Usually, hippoboscids infest animal hosts, but they can also bite humans, creating a consequent health risk, which needs to be verified with further studies, that these insects may transmit some zoonotic pathogens [24–30]; however, no overt form of these diseases has yet been detected in deer hosts. Moreover, the bites of deer keds on humans can result in persisting and itching papules, in addition to dermatitis [31–34]. In countries where ked density is particularly high, people frequenting forests and natural areas complain of the great nuisance of keds, which ultimately leads to a reduction in recreational activities and hunting in this habitat [32].

Understanding the relationship between animals and their parasites is crucial; in fact, the spread of deer keds seems to be strongly related to the availability and density of potential hosts, whose spatiotemporal variation is considered one of the most important factors affecting the dispersal of ectoparasites [35]. Other factors also affect the presence and distribution of these insects, such as cold tolerance, habitat, and predation [22]. In particular, climate change is suggested to support the increase in deer ked populations, since temperature has a positive effect on the duration of the host-seeking period, extending the possibility of host acquisition [36,37]. The risks related to the general increase in the density of cervid populations and

global warming, which could facilitate the expansion of ectoparasites, make further investigations on these flies necessary.

*Lipoptena cervi* and *L. fortisetosa* have been recently investigated under different points of view, such as morphology, distribution, or disease transmission, but no studies on the parasitic dynamics of these flies related to their hosts have been carried out in Italy to date.

The aim of this research was to provide an insight into the presence and epidemiology of *L. fortisetosa* on cervids (*Cervus elaphus*, *Dama dama*, and *Capreolus capreolus*) in some areas of the Tuscan-Emilian Apennines, central Italy, and any differences in infestation with respect to the native parasite, *L. cervi*.

In particular, the goals were to investigate (a) the distribution of these parasitic flies in the study area; (b) the level of infestation on the three examined ungulate species; (c) the possible preference of the ectoparasites for a definite host species; and (d) the potential predilection of deer keds toward host sex and age class.

## Material and methods

### Study area

Samples were collected in the Tuscan-Emilian Apennines (central Italy) from an area extending along approximately 207 km of surrounding territories encompassing four provinces of Tuscany (Arezzo, Florence, Pistoia, and Prato) and three provinces of Emilia Romagna (Bologna, Modena, and Reggio Emilia). In the study area, the landscape is characterized by different altitudes, with hills and mountains ranging from 500 to a maximum of approximately 2000 m. This large region is covered by a variety of vegetation zones, and inhabited areas of different extents intersect valleys. In general, large

cervid populations, managed through wildlife hunting programs, exist in the study area.

### Sample collection and taxonomic identification of Hippoboscidae

Deer keds were collected from the fur of free-ranging cervids of three different species: *Cervus elaphus*, *Capreolus capreolus*, and *Dama dama* (Figure 6.1).

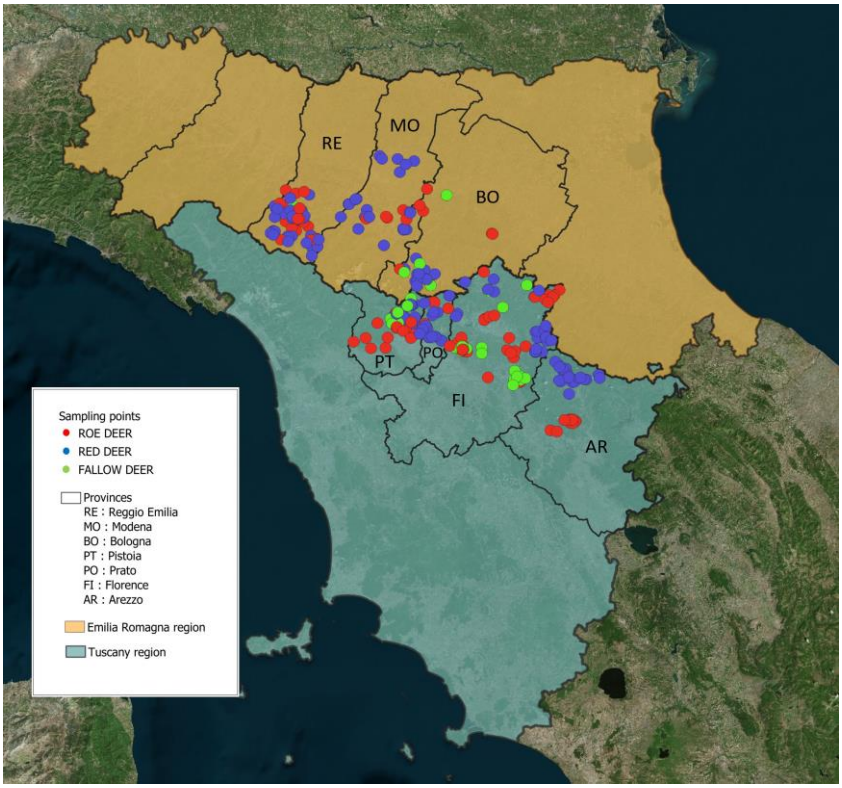


Figure 6.1. Map of host sampling sites in the Tuscan-Emilian Apennines (central Italy). (Map created using the Free and Open Source QGIS).

All ectoparasites were obtained from animals hunted during the culling seasons of 2018–2020. The examined cervids were sampled almost continuously, depending on the specific hunting period, from November 2018 to March 2020, except for May 2019. A total of 326 animals (181 red deer, 107 roe deer, and 38 fallow deer) were

sampled, and insects were picked off from two pieces of cervid skin that were voluntarily provided by hunters and local wildlife technicians, who were carefully instructed of this process before the hunting period. They were required to cut two skin samples, 20 × 20 cm each, one from the neck and the other from the groin region [10,38], and to store them in a plastic bag at −20°C until they were transferred to the staff of the Department of Agriculture, Food, Environment and Forestry (DAGRI), University of Florence.

Each skin sample was accompanied by a form containing detailed information such as animal species, sex, and age class. Age classes were the following:

- Fawn (<1 year for all cervid species);
- Subadult (between 1 and 4 years for fallow deer; 1 and 2 years for row deer; 1 and 3 years for female red deer and 1 and 5 years for male red deer);
- Adults (>4 years for fallow deer; >2 years for roe deer; >3 years for female red deer and >5 years for male red deer).

Fur samples were thawed and then visually examined for deer keds. All the parasites were manually removed with forceps and morphologically observed under a stereomicroscope (Leica/Wild MZ16, equipped with an L2 illuminator; Leica Microsystems, Wetzlar, Germany) for taxonomic identification using keys and previously described characters [6,9,39,40]. Subsequently, all the insects were separated by species and sex, counted, and stored in 70% ethanol or frozen at −20°C, pending further analyses.

All cervid handling procedures followed the regional, national, and institutional guidelines.

Data were transcribed and reported into different software programs: QGIS 3.16.3 Hannover (QGIS Geographic Information

System, open source software available at http://www.qgis.org, accessed on 25 September 2021); GRASS 7.8.5 (Geographic Resources Analysis Support System, open source software available at grass.osgeo.org, accessed on 25 September 2021), and Excel (Microsoft 365, 2016, Microsoft Italia, Milano, Italy) to be used for further analyses.

From here onward, any mention of parasites per host animal is always referred to as the sum of the keds collected from the two skin samples described above.

### Parasitological index

The infestation of deer keds on the three different host species was described using parasitological indices according to Margolis *et al*. [41]. All the descriptors were stratified by host and ectoparasite species. The distribution of *Lipoptena* spp. was evaluated by the parasitological index of density (average number of parasites per unit area ($cm^2$) of the host body), prevalence (percentage of infested deer); abundance (average number of parasites per host), mean intensity (average number of parasites per infested host), and minimum and maximum intensity (*I*min-*I*max). Moreover, the variance-to-mean ratio (variance of infestation divided by mean abundance) was calculated as the aggregation index, considering the distribution as overdispersed (or aggregated) if the value was >1, as demonstrated by Barbour and Pugliese [42].

### Statistical analyses

Data were statistically analyzed using R software [43]. Preliminary analysis highlighted aggregated parasite distribution; therefore, generalized linear models (in particular negative binomial regression)

with the abundance of each parasite species as the dependent variable were built using the MASS package [44–46].

First, the chi-square test was used to compare parasite prevalence among the three host species.

Differences in parasite abundance among the three host species were evaluated using a univariable model to determine the primary host species; therefore, multivariable models were used to evaluate the influence of host-related variables on parasite abundance in the formerly determined host species.

## Result

Out of the 326 examined cervids, 287 harbored deer keds (88.0%). The morphological analyses of the 23,074 collected flies revealed the presence of two hippoboscid species, identified as *L. fortisetosa* and *L. cervi*. Of the total insects, 18,441 were *L. fortisetosa*, and 4633 were *L. cervi*; even though the total highest number of insects was *L. fortisetosa*, some host animals were more infested with *L. cervi*. Of the total examined hosts, 127 cervids carried both the *Lipoptena* species, while 26 out of 107 tested roe deer, 45 out of 181 tested red deer, and two out of 38 tested fallow deer did not harbor any parasites. The data on the overall deer ked infestations in the three host species are given in Table 6.1.

Table 6.1. Epidemiological descriptors of *Lipoptena* spp. infestation on different hosts.

| Host Species | Total Number of Deer Keds Collected on Infested Hosts | Density (n. Parasites/cm$^2$) | Prevalence of Infestation % (n. Infested Hosts/n. Tested Hosts) | Prevalence of Mixed Infestation % (n. Hosts Infested by Both Parasites/n. Tested Hosts) | Mean Abundance (n. Parasites/n. Tested Hosts) | Mean Intensity of Infestation (±sd) (n. Parasites/n. Infested Hosts) | Intensity of Infestation (Imin-Imax) |
|---|---|---|---|---|---|---|---|
| Red deer ($n$ = 181) | 21,548 | 0.149 | 93.9 | 52.5 | 119.05 | 126.75 (±235.67) | 0–1,844 |
| Fallow deer ($n$ = 38) | 645 | 0.021 | 94.7 | 21.0 | 16.97 | 17.92 (±37.37) | 0–214 |
| Roe deer ($n$ = 107) | 881 | 0.010 | 75.7 | 22.4 | 8.23 | 10.88 (±16.04) | 0–123 |

The number of males and females of the two ectoparasites, together with the values of the parasitological parameters for each of the three host species, are reported in Table 6.2.

The highest number of deer keds on a host subject was found in red deer, while the number of parasites obtained from roe deer and fallow deer was much lower. A maximum of 1,844 *L. fortisetosa* were collected from a host specimen, while a maximum of 398 flies of *L. cervi* were picked off a single red deer.

The chi-square test highlighted significant differences among the prevalence of both parasite species in the three hosts ($p = 0.000$ and $p = 0.000$, respectively for *L. fortisetosa* and *L. cervi*). In particular, fallow deer showed the highest prevalence for *L. fortisetosa* (94.8%), while it displayed the lowest prevalence value for *L. cervi* (21.5%). Although fallow deer was infested more often with *L. fortisetosa* than the other two hosts, the abundance of this parasite was highest for red deer, as confirmed by the univariable negative binomial regression ($p = 0.000$). Additionally, *L. cervi* abundance was higher in red deer than in the other two host species ($p = 0.000$). Notably, only 17 *L. cervi* were found on the 38 analyzed fallow deer (Table 6.2).

The aggregation index was >1 for all the cervid species for both keds, meaning that the parasites were aggregated over the host populations, as illustrated in Figure 6.2. Further details on the epidemiological parameters stratified by the sex and age of the hosts are provided in Table 6.3.

Table 6.2. Epidemiological descriptors of the ectoparasites *Lipoptena fortisetosa* and *Lipoptena cervi* on their three main hosts in central Italy.

| Deer Keds and Host Species | Total Number of Deer Keds Collected on Infested Hosts and Sex Ratio | Density (n. Parasites/cm$^2$) | Prevalence of Infestation % (n. Infested Hosts/n. Tested Hosts) | Mean Abundance (n. Parasites/n. Tested Hosts) | Mean Intensity of Infestation (±sd) (n. Parasites/n. Infested Hosts) | Intensity of Infestation (*I*min-*I*max) | Aggregation Index (Variance/Mean Abundance) |
|---|---|---|---|---|---|---|---|
| *Lipoptena fortisetosa* | | | | | | | |
| Red deer ($n$ = 181) | 17,194 (6999/10,195) | 0.118 | 71.3 | 94.99 | 133.28 (±243.88) | 0–1,844 | 500 (>1) |
| Fallow deer ($n$ = 38) | 628 (331/297) | 0.021 | 94.7 | 16.53 | 17.44 (±37.96) | 0–214 | 83 (>1) |
| Roe deer ($n$ = 107) | 619 (345/274) | 0.007 | 53.3 | 5.79 | 10.86 (±19.80) | 0–123 | 50 (>1) |
| *Lipoptena cervi* | | | | | | | |
| Red deer ($n$ = 181) | 4354 (1610/2744) | 0.030 | 75.1 | 24.06 | 32.01 (±57.39) | 0–398 | 111 (>1) |
| Fallow deer ($n$ = 38) | 17 (9/8) | 0.001 | 21.0 | 0.45 | 2.12 (±2.03) | 0–7 | 3.5 (>1) |
| Roe deer ($n$ = 107) | 262 (109/153) | 0.003 | 44.9 | 2.45 | 5.46 (±7.3) | 0–37 | 12.5 (>1) |

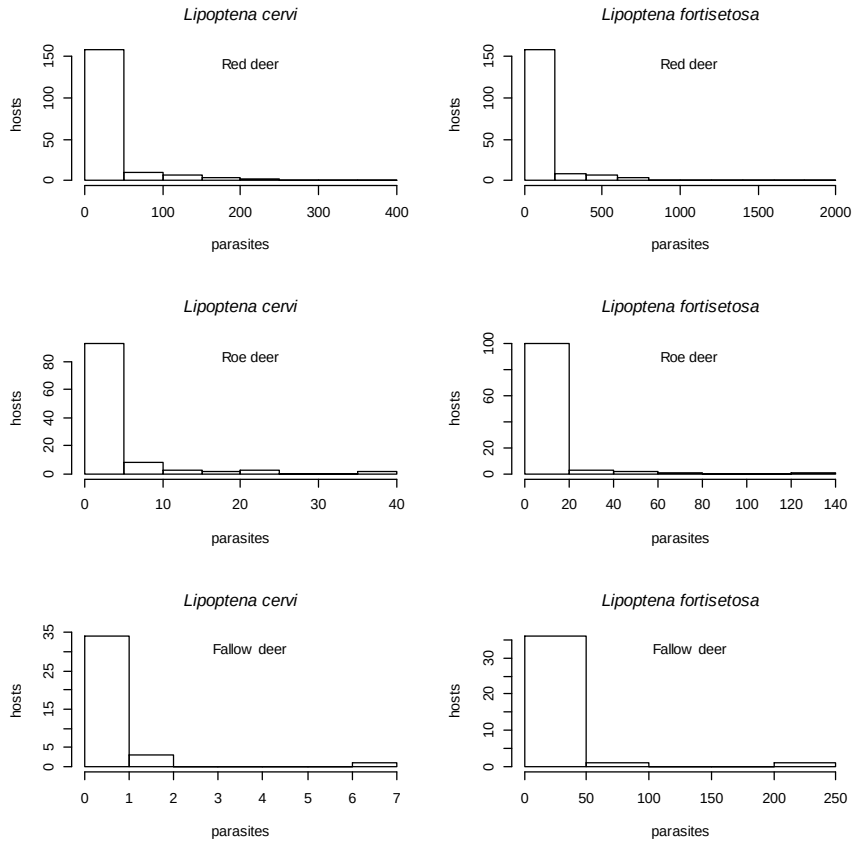


Figure 6.2. Histograms of parasite distribution: both parasite species are aggregated on all three host species.

Table 6.3. Epidemiological descriptors, stratified by host sex and age class, of the ectoparasites *Lipoptena fortisetosa* and *Lipoptena cervi* on their three main hosts in central Italy.

| Deer Ked Species | Host Species and Sex | Age Class and Number of Sampled Hosts | Total Number of Deer Keds Collected on Infested Hosts | Density (n. Parasites/cm$^2$) | Prevalence of Infestation % (n. Infested Hosts/n. Tested Hosts) | Mean Abundance (n. Parasites/n. Tested Hosts) | Mean Intensity of Infestation (± sd) (n. Parasites/n. Infested Hosts) | Intensity of Infestation (Imin-Imax) | Aggregation Index (Variance /Mean Abundance) |
|---|---|---|---|---|---|---|---|---|---|
| *Lipoptenafortisetosa* | | | | | | | | | |
| | Red deer | | | | | | | | |
| | female | *adult (n = 49)* | 6274 | 0.16 | 71.4 | 128.04 | 179.26 (±293.29) | 0–1,048 | 500 (>1) |
| | | *subadult (n = 11)* | 1050 | 0.12 | 81.8 | 95.45 | 116.67 (±158.88) | 0–419 | 250 (>1) |
| | | *fawn (n = 20)* | 2714 | 0.17 | 60.0 | 135.70 | 226.17 (±397.09) | 0–1,311 | 1000 (>1) |
| | male | *adult (n = 36)* | 773 | 0.03 | 63.9 | 21.47 | 33.61 (±58.02) | 0–195 | 100 (>1) |
| | | *subadult (n = 49)* | 5078 | 0.13 | 73.5 | 103.63 | 141.06 (±330.45) | 0–1844 | 2.8 (>1) |
| | | *fawn (n = 16)* | 1305 | 0.1 | 87.5 | 81.56 | 93.21 (±133.68) | 0–474 | 200 (>1) |
| | Fallow deer | | | | | | | | |
| | female | *adult (n = 8)* | 73 | 0.01 | 100.0 | 9.13 | 9.13 (±15.06) | 1–46 | 25 (>1) |
| | | *subadult (n = 3)* | 22 | 0.01 | 100.0 | 7.33 | 7.33 (±4.51) | 3–12 | 2.8 (>1) |
| | | *fawn (n = 0)* | | | | | | | |
| | male | *adult (n = 10)* | 288 | 0.04 | 100.0 | 28.80 | 28.8 (±65.36) | 1–214 | 142.9 (>1) |
| | | *subadult (n = 12)* | 203 | 0.02 | 91.7 | 16.92 | 18.45 (±27.75) | 0–95 | 43.4 (>1) |
| | | *fawn (n = 5)* | 42 | 0.01 | 80.0 | 8.40 | 10.50 (±9.95) | 0–23 | 11.1 (>1) |
| | Roe deer | | | | | | | | |
| | female | *adult (n = 25)* | 67 | 0.003 | 36.0 | 2.68 | 7.44 (±13.46) | 0–43 | 27.8 (>1) |
| | | *subadult (n = 10)* | 62 | 0.01 | 50.0 | 6.20 | 12.4 (±14.88) | 0–37 | 22.7 (>1) |
| | | *fawn (n = 9)* | 3 | 0.0004 | 22.2 | 0.33 | 1.5 (±0.71) | 0–2 | 1.5 (>1) |
| | male | *adult (n = 37)* | 360 | 0.01 | 64.9 | 9.73 | 15 (±27.84) | 0–123 | 50 (>1) |
| | | *subadult (n = 14)* | 80 | 0.01 | 78.6 | 5.71 | 7.23 (±7.78) | 0–27 | 10 (>1) |
| | | *fawn (n = 12)* | 47 | 0.0005 | 50.0 | 3.92 | 7.83 (±7.68) | 0–21 | 10 (>1) |
| *Lipoptena cervi* | | | | | | | | | |
| | Red deer | | | | | | | | |
| | female | *adult (n = 49)* | 738 | 0.02 | 83.7 | 15.06 | 18 (±31.02) | 0–171 | 50 (>1) |
| | | *subadult (n = 11)* | 47 | 0.01 | 36.4 | 4.27 | 11.75 (±6.60) | 0–19 | 11.1 (>1) |
| | | *fawn (n = 20)* | 45 | 0.003 | 45.0 | 2.25 | 5 (±5.77) | 0–19 | 10 (>1) |
| | male | *adult (n = 36)* | 1006 | 0.03 | 77.8 | 27.94 | 35.93 (±54.88) | 0–204 | 83.3 (>1) |
| | | *subadult (n = 49)* | 2453 | 0.06 | 91.8 | 50.06 | 54.51 (±80.48) | 0–398 | 125 (>1) |
| | | *fawn (n = 16)* | 65 | 0.01 | 56.3 | 4.06 | 7.22 (±6.04) | 0–18 | 7.7 (>1) |
| | Fallow deer | | | | | | | | |
| | female | *adult (n = 8)* | 7 | 0.001 | 12.5 | 0.88 | 7 | 0–7 | 7.1 (>1) |
| | | *subadult (n = 3)* | 0 | | | | | | |
| | | *fawn (n = 0)* | | | | | | | |
| | male | *adult (n = 10)* | 2 | 0.0003 | 20.0 | 0.2 | 1 | 0–1 | 0.91 (<1) |
| | | *subadult (n = 12)* | 8 | 0.001 | 41.7 | 0.67 | 1.6 (±0.55) | 0–2 | 1.2 (>1) |
| | | *fawn (n = 5)* | 0 | | | | | | |
| | Roe deer | | | | | | | | |
| | female | *adult (n = 25)* | 147 | 0.01 | 52.0 | 5.88 | 11.31 (±11.4) | 0–37 | 16.7 (>1) |
| | | *subadult (n = 10)* | 15 | 0.002 | 30.0 | 1.5 | 5 (±4.58) | 0–10 | 7.1 (>1) |
| | | *fawn (n = 9)* | 8 | 0.001 | 55.6 | 0.89 | 1.6 (±1.34) | 0–4 | 1.8 (>1) |
| | male | *adult (n = 37)* | 46 | 0.002 | 43.2 | 1.24 | 2.87 (±3.03) | 0–10 | 4.8 (>1) |
| | | *subadult (n = 14)* | 22 | 0.002 | 35.7 | 1.57 | 4.4 (±3.29) | 0–8 | 5.3 (>1) |
| | | *fawn (n = 12)* | 24 | 0.003 | 50.0 | 2 | 4 (±3.69) | 0–11 | 5.3 (>1) |

The multivariable negative binomial regression, taking into consideration sex and age, was constructed for red deer only since it was the primary host species for both parasites. The results are reported in Tables 6.4 and 6.5 for *L. cervi* and *L. fortisetosa*, respectively. *Lipoptena cervi* was significantly less abundant in females than in males and in fawns than in subadults, while *L. fortisetosa* was significantly less abundant in adults than in subadults.

Table 6.4. Results of the multivariable negative binomial model with *Lipoptena cervi* as the dependent variable and red deer age and sex as covariates.

| *Lipoptena cervi* | Coefficient | Std. Error | z | *p* Value |
|---|---|---|---|---|
| Intercept | 3.913 | 0.233 | 16.801 | 0.000 |
| Sex | | | | |
| Male | Reference | | | |
| Female | −2.461 | 0.563 | −4.382 | 0.000 |
| Age | | | | |
| Subadult | Reference | | | |
| Adult | −0.583 | 0.359 | −1.626 | 0.104 |
| Fawn | −2.511 | 0.484 | −5.186 | 0.000 |
| Interactions | | | | |
| Adult-Male | Reference | | | |
| Adult-Female | 1.843 | 0.667 | 2.763 | 0.006 |
| Fawn-Female | 1.870 | 0.806 | 2.320 | 0.020 |

Table 6.5. Results of the multivariable negative binomial model with *Lipoptena fortisetosa* as the dependent variable and red deer age and sex as covariates.

| *Lipoptena fortisetosa* | Coefficient | Std. Error | z | *p* Value |
|---|---|---|---|---|
| Intercept | 4.641 | 0.324 | 14.324 | 0.000 |
| Sex | | | | |
| Male | Reference | | | |
| Female | −0.082 | 0.757 | −0.109 | 0.914 |
| Age | | | | |
| Subadult | Reference | | | |
| Adult | −1.574 | 0.499 | −3.155 | 0.002 |
| Fawn | −0.240 | 0.653 | −0.367 | 0.714 |
| Interactions | | | | |
| Adult-Male | Reference | | | |
| Adult-Female | 1.868 | 0.906 | 2.061 | 0.039 |
| Fawn-Female | 0.591 | 1.073 | 0.551 | 0.582 |

The interactions between sex and age classes were significant for both parasite species, as evident in Figure 6.3. The plots of the residuals in Figure 6.4 show a good residual pattern, with similar residual distributions across the levels of the predicted values.

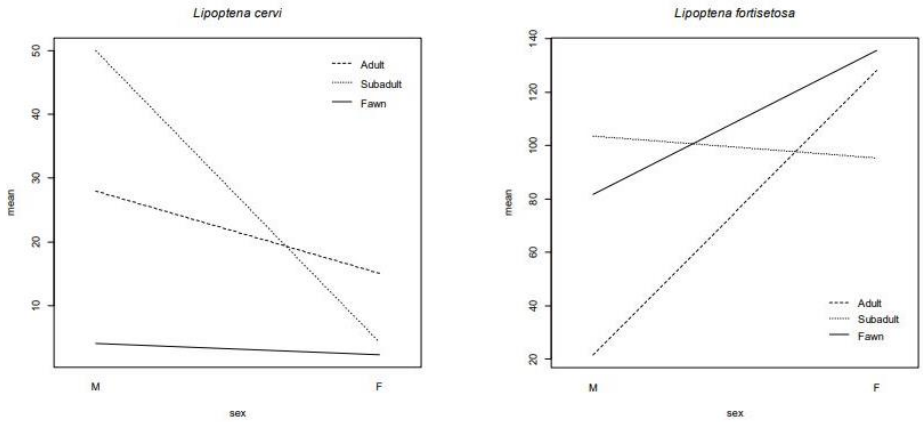


Figure 6.3. Interactions between sex and age in determining the mean abundance of *Lipoptena cervi* and *Lipoptena fortisetosa* on red deer.

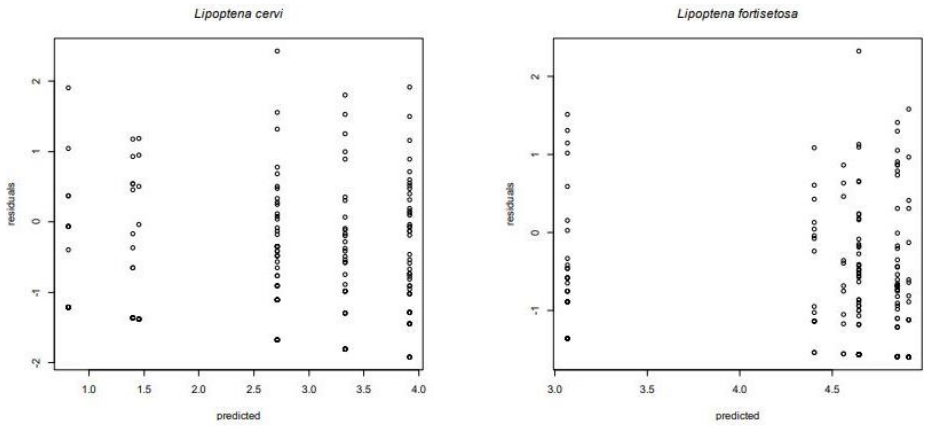


Figure 6.4. Plot of residuals vs. predicted values of the multivariable model with *Lipoptena cervi* and *L. fortisetosa* as the dependent variable and red deer age and sex as covariates.

## Discussion

This study documents the presence of both native *L. cervi* and allochthonous *L. fortisetosa* in the Tuscan-Emilian Apennines (central Italy). These hippoboscids have already been documented in Italy, but the literature is still limited to local areas [6]. *Cervus elaphus*, *C. capreolus,* and *D. dama* were hunted and sampled for ectoparasites, revealing a considerable distribution of these flies. All three host species were infested with both parasites, showing the adaptability of these parasites to the examined cervids. Although *L. cervi* seems to have a greater worldwide distribution than that of *L. fortisetosa* [47], our survey proves that locally allochthonous species may be were largely more abundant than autochthonous species, demonstrating that the introduced *L. fortisetosa* is numerous in the study area and strongly competes with native hippoboscids which not only live in the same geographic territories but also share the same host species. Our study confirms the coexistence of *L. cervi* and *L. fortisetosa* in the same area, as evidenced in other European regions, such as northeastern Poland and Lithuania [19,48]. Moreover, *L. fortisetosa* was found to share the same host with other dipteran ectoparasite species in Japan, where it was sampled on Japanese deer with *Lipoptena sikae* [49].

Although many cervid species have been reported as suitable definitive hosts for *L. cervi*, red deer and moose seem to be the favored species in Europe, while the Japanese deer, *C. nippon*, is considered the main and original host for *L. fortisetosa* [15]. On red deer, *L. cervi* can reach a very high frequency of infestation, ranging between 78% and 100% [19,50], while it is less abundant on fallow deer [10]. A heavy infestation of *L. cervi* in four hunted roe deer was recorded in Romania, with the average number of flies exceeding 2500 parasites per host [51]. Nevertheless, in other countries, a lower infestation prevalence

was noted for *L. cervi* on the same host species, varying from 36% to 64% [52–54]. In Lithuania, *L. cervi* was less abundant on roe deer specimens than on in the other two examined host species, red deer and moose [19]. Additionally, *L. fortisetosa* showed a preference for attacking red deer over roe deer since the prevalence of infestation was 49% [55] or 100% [19], and 23% [54] or 90% [19], respectively. To the best of our knowledge, no studies balancing *L. fortisetosa* infestation on red deer, roe deer, and fallow deer have been carried out, but our data are consistent in stating that both parasites prefer red deer hosts over the other two cervids.

Interestingly, although some host species showed a high overall fly infestation prevalence (i.e., 94.7% *L. fortisetosa* on *D. dama*), no one species reached the 100% prevalence recorded on moose by several authors [19,38,56]. Moreover, in our survey, the overall density of *Lipoptena* spp. was higher on red deer than on the other two host species, at 0.12/cm$^2$ for *L. fortisetosa* and 0.03/cm$^2$ for *L. cervi*. Yet, a much greater number of deer keds was counted on moose, on which these parasites reached as many as 17,500 specimens on a single bull [56]. Since moose generally harbors a large number of hippoboscids, we could deduce that this species is more suitable for these parasites. Kadulski [52] found that among moose, red deer, roe deer, fallow deer, and Sika deer, the prevalence and intensity of infestation were directly proportional to the size of the host. Our findings are consistent with this conclusion since roe deer are smaller than red deer and fallow deer. Visual stimuli are considered important during the host location behavior of hippoboscids [57], and these parasites probably tend to attack larger species that are more easily detectable because of their size. In addition, several hematophagous ectoparasites use chemical cues ($CO_2$ or odors) during the host-finding process [58]. Lourenço

and Palmeirim [59] found that two Nycteribiidae species (Hippoboscoidea superfamily) mainly used carbon dioxide for long-distance host locations. This cue is emitted by all vertebrates, and animals larger in size tend to release it in larger amounts [60]. The antennae of *L. cervi* and *L. fortisetosa* are equipped with a well-developed sensory pattern on the external surface of the pedicel, suggesting that these different types of sensilla are likely able to perceive the chemical cues emitted by the hosts, supporting the hypothesis that more than visual signals alone are responsible for identifying host locations [6,61]. We can speculate that the large amount of $CO_2$ released by the hosts may contribute to explaining how the roe deer are attacked less than the other two species. Haarløv [10] suggested that red deer species occur in habitats that are more suitable for the development of pupae that fall on substrates that are more suitable for their survival. In our case, red deer and fallow deer coexist in the study area and share the same territories, making it difficult to hypothesize that the local habitat strictly affects host choice. Most likely, instead, preference for red deer could be due to the physical features of this host species, such as the structure of its coat. In fact, the host fur represents the environment in which hippoboscids live, so it should have the conditions they need to survive. Red deer have long and robust guard hairs with a dense layer of underhairs at the base; however, roe deer and fallow deer have shorter hairs forming a softer but thicker covering that may obstructed parasites from reaching the skin, making trophic activity more difficult [10,61].

In this paper, the objective of verifying the possible differences between host age classes and sex was determined only for red deer since this species was the favorite host for both *L. cervi* and *L. fortisetosa*.

Kadulski [52] observed that the intensity of infestation increases with the size of a host. Our results show significant differences in the choice of host age classes by both deer keds. Other authors highlighted that fawns are attacked less than subadults or adults [38,56]. This preference could be due to fawn behavior since they follow dams during their first year of life. Given that fawns are together with the adult females, the flies are more likely to choose the larger subject since it is more visible and releases a greater amount of $CO_2$. Regardless, body size alone cannot explain the host choice displayed by these hippoboscids; in fact, other aspects, such as the behavior and ecology of the host species, interact to affect this selection. Madslien *et al*. [38] hypothesized that *L. cervi* prefers parasitizing subadults over adult moose since the former moves more, increasing the chance of encountering a deer ked. Another explanation for this preference could be the resistance that some host species seem to develop toward hippoboscid attacks, as suggested for reindeer [62] and moose [38]. However, deer do not show similar resistance in the case of severe infestations [56].

According to our results, *L. cervi* is significantly more abundant in males. Additionally, in this case, the larger size and the more intense motility of host males may explain this preference, but it is also possible to hypothesize that odor secretions emitted by red deer can affect host choice as well. Deer have specialized regions with glands, whose activity may change between sexes, producing secretions to mark their territory. As demonstrated for white-tailed deer (*Odocoileus virginianus*), this glandular activity is higher in males, especially in dominant subjects [63]. Additionally, in *C. elaphus*, there are quantitative and qualitative differences between males and females in terms of their released compounds [64]. As reported by Johnson and

Leask [65] for *C. capreolus*, glandular activity and active testosterone metabolism can increase just prior to and during the mating season. In Italy, the breeding season of red deer occurs from late summer to early fall, overlapping with the host seeking period of *L. cervi*, possibly affecting the preference of the parasite for males. Further studies are needed to confirm the influence of sex differences in the odor secretions on *L. cervi* host selection.

Although *L. cervi* and *L. fortisetosa* are restricted to a limited group of species, they are able to adapt to new hosts and do not appear to strictly follow a parasitization scheme. In fact, we found both hippoboscids on all three examined deer species and on all host age classes and sexes. Apparently, these flies cannot be too selective in terms of sex and age classes since they are obligate ectoparasites and need to find a host shortly after emergence. The host species, however, seems to be an important prerequisite for *Lipoptena* spp.; in fact, red deer are favored by both flies.

Host density is one of the most important factors that needs to be considered when studying the distribution of hippoboscid ectoparasites, even if it does not explain all of the variation in the expansion of these flies. For instance, Meier *et al*. [35], suggested that a local increase in host density may allow for the rapid establishment of allochthonous ectoparasites. Additionally, *L. cervi* occurred in Finland in 1960 when the moose density experienced a large population growth [66]. The study of the relationship between parasites and hosts is fundamental, especially when it concerns allochthonous species which are able to adapt to new hosts, competing with native species/fauna. Hosts can represent the easiest transfer option for ectoparasites so that they can be disseminated in new territories during host movements and introductions. Just as the

expansion of *L. cervi* in the northeastern United States is considered to be due to the anthropogenic introduction of European deer [9], it is likely that *L. fortisetosa* spread to Europe due to the relocation of its original host, *Cervus nippon*. However, the possible hybridization between sika and red deer, or the translocation of *C. elaphus*-related subspecies to Europe cannot be ignored. Currently, *C. nippon* is recorded in 20 European countries, while *L. fortisetosa* is present in 13 European countries [67]. In Italy, a great increase in cervid abundance has been recorded in recent years, and the presence of *C. nippon* has been recently documented [3,5]. This situation confirms the risk related to the increase in the abundance of native ectoparasites, together with the spread of alien parasitic species further favored by global warming.

## Conclusion

The results of the present study show the great expansion of the allochthonous parasite *L. fortisetosa*, recently detected in Italy. This fly, originally restricted to the main host *C. nippon*, has a strong adaptability to other host species, such as red deer, fallow deer, and roe deer. Moreover, it seems to strongly compete with the autochthonous hippoboscid *L. cervi*, being more numerous in the study area. The favored host of both flies was red deer, even if all three examined host species harbored parasites. Different preferences for sex and age classes of the hosts were observed in the two hippoboscids. Although some explanations were hypothesized for these outcomes, at present, it is difficult to provide a specific explanation, since each choice occurred due to the interactions of many factors. Thus, further investigations are ongoing. Another aspect

worthy of attention is related to the possible health risk implicated in the expansion of allochthonous species as potential vectors of harmful pathogens. Therefore, hippoboscid populations should be continuously monitored to promptly identify possible substantial expansion or adaptation to other host species, which can lead to further spread with negative consequences from both ecological and health perspectives. Regular monitoring of deer keds should also be carried out to improve the knowledge of these parasites and establish specific management strategies to limit hippoboscid expansion.

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# 7. Asia and Europe: so distant so close? The case of *Lipoptena fortisetosa* in Italy

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## Abstract

In Europe, 5 *Lipoptena* species have been recorded, including *Lipoptena fortisetosa*. This species, native to Asian countries, was described as a parasite of sika deer and its appearance in Europe dates back to more than 50 years ago. *Lipoptena fortisetosa* has been recently reported in Italy, sharing its hosts with *Lipoptena cervi*. A morpho-molecular approach was developed to determine the phylogenetic interrelationship of Italian and Asian CO1 haplotypes sequenced from *Lipoptena* fly individuals collected in Italy, and their DNA sequences were compared with conspecifics available in GenBank; morphological key-characters (terminalia) of *L. fortisetosa* were compared with the original description. Two haplotypes were recorded from Italy and assigned to *L. cervi* and *L. fortisetosa*, respectively. The latter was part of the monophyletic clade *L. fortisetosa*, along with 2 Central European and 2 Korean haplotypes (100% identical to one of the Korean haplotypes); moreover, Italian *L. fortisetosa* female terminalia were consistent with the original description of Asian individuals. Pending more in-depth investigations, this study provides a first answer to the hypothesis of the recent colonization of Italy by *L. fortisetosa* from Asia as we did not detect any

obvious and stable morphological and molecular differences in specimens from the 2 geographical areas. The presence of the sika deer in Europe was retraced and the possible route travelled by the parasite from Asia and the eco-biological factors that may have enhanced its settlement are discussed.

## Introduction

Deer keds *Lipoptena* spp. (Hippoboscidae, Lipopteninae) are blood-sucking obligate ectoparasites of almost exclusively Cervidae (deer) and Bovidae (cattle, goats, chamois, antelopes, etc.), and can occasionally bite humans [1-3]. Indeed, the *Lipoptena* genus includes about 30 species spread worldwide [1]: most of them occur in the Palearctic region, mainly in continental Europe and Asia, while 8 species are native to far East Asian countries. Five species have been recorded in America, with 4 of them native to this continent [4]. Fragmentary information is available on the species accounted for African countries [5,6] (Supplementary Table S1). In severe infestations, *Lipoptena* spp. may be responsible for anemia and skin lesions and may be involved in the transmission of several pathogens [7,8]. In Europe, 5 species of *Lipoptena* have been recorded: *Lipoptena capreoli* Róndani, 1878, *Lipoptena couturieri* Séguy, 1935, *Lipoptena arianae* Maa, 1969, *Lipoptena cervi* (Linnaeus, 1758), and *Lipoptena fortisetosa* Maa, 1965 [9]; however, the presence and the geographical range of the first 3 species need to be confirmed. *Lipoptena cervi* may be considered the oldest deer ked in Europe as the relationship of this species with wild ungulates dates back to more than 5,000 years ago, being found in the remains of a Late Neolithic human mummy discovered in a glacier in the Alps [10]. It has a wide

distribution in Europe and has currently been reported from more than 20 countries [11]. *Lipoptena cervi* has been recorded on *Cervus elaphus*, *Dama dama*, *Alces alces*, *Rupicapra rupicapra*, *Capreolus capreolus*, *Moschus moschiferus* [9] and it has the potential to transmit bacteria e.g., *Bartonella* spp., *Borrelia* spp. [12-14], *Anaplasma* spp., *Ehrlichia* spp., and *Rickettsia* spp., and protozoans, e.g., *Babesia* spp., *Theileria* spp., *Hepatozoon* spp. [15-17]. *Lipoptena fortisetosa* was originally recorded in Japan from the sika deer *Cervus nippon* [18-20], and is considered quite restricted to this ungulate, although it has been occasionally collected from a bird *Emberiza spodocephata* [21]. It has also been reported from the Siberian roe deer *Capreolus pygargus* in South Korea [22] and in *Capreolus capreolus* in Kazakhstan [23]. This species was first reported in Europe about 50 years ago when it was found in the Czech Republic [24] and later in the Moscow district in Russia [25]. Afterward, from the 80s' to date, it has been confirmed and/or recorded in 12 countries, i.e., Czech Republic, Poland, Moldavia, Germany, Switzerland, Lithuania, Romania, Austria, Belarus, Slovak Republic, Moscow-district, and Estonia [26]. The range of *L. fortisetosa* has expanded in the southern part of Europe, including Italy, where it has very recently been reported [27,28]. In Europe, *L. fortisetosa* attacks mainly deer [29,30], and occasionally cattle [31], goats, sheep [29,30], dogs [32,33], and humans, as reported in Germany [34], Estonia [26], and Slovakia [35]. *Lipoptena fortisetosa* has been found to mechanically carry pathogens, i.e., *Coxiella-like* bacteria (CLB), *Theileria luwenshuni*, and *Theileria ovis* [36]. In addition, very recently, both *L. cervi* and *L. fortisetosa* specimens from Poland were found positive to *Trypanosoma* DNA [37]. In Europe, *L. fortisetosa* appears to share with *L. cervi* approximately the same ungulate species as the host group (above listed) and roughly the same territory [26].

However, it is unclear whether the native host (i.e., the sika deer *Cervus nippon*) of *L. fortisetosa* played a role in spreading the Asian species to Europe or the parasite propagated independently, as already speculated [38], or to which extent human activities might have helped the ked expansion. *Lipoptena fortisetosa* likely dispersed widely and settled in Europe quite quickly, so that possible changes in genetic constitution compared to the Asian indigenous populations may be hypothesized. Molecular investigations coupled to morphological analysis, as well as the ecological requirements, help to provide more insight into the perspective of the integrated taxonomy concept [39]. In order to state whether there are sharp differences between Italian and Asian individuals, we developed a morpho-molecular approach. In particular, we investigated the phylogenetic interrelationship of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene haplotypes of *Lipoptena* individuals sampled in Italy-analyzing them in a wider context together with the DNA sequences of conspecifics available in the NCBI GenBank and morphologically analyzed the Italian population of *L. fortisetosa*. We also focused on the observation of some stable key-characters (terminalia) and compared them with the original description of the species, as well as with the indigenous *L. cervi* species, as additional documentation. Finally, the presence of the sika deer in Europe was retraced in order to discuss the possible route travelled by the ectoparasite from Asia and the eco-biological factors that may have enhanced its settlement.

## Materials and methods

### Ethics statement, specimens and processing

All animal handling procedures followed all regional, national, and institutional guidelines.

From a total of 312 *Lipoptena* specimens, previously collected from 3 species of wild ruminants [28], belonging to *Lipoptena cervi* and *Lipoptena fortisetosa*, the following were selected from different host species: 10 flies each from 5 *Cervus elaphus* hosts (total 50 specimens: 30 and 20 specimens belonging to *L. cervi* and *L. fortisetosa*, respectively); 10 flies from 3 *Capreolus capreolus* hosts (total 30 specimens, all belonging to *L. fortisetosa*), 10 flies from 1 *Dama dama* host (total 10 specimens, all belonging to *L. fortisetosa*), and frozen at –20˚C, until DNA extraction. Genomic DNA was extracted individually from the abdomens using the Nucleospin Tissue kit (Macherey-Nagel, Amsterdam, Netherlands) in accordance with the manufacturer's instructions. The extracted DNA was eluted in 50 μl of distilled water and the samples were stored at −20˚C, pending molecular analysis. PCR amplifications were performed in a CFX96 thermal cycler (Bio-Rad, Hercules, California, USA.) using 10 μl of Phire Reaction Buffer 5X (Thermo Scientific, Waltham, Massachusetts, USA), 0.4 μl of dNTPs (200 μm) (Qiagen, Germantown, Maryland, USA), 1 μl of specific primer pairs (10 μm), 0.4 μl of Phire Hot Start II DNA Polymerase 1 U (Thermo Scientific), and 5 μl (approximately 100 μg) of genomic DNA per reaction. A blank control (pure water instead of genomic DNA) was included in each PCR run.

An approximately 710-bp gene fragment of CO1 was amplified using primers LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) [40]. The cycling parameters were: 2 min denaturing at 94˚C, followed by 35

cycles of 30 sec at 94˚C, 30 sec at 56˚C and 60 sec at 70˚C, and final extension of 7 min at 70˚C.

PCR products were run on 1.2% agarose gel, and positive samples purified with exonuclease I (EXO I) and thermo- sensitive alkaline phosphatase (FAST AP) (Fermentas, Waltham, Massachusetts, USA) enzymes, in accordance with the manufacturer's instructions. PCR products were directly sequenced in both directions using the ABI PRIMS BygDye Terminator v. 3.1Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) with the same primers as the respective PCR reactions, in accordance with the manufacturer's instructions. The sequences obtained were determined using an ABI PRISM 3130 Genetic Analyser (Applied Biosystems), chromatograms were inspected by eye using FinchTV (https://digitalworldbiology.com/FinchTV) and primer regions plus bad-quality regions were removed. Once the sequences were cleaned up, each sequence was compared with the *Lipoptena* spp. homologous nucleotide sequences available in the GenBank database using the BLAST program (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.
gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_
L+OC=blasthome). The 19 sequences having the highest percent similarity with our sequences and labelled as *Lipoptena* CO1 in GenBank were then sampled and gathered in a FASTA file with our own sequences. The new sequence dataset was aligned using the CLUSTALW implementation of BIOEDIT, version 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the alignment adjusted manually, if necessary. Once the sequences were aligned, the absence of stop codons was checked. Phylogenetic analysis of the obtained sequences and homologous sequences from GenBank were

performed using the maximum likelihood method in MEGA, version 7.0.9 (https:// www.megasoftware.net). Bootstrap confidence values for the branching reliability were calculated with 10,000 replicates.

All specimens of *L. fortisetosa* and *L. cervi* intended for molecular analysis were morphologically identified, based on Maa's original description [19] and a recent taxonomic key [9]. Other specimens were processed for Scanning Electron Microscope (SEM) observations, according to the procedures previously described [28] to further examine male and female terminalia. Description of terminalia features follows the terminology and nomenclature reported by Maa and Peterson [41].

## Results

A total 60/90 (66.7%) of the specimens provided good quality PCR fragments and sequences for the CO1 gene. After alignment with the homologous sequences of *Lipoptena* spp. available in GenBank, 2 sets of sequences were identified, one with the mean percentage of identity of 89% with *L. cervi* and 92% with *L. fortisetosa*, and one with 97% with *L. cervi* and 86% with *L. fortisetosa*. Two haplotypes were recorded from the 60 specimens sequenced in the present study and the phylogenetic analysis confirmed that these 2 haplotypes belonged to *L. cervi* and *L. fortisetosa*, respectively. The data matrix comprised 14 haplotypes of *L. cervi* (one haplotype from the present study, 13 downloaded from GenBank) and 7 haplotypes of *L. fortisetosa* (one haplotype from the present study, 6 downloaded). The genetic distances ranged from 0.081-0.084 for *L. cervi* group sequences and 0.003-0.018 for *L. fortisetosa* group sequences. While the clade *L. fortisetosa* appears to be monophyletic, the internal 2-subclade

structure of this clade is poorly supported (low bootstrap values) as expected within the species. We will thus note essentially the following points: the single haplotype recorded from the Italian *L. fortisetosa* specimens was closely related to 2 Central European haplotypes and 100% identical to one of the 2 haplotypes (KU356895) found in Korea (Figure 7.1). Morphological investigations showed that female terminalia of *L. fortisetosa* are characterized by a typical pregenital sclerite that is peg-like and bears 2 or 3 strong bristles (Figure 7.2 A, C). The pregenital plate is elongated and lozenge-shaped, and the underlying hypoproct is covered by several bristles interspersed with an area densely hairy. The genital opening is clearly visible between the pregenital sclerite and the pregenital plate (Figure 7.2 C). Female terminalia of *L. cervi* showed the presence of 3 pregenital sclerites bearing several differently sized bristles (Figure 7.2 B, D). The central sclerite is bigger and with more numerous setae than the external 2; the pregenital plate shows many series of long setae arranged in the distal portion, while the hypoproct is completely bare. Male terminalia of *L. fortisetosa* (Figure 7.2 E) consist of 2 well-sclerotized and slender gonopods that guide the aedeagus. This latter is wider in the proximal part and ends with a bilobate tip provided with spines. In *L. cervi*, the gonopods (Figure 7.2 F) are similar to those of *L. fortisetosa*. However, the aedeagus is membranous in the proximal and lateral parts, while in the middle it is formed by 2 fused and sclerotized strips ending in a ridge tip. Morphological observations of *L. cervi* and *L. fortisetosa* strengthened the strong diversity between these 2 species [28] but, more importantly, demonstrate that the features of *L. fortisetosa* female terminalia are consistent with the original description by Maa [18] (Figure 7.3). Taking into account that terminalia are considered stable features that allow a correct morphological identification at

species-specific level in Diptera [42], including hippoboscids [19,43], this supports the above confirmation that the boundaries between the 2 species (*L. fortisetosa* and *L. cervi*) are correct based on morphology and molecular data. Therefore, we can conclude that the individuals of which we only have the sequences (GenBank) belong to the same species. *Lipoptena fortisetosa* has been introduced in Europe probably with *Cervus nippon*, the sika deer [28,33,44], during the last 150 years of restocking of deer in the Continent, apparently, since 1893, and probably a number of times [44]. Sika deer has successfully settled in the European fauna thanks to its high potential to compete with autochthonous species and readiness to hybridize with native red deer, as demonstrated by the presence of hybrids of sika with red deer in several countries [45-49]. Sika deer (or hybrids) is currently present in 20 European countries, including Italy, where it was recently reported [50]. Spreading through Europe, sika deer has likely carried and disseminated its ectoparasites, including *L. fortisetosa* that is currently recorded in 13 European countries (Figure 7.4), Italy included [27].

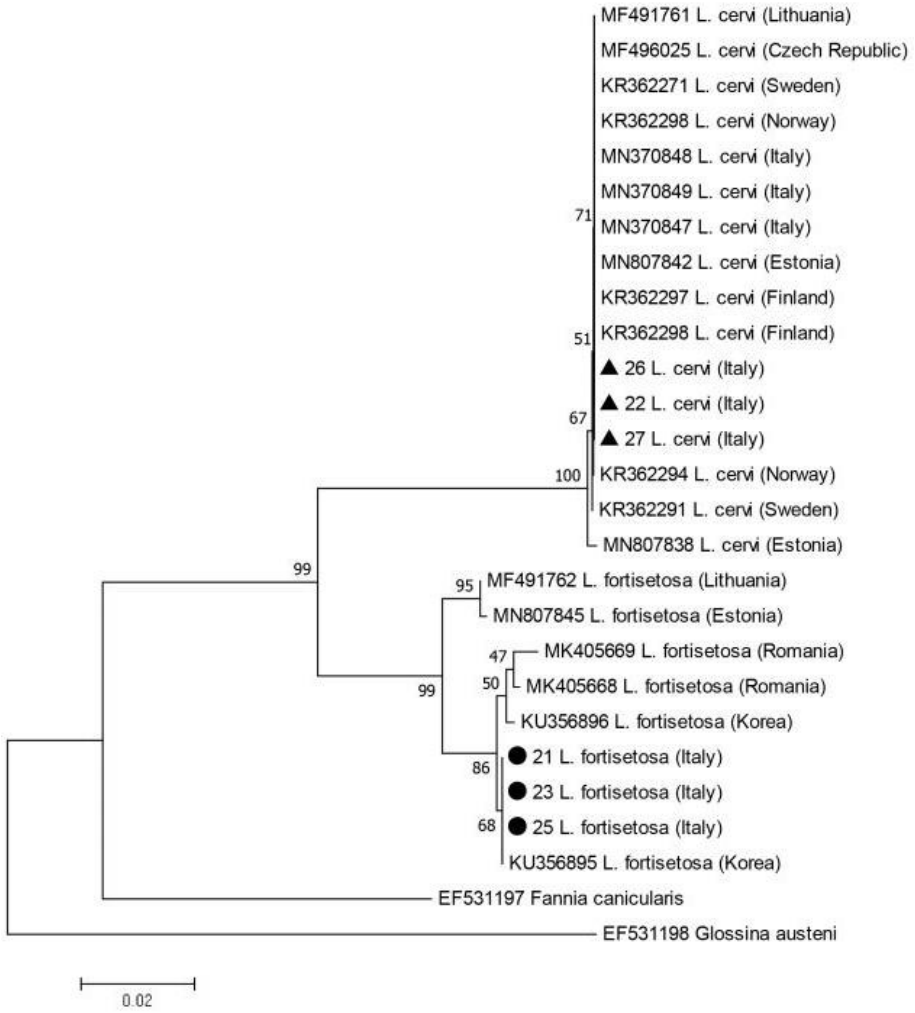


Figure 7.1. Phylogenetic topology based on the analysis of the maximum likehood of the partial CO1 gene sequences from *Lipoptena* individuals from the present study and *Lipoptena* sequences available from GenBank. Labels include accession numbers, species identity and country origin. The 2 haplotypes found from the present study are labelled with a black triangle for *Lipoptena cervi* and a black circle for *Lipoptena fortisetosa*. *Fannia cunicularis* and *Glossina austeni* sequences were used as outgroups. The percentage of trees in which the associated individuals clustered together is shown next to the branches.

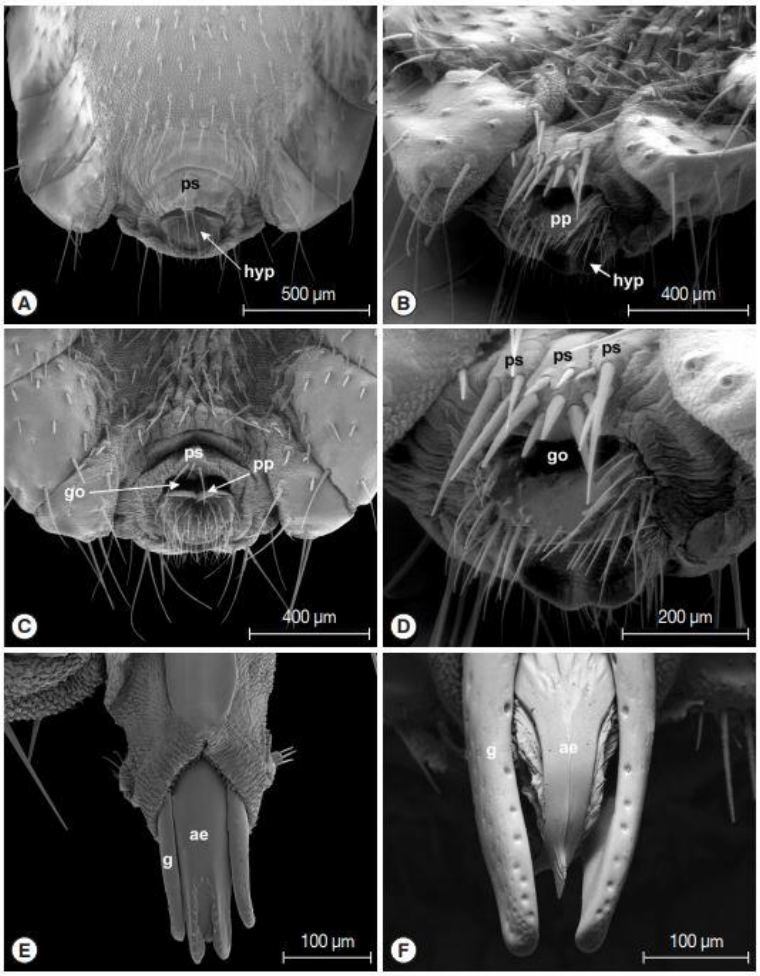


Figure 7.2. Terminalia of *Lipoptena fortisetosa* (A&C, female; E, male) and *Lipoptena cervi* (B&D, female; F, male). ps, pregenital sclerite; hyp, hypoproct; go, genital opening; pp, pregenital plate; ae, aedeagus; g, gonopod.

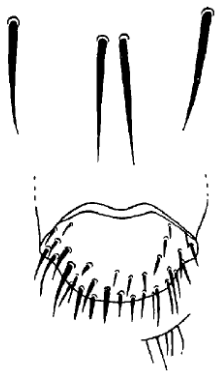

Figure 7.3. Female terminalia of *Lipoptena fortisetosa*, drawing from Maa [18].

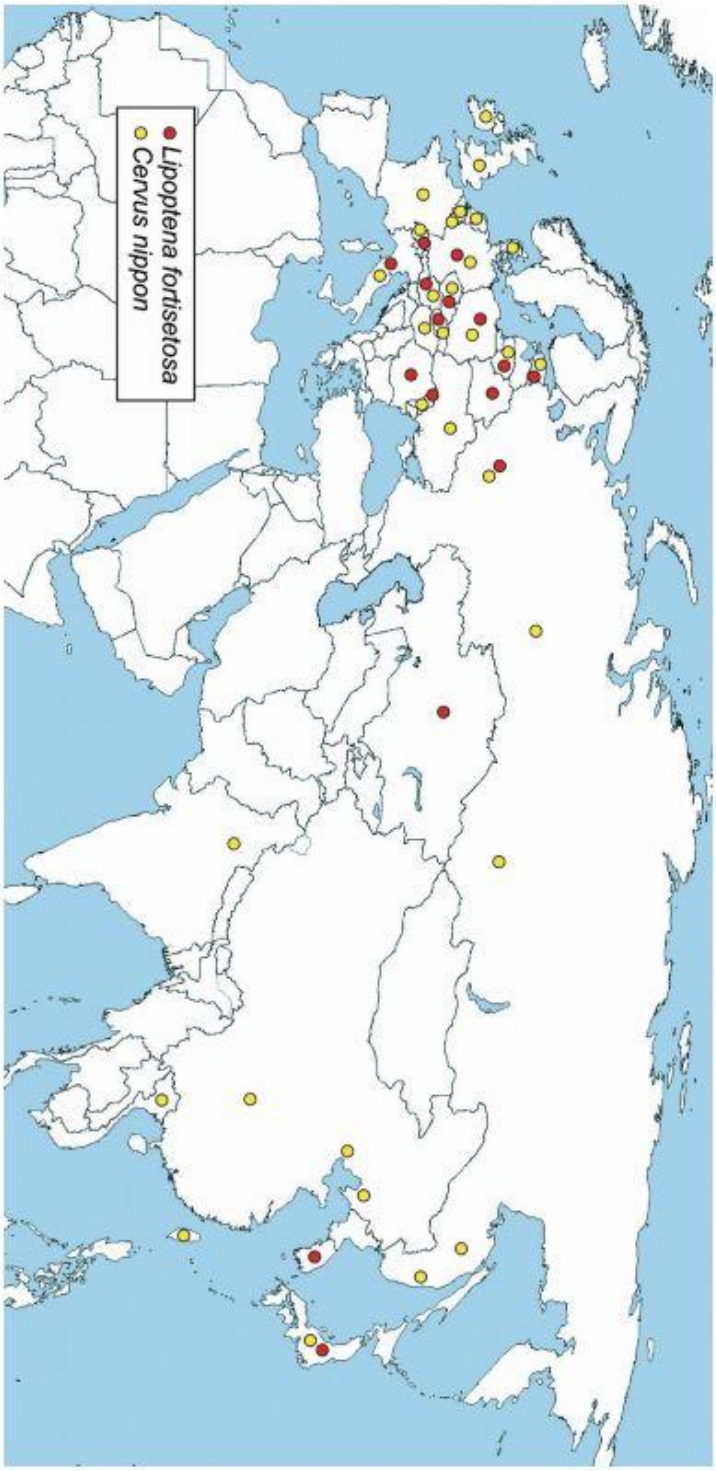


Figure 7.4. Distribution map of *Lipoptena fortisetosa* (red dot) and *Cervus nippon* (sika deer) (yellow dot). Data from different sources [11,19,23,26,44,49,50].

## Discussion

According to the present results, while *L. cervi* confirms previous findings [9], we cannot exclude a scenario depicting a recent colonization(s) of *L. fortisetosa* in Europe. According to the latest studies, the hypothesis of geographically distinct CO1 lineages should be rejected at the Central Europe level [26]. Our study suggests that this hypothesis should also be rejected at a global level. However, we were unable to determine the time of colonization or to identify the actual host that introduced *L. fortisetosa* from Asia to Europe. In fact, this parasite has been collected from many different cervids, e.g., red deer (*Cervus elaphus*) [27,28], Manchurian elk (*Cervus elaphus xanthopygus*), Maral red deer (*Cervus elaphus maral*), fallow deer (*Dama dama*) [34], Korean water deer (*Hydropotes inermis*) [36], roe deer (*Capreolus capreolus*) [23], Siberian roe deer (*Capreolus pygargus*) [22], both in Europe and in Asia. The possibility of recent colonization by *L. fortisetosa* specimens from cervids purchased for the restocking of deer farms, or in captive hosts in the Oriental region transferred to fenced areas for recreational or conservation purposes, cannot be excluded. We also suggest, as a minor hypothesis, the relatively recent migration to Italy of sika or hybrid individuals from neighboring countries that may have transmitted the ectoparasite joining red deer groups. This study doesn't reject the hypothesis of the recent colonization of Italy by *L. fortisetosa* from Asia as no obvious and stable morphological and molecular differences were observed in the populations from the 2 regions. However, such a hypothesis requires further study, including a straightforward analysis of numerous specimens from Asian and European countries. In line with other authors' suggestions [26], population genetic analyses of Asian and European keds' populations are required to evaluate whether the

colonization scenario is likely or not. Moreover, wider investigations aimed at comparing genetic make-up of populations from different countries, host and parasite distribution, together with morphological differentiation, might resolve phylogenetic relationships of this neglected group within a desirable integrated taxonomic framework as applied in other groups of insects [51,52]. Due to the ability of *L. fortisetosa* to parasitize a wide range of homeothermic animals, there are no obvious limitations to its further expansion. Given such possibility, the potential for transmitting pathogens, and the frequent attacks reported among humans [26], more in-depth investigations are required on *Lipoptena* species with a One Health perspective.

# Supplementary material

**Supplementary Table S1.** Species of the genus *Lipoptena* spp. described in the world with their distribution and hosts [1,4-6,19,43]

| Species | Distribution | Hosts |
|---|---|---|
| species group "a" | | |
| *Lipoptena axis* Maa, 1969 | India, Sri Lanka, Nepal | *Axis axis, Tetracerus quadricornis* |
| *Lipoptena cervi* (Linnaeus, 1758) | Europe, Asia, Eastern states of USA | *Cervus elaphus, Cervus nippon, Cervus canadensis, Capreolus capreolus, Dama dama, Rupicapra rupicapra, Ovis musimon, Alces alces, Rangifer tarandus fennicus, Odocoileus virginianus* |
| *Lipoptena efovea* Speiser, 1905 | Sri Lanka | *Axis axis ceylonensis, Muntiacus muntjak malabaricus* |
| *Lipoptena fortisetosa* Maa, 1965 | South Korea, Japan, Europe, Kazakhstan | *Cervus nippon, Cervus elaphus, Capreolus capreolus, Capreolus pygargus, Dama dama, Emberiza spodocephata* |
| *Lipoptena japonica* Bequaert, 1942 | Japan | *Capricornis crispus* |
| *Lipoptena nirvana* Maa, 1969 | Vietnam | unknown |
| *Lipoptena pauciseta* Edwards, 1919 | India, China, Thailand, Laos, Vietnam, Indonesia | *Muntiacus muntjak* |
| *Lipoptena rusaecola* Bequaert, 1942 | Philippines | *Rusa unicolor* |
| *Lipoptena saepes* Maa, 1969 | Nepal | *Axis porcinus* |
| *Lipoptena sigma* Maa, 1965 | Taiwan | *Rusa unicolor swinhoii* |
| *Lipoptena timida* Maa, 1969 | Nepal | *Axis porcinus* |
| species group "b" | | |
| *Lipoptena pteropi* Denny, 1843 | Thailand, Vietnam, Myanmar, Malaysia, Indonesia | *Tragulus napu, Tragulus javanicus* |
| species group "c" | | |
| *Lipoptena arianae* Maa, 1969 | Europe, Turkey, Iran, Afghanistan | Wild *Ovis* sp. |
| *Lipoptena capreoli* Rondani, 1878 | Serbia, Greece, Cyprus, Turkey, Syria, Palestine, Iraq, Iran, Pakistan | *Capra hircus hircus* |
| *Lipoptena chalcomelaena* Speiser, 1904 | Egypt, Turkey, Sudan | *Capra nubiana, Capra hircus aegagrus* |
| *Lipoptena couturieri* Seguy, 1935 | Pyrenees Mountains (France, Spain) | *Rupicapra rupicapra* |
| *Lipoptena grahami* Bequaert, 1942 | China | unknown |
| *Lipoptena saltatrix* Maa, 1969 | India | *Hemitragus jemlahicus, Naemorhedus goral* |
| *Lipoptena weidneri* Maa, 1969 | India | *Naemorhedus goral* |
| species group "d" | | |
| *Lipoptena binocula* (Speiser, 1908) | South Africa, Mozambique, Botswana | *Raphicerus campestris, Antidorcas marsupialis* |
| *Lipoptena hopkinsi* Bequaert, 1942 | Kenya, Uganda, Congo (DRC) | *Tragelaphus scriptus, Cephalophus monticola, Cephalophus nigrifrons, Nesotragus moschatus* |
| *Lipoptena iniqua* Maa, 1969 | India | *Antilope cervicapra* |
| *Lipoptena paradoxa* Newstead, 1907 | Ghana, Congo (DRC), Angola, Burundi, Uganda, Ethiopia, Kenya, Tanzania, Zimbabwe, Malawi, Mozambique, South Africa | *Tragelaphus angasii Tragelaphus scriptus, Tragelaphus imberbis, Tragelaphus strepsiceros, Taurotragus oryx, Sylvicapra grimmia, Kobus ellipsiprymnus, Redunca arundinum, Hippotragus equinus, Ourebia ourebi, Raphicerus melanotis, Aepyceros melampus* |
| *Lipoptena annalizeae* Visagie, 1992 | South Africa | *Antidorcas marsupialis* |
| *Lipoptena sepiacea* Speiser, 1905 | Gambia, Ghana, Nigeria, Sudan, Eritrea, Kenya, Uganda, Tanzania, Malawi | *Tragelaphus scriptus, Tragelaphus strepsiceros, Cephalophus monticola, Cephalophus rufilatus, Sylvicapra grimmia, Damaliscus korrigum, Ourebia ourebi, Gazella rufifrons, Gazella thomsonii, Gazella tilonura, Gazella granti* |

(*Continued to the next page*)

**Supplementary Table S1.** Continued

| Species | Distribution | Hosts |
|---|---|---|
| species group "e" | | |
| *Lipoptena depressa depressa* (Say, 1823) | Western regions of Canada and USA | *Odocoileus hemionus, Odocoileus virginianus* |
| *Lipoptena depressa pacifica* Maa, 1969 | Western regions of Canada and USA | *Odocoileus hemionus, Odocoileus virginianus* |
| *Lipoptena guimaraesi* Bequaert, 1957 | Brazil | *Ozotoceros bezoarticus* |
| *Lipoptena mazamae* Rondani, 1878 | Southern-eastern states of USA, central and south America | *Mazama americana, Mazama guazupita, Mazama simplicicornis, Mazama tema, Odocoileus virginianus* |
| "Incertae sedis" | | |
| *Lipoptena doszhanovi* Grunin & Doszhanov, 1974 | Kazakhstan | *Passer hispaniolensis* |
| *Lipoptena pudui* Peterson & Maa, 1970 | Chile | *Pudu pudu* |
| *Lipoptena sikae* Mogi, 1975 | Japan | *Cervus nippon* |

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# 8. Analysis of the microbiota associated to pupae, winged and wingless adults of *Lipoptena fortisetosa* collected from cervids in Italy

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## Abstract

The hippoboscid *Lipoptena fortisetosa* Maa, 1965 is a hematophagous ectoparasites of cervids and can bite humans. This fly is enlarging its geographical range with concern for animal and human health since it has been found to harbour potentially harmful microorganisms. This study aimed at characterizing the microbiota of *L. fortisetosa* in its different life-cycle stages. Pupae and wingless adults were collected from hunted cervids and pooled into three and 142 samples of ten specimens, respectively. Moreover, winged flies were swept from the environment and separated into five pools of ten insects. After DNA extraction, samples have been analysed through Next Generation Sequencing using a 16S metabarcoding approach. Results revealed that community composition and relative abundance of different taxa greatly differed in the three analysed stages. Particularly, wingless adults showed a high presence of *Bartonella* (33.07%), which is, instead, almost absent in winged flies and pupae. Among the detected pathogens, five genera of concern for human health were found: *Bartonella*, *Moraxella*, *Mycobacterium*, *Arsenophonus*, *Rickettsia*. Interestingly *Bartonella bovis*, *Moraxella*

*osloensis* and *Arsenophonus lipopteni* have been detected. These findings suggest the possible role of *L. fortisetosa* as reservoir of pathogenic microorganisms, confirming the need of further investigation to ascertain its vector capacity.

## Introduction

Many arthropods are known as vectors of pathogens responsible of diseases. The most renowned groups that caused vector-borne diseases are mosquitoes, together with ticks, fleas, lice, and flies. Especially viruses and bacteria (including rickettsiae) are transmitted by arthropods, followed by protozoa and filarial nematodes (Mullen and Durden, 2019). Among the species with vector capacity, flies of the family Hippoboscidae are currently receiving great attention and several etiological agents have been detected in many species, making hippoboscids worthy of further investigations from a public health perspective. Recently, the importance and the role of these ectoparasites as nuisance for animal hosts and humans have been overviewed highlighting that the health risk concerning these dipterans is probably much greater than presently known (Reeves and Lloyd, 2019; Bezerra-Santos and Otranto, 2020).

Hippoboscidae family is composed by obligatory hematophagous ectoparasites thriving on different species of mammals and birds (Hutson, 1984). Into the family, the *Lipoptena* genus (subfamily Lipopteninae) specifically targets cervids. Currently, *Lipoptena cervi* (Linnaeus 1758) and *L. fortisetosa* Maa, 1965 are considered to be the only two members of this genus present in Italy, where they infest predominantly *Cervus elaphus* Linnaeus, 1758, *Capreolus capreolus* (Linnaeus, 1758), and *Dama dama* (Linnaeus,

1758) (Andreani *et al*., 2019). In other countries *L. cervi* has also been found on *Alces alces* Linnaeus, 1758, *Moschus moschiferus* Linnaeus, 1758, *Rangifer tarandus* (Linnaeus, 1758), *Cervus nippon* Temminck, 1838, *C. canadensis* Erxleben, 1777, and *Odocoileus virginianus* Zimmermann, 1780 (Bequaert, 1942; Maa, 1969), while *L. fortisetosa* has been reported on a few other species: its original host *C. nippon*, *Capreolus pygargus* Pallas, 1771, and *A. alces* (Maa, 1965; Choi *et al*., 2013; Klepeckienė *et al*., 2020). Both these ectoparasites can also feed on some occasional hosts: *L. cervi* has been found on domestic horse, cattle, European badger, and dog specimens (Bequaert, 1942; Hermosilla *et al*., 2006), while *L. fortisetosa* on fox and dog (Kadulski, 2007; Sokół and Gałęcki, 2017; Mihalca *et al*., 2019). Additionally, it must be taken into consideration that these hippoboscids can accidentally bite humans with possible consequent health risks (Bequaert, 1942; Schumann and Messner, 1993; Buczek *et al*., 2020).

Like all the hippoboscid representatives, also *Lipoptena* spp. reproduces through the adenotrophic viviparity strategy, larvipositing a single fully-grown larva at a time that subsequently pupate. Adults emerge from the pupae as winged imago and spend the first period seeking for a proper host, that they need to find in about a month. When they locate a suitable subject, they settle into the fur of the victim and live continuously on it reproducing and feeding. Both sexes of these flies are hematophagous and feed thanks to a perfectly adapted piercing mouth apparatus equipped with rows of teeth able to scratch the skin allowing the bleeding (Snodgrass, 1943). Although the amount of drawn blood is exiguous, 0.0002–0.0003 g, meals occur repeatedly: each adult feeds up to 20 times a day, causing annoyance and skin irritation on their hosts (Ivanov, 1974). Adults shed wings once on-host remaining into its fur during their whole life. Due to wing loss,

switching to other victims is difficult and parasites are strictly associated with a single subject, although they can be transferred during the breeding season of cervids or from the mothers to the fawns (Samuel and Trainer, 1972; Davis, 1973).

Deer keds seem to have no heavy negative impact on wildlife populations, and to date no evidence of disease has been found in animals in which pathogens have been detected (Allan, 2001). Nevertheless, parasite abundance on a single host can be very high, for example *L. cervi* reached an intensity of more than 17,500 individuals on a single moose bull (Paakkonen *et al*., 2010). It is reasonable that such a heavy infestation can be detrimental to the hosts, in fact cases of anaemia, dermatitis, severe bleedings, and behavioural alterations in animals have already been ascertained (Kaunisto *et al*., 2009; Madslien *et al*., 2011; Kynkäänniemi *et al*., 2014). Humans can be occasionally attacked by deer keds, and symptoms induced by their bites include the onset of a variable number of papules persisting for several weeks or up to a year. The allergic reaction comprises an intense pruritus, erythema, and sometimes secondary infection (Härkönen *et al*., 2009; Buczek *et al*., 2020; Maślanko *et al*., 2020). Cases of occupational allergic rhinoconjunctivitis and chronic deer ked dermatitis have been reported as well (Rantanen *et al.*, 1982; Laukkanen *et al*., 2005). Additionally, these ectoparasites can be bothersome to people, especially those who work in or visit natural habitats. In Finland the occurrence of these flies was one of the most important reasons for controlling moose numbers, together with road accidents and forest damages (Härkönen *et al*., 2009). Besides, it is noteworthy that hippoboscids are possible vectors of etiological agents responsible for zoonoses (Baker, 1967). Many potentially harmful microorganisms

have been detected in keds of *L. cervi*, *Lipoptena mazamae* Rondani, 1878, *Hippobosca equina* Linnaeus, 1758, and *Melophagus ovinus* (Linnaeus, 1758) species (Böse and Petersen, 1991; Dehio *et al*., 2004; Halos *et al*., 2004; Reeves *et al*., 2006; Hornok *et al*., 2011; Duodu *et al*., 2013; De Bruin *et al*., 2015; Korhonen *et al*., 2015; Buss *et al*., 2016; Szewczyk *et al*., 2017; Regier *et al*., 2018). In last years, the vector capacity of *L. fortisetosa* for *Coxiella* spp., *Theileria luwenshuni*Lee *et al*., 2016, *T. ovis* Rodhain 1916, *Bartonella* spp., *Trypanosoma* spp., *Anaplasma phagocytophilum* (Foggie, 1949) Dumler *et al*., 2001, *Babesia* spp., *Borrelia* spp., *Francisiella tularensis* Gałęcki *et al*., 2021, *Mycoplasma* spp., and *Rickettsia* spp. has been suggested by several authors (Lee *et al*., 2016; Werszko *et al*., 2020; Bartosik *et al*., 2021; Gałęcki *et al*., 2021; Sato *et al*., 2021).

Wildlife species are the main reservoir of infectious agents since they support an impressive number of macro and micro parasites which can transfer microorganisms. Moreover, pathogen infection in deer ked can be positively correlated with the infestation of deer (Izenour *et al*., 2020). Pathogenic organisms originating from wild fauna are becoming even more important since cases of zoonotic diseases are increasing throughout the world (Bengis *et al*., 2004). As deer keds can feed on animals considered as reservoirs of anthropozoonotic potential agents, the risk of pathogens transmission to humans cannot be ignored.

Allochthonous cervids are possible ways for the diffusion of their ectoparasites, which in turn can cause the spread in new territories of pathogens responsible of zoonoses. *Lipoptena fortisetosa* is continuously dispersing in Europe where it seems to have been introduced via its original host *C. nippon*. To date the possible vector capacity of this fly has not been thoroughly studied in Italy, but

biological and behavioural characteristics of this species, similarly to other deer keds, make it a potential suitable reservoir for the multiplication and transmission to hosts of etiological pathogens (Bezerra-Santos and Otranto, 2020).

For these reasons, this study aimed at getting further information on the microbiota of this adventive ectoparasite to evaluate the possibility that *L. fortisetosa* is a transfer of harmful microorganisms. To achieve this aim, specimens of *L. fortisetosa* collected from wild deer living in different areas of the Tuscan-Emilian Apennines (central Italy) have been analysed through Next Generation Sequencing (NGS) using a 16S metabarcoding approach.

## Materials and methods

### Hippoboscid collection

During an ectoparasite survey conducted in the Tuscan-Emilian Apennines, flies belonging to *L. fortisetosa* were manually collected from the fur of the host animals hunted during the culling seasons 2018-2019. The specimens were taxonomically identified following different keys and descriptions of *Lipoptena* spp. (Bequaert, 1942; Maa, 1965; 1967; Andreani *et al*., 2019) and kept at -20°C till further processing.

Hippoboscids were picked up from 71 cervids hunted during the culling seasons 2018-2019, specifically, *Cervus elaphus* (n=61), *Capreolus capreolus* (n=5), and *Dama dama* (n=5). Samples came from different territories of the study area, further details and procedures are described by Andreani *et al*. (2021). From every single host, 20 flies (ten males and ten females) were randomly selected and pooled according to fly sex. In addition, 30 pupae were collected from

the fur of different red deer and processed as three pools of ten samples each. Finally, 50 winged adults (grouped into five pools of ten insects, without selecting males and females), swept in a deer ked highly infested area during their host location behaviour in the 2018 and 2019 springs, were tested as well.

### DNA extraction

DNA extraction was performed using the QIAamp PowerFecal Pro DNA Kit (Qiagen) following the manufacturer instruction, except for the homogenization step. The samples were transferred in grinding tubes with ceramic beads, added with 800 µL of CD1 lysis buffer and ground in a FP120 FastPrep homogenizer (Sartorius) with seven cycles of 1 min at 10 m/s. After extraction, DNA yields and purity were determined by spectrophotometric (VivaSpec LS, Sartorius) and fluorimetric (Qubit 3.0 and Qubit dsDNA HS, Life Technologies) measurements.

### DNA metabarcoding

The Illumina protocol for the 16S Metagenomic Sequencing Library Preparation was used for the metabarcoding analysis of the microbial community in *L. fortisetosa*. The primers 515FB (Parada *et al*., 2016) and 806RB (Appril *et al*., 2015) were used for the amplification of the V4 region of the 16S rRNA gene.

PCR assay was performed using 12.5 µL NEBNext Q5 Hot Start HiFi 2X master mix (BioLabs), 1.25 µL of each primer 10 µM, 7.5 µL $H_2O$, 2.5 µL DNA (5ng/µL), with the following thermal profile: 98°C for 30 s; 30 cycles at 98°C for 10 s; 55°C for 30 s; 72°C for 30 s; 72°C for 2 min. Negative controls were performed using pure water. In addition, microbial mock communities (ZymoResearch) were run along as a

standard and as a quality control for determining contamination bias from DNA extraction.

All the PCR were visualized on agarose gel to check the amplification of the expected products. The amplified DNA was purified by magnetic beads (AgencourtAMPure XP, Beckman Coulter) and used as a template for the index PCR. The reaction was prepared in a final volume of 50 µL using 5 µL DNA, 5 µL Nextera XT Index Primer 1 (N7xx), 5 µL Nextera XT Index Primer 2 (S5xx), 25 µL NEBNext Q5 Hot Start HiFi 2X master mix (BioLabs), 10 µL ultrapure $H_2O$, following this thermal profile: 98°C for 30 s; 12 cycles at 98°C for 10 s, 55°C for 30 s, 72°C for 30 s; 72°C for 2 min.

The PCR products were purified again using magnetic beads and analyzed on a Bioanalyzer 2100 (Agilent) with the high sensitivity DNA kit to verify the library size. The amplified fragments were also quantified with the Qubit DNA HS kit on a Qubit 2.0 fluorimeter (Life Technologies) for normalization of the library at 4 nM. Library concentration was checked by qPCR using the NEBNext Library Quant Kit for Illumina (New England BioLabs). The library was then sequenced on an Illumina MiSeq platform using a MiSeq Reagent Kit v3 (600-cycle) and paired-end 2x200 bp sequencing.

### Bioinformatic and statistical analysis

The raw fastq data were analysed with tools of the Microbial Genomics Module in the CLC Genomic Workbench (Qiagen). The paired end reads were joined and trimmed for low quality score (Qscore < 0.05), nucleotides ambiguity (max 2 nucleotides allow), adapter sequences and length. Duplicate sequences were merged and aligned against the SILVA database 97%. Chimeric reads were removed and taxonomy was assigned, with the creation of an OTU

table. The profiles of the negative control and the mock communities were analysed to check for correct procedures and cross-contamination, then they were removed. The alpha-diversity analysis (observed OTUs, Chao1 index, Shannon index and Simpson index) was performed for life cycle stages and sex of wingless ectoparasites, while the beta-diversity (Bray-Curtis index with PCoA) was evaluated for sex of wingless ectoparasites.

## Results

DNA yields ranged from 12.5 ng/μL to 63 ng/μL, except for four samples with a range between 1.5 ng/μL and 7.07ng/μL. All the samples showed a 350 bp amplicon on agarose gel, except the negative control.

The high throughput sequencing of the V4 region of the 16S rRNA gene gained 111,566,480 reads; after quality filtering 41,811,165 paired reads were obtained for the OTU clustering. The 1,202,846 unique non chimeric sequences were assigned to 12,428 OTUs. The data from the mock community analysis matched the expected results, while the negative controls showed a low number of reads assigned to OTUs absent or with a very low abundance in the samples.

The most abundant families in wingless adults were Bartonellaceae, Moraxellaceae, Staphylococcaceae, Pseudomonaceae, Corynebacteriaceae. The winged adults showed a higher presence of Mycobacterium while Staphylococcaceae and Moraxellaceaewere abundant in pupae (Figure 8.1).

Considering the genus level (Figure 8.2), the community composition and the relative abundance of different taxa greatly differed in the three analyzed groups. In wingless adults a high

presence of *Bartonella* (33.07%) was highlighted, followed by *Pseudomonas* (9.49%), *Staphylococcus* (8.13%), *Acinetobacter* (7.18%), and *Corynebacterium1* (6.54%). Otherwise, in winged adults 67.93% of OTUs corresponded to a bacterial ambiguous taxon, 8.08% to *Mycobacterium* and 4.64% to an *Uncultured-123*. Nearly half (44.48%) of the microbiota identified in pupae was represented by *Staphylococcus*, followed by *Psychrobacter* (22.96%), *Acinetobacter* (12.40%), *Pantoea* (6.98%), and *Macrococcus* (4.01%). Both winged adults and pupae harboured a negligible quantity of *Bartonella* (0.16% and 0.19% respectively).

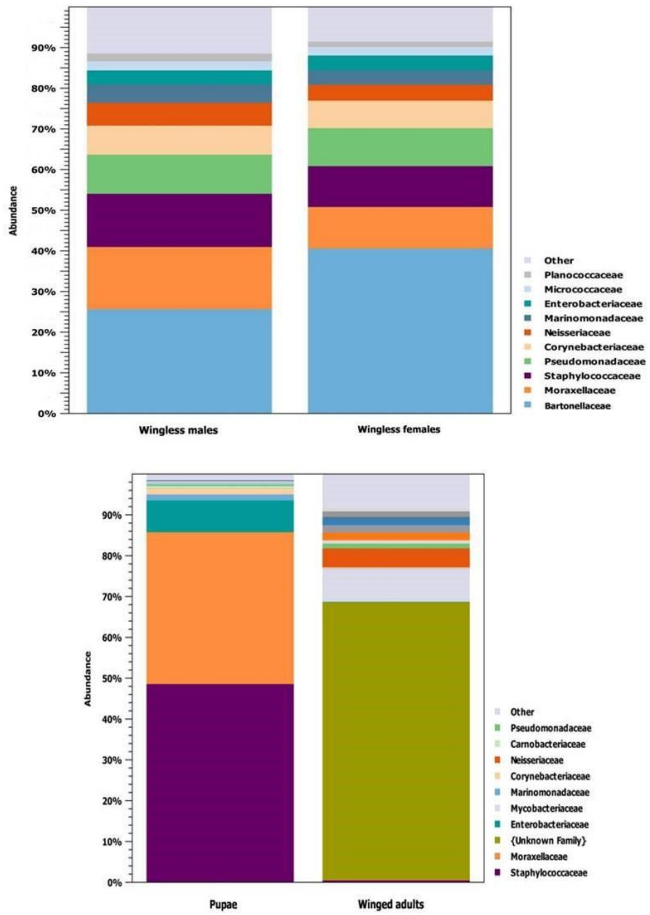


Figure 8.1. Relative abundance of bacterial families detected in male (M) and female (F) wingless adults, pupae, and winged adults of *L. fortisetosa*.

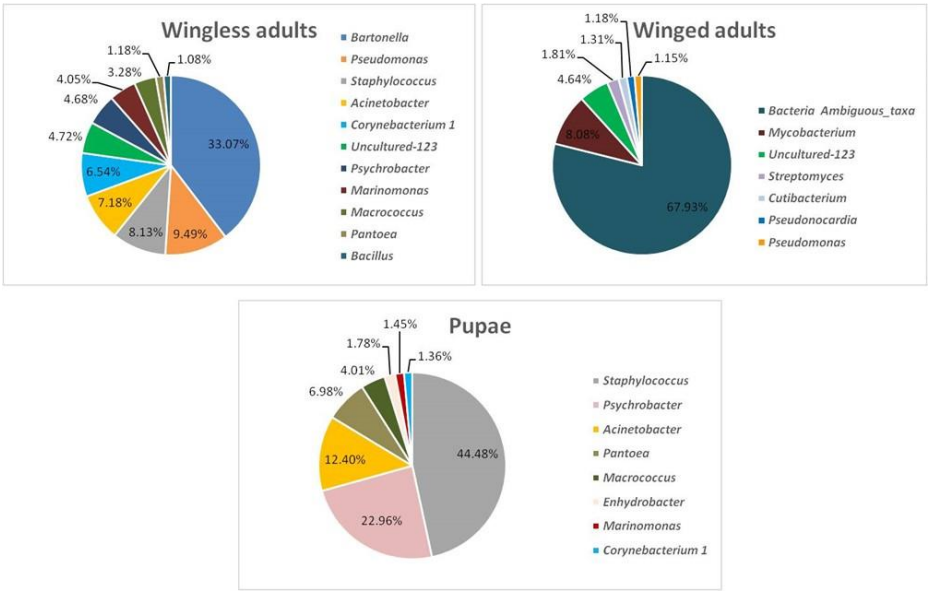


Figure 8.2. Composition of the microbiota community of wingless adults, winged adults, and pupae of *L. fortisetosa* at genus level (only genera > 1% are represented)

Considering the sex of the wingless adult ectoparasites, the microbial composition was almost identical in terms of genus, with high abundance of *Bartonella*, followed by *Pseudomonas*, *Staphylococcus*, *Acinetobacter*, *Corynebacterium 1*, *Uncultured-123*, *Psychrobacter*, *Marinomonas*, *Macrococcus*, *Pantoea* and *Bacillus*. Taking into account only genera with over 1% of relative abundance, the only difference was the presence of 1.01% of *Flavobacterium* and 1% of *Kurthia* in the male group, and *Enterobacter* 1% in the female group. Differences in the relative abundance of the genus in the two groups were highlighted as shown in Figure 8.3.

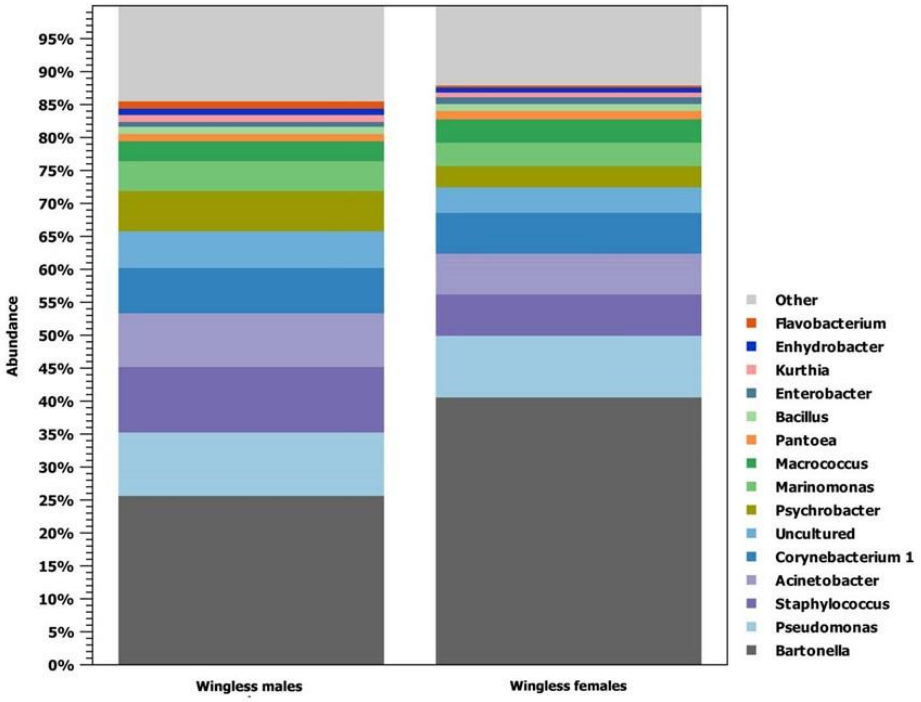


Figure 8.3. Relative abundance of bacterial genera detected in male (M) and female (F) wingless adults of *L. fortisetosa*.

The alpha-diversity analysis confirmed these data (Figure 8.4). The evaluation with total number and Chao-1 bias-corrected indexes was not significant, while the Kruskal-Wallis p-values for Shannon entropy and Simpson's index were 0.005 and 0.01, respectively. The two last indexes consider the presence/absence and the abundance of different genera. The comparison between genera identified in males and females did not reveal differences (Figure 8.5): the Permanova analysis on the results of the beta-diversity calculated with Bray-Curtis index was not significant. Regarding the presence of potential human pathogens (Figure 8.6), the high abundance of *Bartonella* was evident. Almost all (99.9%) the *Bartonella* OTUs were assigned to *B. bovis* Bermond *et al*. 2002. In four samples the presence of *Moraxella osloensis* Bøvre & Henriksen, 1967 was identified ranging from 9.13%

to 24.99%. One sample showed a high relative abundance (13.28%) of *Arsenophonus*, an endosymbiont of hippoboscids. Only two OTUs were identified as *Candidatus A. lipopteni* One pool of winged adults showed 45.16% of *Mycobacterium*, while *Rickettsia* was detected in trace in some samples, with a maximum of 0.0022 %.

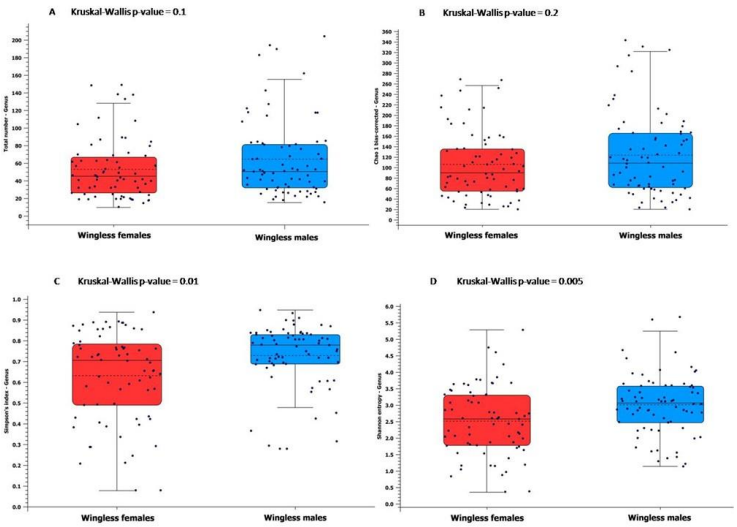


Figure 8.4. Box plots representing the alpha-diversity calculated with Total number (A), Chao 1 bias-corrected (B), Simpson's index (C), and Shannon entropy (D) for male (M) and female (F) wingless adults of *L. fortisetosa*.

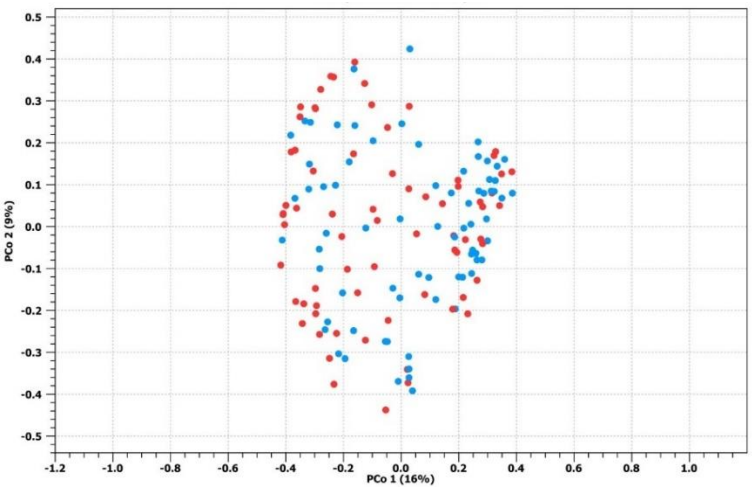


Figure 8.5. Scatter plot of the beta-diversity analysis of the male (blue) and female (red) wingless adults of *L. fortisetosa*.

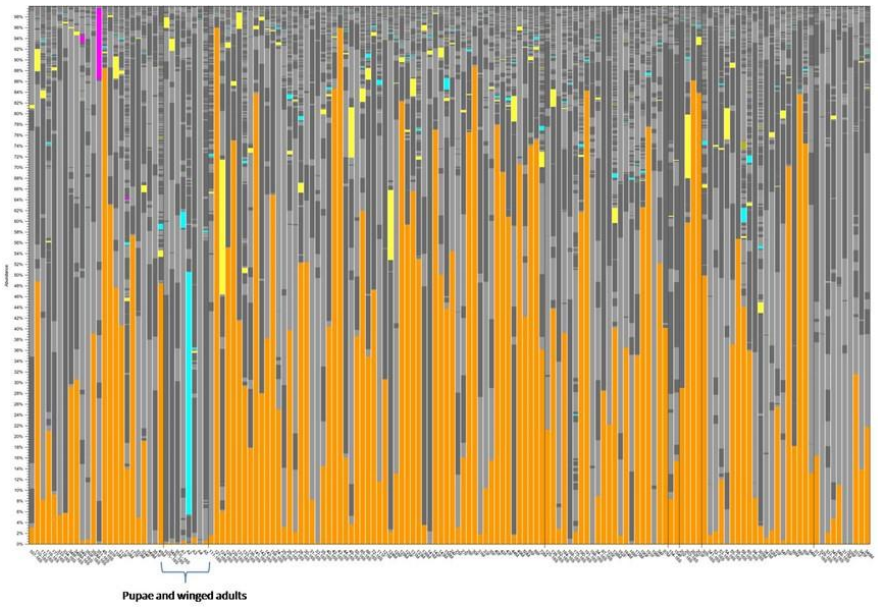


Figure 8.6. Microbial community composition at genus level for all the analysed samples (orange: *Bartonella* spp.; yellow: *Moraxella osloensis*; purple: *Arsenophonus* spp.; light blue: *Mycobacterium* spp.)

## Discussion

In this study, we analysed samples of *L. fortisetosa* collected from different areas of the Tuscan-Emilian Apennines (central Italy) in order to characterize the ectoparasite microbiota and to investigate the presence of microorganisms dangerous for animals or humans.

Differences were highlighted in the microbial community composition of adults, winged adults, and pupae. In particular, the most remarkable result was the great abundance of Bartonellaceae in wingless adults. On the contrary, in winged adults and pupae Bartonellaceae was almost absent with a major presence of Mycobacteriaceae in winged adults while Staphylococcaceae and Moraxellaceae in pupae. Regier *et al*., (2018) analysed the microbial composition in *L. cervi* collected from roe deer and fallow deer

showing a great abundance of Bartonellaceae and Enterobacteriaceae, which counted for almost the 90% of the microbial composition. Our data did not show high level of Enterobacteriaceae in any developmental stage, while the great amount of Bartonellaceae was confirmed also for our samples, although a higher diversity of bacterial composition in *L. fortisetosa* was evidenced in comparison to *L. cervi* in Germany (Regier *et al*., 2018).

Considering the genus level, despite a high abundance of *Bartonella* (33.07%) in adults of *L. fortisetosa*, other nine genera counted over 1%, ranging between 1.08% and 9.49%. In *L. cervi* the microbiome mainly consisted of *Arsenophonus* spp. and *Bartonella* spp., referred in particular to *A. lipopteni* and *Bartonella schoenbuchensis* Dehio *et al*., 2001 (Regier *et al*., 2018). Our data obtained by *L. fortisetosa* were classified mainly as *B. bovis*, while only two OTUs were detected for *A. lipopteni*.

Comparing the microbial community of male and female wingless adults, there was no difference in terms of identified OTUs. Only the Shannon entropy and the Simpsons' index were significant: these two indices of diversity take into account also the abundance of the OTUs in the groups beside the OTU composition of the communities. Nevertheless, the beta diversity analysis did not show any difference between the two groups.

Among the detected pathogens, five genera of concern for human health were found: *Bartonella*, *Moraxella*, *Mycobacterium*, *Arsenophonus*, and *Rickettsia*.

In general, our results showed a relevant presence of *Bartonella* spp. in the microbiota of *L. fortisetosa* samples. These microorganisms are facultative aerobic or microaerophilic, fastidious, Gram-negative

bacteria. Until now 35 species and subspecies have been identified, of which 13 have been considered involved in human diseases (Okaro *et al*., 2017). *Bartonella bacilliformis* (Strong *et al*. 1913) Strong *et al*. 1915, *B. henselae* (Regnery *et al*., 1992) Brenner *et al*., 1993, and *B. quintana* (Schmincke, 1917) Brenner *et al*., 1993 are the most likely implicated species in human infections, but it is plausible that all *Bartonella* species found in animals are able to infect humans. Actually, *B. schoenbuchensis* is considered a possible aetiological agent of deer ked dermatitis (Dehio *et al*., 2004, Korhonen *et al*., 2015). Many mammals, including canines, felines, rodents, bats, and ruminants are known as reservoirs for these bacteria (Breitschwerdt and Kordick, 2000; Chomel *et al*., 2006). Within the wide range of animal hosts, these pathogens have been isolated or detected in free-living cervid species, mainly attacked by deer keds, as red deer (*C. elaphus*), moose (*A. alces*), roe deer (*C. capreolus*), white-tailed deer (*O. virginianus*), and Sika deer (*C. nippon*) (Dehio *et al*., 2001; Víchová *et al*., 2011; Sato *et al*., 2012; Duodu *et al*., 2013; Korhonen *et al*., 2015; Razanske *et al*., 2018; Regier *et al*., 2018; Izenour *et al*., 2020). *Bartonella* spp. are especially transferred from reservoir hosts to susceptible uninfected ones via arthropod vectors or through direct inoculation via blood-to-blood, depending on the species. Several hematophagous species, as sandflies, lice, fleas, ticks, and biting flies, are known to be carriers of these bacteria (Chomel *et al*., 2009a; Tsai *et al*., 2011). In the Hippoboscidae family, *L. cervi*, *H. equina*, and *M. ovinus* have been recognized to be possible vectors of *Bartonella* spp. to ruminants (Halos *et al*., 2004). As well *Bartonella* spp. have been detected in *L. mazamae* samples collected from white-tailed deer in Georgia, South Carolina, and Massachusetts (Reeves *et al*., 2006; Matsumoto *et al*., 2008). Similar to our results, *L. fortisetosa* tested positive for these

microorganisms also in other European countries (Bartosik *et al*., 2021; Gałęcki *et al*., 2021; Sato *et al*., 2021). Such findings are consistent with the hypothesis that these keds are competent for *Bartonella* spp. transmission. The detection of *Bartonella* DNA in hippoboscid flies suggests that these ectoparasites may play a significant role in the circulation and maintenance of these vector-borne pathogens, which have been linked to emerging and re-emerging diseases for humans and animals (Chomel *et al*., 2003; 2009b).

Despite ruminant-infesting *Bartonella* species seem to be of little clinical importance on animal hosts, the detection of these pathogens in the blood of deer ked-infested moose indicates that these microorganisms cause a persistent and systemic infection in this cervid (Duodu *et al*., 2013). Additionally, these pathogens can be detrimental to humans. These bacteria, in fact, cause a complex disease known as Bartonellosis which resolves in a variety of signs from mild symptoms such as fever, headache, weight loss, and muscle fatigue, to more severe symptoms such as hallucinations, partial paralysis, and other neurological manifestations. Several disease syndromes are associated with *Bartonella* infection: Carrion's disease, cat-scratch disease, chronic lymphadenopathy, trench fever, chronic bacteraemia, bacillary angiomatosis, bacillary peliosis, vasculitis, uveitis and endocarditis, whose cases are rapidly rising (Chomel *et al*., 2003; Okaro *et al*., 2017; Cheslock and Embers, 2019).

Our analyses revealed that almost all the detected *Bartonella* belonged to the species *B. bovis*. It is known that this microorganism can cause bovine endocarditis (Maillard *et al*., 2007; Erol *et al*., 2013), but it seems to be associated also with human Bartonellosis (Garcia-Esteban *et al*., 2005). The presence of pathogen DNA in hippoboscids does not demonstrate itself their vector competence, but, as

highlighted by Dehio *et al*. (2004) and Sato *et al*. (2021), the detection of *Bartonella* in the midgut of *L. cervi* and *L. fortisetosa* suggests that deer keds may serve as biological vector for these bacteria. Such hypothesis is further supported by the evidence that *Bartonella* spp. can be vertically transmitted both transgenerationally from adults to their offspring and transstadially through the life-cycle stages. In fact, de Bruin *et al*., 2015 and Korhonen *et al*., 2015 underlined the presence of *Bartonella* spp. in winged adult and larval stages of *L. cervi*, and Duodu *et al*., 2013 tested ten pools of *L. cervi* pupae, finding a high prevalence of *Bartonella* spp. in one pool, but a scarce or absence occurrence in the others. Gałęcki *et al*. (2021) detected the presence of several pathogens, including *Bartonella* spp., in *L. fortisetosa* winged adults swept from the environment. Differently, in the present study, winged specimens collected into the wild and pupae picked up directly from cervids revealed that *Bartonella* spp. was almost absent, with a relative abundance ranging between 0.16% and 0.19%. Contrarily, our outcomes highlighted that in wingless adults these microorganisms are the most frequent pathogens representing 33.07% of the microbiota. These results might suggest that vertical transstadially transmission of these microorganisms in *L. fortisetosa* is not very likely, however further data are needed because of the small number of pupae and winged adults tested.

The presence of *Mycobacterium* spp. in one pool of wingless samples is noteworthy since this genus comprises several species associated with human tuberculosis. Besides, it counts also nontuberculosis species able to cause other human infections (Falkinham, 2002). As a matter of fact, these pathogens can infect and cause diseases, such as respiratory disorder, skin, and joint infections in humans, mammals, and birds. The *Mycobacterium* genus was

isolated from ticks collected in Hungary, inducing the hypothesis that these pathogens could enter the tick body, replicate, and be spread by the vector (Egyed and Makrai, 2014).

Belonging to the Enterobacteriaceae, we identified the presence of *Arsenophonus* genus in one pool of wingless adults collected from a red deer. This genus encompasses obligate intracellular symbionts often associated with hippoboscids. In fact, it has been already detected in the sheep ked *M. ovinus* (Nováková *et al*., 2015), in the louse fly *Crataerina pallida* (Latreille, 1812) (Cerutti *et al*., 2018), and in the deer ked *L. cervi* (Nováková *et al*., 2016; Regier *et al*., 2018). Even though *Arsenophonus* can be considered an insect endosymbiont of unknown pathogenicity in humans, a co-infection with *A. nasoniae* Gherna *et al*., 1991 and *Orientia tsutsugamushi* Tamura, 1995 has been reported in a traveller's skin eschar (Edouard *et al*., 2013). Interestingly, *Bartonella* was absent in the pool where *Arsenophonus* was detected, but no hypothesis can be raised at this point to explain this intriguing observation.

Our analyses detected also the presence of *Rickettsia* genus, although in low quantity, in a pool of wingless adults collected from a red deer. *Rickettsia* spp. are non-motile, Gram-negative, obligate intracellular bacteria with worldwide distribution. The genus comprises 27 recognized species and is classified into different groups: the spotted fever group (SFG); the ancestral group; the typhus group (TG). The main vectors are blood-feeding arthropods such as ticks, lice, and fleas, but also *M. ovinus* and *L. cervi* have been recently recognized to be competent vectors of these agents (Hornok *et al*., 2011; Merhej *et al*., 2014; de Bruin *et al*., 2015; Liu *et al*., 2016). Different animals such as cattle, sika deer, roe deer, and red deer have been evidenced to be reservoirs of rickettsiae (Jilintai *et al*., 2008; Hornok *et al*., 2011). Several

*Rickettsia* spp. can cause illnesses in humans. The most common symptoms are fever, headache, rash, or eschar, but these pathogens have been associated also with perimyocarditis and meningitis (Nilsson *et al*., 1999; Fournier *et al*., 2000; Nilsson, 2009; Nilsson *et al*., 2010). Differently from the results obtained by Gałęcki *et al*., 2021 on *L. fortisetosa*, our analyses did not reveal the presence of these species in winged samples. Additionally, De Bruin *et al*., 2015 did not detect these pathogens in winged *L. cervi*, suggesting that a vertical transmission is not very likely. However, a greater amount of samples should be analysed to confirm this hypothesis.

Noteworthy, *Moraxella* genus was detected in the microbiota of *L. fortisetosa*. These microorganisms are aerobic, oxidase-positive, Gram-negative, commensal coccobacilli, which are part of the normal flora of respiratory tract of humans. However, these bacteria have also been reported as rare causative pathogens in human diseases. The genus encompasses above 20 species, including *M. osloensis*, which has been isolated from nasopharynx. This species can be involved in rare human infections, harmful for adults or children, outpatients, as well as people without pre-existing pathologies. Diseases include endocarditis, meningitis, osteomyelitis, endophthalmitis, pneumonia, septic arthritis, central venous catheter-related infections, and bacteraemia (Shah *et al*., 2000; Roh *et al*., 2010; Bard *et al*., 2011; Sung *et al*., 2014).

## Conclusions

Overall, these first findings on the microbiota of *L. fortisetosa* in central Italy, provide a draft scenario of the possible role of this invasive ectoparasite as a vector of pathogens potentially harmful for animals

and humans. Our analyses revealed the presence of some microorganisms known as aetiological agents, including *Bartonella* and *Rickettsia* genera. Even though the vector capacity of *L. fortisetosa* has yet to be ascertained, it should not be ignored that this deer ked may transmit dangerous microorganisms, particularly in highly infested environments. These investigations provide a contribute to enlarge the knowledge on medical and veterinary importance of this species actively spreading throughout Europe. Moreover, such results are especially valuable for people working or going round natural environments for recreational purposes or hunting activities.

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# Conclusion

The present research intended to study two species of deer keds, *Lipoptena cervi* and *L. fortisetosa*, under different aspects, especially those related to the ectoparasitic lifestyle they evolved to successfully exploit their hosts. Hippoboscids have been extensively explored, both for morphological and behavioural adaptations which have ensured them to build a close association with suitable hosts.

The study was focused on *L. cervi* and *L. fortisetosa* since they are currently receiving great attention especially for possible implications in the transmission of pathogenic microorganisms. For this reason, the bacterial community of *L. fortisetosa* adults and pupae has been characterized in order to detect the eventual presence of etiological agents harmful for animals and especially humans. This hippoboscid was found to harbour pathogens of medical interest, e.g. *Bartonella* spp., *Moraxella* spp., *Arsenophonus* spp., *Mycobacterium* spp., and *Rickettsia* spp., raising concerns that this spreading ectoparasite may be a vector for zoonotic microorganisms. However, other aspects related to hippoboscids were worthy to be investigated, like the relationship they established with their hosts, how they locate the victims, and the dynamics they use to parasitize animals. Since humans could be occasionally bitten with health risks, understanding these ectoparasite behaviours was important to increase the knowledge on deer keds and provide insights into how to limit attacks on humans.

First of all, the present research allowed to determine the spread of both these ectoparasites in the Tuscan-Emilian Apennines in central Italy. An extensive sampling of hunted cervids permitted to confirm the establishment of the allochthonous *L. fortisetosa* in the study area and to assess that the species is strongly competing with the

autochthonous *L. cervi*. The infestation preference on three cervid species has been analysed concluding that both the ectoparasites target mainly red deer, although they are able to live also on roe deer and fallow deer. Regarding the host seeking activity, field experiments supported by microscopy observations on insect sensory organs have been carried out to understand which kind of stimuli (visual, chemical or both) are used by these ectoparasites to move around in the habitat, searching for a suitable host during their winged adult period. Interestingly, results showed that *L. fortisetosa* has a colour ranking of preference, with blue as the most attractant similarly to many other hematophagous insects. Responses clearly demonstrated that these flies use visual stimuli and are attracted to a specific colour, suggesting that it could be possible to set up traps *ad hoc* to monitor populations or to limit attacks to humans. Additionally, morphological and ultrastructural investigations on different hippoboscid species, including *L. fortisetosa* and *L. cervi*, revealed that they evolved a peculiar conformation of the antennae useful to protect these important appendages from the harsh environment in which the ectoparasites live (fur or plumage of hosts). Besides, the studied hippoboscids displayed a sensory pattern constituted by two types of sensilla (grooved coeloconic and basiconic sensilla) that seem to be involved in a thermo- hygro- carbon dioxide reception, and in the host odor perception, respectively. As well, other antennal structures appeared to be implied in the host location: the unarticulated arista could detect temperature variations, while microtrichia together with the reticulated cuticular surface could aid convey volatile compounds towards the internal sensory area. Such investigations allowed to confirm that hippoboscids use the chemoreception, together with visual stimuli, in the important activity of location of hosts. The peculiar

conformation of antennae with the total concealment of the third segment (flagellum) inside the other two elements (scape and pedicel) responds to the general morphological modification of the body of these insects, which are perfectly adapted to live together with specific host animals. Other microscopy observations have been performed in order to verify if Hippoboscidae members belonging to the three subfamilies (Ornithomyinae, Hippoboscinae, Lipopteninae) evolved some body features in response to the different infested species and the diverse parasitic behaviour. Outcomes revealed that the association level with the host, as well as the environment in which the ectoparasites live (animals' coat), strongly affected insect morphology. In fact, body structures such as legs, wings, and external sensory pattern of antennae are divergent features being different among the species. Further morphological studies enabled also to identify those body characters useful to taxonomically discriminate *L. cervi* and *L. fortisetosa*, which are often confused for their morphological resemblances.

All the conducted studies suggested that ectoparasite and host are closely associated. It is undoubted, in fact, that hippoboscids have been undergone to an evolutionary pressure exerted by the hosts (in terms of lifecycle and micro niche environment) which has led the ectoparasites to evolve themselves in order to co-exist with the victims. Besides to affect ectoparasite morphology and behaviour, the host can play an important role also on its spread. Phylogenetical studies are useful to trace the route covered by an alien species. Analyses performed on *L. fortisetosa* samples collected in Italy revealed that these populations are more closely related to those from Asia, the native area of *L. fortisetosa*, compared to those living in Europe. It is possible that this allochthonous hippoboscid species travelled from

Japan to Italy carried by its main and original host *C. nippon*, which has been recently detected in Italy as well.

The present research allowed to increase the general knowledge on two ectoparasitic species of veterinary and medical importance. Additionally, it permitted to acquire fundamental information about morphological, ultrastructural, and behavioural aspects poorly investigated, providing insights to set up monitor and control strategies against these annoying flies. However, other paths should be explored in order to complete the framework on *L. fortisetosa* and *L. cervi*. In particular, electrophysiological and behavioral bioassays should be carried out to further clarify host location responses. Additionally, parasitism dynamics of these hippoboscids should be analyzed considering also environmental variables and abundance of host species (for example land cover, altitude, slope, vegetation, climate, host density in the study area etc.). Finally, more-in depth investigations on potential vector competence of deer keds would be helpful in a public health perspective.

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