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**Morphological and molecular analyses of flies and blood-
sucking lice of veterinary importance from Malta**

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1. Introduction

Malta consists of three larger and several smaller islands that are situated in the middle of the Mediterranean region, south of Italy- between Africa and Europe (Alexander, 1988). This geographical position makes these islands very important from the veterinary-medical point of view, both economically (Bland, 1994) and ecologically. Considering the latter, Malta is an important stop-over site for migratory birds, flying in the autumn from Europe to their wintering grounds in Africa, then returning from Africa to Europe in the spring. Thus, during migratory activity as a natural phenomenon birds transport epidemiologically important tick species via Malta intercontinentally (Hornok et al., 2022). However, Africa and Europe are also connected through these islands as a consequence of human activity, for instance during trading and transportation of livestock and pet animals.

During the past few years several new tick species (Acari: Ixodidae, Argasidae) were discovered in Malta, in part probably as a consequence of animal transportation as reflected by the history of their finding on domestic animals (chicken and rabbit: Hornok et al., 2020). In another work, in the context of the presence of sucking lice (Phthiraptera: Anoplura) on goats in Sardinia, it was noted that this may have been related to the introduction of Maltese breed goats to that island (Fois et al., 2013/2014). However, focusing on arthropod ectoparasites of veterinary-medical importance in general, hitherto only the tick fauna and flea-borne pathogens appear to be updated in Malta (Hornok et al., 2018, 2020, 2022). Although the Diptera fauna of Malta has been extensively reviewed recently, (Ebejer and Gatt, 2021) it is not certain whether all muscoid and myiasis-causing flies have been surveyed on these islands or there are species that might still be indigenous but not yet found. For example, *Wohlfahrtia magnifica* (Diptera: Sarcophagidae) is known to occur in Sicily (Gaglio et al., 2011) less than 200 km from Malta. However, this fly species has never been reported in Malta (Ebejer and Gatt, 2021).

Thus, the aim of this study was to compensate for the lack of updated information on ectoparasites associated with domestic (mainly livestock) animals in Malta, with special emphasis on muscoid flies (Diptera: Muscidae), myiasis-causing flies (Diptera: Sarcophagidae, Calliphoridae) and blood-sucking lice (Anoplura: Haematopinidae, Linognathidae). It was also within the scope of this study to analyze collected flies and lice with molecular biological tools, to our knowledge for the first time in the context of Malta.

2. Literature Review

2.1. The Mediterranean Basin

The Mediterranean Sea has a total area of approximately 2.5 million km². It stretches a length of 3,600 km across, with the Straits of Gibraltar found to the west, and the Lebanese shores to the east. At the northern border lies the European continent while to the south is the continent of Africa, spanning a distance of 1,600 km. Due to the presence of such large landmasses to both the north and south of the Mediterranean basin, they have a significant effect on the climate associated with islands of this sea. The continental tropical air mass, which originates from Africa, is generally dry and warm. However, although such wind currents provide the islands with long-term stable weather throughout the summer months, it also produces high humidity, and thus causes these summer months to be both hot and humid.

From the north, two main air masses counter these southern winds. The first originating from mainland Asia is known as the continental polar air mass (which is relatively dry), while the other is the maritime polar air mass which originates from the North Atlantic. These previously mentioned air masses reach the centre of the Mediterranean basin - primarily during the winter and spring seasons, thus allowing temperatures to cool as well as promoting precipitation during the autumn. The collision of the northern and southern air masses produces the Mediterranean Front which results in temperatures across the Mediterranean basin - ranging in the winter between 6.1-10.0 °C, while inland temperatures in the summer may be as high as 32.2 °C (Schembri, 2019).

The Mediterranean basin is home to almost 3,000 islands, but for the study below, the Maltese archipelago is of particular importance. The Maltese islands are located 35° 55' 4.7028"N and 14° 24' 35.7948"E within the Sicilian channel, where the eastern and western Mediterranean basins connect. Although these islands are generally described as being in the centre of the Mediterranean basin and geographically equidistant from either end, they are about 93 km south from the island of Sicily, and 290 km from the north African coast. The Maltese islands spread

over a length of 45 km and possess an area of 316 km². These consist of a number of islands, islets and rock groups. The main inhabited islands are Malta, Gozo and Comino, which have an area of 245.8 km², 67.1 km² and 2.8 km² respectively. The length of the channel that separates the two main islands (Malta and Gozo) is 5 km. Although the two main islands are in relative vicinity of each other, the habitats on Gozo are somewhat different, lacking the urbanization and industrial development observed in Malta (Schembri, 2019).

2.2. Occurrence and veterinary-medical significance of the common housefly (*Musca domestica*) in the Mediterranean Basin

The order Diptera includes a number of fly and mosquito species. The flies in particular have a life cycle that has become closely associated with that of humans and have even adapted to the domestic environment of humans. Due to their close association, they have become of particular importance as potential pests and carriers of diseases, namely of those enteric in nature. Such dipterans are considered to be public health pest with 44 species of Calliphoridae, Fanniidae and Muscidae being identified on the Maltese islands (Savona-Ventura, 2002; Schembri et al, 1991). Amongst these pests is the Muscidae, including *Musca domestica*. (Savona-Ventura, 2002).

The common housefly, *M. domestica* (Linnaeus, 1758), is ubiquitous in the Maltese islands. These flies breed during a period spanning between April and November (Harris et al, 1976). As stated previously, primary breeding habitat for this fly is manure, which attracts adults and if not completely dry, acts as a substrate in which dipterous larvae may survive. Pig manure in particular, when not too fluid in consistency, was of particular favorite of the housefly (Grose et al, 1977; Harris et al, 1976). Within piggeries and cowsheds, *M. domestica* is primarily found resting on strings and wires as well as on the edges of horizontal roof-beams and the upper vertical surfaces of low walls, which may separate the animals. Spilled feed such as bran or that of milk origin, as well as unwashed utensils and milk coolers within dairies were also common locations for the species to reside, albeit not for breeding. In the case of poultry houses, similarly to piggeries and cowsheds, flies congregated on the lower levels of cages and on the floors as well as within

feed containers, and in locations where feed was spilled. However, in this case, less flies were found around the birds as well as their droppings. It has been suggested that such an observation may be due to the fact that *M. domestica* may prefer well-lit areas while poultry houses, during this study, were more dimly lit. Outside the stables, the houseflies resided on the farm walls, manure heaps as well as upon nearby vegetation, with the flies preferring low walls and areas generally protected from strong winds, but with no preference between sunlit and shaded areas (Grose et al, 1977).

In a study carried out by Kjærsgaard et al (2015), a number of populations of *M. domestica* were studied across Europe (Spain, Switzerland and Denmark) and their adaptability to temperature stress was emphasized. The species' ability to adapt to heat stress was based on the distance the different populations could travel under varying temperatures. From the results, it was concluded that the Spanish flies had a greater resistance to heat when compared to those of Swiss and Danish origin, as they had flown the furthest and were also capable of remaining more active at the temperatures being studied. This study suggested that *M. domestica* populations are capable of adaptive differentiation for heat resistance, allowing them to survive relatively hot and humid environments (Kjærsgaard et al, 2015).

Within the Maltese islands, *M. domestica*, although closely related to animal farms and human habitats, has not been recorded to be the vector of any specific human or animal diseases. However, due to the housefly's incremental manner of feeding, it may be partly responsible for some enteric infections in humans (Grose et al 1977). Despite this observation in the Maltese islands, *M. domestica* is generally known to be the carrier of a number of pathogens that cause disease in both humans and animals. The main means of transmission of pathogens is through mechanical transmission through its vomit or excreta. The type of pathogens transmitted by houseflies range from bacteria (e.g. causing anthrax and cholera), viruses (infantile diarrhea), fungi (*Aspergillus flavus* (Phoku et al, 2014)), helminth eggs (*Diphyllobothrium* sp.), protozoan cysts and trophozoites (*Trichomonas* spp., *Entamoeba histolytica* and *Giardia duodenalis*) (Issa, 2019).

2.3. Occurrence and veterinary-medical significance of the stable fly (*Stomoxys calcitrans*) in the Mediterranean Basin

Stomoxys calcitrans (Linnaeus, 1758), also known as the stable fly is another member of the Diptera order which is of particular importance to veterinary medicine. *Stomoxys* are members of the Muscidae family, which are obligate blood-sucking insects. Both males and females of the species feed on blood and are both aggressive and persistent feeders, known to even attack humans in the absences of their preferred hosts. A number of species have been recognized to be significant economic pests of livestock and other warm-blood animals. *Stomoxys calcitrans* is one such species with cosmopolitan distribution (Zumt, 1973). This species has been identified to be the most widely distributed members of the genus, being found in the western hemisphere and old world countries. Phenotypically, the stable fly greatly resembles the housefly mentioned before, *M. domestica*, as well as the horn fly, *Haematobia irritans*, however they possess a characteristic greenish-yellow color with four black stripes present upon their thorax and black checkering upon their abdomen (Showler and Osbrink, 2014).

Stomoxys calcitrans, within the Maltese islands, has been observed to remain in the vicinity of farm buildings and are more prevalent on the island of Gozo than the main island of Malta (Saliba et al, 1976). However, although found mainly near farms, they were also located in residential and business areas on the islands. It was also observed that the species was predominantly found in the vicinity of cattle and horses and, in a relatively small population near pigs, goats and dogs (Grose et al, 1977). The stable fly is a multivoltine species with the generation time being temperature dependent (Dsouli-Aymes, 2010). In a study conducted by Jacquiet et al (2014), it was observed that stable fly populations peaked in early summer and fall, but were nearly nonexistent in winter and relatively lower in summer. Such results indicate that temperature may be a determining factor of the life history parameters of *S. calcitrans*. In a study conducted by Lysyk (1998), it was found that the median immature development times varied depending on temperatures - with more than 60 days being needed at 15 °C and less than 12 days being needed at temperatures of 30 °C. In another study more specifically conducted to test *S. calcitrans* activity

pattern in Mediterranean semi-arid climates around cattle farms in Tunisia, it was deduced that, within such a habitat, the species exhibited two main peaks of activity. A peak was observed in March to July while a smaller peak was observed in November to January. The study also indicated that the trapping of the fly was positively influenced by temperature but negatively affected by rainfall and humidity, while no correlation was observed with wind speed. Within a day, the fly was observed to exhibit a bimodal activity pattern with a pick being observed between 11:00 am and 1:00 pm, however this varied greatly between the months and seasons (Khalifa et al, 2022).

Stable flies' larval stages mainly develop in moist soils and similar substrates, and these cause severe problems in dairies and feedlot. The resulting biting activity of the developing flies can cause decreases in both milk and meat production in cattle (Harris et al, 1976; Wall and Shearer, 1997). This species is also responsible for skin lesions which are a necrotic type to dermatitis on the tips of ears in dogs, an exudative dermatitis on the legs of horses (Wall and Shearer, 1997) and “hair whirlpools” on the backs of calves (Baldacchino et al. 2013) . As with other blood-sucking insects, they are the cause of a number of general issues related with veterinary medicine including pain and toxicity of skin during skin puncture and saliva injection, blood loss as well as being nuisances to the animals upon which they feed (Baldacchino et al. 2013).

As stated previously, *S. calcitrans* is of particular significance due to its ability to be a vector for disease. Due to the blood-sucking life cycle of these flies, they have been utilized as carriers of vector borne diseases, mainly through the mechanical transmission of pathogens. This mainly occurs prior to feeding, whereby saliva is injected into the host. During this time, it is possible for the stable flies to inoculate the host with some of the blood that remained in their mouthparts which may be infected with some disease. Their crop, which may also store blood, can also provide an ideal environment in which pathogens may thrive. Equine Infectious Anemia, African Swine Fever, West Nile virus and Rift Valley virus are just a few of the viruses which have been confirmed to utilize *Stomoxys* flies as their vectors for transmission while a few other pathogens, such a *Rickettsia*, *Trypanosoma* spp. and *Besnoitia* have also been considered as possible pathogens transmitted by this fly. *Stomoxys* has also been identified as the intermediate

host of a number of helminth parasites - including *Habronema microstoma* and may also be involved in *Onchocerca* and *Dirofilaria* species transmission (Baldachino et al, 2013).

In 2015, the African Swine Fever virus was detected in Europe and spread from wild and domestic swine. Initially it was detected in the Baltic regions and Poland, but by 2018 it had spread to the Czech Republic, Romania and Hungary (Olesen et al, 2018). African Swine Fever virus is a double-stranded DNA virus of the Asfarviridae family. This virus is responsible for causing a hemorrhagic disease of high lethality, mainly in domestic pigs and Eurasian wild boars. Although the host preference is particularly limited and, thus far, the virus has exhibited no zoonotic potential, it is classified as a notifiable disease by the World Organization of Animal Health (OIE). Due to its high mortality rate in the aforementioned swine, it is of particular socio-economic importance (Blome et al, 2020). Although its most likely means of transmission is direct contact with infected individuals, studies by Olesen et al (2018) and Vergne et al (2021) discussed the possibility of the virus being transmitted by *S. calcitrans* which had ingested infected blood and were, in turn eaten by other swine.

However, despite being a known vector of a number of diseases, within the Maltese archipelago *S. calcitrans* has not yet been identified as the transmitter of any important pathogens (Grose et al, 1977). Although they have not been associated with the transmission of disease in the Maltese islands, they are still considered as public health pests (Savona-Ventura, 2002).

2.4. Occurrence and veterinary-medical significance of green blowflies (*Lucilia* spp.) in the Mediterranean Basin

Lucilia species are members of the Calliphoridae family. Amongst the members of this family two main species are of notable importance in the Mediterranean region, the first being *Lucilia sericata* (Meigen, 1826), which is also known as the common green bottle fly. The second main species found is *Lucilia cuprina* (Wiedemann, 1830), commonly known as the Australian sheep blowfly. These two species may be differentiated morphologically with *L. cuprina* having a coppery (metallic) green coloration of the abdomen, while *L. sericata* has a metallic blue-green colour

(Tourle et al, 2009). The metasternal area is also distinguishable between the two species; with *L. sericata*'s being hairy, while that of *L. cuprina* is bare. The contour of the last abdominal tergite may also be used to differentiate the two species, with an irregular depression being present on *L. sericata* while it is generally smooth in *L. cuprina* (Williams and Villet, 2014).

Both *Lucilia* species previously mentioned are facultative ectoparasites of a number of endotherms, namely domestic sheep. Due to this preference of host, the *Lucilia* species are of economic importance as they are usually considered agricultural pests. *Lucilia cuprina* is often considered the main cause of myiasis or 'strike' in Australian sheep flocks. Sheep strike may be defined as the process during which flies lay their eggs on living organisms - in most cases sheep, and the maggots which hatch cause damage to the wool and skin by feeding on the sheep (Williams et al, 2014). However, in New Zealand it has been shown that both species are responsible for sheep myiasis (Tourle et al, 2009). Although *L. cuprina* is thought to have originated from the oriental regions, and *L. sericata* is thought to be Palaearctic in origin, both species have been identified in South Africa. However *L. cuprina* appears to be a far more problematic pest than *L. sericata*, with the latter being a major pest in Europe (Tourle et al, 2009; Stevens and Wall, 1996; Williams and Villet, 2014).

In the Maltese islands, it is *L. sericata*, which is considered of particular importance, as it is a recognized public health pest (Savona-Ventura, 2002). What is interesting to note is that, although *L. sericata* is found throughout Europe, in the south of Europe, contrary to what is observed in the more northern regions, it is not usually associated with myiasis and is found almost exclusively in carrion and in smaller populations. However, the exact reason behind this observation is as yet unknown. One possibility is the variation in population numbers of *L. sericata*'s natural competitors and predators (*W. magnifica* and *Chrysomya albiceps* respectively) between the northern and southern European regions (Martinez-Sanchez et al, 2006).

Lucilia sericata is a synanthropic fly, which is commonly found around human habitats across the globe (Diakova et al, 2018; Grassberger and Reiter, 2001). *Lucilia cuprina*, on the other hand, has been noticed to prefer rural environments and is more rarely found near urban

environments. However, irrespective of the species' respective habitats, they both possess the potential to spread disease to humans and animals alike due to the fact that they breed on decaying and rotting organic material (Williams et al, 2014). With the urbanization of land, a number of Mediterranean communities have changed over the years, with wooded areas being converted into open, clear-felled areas. Such locations were established for grazing purposes and have thus become closely associated with cattle dung and carrion and, of course, the insect fauna associated with such substrates. Within Europe, the blowflies dominate carrion communities as this substance acts as an important breeding habitat or a good source of proteins (Martinez-Sanchez et al, 2000).

However, some seasonal variation has been observed with the *Lucilia* species, with populations being more abundant in pastures during summer and autumn than in woodlands but with their populations becoming relatively smaller in winter and spring. In the summer, *L. sericata* was one of the most abundant species present but preferred cooler and less dry conditions when compared to other Calliphoridae species (Martinez-Sanchez et al, 2000). With the rise of global temperatures due to global climate change, however, spatial patterns of *Lucilia* species and their associated diseases may also change. A study by Rose and Wall (2011), predicted that, with the rate at which current global temperatures are rising, an increased incidence of 'Strike' as well as elongated blowfly seasons may develop. Although they theorized that Strike would still remain relatively rare in the winter, the temperatures in the midsummer may become too high, and the environment too dry for *L. sericata* to continue to thrive. However, this does not negate the possibility that other more pathogenic Mediterranean agents of myiasis, such as *W. magnifica*, may take advantage of these climatic changes to increase their population numbers and so become a more significant health risk during these times.

Both species of *Lucilia* are of medical importance due to the major health risks they may cause in humans (such as Shigella dysentery) (Tourle et al, 2009). However, despite these problems, *L. sericata* has been used medically in the cleaning of dead tissue around wounds known as maggot debridement therapy (Tourle et al, 2009). *Lucilia sericata* has also been utilized within forensic entomology, whereby their larvae are utilized to estimate post mortem intervals

(Grassberger and Reiter, 2001). However, one defining factor that differentiates *L. sericata* from *L. cuprina* is that the latter has little to no medical usage as, unlike *L. sericata*, if applied to necrotic tissue to clean wounds, it will consume this tissue along with the healthy tissue and which, in turn may result in bacterial infection arising within the wound (Tourle et al, 2009).

2.5. Occurrence and veterinary-medical significance of blood-sucking lice (*Haematopinus* and *Linognathus* spp.) in the Mediterranean Basin

Blood sucking lice are members of the parvorder Phthiraptera and the superfamily Anoplura. Such lice are permanent, host-specific ectoparasites of mammals. Such species are of veterinary importance due to two main reasons;

Firstly, they are responsible for a number of economic losses due to the induction of pathophysiological changes in their hosts (weight loss, anemia, hide damage etc). Mild to severe anemia has been most commonly reported in cattle infested with blood-sucking lice (Hornok et al, 2010). Since such ectoparasites may live on, puncture or burrow through the host's epidermis to feed or for shelter they often result in direct damage to the host's skin and subcutaneous tissue. The presence of salivary and fecal antigens of these ectoparasites may stimulate the immune response of the host which may, in turn, results in hypersensitivity in certain individuals. Apart from this, during the feeding of such ectoparasites, significant blood loss may be observed, as well as secondary infection, pruritus, excoriation, alopecia and, ultimately death in some cases (Colebrook and Wall, 2004). An overall decrease in production in both breeding and fattening in the case of pigs (Damriyasa et al, 2004) and a decreased milk and meat productivity in buffalo (Veneziano et al, 2013) is also seen.

The second reason for their importance to veterinary medicine is their ability to act as vectors through which louse-borne pathogens may be transmitted to susceptible hosts. They may act as biological vectors, whereby the development of disease agents occurs in their gut cells. They may be, in such situations, capable of inoculating the pathogen during consecutive blood-sucking feeds, however pathogens may be rubbed into the host's skin as well. Louse are also capable of

acting as short term mechanical vectors, but it must occur within hours of prior feeding, whereby the inoculation pathogenic agents attach to their mouthparts. However, irrespective of the method of pathogen transmission, it is required for the lice to travel between hosts to transfer the pathogens between hosts, which is, in fact, an integral aspect of their behavior (Hornok et al, 2010).

Two groups of the main blood sucking lice found along the north African coast and the Mediterranean basin are *Haematopinus* spp. (Leach, 1815) and *Linognathus* spp. (Enderlein, 1905) (Colebrook and Wall, 2004; Gabaj et al, 1993). The main hosts of *Haematopinus* spp. have been described to be Bovidae, Cervidae, Suidae and Equidae while those of *Linognathus* spp. are Bovidae, Cervidae, Giraffidae and Canidae (Durdan and Musser, 1994).

As stated previously, blood sucking lice may act as vectors of pathogenic bacteria such as members of the genus *Anaplasma*. It has been recorded that *Haematopinus suis* and *Linognathus stenopsis* have been shown to be vectors for this bacteria, namely in the suis and caprine populations respectively. *Linognathus vituli*, *L. stenopsis* and *H. suis* have also been reported to possess bovine and ovine *Anaplasma* spp. A number of *Linognathus* species, as well as species of *Haematopinus eurysternus* were also detected to carry *Rickettsia* species (Hornok et al, 2010). *Haematopinus tuberculatus* has also been identified as the vector of anaplasmosis in buffalo (Da Silva et al, 2013). *Haematopinus quadripertusus* and *H. eurysternus* are ectoparasites of cattle, and found to be the cause of skin damage leading to keratoconjunctivitis and periorbital papillomatosis during heavy infections (Yeruham et al, 200). *Haematopinus eurysternus* has also been identified as the cause of anemia, unthriftiness and a decrease in vigor in highly infected animals (Shemanchuk et al, 1960). In experimentally infected droves of pigs, *Haematopinus* has been observed to also be a carrier of African Swine Fever (Guinat et al, 2016) while *H. tuberculatus* has been suggested to be the carrier of *Brucella abortus* (Neglia et al, 2013). *Acanthocheilonema reconditum* (causative agent of canine subcutaneous filariasis) follows an indirect life cycle in which the infective larvae are carried by lice - including those of the genus *Linognathus* within the Mediterranean basin (Tahir et al, 2019).

3. The Study

3.1. Materials and Methods

3.1.1. Sample collection, morphological identification

During the summer and autumn of 2020 and 2021, altogether 3095 flies were collected on repeated (mostly monthly) occasions in rural areas: at four cattle farms, three sheep and goat farms, as well as a pig farm, and near dogs in two locations (Figure 1, Table 1). Sampling took place between 9 a.m. and 11 a.m, with fish nets. Flies were caught from the walls and gates of stables and pens below 1 m height (e.g. where the animals put through their head to feed). Samples were also collected besides the calves in a similar manner. In case of dogs the area outside the kennel cages was sampled. For comparison, after evaluating the species of blowflies caught with fish net near livestock animals at farms in July and August, 2020-2021, 37 blowflies were collected at a rural site (void of farms) in the northern part of Gozo and at an urban site, in Swieqi in Malta in July, 2022. Bait traps made of plastic bottles (containing pieces of decomposing chicken meat and pork) were used for this purpose. In addition, lice were removed from 20 goats with soft tweezers at two farms.

All flies, lice were stored in 96% ethanol, and their species/sexes were morphologically identified according to the following sources: Williams and Villet, 2014; Vockeroth, 1953; Sztankay-Gulyás, 1953; Złotorzycka et al., 1974. Pictures of representative specimens (were made with a VHX-5000 digital microscope (Keyence Co., Osaka, Japan). Molecular identification of selected flies was performed as outlined below (all individuals from the genus *Stomoxys*, 17 *Lucilia*, three *Musca* and four *Linognathus* specimens).

Figure 1 Map of the Maltese islands indicating sampling locations



3.1.2. DNA extraction

From 28 muscoid flies and blowflies that served as voucher for species identification, DNA was extracted from two legs (including all eight *Stomoxys* and three *Lucilia*, as well as three *Musca*) individually. After successful species identification, this was followed by DNA extraction from the whole body for pathogen screening in case of six specimens of *Stomoxys* and 13 blood-sucking lice (six males, seven females), with the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction, including an overnight digestion in tissue lysis buffer

and Proteinase-K at 56 °C, as reported by Hornok et al. (2014). An extraction control was also processed in each set of samples.

3.1.3. Molecular analyses of flies and blood-sucking lice for species identification

A 710 bp-long fragment of the *cox1* (cytochrome c oxidase subunit I) gene was amplified from fly DNA extracts with a conventional PCR modified from Folmer et al. (1994). The primers HCO2198 (forward: 5' -TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (reverse: 5' -GGT CAA CAA ATC ATA AAG ATA TTG G-3') were used in a reaction volume of 25 µl, containing 1 U (0.2 µl) HotStarTaq Plus DNA polymerase, 2.5 µl 10x CoralLoad Reaction buffer (including 15 mM MgCl₂), 0.5 µl PCR nucleotide Mix (0.2 mM each), 0.5 µl (1 µM final concentration) of each primer, 15.8 µl ddH₂O and 5 µl template DNA. During the amplification, the initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 94 °C for 40 s, annealing at 48 °C for 1 min and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min.

For the identification and molecular analyses (pathogen screening) of blood-sucking lice another PCR was used, which amplifies an approx. 420 bp-long-fragment of the *cox1* gene of Phthiraptera with the primers L6625 (5'- COG GAT CCT TYT GRT TYT TYG GNC AYC C -3') and H7005 (5' – CCG GAT CCA CNA CRT ART ANG TRT CRT G -3') (Hafner et al., 1994). During the amplification, the initial denaturation step at 95 °C for 5 min was followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 45 °C for 1 min and extension at 72 °C for 1 min. Final extension was performed at 72 °C for 10 min.

3.1.4. Molecular analyses of flies and blood-sucking lice for pathogen screening

Samples were screened for the presence of **piroplasm**s by a conventional PCR modified from Casati et al. (2006) as reported (Hornok et al., 2022). The primers BJ1 (forward: 5'-GTC TTG TAA TTG GAA TGA TGG-3') and BN2 (reverse: 5'-TAG TTT ATG GTT AGG ACT ACG-3') were used to amplify an approximately 500-bp-portion of the 18S rRNA gene of *Babesia/Theileria*

spp. The reaction volume was 25 µl, i.e., 5 µl of extracted DNA was added to 20 µl reaction mixture containing 1 U of HotStarTaq DNA Plus polymerase (Qiagen), 200 µM of PCR nucleotide mix, 1 µM of each primer and 2.5 µl of 10× CoralLoad PCR buffer (15 mM MgCl₂ included) (Qiagen). Cycling conditions included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s and extension at 72°C for 40 s. The final extension was performed at 72°C for 5 min.

For the assessment of samples for the presence of **Anaplasmataceae**, the primers EHR16SD (5'-GGTACC YAC AGA AGA AGT CC-3') and EHR16SR (5'-TAG CAC TCATCG TTTACA GC-3') were used, which amplify an approximately 345 bp fragment from the 50 region of the 16S rRNA gene from various members of this family (Brown et al., 2001). One microliter of extracted DNA was included in a 20 ml reaction mixture comprised of 1.0 unit of HotStartTaq DNA polymerase, 200 mM of each dNTP, 10 pmol of each primer and 1.5 mM MgCl₂ (HotStart-Taq Master Mix, QIAGEN, Hilden, Germany). An initial denaturation step at 94°C for 15 min was followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s. Final extension was done at 72°C for 5 min.

In addition, a 380 bp-long fragment of the citrate synthase (gltA) gene of **rickettsiae** was also attempted, with the primers RpCs.877p (5'-GGG GGC CTG CTC ACG GCG G-3') and RpCs.1258n (5'-ATT GCA AAA AGT ACA GTG AAC A-3') (Regnery et al., 1991). During the PCR, the initial denaturation step at 95°C for 5 min was followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 48°C for 30 sec and extension at 72°C for 1 min. Final extension was performed at 72°C for 5 min.

3.1.5. PCR controls, sequencing and phylogenetic analyses

All above pathogen screening PCRs were run with appropriate sequence-verified positive controls and negative control (non-template reaction mixture). Extraction controls and negative controls remained PCR negative in all tests.

Conventional PCR products were subjected to electrophoresis and consequently stained, visualized in 1% standard agarose gel (SeaKem LE Agarose, Lonza Inc.). Purification and

sequencing of the PCR products were done by Biomi Ltd. (Gödöllő, Hungary). Obtained sequences were manually edited, then aligned with GenBank sequences by nucleotide BLASTN program (<https://blast.ncbi.nlm.nih.gov>). Representative sequences will be submitted to GenBank. Sequences from other studies (retrieved from GenBank) were included in the phylogenetic analyses only if they had nearly 100% coverage with sequences from this study. This dataset was resampled 1,000 times to generate bootstrap values. Phylogenetic analyses were conducted with the Maximum Likelihood method by using MEGA version 7.0.

3.1.6. Statistical analysis

Abundance data were analyzed by Fisher's exact test and differences were regarded as significant if $P < 0.05$.

3.1.7. Ethical permission

Ectoparasites used in this study were collected under veterinary supervision, either from the environment of livestock animals without restraint (relevant to flies), or from small ruminants during regular care. Therefore, no ethical permission was needed.

3.2. Results

3.2.1. Morphological identification of species

Altogether, 3095 flies (Diptera: Muscidae, Calliphoridae) were collected at farms and kennels near domestic animals, as well as 37 blowflies (Calliphoridae) in rural and urban areas without animals nearby. Regarding Muscidae, the great majority of flies ($n = 3084$) were identified as the common housefly *Musca domestica*, based on the position of eyes (wide frons), shape of the abdomen (longitudinally flattened, pointed at the end) and setation of the propleuron. Eight flies represented the stable fly *Stomoxys calcitrans* (Figure 2.a.) based on the forward projecting proboscis and dotted abdominal pattern. In addition, three blowflies collected near animals belonged to *Lucilia cuprina* based on the dark color of the frontoclypeal membrane and the number of setae on the scutellum (Figure 2.b.). By contrast, all 37 blowflies collected with bait traps, without domestic animals nearby, were identified as *L. sericata*.

Figure 2.a Images of *Stomoxys calcitrans* collected in Malta

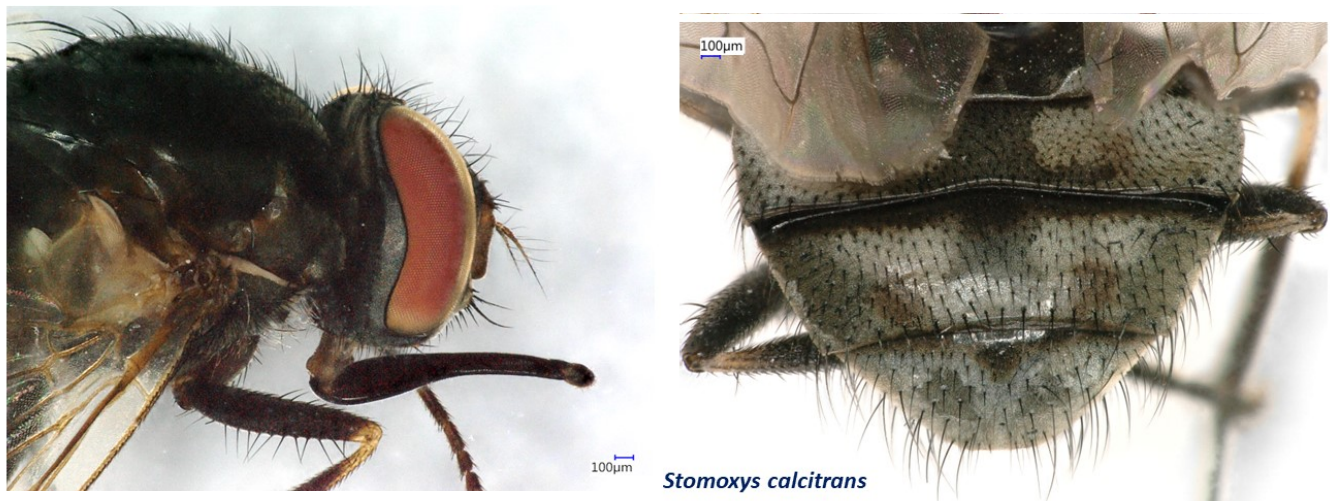
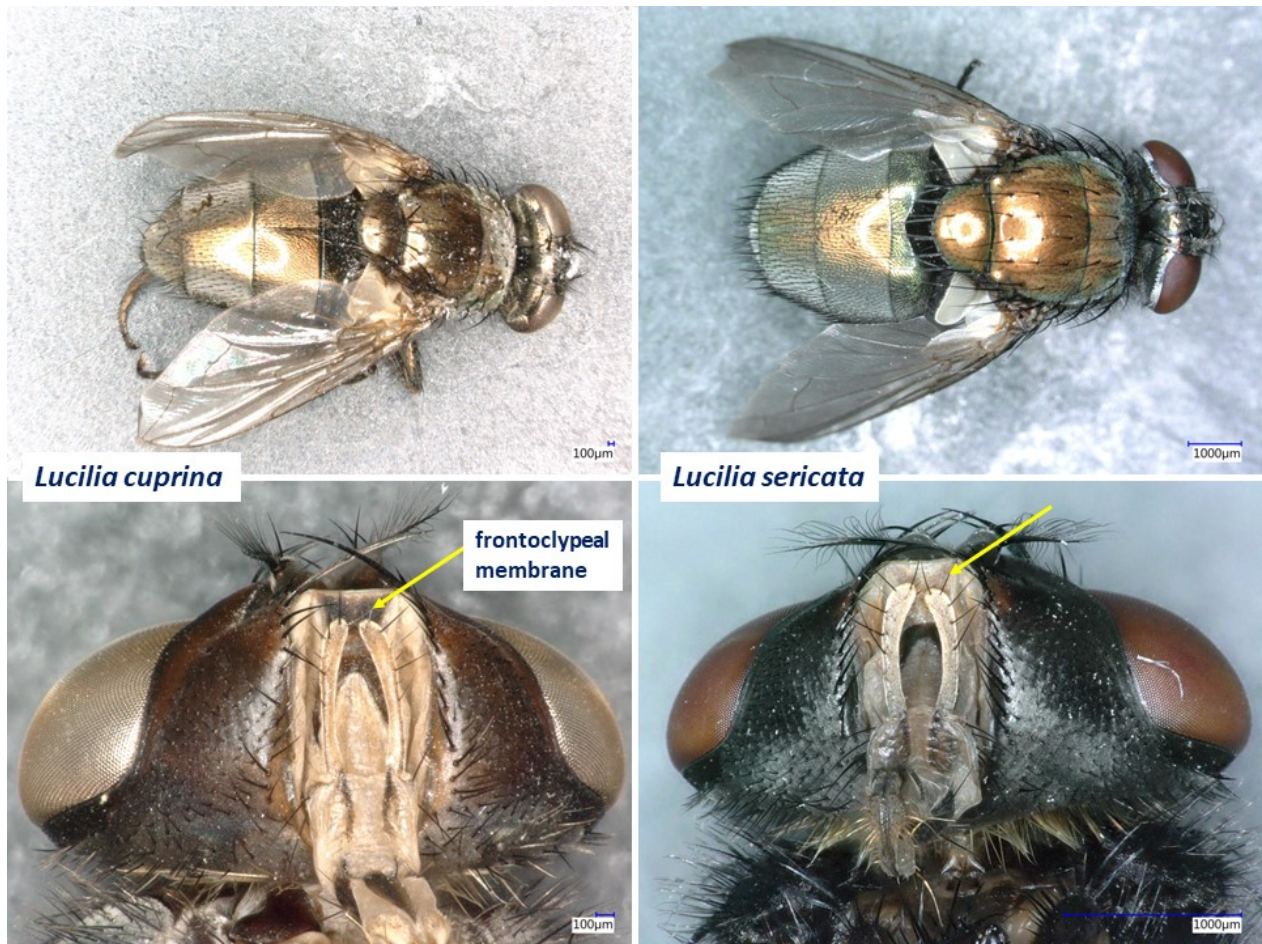
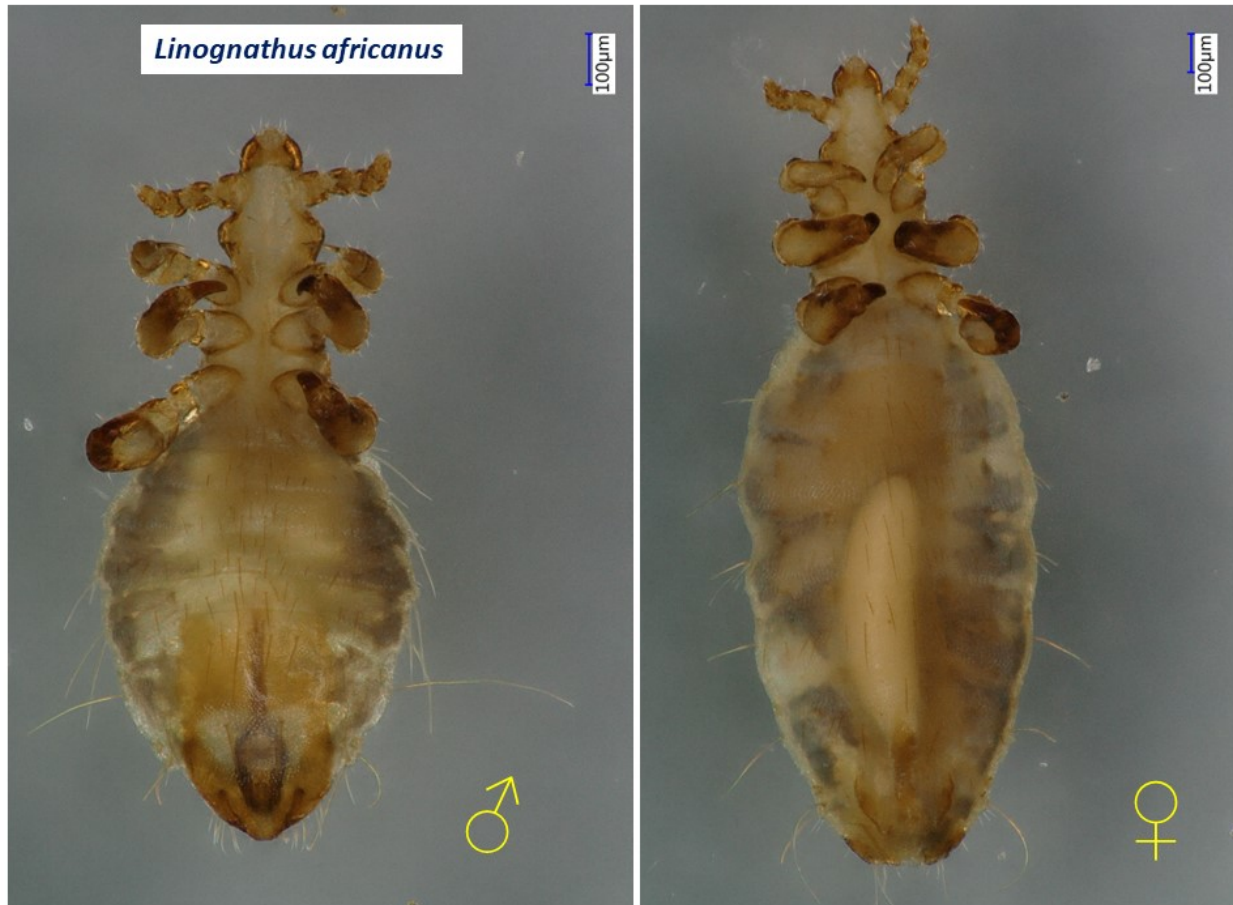


Figure 2.b Images of *Lucilia cuprina* (left) collected in Malta, in comparison with *Lucilia sericata* (right)



All sucking lice (n=22) removed from goats on three farms belonged to *Linognathus africanus*, based on the semicircular, sclerotized "ocular" elevation behind the antennae and the structure of the last abdominal segment (Figure 3).

Figure 3 *Linognathus africanus* collected in Malta, showing sexual dimorphism: male (left) and female (right)



3.2.2. Molecular-phylogenetic identification of species

In general, molecular identification confirmed the results of morphological identification. Among the flies, three *M. domestica* had two *cox1* haplotypes, one with 100% (636/636 bp) sequence identity to specimens reported from both the Old and the New Worlds, e.g. from the UK (GenBank: HE614023). The other *M. domestica* *cox1* sequence was new, with 99.8% (635/636 bp) maximum identity to any other sequence of this species available in GenBank.

Similarly, the majority (7 of 8) of *S. calcitrans* cox1 sequences were 100% (636/636) identical to several sequences in GenBank (e.g. from Canada: KM571650, Brazil: JQ246704, Portugal: MN555676) but one haplotype was new, with a maximum of 99.8% (635/636 bp) identity to the closest matches in GenBank. All three *L. cuprina* from Malta had cox1 sequences 100% (636/636 bp) identical to *L. cuprina* from South Africa (MW222990) and Portugal (KY859990). *Lucilia sericata* individuals that were analyzed for the cox1 gene (n=14) belonged to four haplotypes, with 99.8-100% (635-636/636 bp) identity to a sequence of this species deposited in GenBank from Malta (MF059331). Phylogenetic analyses showed that the cox1 sequences of all flies collected in Malta clustered with conspecific flies (Figure 4).

The cox1 sequences of four *L. africanus* lice were identical, and showed only 99.4% (358/360 bp) identity to the only sequence (EU375760) of this species available in GenBank from a specimen removed from a goat.

3.2.3. Observations on the host-association, sex ratio and monthly distribution of flies

Considering the sex ratio of *M. domestica* among samples collected randomly at cattle farms, females predominated in the whole study period (n = 1566 vs 852 males), but the abundance of males increased significantly from July to August (P = 0.009) and August to September (P = 0.0006) at one place, and from July to October, then from October to November (P < 0.0001) at another place where repeated samplings were performed. On the contrary, the sex ratio of this fly species remained relatively constant in one place where small ruminants were kept, but the ratio of males increased from July to August or to September at another sampling site with sheep and goats (P < 0.0001 and P = 0.0002, respectively). This phenomenon was also observed at the dog kennel (P = 0.003 and 0.004, respectively) (Table 1).

Stomoxys calcitrans was associated with cattle and dogs, whereas *L. cuprina* was found near small ruminants and dogs (Table 1). The latter species was only collected in the summer time, but the former was more likely to occur in the autumn.

Figure 4 Phylogenetic analyses of flies collected in Malta

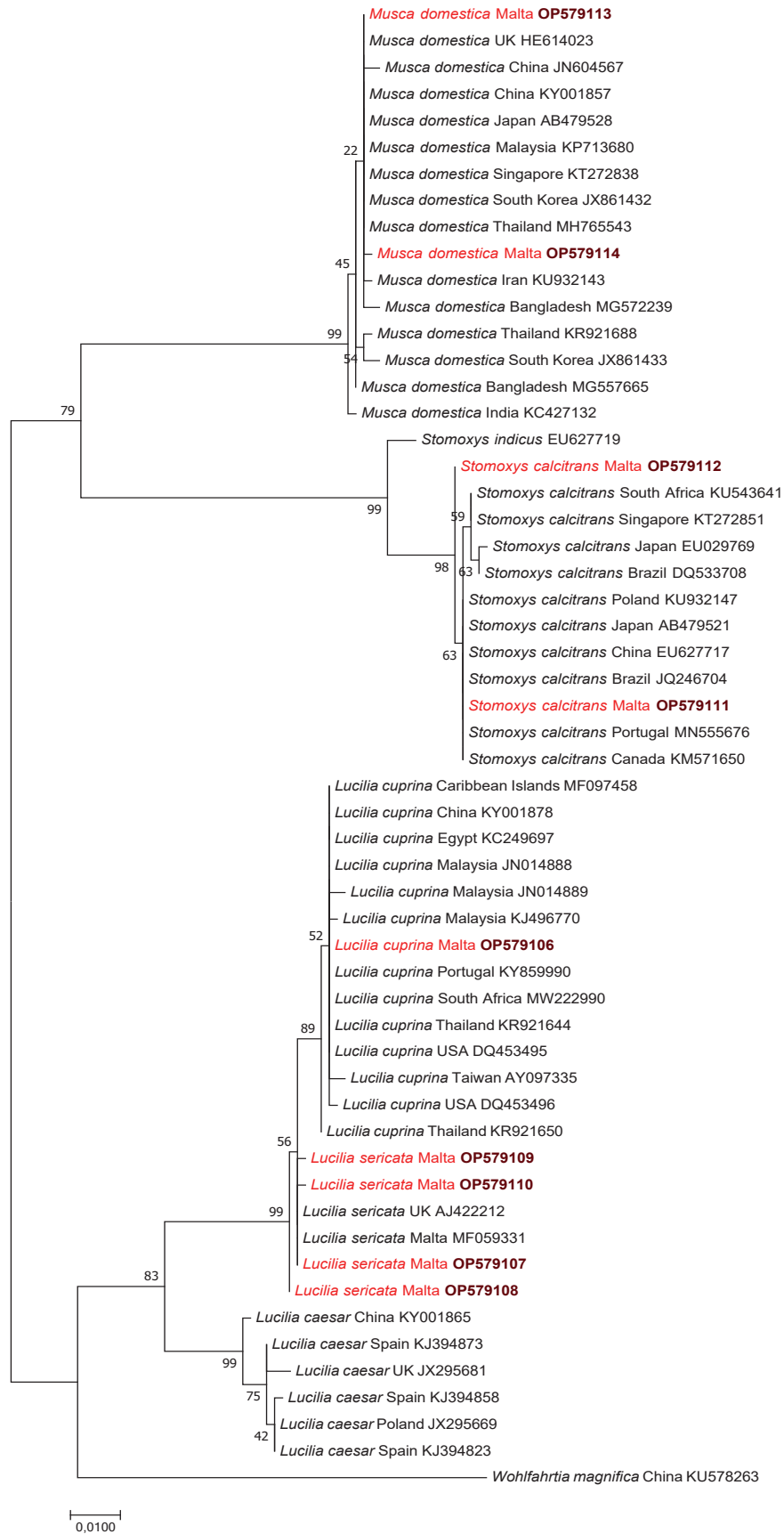


Table 1.a (M: males, F: females) collected in pens and stables at **cattle** farms. The location is also shown on the map (Figure 1) according to the code in parentheses.

Location (code)	Month (year)	<i>Musca domestica</i>	<i>Stomoxys calcitrans</i>	<i>Lucilia cuprina</i>
Maghtab (MH)	July (2020)	45M + 123F	-	-
	August (2021)	87M + 131F	-	-
	September (2021)	366M + 318F	-	-
Hal' Ghaxaq (HG)	July (2020)	116M + 228F	1M	-
San Gwann (SG)	July (2020)	27M + 199F	-	-
	October (2020)	63M + 109F	-	-
	November (2020)	54M + 101F	-	-
Sannat (GO)	July (2020)	94M + 357F	1F	-
total =		2418	2	-

Table 1.b Flies (M: males, F: females) collected near **goats/sheep, pigs or dogs**. The location is also shown on the map (Figure 1) according to the code in parentheses.

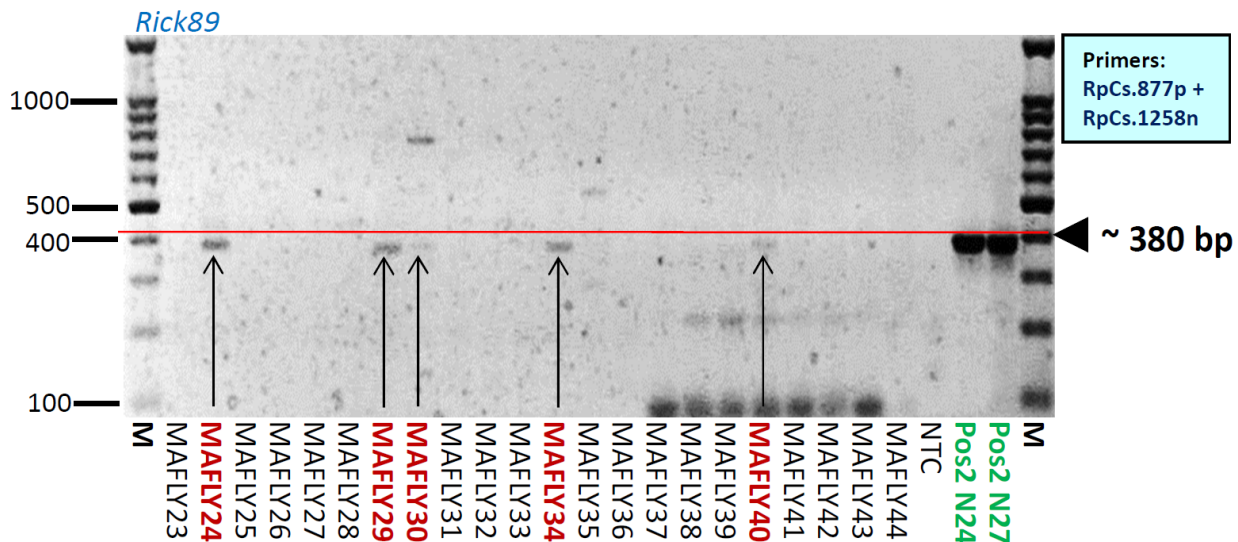
Animals kept (code or location)	Month (year)	<i>Musca domestica</i>	<i>Stomoxys calcitrans</i>	<i>Lucilia cuprina</i>
Goats, sheep (1)	July (2021)	20M + 67F	-	-
	August (2021)	26M + 63F	-	-
	September (2021)	13M + 35F	-	-
Goats, sheep (2)	July (2021)	26M + 136F	-	1M + 1F
	August (2021)	20M + 18F	-	-
	September (2021)	26M + 40F	-	-
Sheep (Maghtab)	September (2021)	11M + 8F	-	-
Pigs (Mgarr)	August (2021)	3M + 2F	-	-
	September (2021)	8M + 5F	-	-
Dogs (Manikata)	August (2020)	15M + 25F	-	1F
Dogs (Noah's ark)	July (2021)	3M + 36F	1F	-
	August (2021)	16M + 28F	-	-
	September (2021)	7M + 9F	5M	-
total =		666	6	3

3.2.4. Molecular screening for pathogens

All flies and lice analyzed for pathogen DNA were negative for piroplasms, and no specific product was obtained in the PCR detecting Anaplasmatocae. However, five samples were positive in the rickettsia gltA PCR (Figure 5). From a female *L. africanus* a *Rickettsia* sp. was amplified which had the highest, 100% (292/292 bp) sequence identity with a genotype reported from a rodent (*Apodemus flavicollis*) in Poland (KY488187), but also high (304/306 bp) identity to *Rickettsia* sp. OM-2012 from human blood in Senegal (JQ674485) as well as 99.7% (288/289 bp) to a rickettsia reported from mosquitoes in Cote d'Ivoire (JN620082) and Gabon (JQ354961). This rickettsia detected in Malta was also very similar in its gltA gene to *Rickettsia hoogstraalii*, with 98.7% (300/304 bp) sequence identity to a sequence (KY570488) reported from a tick in Greece.

Considering aspecific PCR products of the latter PCR, from a male louse, the sequence of a *Moraxella* sp. was amplified, which had the closest, 86.3% (264/306 bp) sequence identity to *Moraxella bovoculi* (CP011381). In addition, two further lice (both females) contained the DNA of a *Neisseria* sp. most closely related to *Neisseria dentiae* (CP059570) with 95.6% (304/318 bp) sequence identity.

Figure 5 Results of PCR targeting the citrate synthase (gltA) gene of rickettsiae.



4. Discussion

To our knowledge, this is the first study focusing on the molecular analysis of flies and lice of veterinary-medical importance in Malta. Regarding muscoid flies, the common housefly, *M. domestica* is a fly species known for its epidemiological role as a carrier of pathogens (Issa, 2019), and significant economic losses can be attributed to its presence and the resultant annoyance (Geden et al., 2021). It is not surprising that *M. domestica* was found to be the most abundant fly species in Malta, taking into account previous data on its occurrence in this country (Harris et al., 1976).

The stable fly, *S. calcitrans* has similarly high veterinary-medical significance, owing to its aggressive, persistent blood-feeding behavior and potential vector role affecting both domestic animals and humans (Baldacchino et al., 2013). This muscoid fly species has also been long known to occur in Malta, where its breeding places are limited (Harris et al., 1976), explaining their low number in the present study. Finding of exclusively this cosmopolitan *Stomoxys* species suggests the absence of the African *S. niger* from the Dipteran fauna of Malta, as already reported (Ebejer and Gatt, 2021). Phylogenetically, one *S. calcitrans* haplotype from Malta clustered separately from conspecific flies of which sequences were available in GenBank. This may indicate that at least some stable fly populations in Malta are reproductively isolated from those in mainland Europe, probably because they are not readily transported with animal hosts and their flight range is within 30 kms (Sprygin et al., 2020).

Among calliphorid flies, *L. cuprina* is a very important causative agent of fly strike (myiasis) particularly affecting sheep and other livestock, but potentially all warm-blooded vertebrates (Stevens and Wall, 1996). Perhaps, the most important finding of this study is the first evidence for the autochthonous occurrence of *L. cuprina* in Malta where even recently it has not been known to occur (Ebejer and Gatt, 2021). This blowfly species most likely started its geographic range expansion from Australia and South Africa (Waterhouse and Paramonov, 1950; Tourle et al., 2009). The only haplotype identified in Malta had the closest *cox1* sequence identity

and phylogenetic clustering to isolates of this species from Portugal and South Africa, most likely in line with this geographical origin.

In Europe, *L. cuprina* has already been reported from Spain (Rognes, 1994), Turkey (and the Middle East: Akbarzadeh et al., 2015), and on single occasions from other countries (Czech Republic: Fischer and Vicha 2003; southern Ukraine: Verves and Khrokalo, 2010; UK: Sivell, 2020). Interestingly, this blowfly species is not known to occur in Italy (i.e., directly north of Malta), because when it was reported in that country, it turned out to be a misidentification (Rognes, 2014). Owing to the preference of *L. cuprina* for warmer climate (Stevens and Wall, 1996), it is unlikely that it will establish north of Mediterranean Basin, but consequent to its emergence in several parts of southern Europe, probably it will become well-established in the latter region.

While *L. cuprina* was exclusively found near domestic animals in Malta, in other types of rural and urban locations only *L. sericata* could be collected with bait traps. This is unlikely a consequence of different collection methods, because both of these species are attracted to baits (Brett et al., 2021), and both are attracted to ovine hosts and their responses to chemical stimuli are broadly similar (Ashworth and Wall, 1994). Rather, this may be related to differences in their habitat preference. For instance, it was reported in South Africa that *L. sericata* occurs in urban areas and is not found in rural settings (Meskin, 1986; Williams et al., 2014).

Regarding host associations, *M. domestica* was reported to predominate near pigs in Malta (Harris et al., 1976) but in the present study it showed very high abundance near cattle, small ruminants and dogs. The present results also confirmed previous data that *S. calcitrans* can be found mainly on or around cattle in Malta (Harris et al., 1976), but in the present study this fly species was also collected near dogs, as reported in other countries and justifying its other name as "dog fly" (Frankhauser et al., 2015). *Lucilia cuprina* was found near dogs and small ruminants in Malta, i.e., close to animals in which it can cause myiasis (Stevens and Wall, 1996; Soundararajan 2020), justifying the need for further evaluation of the country-wide veterinary significance of this species.

In the context of seasonality, it is not surprising that *M. domestica* was found to be the most abundant fly species during the summer and autumn months at farms, because it is known to be active between April and November in Malta (Harris et al., 1976). It has been shown that males and females are different in their physical activity and mating behavior (Ragland and Sohal 1973), plus males and females mature at different ages (Michelsen 1960; Sacca 1964). Therefore, in *M. domestica* populations different sex-ratios and densities can be expected to occur under natural conditions (Bahrndorff et al., 2012). Similarly to the present survey, predominance of females was reported in other studies (Aigbodion and Omobhude, 2005; Chaiwong et al., 2012). This was modified towards the autumn when the shift towards a higher ratio of males might reflect decreasing activity which is in favor of their longer survival (Buchan and Sohal, 1981). The sex ratio of *M. domestica* is crucial to know properly, because it will influence locomotor activity (Bahrndorff et al., 2012), feeding behavior and thus the role in pathogen transmission, as well as the sensitivity to biological means of control (Geden et al., 2021). At the same time, the individual number of *S. calcitrans* was too low in this study to assess seasonality.

The sucking louse species *L. africanus* was already reported as established not only in the Mediterranean region (Sardinia: Fois et al., 2014) but also in the Balkans, in Bulgaria (Nizamov and Prelezov, 2021). Therefore, it can be reasonably assumed that it had been present for some time but was not yet reported in Malta. Based on the three examined goat herds, the situation was similar to northern Africa (Algeria) where the exclusive presence of *L. africanus* was reported (Meguini et al., 2018), unlike north of Malta in the Mediterranean Basin (Sardinia) where populations of this species are mixed with *L. stenopsis* (Fois et al., 2014).

Regarding molecular detection of pathogens from *L. africanus*, three sequences were successfully amplified. The first was a *Rickettsia* genotype closely related to isolates detected in rodent blood in Europe, in mosquito in Africa and in ticks in the Mediterranean region. The latter corresponds to *R. hoogstraali* which is a spotted fever group rickettsia associated with hard and soft ticks (Duh et al., 2010). Its pathogenic role is unknown. To our knowledge, this is the first finding of a similar rickettsia in any blood-sucking louse. The significance of this finding is

illustrated by data according to which lice can acquire, maintain and even inoculate tick-borne rickettsiae (Houhamdi et al., 2003; Houhamdi and Raoult, 2006).

The genera *Moraxella* and *Neisseria* detected here for the first time in anopluran lice are closely related to each other (Enright and McKenzie, 1997). These findings may either indicate bacteraemia of relevant hosts (goats) or the symbiotic nature or vector-borne transmission of these agents by *L. africanus*. It is highly relevant to note here that a novel symbiotic bacterial lineage has recently been discovered in blood-sucking lice (*Polyplax* and *Hoplopleura*), closely related to *Neisseria* (Říhová et al., 2021). Therefore, this is the first indication that similar bacteria might exist in association with lice in the genus *Linognathus*.

5. Summary

In order to obtain new data on the occurrence and potential vector role of flies and blood-sucking lice of veterinary importance in Malta, ectoparasites were collected at cattle, sheep and goat farms, near pigs and dogs, as well as in two places in the absence of domestic animals using bait traps. Altogether 3095 flies (Diptera: Muscidae, Calliphoridae) were collected at farms and kennels near domestic animals, as well as 37 blowflies (Calliphoridae) in rural and urban areas without animals nearby. Regarding Muscidae, the great majority of flies (n = 3084) were identified as the common housefly *Musca domestica*. Eight flies represented the stable fly *Stomoxys calcitrans*. Three blowflies associated with dogs and small ruminants belonged to *Lucilia cuprina*. By contrast, all 37 blowflies collected with bait traps, without domestic animals nearby, were identified as *L. sericata*. In addition, sucking lice (n=22) belonged to *Linognathus africanus*. Molecular identification of 28 specimens confirmed these results.

Considering the sex ratio of *M. domestica* among samples collected randomly at cattle farms, females predominated in the whole study period, but the abundance of males increased significantly towards the autumn. *Stomoxys calcitrans* was associated with cattle and dogs, whereas *L. cuprina* was found near small ruminants and dogs. Pathogen screening demonstrated the presence of a rickettsia closely related to *Rickettsia hoogstraalii*, as well as of a *Moraxella* and a *Neisseria* sp. in *L. africanus*.

To our knowledge, this is the first study focusing on the molecular analysis of flies and lice of veterinary-medical importance in Malta. The most important finding of this study is the first evidence for the autochthonous occurrence of *L. cuprina* in Malta. Owing to the preference of *L. cuprina* for warmer climate, it is unlikely that it will establish north of Mediterranean Basin, but consequent to its emergence in several parts of southern Europe (now including Malta), probably it will become well-established in the latter region. While *L. cuprina* was exclusively found near domestic animals in Malta, in other types of rural and urban locations only *L. sericata* could be collected with bait traps. This may be related to differences in their habitat preference. Based on the sucking-louse burden in the examined goat herds, the situation in Malta was similar to northern

Africa where the exclusive presence of *L. africanus* was reported, unlike towards the north in the Mediterranean Basin where populations of this species are mixed with *L. stenopsis*. Based on the molecular evidence of bacteria not detected before in *L. africanus*, its bacteriome deserves further evaluation in the near future.

6. Összefoglaló

Azzal a céllal, hogy új adatokhoz jussunk a legyek és a vérszívó tetvek előfordulásáról és kórokozóterjesztő szerepéről Máltán, külső élősködőket gyűjtöttünk szarvasmarha, juh és kecske farmokon, sertések és kutyák közelében, valamint két helyen csalétkes csapdával. Összesen 3095 legyet (Diptera: Muscidae, Calliphoridae) fogtunk farmokon és kenneleknél háziállatok közelében, míg 37 döglégyet (Calliphoridae) vidéki és városi területen állatoktól távol. Az igazi legyek nagy többsége (n = 3084) közönséges házilégy (*Musca domestica*) volt, nyolc példány szuronyos istállólégy (*Stomoxys calcitrans*), három döglégy kutyák és kiskérődzők közelében pedig a *Lucilia cuprina* fajba tartozott. Ezzel szemben mind a 37 csapdázott példány selymes döglégynek (*L. sericata*) bizonyult. A vérszívó tetvek (n = 22) mindegyike *Linognathus africanus* volt. A 28 példányból elvégzett molekuláris azonosítás igazolta a fentieket.

A szarvasmarhák mellett gyűjtött házilégyek ivararányát összességében a nőstények túlsúlya jellemezte, azonban a hímek aránya jelentősen nőtt az ősz közeledtével. A szuronyos istállólégy kedvelt gazdái a szarvasmarha és a kutya voltak, míg a *L. cuprina* kiskérődzők és kutyák közelében fordult elő. A kórokozókimutatás egy, a *Rickettsia hoogstraalii* fajjal rokon rickettsia és egy-egy *Moraxella* és *Neisseria*-faj jelenlétét igazolta *L. africanus*-ban.

Tudomásunk szerint ez az állategészségügyi szempontból fontos legyek és tetvek első molekuláris vizsgálata Máltáról, amelynek legfontosabb eredménye a *L. cuprina* autochthon előfordulásának első bizonyítéka. E faj melegkedvelő, így nem valószínű, hogy a mediterrán térségtől északra megtelepszik, de Dél-Európa több részén való megjelenése következtében ott széles körben elterjedhet. Míg a *L. cuprina* Máltán csak állatok közelében volt gyűjthető, a más típusú vidéki és városi élőhelyen kizárólag *L. sericata* került elő a csalétkes csapdákból. Ez az eltérő élőhelypreferenciájuknak tulajdonítható. A *L. africanus* máltai kecskeállományokban tapasztalt kizárólagos előfordulása az észak-afrikaihoz hasonló, ahonnan genusából szintén csak e tetűfajt jelentették, szemben az észak-mediterrán régiótól, ahol a *L. stenopsis* fajjal együtt fordul elő. A *L. africanus* fajban eddig ki nem mutatott baktériumok molekuláris bizonyítéka alapján érdemes lesz a közeljövőben tovább vizsgálni bakteriomját.

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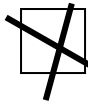
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DECLARATION

I hereby declare that the thesis entitled “**Morphological and molecular analyses of flies and blood-sucking lice of veterinary importance from Malta**” is identical in terms of content and formal requirements to the TDK research paper submitted in 2022.

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Thesis progress report for veterinary students

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Neptun code of the student: WQLLZ3

Name and title of the supervisor: Dr. Sándor Hornok - Head of Department, professor

Department: Department of Parasitology and Zoology

Thesis title: Morphological and molecular analyses of flies and blood-sucking lice of veterinary importance from Malta

Consultation – 1st semester

Timing				Topic / Remarks of the supervisor	Signature of the supervisor
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1.	2022.	06.	23.	Sample processing, identification	<i>Hok sár</i>
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