University of Modena and Reggio Emilia

PhD in AGRI-FOOD SCIENCES, TECHNOLOGIES AND BIOTECHNOLOGIES

Innovative strategies for sustainable management of *Musca domestica* in livestock farms.

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XXXVI CYCLE

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Contents

List of Figures

List of Tables

Summary

The housefly, *Musca domestica*, is a common pest on livestock farms. Its abundance poses a health problem for both humans and animals, as it can carry around 200 pathogens. Therefore, it is crucial to study sustainable alternative strategies for its management, as the housefly can develop resistance to insecticides within a few generations. The documentation of this pest's presence on farms in production of Parmigiano Reggiano cheese area, in Reggio Emilia is limited. Research has indicated that the presence of houseflies on dairy farms can result in a higher incidence of mastitis in cows. This study examines new strategies for the sustainable management of houseflies, utilizing pupal parasitoids in this region. The study investigates the resistance of houseflies to Imidacloprid insecticide, specifically whether discrimination of this insecticide occurs at the tarsi or proboscis level in the resistant Californian strain. In addition, the abundance of houseflies and their pupal parasitoids was monitored in six dairy farms in the Reggio Emilia area. The study was carried out in two types of farms: organic, which used pupal parasitoids, and conventional, which applied insecticides to manage housefly populations. The species that emerged from the collected pupae were identified using a molecular and taxonomical approach. After identification, the parasitizing performance among the commercial and wild populations/species was analyzed. Parasitization performance of two common species of pupal parasitoids of houseflies: *Spalangia cameroni* and *Muscidifurax zaraptor,* was analyzed over a two-week period. This study is the first of its kind in Emilia Romagna region and provides an alternative solution to reduce the population of houseflies to acceptable levels.

Key words: biocontrol, *Musca domestica*, houseflies, parasitoids, management, farms

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Dedication

To my partner Manuel, my family, and friends, Laura & Giulia who have believed in me.

The wing structure of the bumble bee, in relation to its weight, is not suitable for flight, but he does not know this and flies anyway.

Albert Einstein

1. Introduction

The housefly, *Musca domestica L*. (Diptera, Muscidae) is native to Central Asian steppe that has spread worldwide. Houseflies have been regarded as pests to humans and animals since ancient times, with research by Gogarten et al. (2019) suggesting that the relationship between humans and flies may predate recorded history. On all continents except Antarctica, houseflies have evolved to adapt to various environmental conditions and food resources. This adaptation has resulted in a relentless opponent that can potentially resist control efforts by avoiding and adapting to them (Gogarten et al., 2019; Geden et al., 2021). The houseflies are often abundant in areas of human activities such as hospitals, food markets, slaughterhouses, food centers or restaurants, poultry and livestock farms, where they constitute a nuisance to humans, poultry, livestock and other farm animals, and also act as potential vector of diseases (Khamesipour et al., 2018; Smallegange and Otter, 2007).

1.1 Houseflies as pests for humans and animals

Houseflies feed on substrates used as human food as well as on manure and any other type of organic waste. Larvae of houseflies require live bacteria for their nutrition; therefore, all stages are associated with microbial-rich environments. The larvae subsist and consume bacteria, which are assimilated via the collective activity of digestive enzymes, lysozymes, and antimicrobial effectors. Adult houseflies are highly mobile and synanthropic, in contrast to the larvae, that develop quickly in the substrate where the eggs were laid. They are gregarious, moving without discrimination between septic environments and domestic sites and can travel for kilometers (Nayduch and Burrus, 2017). Adult flies are unable to chew or bite, so they consume solid food by regurgitation. When they land on a potentially nutritious substrate, they first regurgitate saliva and crop contents containing digestive juices and enzymes that dissolve the food. Afterwards, the fly can consume the liquefied food using its proboscis (Malik et al., 2007). If flies feed on food contaminated with harmful microorganisms, they can potentially transmit diseases to other organisms. Pathogens can attach to the mouth or other body parts of the fly and be transferred when the fly comes into contact with other organisms or surfaces (Malik et al., 2007). Adult houseflies consume bacteria from septic substrates directly or indirectly via self-cleaning. Ingested microorganisms can survive digestion in adult houseflies, with some even proliferating and exchanging genetic material (Yin et al., 2022). The persistent of ingested microbes within the housefly gut can lead to the spread of such microorganisms in their excretions. As a result, adult flies can spread pathogens as well as antibiotic resistance and virulence genes (Nayduch and Burrus, 2017).

Houseflies are known to transmit over 65 diseases found in human and animal intestines, including protozoan infections such as amoebic dysentery, bacterial infections such as shigellosis, salmonellosis, and cholera, and helminthic infections such as roundworms, hookworms, pinworms, and tapeworms (Greenberg, 1965; Shono and Scott, 2003). Additionally, they have been found to spread eye diseases such as trachoma and infect wounds and skin with various diseases (Shono and Scott, 2003).

Cutaneous diphtheria, mycoses, yaws and leprosy are some of the diseases transmitted by house flies (Shono and Scott, 2003). In addition, fly larvae ingested with food can survive in the human intestine and cause intestinal myiasis, resulting in pain, nausea, and vomiting (Hill, 2005; Malik et al., 2007). Flies can carry enteric bacteria, including enteropathogenic strains such as *Escherichia coli, Vibrio cholerae,* and *Bacillus anthracis*, which can cause enteric diseases (Khamesipour et al., 2018). Antibiotic-resistant bacteria, such as *E. coli, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, have been found in hospitals and livestock farms, with *M. domestica* playing a role in their spread (Arsenopoulos et al., 2018). Houseflies commonly carry fungi and viruses, including *Penicillium* sp., *Aspergillus* sp*.,* and *Candida* sp*.* (Khamesipour et al., 2018). Cladosporium is found in stables and human environments, while Penicillium is found in animal pens and human habitation (Sales et al., 2002). *Aspergillus* sp*.* is mostly associated with adult flies (Sales et al., 2002; Kassiri et al., 2015), while *Candida* sp. is collected from human habitation areas (Kassiri et al., 2015; Khamesipour et al., 2018). Viruses such as Filoviridae (common Ebola virus), Picornavirus, and Orthomyxoviridae have been isolated from houseflies' internal organs (Cranfield et al., 1999; Otake et al., 2003; Haddow et al., 2017)

1.2 Importance of sustainability in production of Parmigiano cheese in Emilia Romagna

Parmigiano Reggiano is a type of Italian hard cheese made from raw cow's milk, partially skimmed by separation, with no additives. It is one of the most significant Italian products exported across the world. Italy produces over 3.5 million wheels of Parmigiano Reggiano Protected Designation of Origin (PDO) cheese every year. Together with Grana Padano PDO cheese, these two products represented nearly 98,000 tons of Italian cheese exported in 2020 (Lovarelli et al., 2022). Parmigiano Reggiano (PR) is subject to rigorous production regulations. Criteria that determine the cheese's authenticity include the geographical location of production and the feeding methods of cows. Specifically, milk production, cheese transformation and a minimum ageing time of 12 months must occur within a small area situated in the North of Italy. The provinces of Parma, Reggio Emilia, Modena, and partially Bologna (left side of Reno River) and Mantua (right side of Po River) are the designated areas of production (Lovarelli et al., 2022). The established regulations of PR cheese production prohibit modifications to the traditional milk production rules. Nevertheless, improvements can be made in the production chain and in the field. According to recent studies, agricultural production can improve in efficiency and environmental sustainability (Lovarelli et al., 2022; Sassenrath et al., 2013) and ongoing implementation of good agricultural practices, such as responsible treatment of manure and the field application (Finzi et al., 2020; Lovarelli et al., 2022; Mariantonietta et al., 2018). These practices are in line with the principles of a circular economy perspective (Lovarelli et al., 2022; Mariantonietta et al., 2018). Efficient conversion of feed into milk is essential for sustainable production. In addition, manure management is a crucial consideration (Lovarelli et al., 2022).

1.3 Impact of houseflies on production of milk and Parmigiano cheese

The presence of flies causes considerable distress to cows, leading to reduced milk production (Arsenopoulos et al., 2018). However, intra-mammary infections in dairy cows are the major issue. Mastitis in cows is the inflammation of the mammary gland caused by bacterial infection (Franceschi et al., 2020) that significantly reduces the quantity and quality of milk, reducing fat and protein concentration and impacting on production value (Arsenopoulos et al., 2018; Ruegg, 2012). Despite numerous control strategies, it remains a significant and economically demanding predicament for dairy cows - according to the National Mastitis Council, it costs dairy farmers over £1.5 billion yearly in USA (Middleton et al., 2014). Research conducted on cattle farms has shown that the housefly is a primary bacterial vector for *Staphylococcus aureus* (Anderson et al., 2012) and *E. coli* (Castro et al., 2016), and a less significant carrier of *Enterobacter, Proteus,* and *Pasteurella* ssp. (Arsenopoulos et al., 2018). These microorganisms are responsible for causing transmissible mastitis, an infection that spreads eight times more easily when transmitted by flies (Anderson et al., 2012). Reducing flies' role in transmitting these pathogens could help mitigate this issue (Arsenopoulos et al., 2018). The reduction in production is due both to the less favorable chemical composition of the milk and to the reduced efficiency of the coagulum in retaining the fat fraction. It follows that the economic impact of the associated reduction in cheese yield is not negligible and could compromise profitability (Franceschi et al., 2020), thus affecting the entire Parmigiano Reggiano production chain.

Furthermore, it must be considered that the milk produced by local dairy farms in Emilia Romagna is not only destined for the production of Parmigiano Reggiano, but also for large distribution companies (Lovarelli et al., 2022). Issues in milk production can result in economic losses in the two related market sectors.

1.4 Monitoring methods for flies

The methods used to monitor housefly activity provide an index of both fly density and behavior, such as flight frequency, landing habits, and response to odors or visual cues. It is possible that monitoring the harmful effects of flies, such as the transmission of pathogens and disturbance, could also provide an indicator of fly activity. To improve the predictiveness of this method, it could consider damage in addition to estimating fly density alone (Gerry, 2020 a). Monitoring methods described below are largely reviewed by Gerry, (2020 a) with specific recommendations by Gerry, (2020b) and general discussion of the importance for monitoring by Geden et al., (2021).

1.4.1 Sampling of larvae

Sampling of animal feces or other developmental substrate for immature flies can provide an early indication of possible future adult fly abundance and activity (Gerry, 2020a). Immature flies can be collected from the substrate using a Berlese funnel (Axtell, 1970a; Schmidtmann, 1988; Stafford and Bay, 1987), by washing the substrate through sieves to retain immatures (Eastwood and Schoenburg, 1966; Schoenburg and Little, 1966;), or by immersing the substrate in high-salinity water (e.g., with magnesium sulfate) causing immature flies to float to the water surface where they can be easily collected (Ladell, 1936; Laurence, 1954; Matthysse and McClain, 1973; Pitts et al., 1998). Methods for sampling immature houseflies are reviewed by Gerry, (2020a). Sampling larvae for routine monitoring of house fly activity is challenging due to variations in immature density and the need to estimate immature mortality (Schoenburg and Little, 1966; Stafford and Bay, 1987; Gerry, 2020a). The use of emergence traps could enhance prediction of adult fly activity. Nevertheless, immature sampling methods are valuable for identifying productive developmental sites for treatment or appraising fly production potential of different substrates or conditions (Eastwood and Schoenburg, 1966; Axtell, 1970a; Stafford and Bay, 1987; Schmidtmann, 1988; Gerry et al., 2005; Gerry, 2020a).

1.4.2 Scudder fly grid

Harvey Scudder created a device called the "Standard Neutral Resting Surface" for adult house flies. The device comprises of wooden strips attached to a frame with open slits, providing a desirable surface for flies to rest on (Scudder, 1947). Placing the grid on the ground in areas of high fly activity, the number of flies on the grid are counted after 30 seconds. The fly grid records an immediate record of the fly activity, not a measure of fly density (Murvosh and Thaggard, 1966; Gerry, 2020a). Although counts are closely correlated with visual counts, the grid establishes a uniform visual standard, consequently increasing the consistency of fly counts among both observers and sites (Gerry, 2020a). The activity of house flies is subject to variation dependent on environmental conditions and time of day, emphasizing the need for instantaneous fly activity records like grid counts (Gerry, 2020a; Zahn and Gerry, 2020). When using the Scudder fly grid, which is frequently inundated with various fly species, identifying flies immediately can be challenging. Moreover, the fly grid and other instantaneous counts of fly activity can be inaccurate due to the changing environmental conditions (Gerry, 2020a).

1.4.3 Sticky Fly Ribbons

The practice of using sticky ribbons, tapes, and papers to capture flies at rest has a lengthy history, dating back to 1908 (Ross, C J, inventor., 1908). Long, slender papers are likely preferred by house flies because of their attraction to surface edges (Scudder, 1947).

By capturing different species of pest flies, fly ribbons can monitor their activity simultaneously (Anderson and Poorbaugh, 1964; Axtell, 1970a, 1970b; Legner et al., 1973; Lysyk and Axtell, 1986; Gerry et al., 2011; Gerry, 2020a). A patent for adhesive fly paper was first documented in 1908 and the sticky fly ribbon is still in use today (Gerry et al., 2020). Fly ribbons are best positioned in areas that are frequented by flies, such as indoors near the roofs of livestock buildings (Anderson and Poorbaugh, 1964; Gerry, 2020a).

They have proven effective for catching flies when suspended from midline roof supports in poultry houses or cattle barns (Anderson and Poorbaugh, 1964; Axtell, 1970a, 1970b; Legner et al., 1973; Lysyk and Axtell, 1986; Gerry, 2020; James et al., 2017). Dusty conditions can diminish the adhesive ability of fly ribbons, rendering them ineffectual for fly monitoring in broiler-breeder poultry installations or dry lot dairies (Gerry et al., 2011). In addition, direct sunlight can also result in the reduced stickiness of the ribbons (Anderson and Poorbaugh, 1964; Gerry, 2020a).

1.4.4 Sticky Fly Traps

Sticky fly traps are covered with an adhesive material to catch flies which encounter the trap during flight or when landing. They possess a wide surface area and are usually equipped with a robust plastic or fiberglass frame (Gerry, 2020a). The Alsynite biting fly trap (Broce, 1988; Gerry, 2020a) is popularly employed for tracking fly movements in animal facilities, although it has also been utilized to track housefly activity in cattle stable (Geden, 2005; Gerry et al., 2011; Urech et al., 2012). Sticky fly traps, such as glue boards, are commonly used in agricultural, residential, and urban settings (Winpisinger et al., 2005; Gerry, 2020a). Other sticky fly traps, such as sticky cards(Black and Krafsur, 1985; Hogsette et al., 1993; Geden et al., 1999; Gerry, 2020a), cans (Black and Krafsur, 1985; Kaya and Moon, 1978), pyramids (Pickens and Miller, 1987), and panels (Kaufman et al., 2001; Zahn and Gerry, 2020), have been developed for similar purposes. However, their efficacy as fly capturing or monitoring devices remains largely untested despite their potential applications (Gerry, 2020a). Sticky traps are a widely used technique for monitoring fly populations, with their positioning and application varying between designs. Longer sampling periods yield a more comprehensive estimate of fly activity compared to 24-hour fly ribbons. The rigid frame and larger surface area of some sticky traps make them suitable for outdoor usage (Gerry, 2020a). Nevertheless, they may lose effectiveness in dusty conditions (Kaufman et al., 2001; Gerry et al., 2011), and activity calculations can be affected by removal of flies by wild birds (Gerry et al., 2011).

1.4.5 Attractant-Baited Traps

In 1872, Harper filed a patent that described a fly trap placed above food waste or other materials that attract flies (Harper, 1872; describe by Gerry, 2020). This invention was timely as it coincided with the recognition by scientists that flies can carry pathogens. In the early 1900s, the inverted cone trap and homemade versions became widely used. Public health agencies and local businesses supported contests for residents to construct and deploy these traps (Gerry, 2020a).

Whilst these traps were successful in capturing many adult flies, they proved ineffective in monitoring adult house fly activity due to the use of bait such as food waste or other attractive materials. Nevertheless, entomologists were able to effectively utilize these traps in early house fly dispersal studies (Gerry, 2020a). Currently, numerous traps design available in the market use odors to attract and trap flies, often drowning them in a smelly liquid solution. These "stinky fly traps" can be effective at catching large numbers of flies, but identifying drowned and decomposing flies is difficult and unpleasant (Gerry, 2020a). The monitoring of fly activity can be made simpler by using a separate collection bag or mesh insert (Urech et al., 2012; James et al., 2017). The effectiveness of attractant-baited traps may vary depending on their proximity to natural attractants or fly development sites. Additionally, their attractiveness can be affected by trap design, bait formulation, age, and environmental conditions (Pickens, and Miller, 1987; Gerry, 2020). The age of the flies can also influence their attraction to baited traps. This variability in attraction could contribute to the lack of agreement in recorded fly activity between baited traps and other trap methods (Geden, 2005; Geden et al., 2009; Gerry, 2020a). A standardized attractant such as vinegar (Qian et al., 2013), molasses (Bishopp and Laake, 1919; Brown et al., 1961; Geden, 2005; Geden et al., 2009), or fly-attractant volatiles (Quinn et al., 2007; Hung et al., 2020) may reduce the variability among individually baited traps and enhance their effectiveness for monitoring fly activity. Also, commercial baits with trimethylamine, indole, + Z-9-tricosene, is very common in US.

1.4.6 Spot Cards

White index cards, termed 'spot cards', can be utilized for observing fly activity by monitoring the deposition of fecal and regurgitation spots (Axtell, 1970b; Lysyk and Axtell, 1986; Gerry et al., 2011). Spot cards of varying sizes can be used, with 3 x 5-inch (7.62 x 12.7 cm) cards being popular. Different sizes can be converted to spots/cm2 of card area for comparative analysis (Gerry et al., 2011). Other white objects, such as paper strips or tiles, may additionally be employed (Matthysse and McClain, 1973; Gerry, 2020a). Spot counts provide a relative estimate of total fly activity(Lysyk and Axtell, 1986), with the proportional representation of each fly species in the overall spot card count determined by their respective densities on fly ribbons or other methods of adult fly sampling (Gerry, 2020a).

1.5 Methods for houseflies' management

Pest insects, such as flies, must be monitored and controlled. Methods currently in use include preventive measures, chemical control, biological control and integrated management. Current practices for management of houseflies are reviewed by (Geden et al., 2021).

1.5.1 Preventive measures

This method incorporates useful practices to prevent infestations. To avoid flies in the domestic environment, one of the most commonly used methods is to screen doors and windows with mosquito nets, while inside the house, flashlights or sticky traps can be used (Abbas et al., 2014).

To prevent the spread of flies, proper disposal of rubbish and organic substrate, which serve as potential breeding and oviposition sites, is one of the most effective preventive measures. It has been estimated that over 50% of urban flies originate from insufficient waste management. Thus, proper disposal techniques and the use of suitable containers with lids should be employed to isolate waste from the external environment (Iqbal et al., 2014). In a zootechnical context, prevention is based on the cleaning and disinfection of premises as well as the elimination of stagnant water. It is crucial to maintain dry conditions, avoid dispersion of feed and separate the solid and liquid fractions of manure. Eliminating adult flies can help reduce infestation, but it is more effective to remove potential breeding sites twice a week. Subsequent development of the larvae can be hindered by drying out manure quickly, rendering it an unsuitable environment for their growth in manure (Sanchez-Arroyo and Capinera, 2017).

1.5.2 Chemical control

Chemical control of *M. domestica* infestations is achieved using insecticides. Organic or inorganic substances, natural or synthetic, which have a toxic effect and act rapidly to reduce high fly densities in a short time. Plants produce a diverse range of secondary metabolites, such as terpenoids, phenols, polyphenols, alkaloids, quinones, and sugars, as a part of their defense mechanism against insects (Pavela, 2013). Plant-derived insecticides have been utilized for centuries. In the 17th century, certain crops were treated with tobacco decoctions containing nicotine. These natural insecticides underwent significant development between the two world wars and have mostly replaced by synthetic insecticides due to their increased cost-effectiveness and efficiency. Among the range of natural molecules possessing insecticidal properties, the mostly utilized ones against the pests are alkaloids, comprising nicotine and pyrethrums (Siegwart et al., 2015).

Insecticides of natural origin include:

• Nicotine, an alkaloid presents in the leaves of several plants. The fixation of nicotine, an acetylcholine mimetic, onto the postsynaptic receptor is accountable for the uninterrupted depolarization of nerve cells, resulting in perpetual excitation. Such excitation brings about the muscular paralysis of the insect, ultimately leading to its demise (Bai et al., 1991; Siegwart et al., 2015). Synthetic neonicotinoids have been developed based on this mode of action to evade some of the issues associated with toxicity to mammals (Siegwart et al., 2015).

• Pyrethrum is a botanical insecticide permitted in organic farming throughout numerous countries worldwide. It is obtained by extracting this compound from dried Chrysanthemum flowers, particularly Dalmatia pyrethrum. These flowers possess insecticidal properties, including a cluster of esters named "pyrethrins," which have the highest relative toxicity. As highly unstable compounds, pyrethrins are swiftly degraded by light, air, and heat.

To enhance the effectiveness of pyrethrum, additional synergistic molecules may be incorporated into the formulation, with piperonylbutoxide (PBO) being the most common synergies. Although pyrethrum containing PBO is permitted in organic farming, there is controversy surrounding its toxicity (Siegwart et al., 2015). Pyrethrins affect the nervous system of insects by blocking sodium channels, releasing acetylcholine, and deactivating postsynaptic receptors. This has a detrimental effect on presynaptic receptors, obstructing acetylcholine release and causing a blockage of synaptic transmission, eventually resulting in paralysis and death (Siegwart et al., 2015)

• Naphthoquinones can be isolated from some plants or obtained in the form of crude plant extracts commonly used in medicine. These botanical insecticides may affect vitality, fecundity and fertility of surviving adults of houseflies (Pavela, 2013)

Synthetic insecticides are categorized as follows:

- Organochlorine compounds, which were initially widely adopted for chemical pest control, are no longer used due to their high toxicity and harm to both humans and the environment. This group includes specific compounds such as DDT (dichlorodiphenyltrichloroethane) or HCH (hexachlorocyclohexane) which act as neurotoxicants (Babers and Pratt, 1950). DDT, pyrethrins, and synthetic pyrethroids act on voltage-gated sodium channel proteins present in insect nerve cell membranes. The proper functioning of these channels is crucial for the normal transmission of nerve impulses, and this process is disrupted by the binding of the insecticides, resulting in paralysis and eventual death (Davies et al., 2007). GABA receptors are the main target of organochlorine insecticide (Rivero et al., 2010).
- Organophosphate compounds are often used to control housefly larvae. From the 1960s to the 1990s, organophosphate and carbamate insecticides were the primary methods employed for the control of houseflies in the United States, and they persist in use today. Among the primary mechanisms of resistance to these insecticides is the alteration of the target site (acetylcholinesterase) (Rivero et al., 2010).

- Carbamate compounds are esters of carbamic acid commonly used as insecticides. These compounds are known as N-methylcarbamates. The toxicity of N-methylcarbamate insecticides occurs through inhibiting the acetylcholinesterase enzyme, resulting in hypercholinergic activity as a toxic sign. Excitotoxicity induced by carbamates also involves the hyperactivation of N-methyl-d-aspartate receptors (Gupta, 2014).

- Neonicotinoid insecticides are artificial derivatives of nicotine, an alkaloid present in the leaves of several plants. They are strong, selective agonists of insect nicotinic acetylcholine receptors, and are extensively used to ensure crop protection and animal health (Byrne et al., 2003; Li et al., 2012; Liu et al., 2005). Imidacloprid, which is the neonicotinoid mainly used for adult flies, was brought into commercial use in 1991 (Li et al., 2012; Liu et al., 2005).

- Pyrethroids are synthetic analogues of pyrethrins. Permethrin (the first field-stable pyrethroid) are used for control of houseflies was approved in the United States and Europe in the early to mid-1980s (Scott, 2017) Despite numerous demonstrations of the capacity of housefly populations to develop high levels of resistance (Qiu et al., 2007) and the ease of detection of pyrethroid resistance in field populations (Qiu et al., 2007; Scott, 2017), pyrethroids are still extensively used for managing house flies (Scott, 2017). Sodium channels are the primary target of pyrethroids. Symptoms of pyrethroid poisoning in insects and generally include hyperexcitability, tremors and convulsions followed by paralysis and death. In insects, the first signs of poisoning are usually incoordination and locomotor instability (Wouters and Van Den Bercken, 1978).

- Insect growth regulators (IGRs) inhibit the development from egg to larvae and from larvae to pupae. Insecticides with growth regulating properties (IGR) can have detrimental effects on insects by regulating or inhibiting essential biochemical pathways or processes crucial for insect growth and development. Exposure to such compounds can result in abnormal regulation of hormone-mediated cell or organ development, leading to the death of some insects. Other insects may be killed either by prolonged exposure at a developmental stage to other mortality factors (increased susceptibility to natural enemies, environmental conditions, etc.) or by abnormal termination of a developmental stage itself. IGR pesticides can hinder the generation of chitin which makes the insects incapable of synthesizing new cuticle and undergoing a successful molt into the subsequent stage (Ijumba et al., 2010). Insect growth regulators can be produced from a mixture of synthetic chemicals or from other natural sources, such as plants (Tunaz and Uygun, 2004).

1.5.2.1. Insecticide resistance

Chemical pesticides have been largely used as the primary means to control insect pests, but consistent exposure has led to insecticide resistance (Gul et al., 2023; Li et al., 2012). The resistance mechanisms that insects have at their disposal can be divided into three general categories. Modified behavioral mechanisms fall under the first category, which effectively decrease an insect's exposure to toxic compounds.

Physiological mechanisms, including alterations to penetration, excretion, transport, or storage of insecticides, constitute the second category. The third category includes biochemical mechanisms such as insensitivity of target sites to insecticides and enhanced detoxification by various metabolic enzymes (Lee et al., 2001). Insects typically resist insecticides through metabolic detoxification, target site mutations, decreased penetration or increased excretion, and behavioral resistance (Kliot and Ghanim, 2012; Gul et al., 2023). Trade-offs exist between biological traits, with higher fitness conferring greater resistance under selection pressure to insecticides but reduced fitness without selection (Gul et al., 2023; Singarayan et al., 2021; Ullah et al., 2020).

Insects exposed to insecticide resistance encounter fitness costs due to energetic and physiological disadvantages. These costs are connected to the insects' adaptation to new habitats, stressors, and toxic secondary metabolites (Gul et al., 2023). This results in fitness costs due to the expenses of reallocating resources and energy (Grigoraki et al., 2017; Gul et al., 2023). Overexposure of humans and animals to pesticides can often lead to poisonings. It is important to minimize exposure to these harmful chemicals to protect public and animal health (Tudi et al., 2022).

1.5.2.2 Resistance of houseflies to insecticides

Dipterans, such as *M. domestica*, are pests of great significance in agriculture, domestic settings, and livestock farms. Their exposure to insecticides has been extensive, with *M. domestica* serving as a prime example of insecticidal resistance (Gul et al., 2023; Roca-Acevedo et al., 2022). It shows resistance to all major insecticide groups, such as organochlorines, organophosphates, carbamates and pyrethroids (Gul et al., 2023). The susceptibility also varies depending on the season, frequency of application, and the insecticidal group, as a result of biological parameters changing or the insecticide exposure being terminated during the off-season (Abbas et al., 2014). Levels of insecticide resistance in housefly populations in the United States were surveyed in 2008–09 (Scott, 2017). The housefly has developed resistance to 62 distinct insecticide active ingredients, with 337 documented cases found internationally. As a consequence, it is currently regarded as the most resistant pest among urban insects worldwide (Devine, 2009; Zhu et al., 2016).

1.5.3 Biological control

Due to increasing resistance to insecticides and public awareness of their harmful effects, alternative control strategies have emerged. Biological control is a technique that exploits the antagonistic relationships between living organisms to reduce populations of pest (Sanchez-Arroyo, and Capinera, 2017). Biocontrol agents for houseflies include both microorganisms, also called entomopathogens (nematodes, fungi, bacteria, viruses and macroorganisms (predators and parasitoids).

1.5.3.1 Nematodes

Steinernematid and heterorhabditid nematodes have been studied for control of flies, but their effectiveness has been mixed (Geden, 2012). Laboratory studies show that fly larvae are highly susceptible to nematodes, but the nematode were not found in the natural substrates. Early reports suggested that nematodes were effective in poultry houses in British Columbia (Belton et al., 1987), but studies show that they perform poorly in poultry and pig manure. Adult flies are less susceptible to parasitism but can be infected by visiting bait stations (Geden, 2012).

1.5.3.2 Fungi

Adult house flies exhibit susceptibility to fungal pathogens such as *Entomophthora muscae* and *E. schizophorae*, resulting in mortality within 4-6 days after exposure to conidia (Geden, 2012).

The release intensity and survival duration of conidia are influenced by temperature and relative humidity (Ruegg, 2012; Geden, 2012). *E. muscae* plays a crucial role as a natural regulator of fly populations, as demonstrated in laboratory, where the flies which were exposed to the pathogen at <36 h of age did not oviposit before they died. Notable, flies exposed at 48-96 h of age laid only 20% as many eggs as uninfected females from the same cohort over their lifespan, in spite of developing and holding mature eggs (Mullens, 1991) . Thus *E. muscae* appears to modify host behavior in *M. domestica,* mass-rearing methods have been successfully developed for infected flies, leading to increased disease prevalence upon field releases of both *E. muscae* and *E. schizophorae* (Geden et al., 1993; Geden, 2012). *Beauveria bassiana* and *Metarhiuzium anisopliae*, though showing low natural prevalence rates in houseflies and stable flies, exhibit high susceptibility in both larvae and adults (Geden, 2012). Virulence levels vary among strains and formulations, with adult houseflies being particularly receptive to sugar baits containing *B. bassiana* conidia (Geden, 2012). Notably, entomopathogenic fungi like *B. bassiana* and *M. anisopliae* coexist compatibly with other natural enemies, including the predator *Carcinops pumilio* and parasitoids such as *Spalangia cameroni* and *Muscidifurax raptor* (Geden et al., 1995; Kaufman et al., 2002). This compatibility highlights the potential for integrated pest management strategies leveraging multiple biological control agents.

1.5.3.3 Bacteria and Viruses

Also, bacteria and viruses can be used for controlling house fly populations. The early use of *Bacillus thuringiensis* (Bt) against flies showed promising results, with significant maggot control achieved by delivering Bt spore formulations to fly breeding sites in manure (Malik et al., 2007). However, the use of exotoxin-producing Bt strains led to the development of resistance in house flies and safety concerns over vertebrate toxicity, resulting in the prohibition of exotoxin-containing Bt products in the US in the mid-1980s (Geden, 2012). Certain strains of Bt with good activity against adult houseflies were identified, suggesting its key role in the activity of these strains for higher flies. Recent research has identified promising new strains of Bt with potential activity against house flies, indicating the need for further study in this area.

Additionally, there has been increased interest in the discovery and use of Bt strains for fly control, with promising new strains identified in Korea as described in Geden's review (Geden, 2012). As for the MdSGHV virus, it is a recently discovered virus that infects house flies, causing hypertrophy of salivary glands and reducing mating success and lifespan in infected flies. The virus has shown potential as a biopesticide, with the ability to infect flies through direct contact with low-dose aqueous virus suspensions (Lietze et al., 2007; Geden et al., 2008). Further research and development of new formulations could greatly improve the prospects for using MdSGHV as an operational biopesticide (Malik et al., 2007; Geden, 2012).

1.5.3.4 Predators

Housefly predation is a biological method of decreasing fly density (Malik et al., 2007). Research indicates that adult *Carcinops pumilio* (Madeira, 1998; Malik et al., 2007) and third instar larvae of *Hydrotaea aenescens* are capable predators of house fly larvae (Malik et al., 2007). Nonetheless, there is no evidence of significant infestations of *H. aenescens* causing disturbance to workers or pigs (Schultka et al., 1986; Malik et al., 2007). Experimental studies demonstrate that *H. capensis* larvae can eliminate up to 17 preys per predator, resulting in a reduction of over 70% in the house fly population (Tsankova and Luvchiev, 1993; Malik et al., 2007). A 14-month field experiment in Florida revealed that *H. aenescens* populations failed to establish in wet poultry manure due to excessive moisture, resulting in a maximum adult emergence of only 20% under field conditions (Hogsette and Jacobs, 1999; Malik et al., 2007). There is potential for *H. capensis* to be an effective biological agent for controlling house flies, although comprehensive field testing is required to confirm its practical efficacy (Malik et al., 2007). Nonetheless, *H. aenescens* larvae, which were released earlier, successfully established themselves at a poultry farm, highlighting the susceptibility of biological control agents to their environment. To achieve anticipated outcomes in the field, it is crucial to adopt a comprehensive approach considering all environmental factors (Malik et al., 2007).

1.5.3. 5 Parasitoids

Srinivasan and Balakrishnan, 1989, investigated four species of parasitoids (Hymenoptera Chalcidoidea) namely *Pachycrepoideus vindemmiae*, *Spalangia cameroni*, *Spalangia nigroaenea*, and *Dirhinus himalayanus*, in different habitats across the district of Pondicherry (Malik et al., 2007; Srinivasan, and Balakrishnan, 1989). Their findings revealed that *D. himalayanus* was solely present in poultry farms, whereas *P. vindemmiae* was present in all the habitats. Srinivasan and Amalraj (2003) investigated the longevity, fertility, efficiency, feeding habits, and stinging behavior of *D. himalayanus*, measuring the rates of parasitism. They discovered that controlling the house fly at varying ratios yielded a range of 50.8% to 95.5% efficacy, with the highest degree of parasitism (75%) occurring in 24-48-hour-old pupae (Srinivasan, and Balakrishnan, 1989). Skovgård and Jespersen (1999) investigated the distribution and relative abundance of *M. domestica* from two pig farms, two dairies, and combined pig and dairy farms in Denmark. They discovered a low rate of parasitism, with *S. cameroni* and *M. raptor* being the most abundant species.

According to Kaufman et al. (2001), pupae of housefly were never parasitized by *M. raptorellus* at a rate higher than 7% in any farm. Mckay and Galloway's investigation into Hymenoptera parasitoids on dairy farms unveiled that *N. vitripennis* accounted for 2.2% of freeze-killed sentinel house fly pupae parasitization while eight other parasitoids accounted for 0.6%. The study disclosed *Phygadeuon fumator* Gravenhorst as the most abundant parasitoid (McKay and Galloway, 1999). Environmental factors have an impact on the abundance and distribution of parasitoids (Malik et al., 2007). Geden's research examined how habitat depth affects host location by five species of parasitoids of house flies in three substrates.

To test this, they used *M. raptor* Girault & Sanders, *S. cameroni* Perkins, *S. endius* Walker, *S. gemina* Boucek, *and D. himalayanus*. *S. cameroni* proved to be the most effective species for identifying hidden pupae in manure. All species searched the hosts in the depth of the fly-rearing medium (Geden, 2002). Skovgard and Nachman's study on Danish dairy farms revealed that *S. cameroni* was the most abundant parasitoid, making up 80.5-93.1% of the population (Skovgård and Nachman, 2004). Inundated releases of *S. cameroni* controlled house flies, leading to a significant reduction, while stable flies remained unaffected. *S. cameroni* emerged as the primary parasitoid, although *M. raptor* also showed to be a significant parasitoid (Skovgård and Nachman, 2004). Parasitoids could provide a cost-effective and efficient control method. However, their use requires confirmation from field experiments before recommendations can be provided (Malik et al., 2007).

1.6 Integrated management

Integrated pest management programs for houseflies, which combine biological, physical, and chemical control alternatives, have become increasingly popular in recent years. These programs can reduce fly densities and address resistance issues, minimizing environmental damage and promoting a healthy environment (Malik et al., 2007).

1.6.1 Biological + Chemical control

Srinivasan and Domic (2003) conducted a study on the efficacy of the parasitoid *D. himalayanus* and the insect growth regulator triflumuron in the control of house flies. The combination of IGR and parasitoids was found to significantly reduce the house fly population. Moreover, a sub-lethal dose of IGR triflumuron delayed the emergence of adults, thereby increasing their exposure time to parasitism. The decrease in pupal and adult density showed a higher rate in areas where both parasitoid-IGR were utilized (Srinivasan, and Dominic, 2003). Geden et al. (1992), implemented an integrated housefly management program in New York and Maryland to manage houseflies. House flies were controlled by releasing *M. raptor*, removing calf bedding weekly and spraying with pyrethrin. The housefly pupal mortality was achieved at rates of 65% and 38% in dairy farms in New York and Maryland respectively, compared to 30% and 26% in control farms. Parasitism was observed at levels of 36% and 18% on release farms as contrasted with 45% and 2% on control farms in New York and Maryland (Geden et al., 1992; Malik et al., 2007).

1.6.2 Cultural + biological + chemical control

Crespo et al. (2002), designed integrated management programs to control houseflies in Argentina's poultry houses using various combinations of cultural, chemical, and biological control strategies. Granular baits containing adulticides were employed in the treatments. The evaluated combinations consisted of control, cultural and chemical control, cultural and biological control with granular bait (IMP 1), and cultural and chemical and biological control with granular bait (IMP 2). The treatments provided maximum control of house flies with minimum use of pesticides.

IMP 2 was most appropriate for farms with large adult house fly populations, while IMP 1 was most effective in situations with low initial adult fly populations and low manure inputs (Crespo et al., 2002)

1.6.3 Combination of biological agents

A study conducted in New York by Kaufman et al. (2005) employed a *Beauveria bassiana* product to counter housefly infestation in comparison to pyrethrin treatments. An integrated fly management program, which included the release of housefly pupal hymenopteran parasitoids, was used at all facilities. The results demonstrate the reduction of adult house fly populations and larvae numbers in *B. bassiana*-treated sites. Pupal parasitism levels of house flies were low but comparable between the two treatments (Kaufman et al., 2005). Adult and larval *Carcinops pumilio* counts were 43% and 66% higher, respectively, in the *B. bassiana*-treated premises than in those treated with pyrethrin. This research concludes that an effective integrated management program can reduce housefly populations (Kaufman et al., 2002).

1.7 Aims of the PhD project

This PhD project aims to promote sustainable housefly management in Italian dairy farms, specifically in the Parmigiano Reggiano cheese production area. The study intends to improve our understanding of insecticide resistance mechanisms and to implement biological control using pupal parasitoids. Several research gaps have been identified throughout the investigation, presenting opportunities for future contributions to the field. Firstly, it is essential to investigate the conduct of houseflies in situations pertaining to specific insecticide resistance. Valuable information can be gained by understanding the complexity of their actions, which will guide the development of targeted and effective management strategies.

In the dairy farms of the Parmigiano Reggiano cheese production area, there is a significant lack of knowledge about the abundance of houseflies and their parasitoids. This gap poses a barrier to designing interventions that are specifically adapted to the dynamics of these agricultural facilities. Furthermore, there is a critical gap in the molecular characterization of different pupal parasitoid species. A specific investigation on the genomic characterization is essential to understand the complex interactions within these populations and to enable informed and strategic interventions.

Furthermore, it is crucial to evaluate the effectiveness of different populations of commercially available and native species of pupal parasitoids in controlling the housefly pests. The assessment is not purely academic since a practical approach to identifying the most efficient and sustainable biological control solutions is strongly needed.

The specific aims were:

1. To investigate whether imidacloprid discrimination occurred in houseflies via the sensory system on their tarsi and proboscis structure and evaluate different behavioral responses by susceptible or resistant fly (Chapter 2).

2. To characterize the abundance of houseflies in dairy farms in the Reggio Emilia area and their natural enemies, pupal parasitoids, considering different dairy farm management and the environmental parameters (Chapter 3).

3. Investigate with molecular and taxonomical approaches, the presence of natural populations of pupal parasitoids of houseflies in the Parmigiano Reggiano production area (Emilia Romagna, Northern Italy) and evaluate the differences in parasitism behavior between commercialized and wild populations of *Muscidifurax zaraptor, Muscidifurax raptor, Nasonia vitripennis* and *Spalangia cameroni* (Chapter 4).

4. Determining the peak of oviposition in the female parasitoids *Spalangia cameroni* and *Muscidifurax zaraptor* in relation to age, whilst verifying the sex ratio of newly emerged parasitoid adults over time and examining their potential for reducing the emergence of houseflies in their presence (Chapter 5)

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Research Article

Chapter 2. Use of the Proboscis Extension Response Assay to Evaluate the Mechanism of HouseFly Behavioral Resistance to Imidacloprid

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2.1 Simple Summary

This study investigates the detection and discrimination of the neonicotinoid insecticide imidacloprid by behaviorally resistant and susceptible houseflies (*Musca domestica* L.). Flies were allowed to contact a sucrose solution containing either a low or a high concentration of imidacloprid with their tarsi alone or with both their tarsi and proboscis. The proboscis extension response (PER) for each housefly was recorded at 0, 2, and 10 s following the start of tarsal contact with the test solution. Following proboscis contact with the sucrose solution containing a high concentration of imidacloprid, behaviorally resistant flies had a significant reduction in PER (within 2 s), while imidacloprid-susceptible flies showed no differences in PER associated with imidacloprid concentration. When only tarsi were allowed to contact either solution, there were no significant differences in PER observed for either fly strain (resistant or susceptible). These results suggest that behaviorally resistant houseflies detect imidacloprid and can discriminate among low and high concentrations following proboscis contact but not tarsal contact with a sucrose solution containing imidacloprid. Understanding the mechanisms responsible for behavioral resistance to insecticides by the housefly is critical for creating sustainable pest management strategies for this fly.

2.2 Abstract

The housefly, *Musca domestica* L., is a significant human and livestock pest. Experiments used female adult houseflies glued onto toothpicks for controlled exposure of their tarsi alone (tarsal assay) or their tarsi and proboscis (proboscis assay) with a sucrose solution containing imidacloprid at either a low (10 µg/ml) or high (4000 µg/ml) concentration. Proboscis extension response (PER) assays were used to characterize the response of imidacloprid-susceptible and behaviorally resistant housefly strains to contact with sucrose solutions containing either a low or high imidacloprid concentration. In each assay, 150 female flies from each fly strain were individually exposed to sucrose solutions containing either a low or high imidacloprid concentration by deliberate contact of the fly tarsi to the test solution. The PER for each fly was subsequently recorded at 0, 2, and 10 s following initial tarsal contact. A significant and rapid reduction in PER was observed only for the behaviorally resistant fly strain and only following contact by the fly proboscis to the sucrose solution containing the high imidacloprid concentration. Results suggest that chemoreceptors on the fly labellum or internally on pharyngeal taste organs are involved in imidacloprid detection and concentration discrimination, resulting in an avoidance behavior (proboscis retraction) only when imidacloprid is at sufficient concentration. Further research is needed to identify the specific receptor(s) responsible for imidacloprid detection.

Keywords: *Musca domestica*, insecticide, chemoreception, discrimination

2.3 Introduction

The housefly (*Musca domestica* L.) is a major pest in confined animal facilities and is a potential mechanical vector of over 200 pathogens (Greenberg, 1965; Nayduch et al., 2023). However, control of this pest has been complicated by the development of insecticide resistance to all major available insecticidal classes, including pyrethroids, organophosphates, carbamates, and neonicotinoids (Geden et al., 2021). Insecticides formulated for housefly control are often applied as a component of sugar-based food bait ("fly bait"). Fly baits containing the neonicotinoid insecticide imidacloprid have been commercially available since 2002, and initially provided good control of houseflies resistant to other available insecticides (Butler et al., 2007). However, as the use of imidacloprid-containing bait for fly control increased, housefly resistance to imidacloprid was soon reported (Gerry and Zhang, 2009; Tan et al., 2015). Gerry and Zhang (2009) suggested that housefly resistance to imidacloprid was due to altered behavior of houseflies following detection of or contact with imidacloprid (Gerry and Zhang, 2009). Later studies confirmed that houseflies express "behavioral resistance" by reduced feeding on the bait (Hubbard and Gerry, 2020). Genetic studies using the F_1 backcross method [(Tsukamoto, 1964)] demonstrated that factors contributing to behavioral resistance to imidacloprid were found on autosomes 1 and 4 of the housefly (Hubbard and Gerry, 2021).

For "food-based" insecticides such as fly baits, consumption of the food material is required to achieve fly mortality, and thus, the mechanisms of "taste" that determine the suitability or palatability of the bait to the fly are important to understand. In flies and other insects, tasting occurs through chemical detection by chemosensory taste receptors (gustatory receptors [GRs]) located within taste sensilla present on the mouthparts, legs, antennae, ovipositor or even the wings (Chen et al., 2021; King and Gunathunga, 2023). In *Drosophila melanogaster* (Meigen), taste sensilla include up to four molecularly and physiologically distinct taste neurons that are selectively activated by palatable (sweet, salty, water) or noxious (high salt, bitter, low pH) tastants (Freeman and Dahanukar, 2015). In Diptera, taste receptors are most reported on the legs (particularly on the fore tarsi) (Adams et al., 1965), the labellum at the tip of the proboscis, and the pharyngeal organs lining the esophagus (Freeman and Dahanukar, 2015; Rajashekhar and Singh, 1994). As flies land on or walk across a surface, the tarsi are often the first body structures to contact a potential food source. When taste receptors on the tarsi are stimulated by food molecules such as sugars, flies initiate an appetitive behavior sequence by extending their proboscis to contact the potential food source to further assess food quality and to initiate feeding if the food is deemed suitable (King and Gunathunga, 2023). Thus, proboscis extension begins the feeding process but follows initial detection of a potential food source by taste receptors on the legs or other body structures (Dunipace et al., 2001; Scott, 2017).

Houseflies are reported to exhibit aversive behaviors to sucrose formulated with imidacloprid only following direct contact with the bait (Hubbard and Gerry, 2020), suggesting flies avoid imidacloprid only following the detection of imidacloprid by gustatory receptors rather than following volatile detection by odorant receptors.

However, the specific mechanism for imidacloprid detection and initiation of a behavioral response by the housefly is not well understood. The present study aims to achieve two objectives. The first is to determine whether imidacloprid detection and concentration discrimination by behaviorally resistant houseflies occurs via the tarsi or the proboscis (labellum) and/or pharyngeal organs lining the esophagus. The second is to determine if susceptible and resistant fly strains vary in their ability to discriminate and respond to imidacloprid at a low or high concentration.

2.4 Materials and Methods

2.4.1 Housefly strains

Two housefly strains (UCR, BRS) were used in this study. The UCR strain is an insecticide-susceptible strain maintained in the laboratory at UC Riverside since 1982 following the collection of pupae from a dairy farm in Mira Loma, California. The BRS strain was selected to exhibit a strong behavioral resistance phenotype to the insecticide imidacloprid (Hubbard and Gerry, 2020) following collection via sweep net from a dairy farm in San Jacinto, CA. Following initial selection for the behavioral resistance phenotype, the BRS fly strain has been maintained under continuous selection pressure (selection every 3 filial generations) to maintain the selected level of behavioral resistance to imidacloprid. Both populations were maintained in insectary rooms under standard environmental conditions (27°C, 14:10 L:D, and 35% RH) and reared following standard practices (Zahn and Gerry, 2018).

2.4.2 Proboscis Extension Response – Proboscis Contact

Proboscis extension response assays were conducted following methods described by (Shiraiwa and Carlson, 2007) with the following modifications: adult house flies (3–6 d old) were aspirated from an adult colony cage, placed into plastic holding cages without food (only water), and held in a standard laboratory room (at 22 ± 2 °C) for a starvation period of 1 d for the UCR strain and 2-3 d for the BRS strain. The length of starvation for each cohort of flies was determined by removing a small number of flies (ca. 10-15) from the holding cage each day to test for a proboscis extension response (PER) to a 30% sucrose solution (as described below). When >60% of tested flies showed a positive PER to the sucrose solution, flies in the cohort were deemed to be sufficiently starved for testing. Following the starvation period flies in the holding cage were chilled in a −20°C freezer to immobilize them for a few minutes and then sorted by sex on an electronic chill table (Catalog #1431, BioQuip Products Inc., Compton, CA).

Female flies were glued to flat wooden toothpicks by placing a drop of fast-drying clear nail polish (Seche Vite, Item 83100, American International Industries) to the flat end of the toothpick and carefully touching the nail polish to the dorsal thorax of the fly (Figure 1).

The glue holding the fly to the wooden toothpick was allowed to dry by placing the narrow end of the toothpick into a microtube rack well such that the flat end of the toothpick with the attached fly was suspended in the air, preventing fly contact to any surface. Glued flies were held in this position for at least 30 min to ensure full recovery from cold knockdown before their use in the feeding assays below.

Glued flies were initially tested for appropriate PER response following contact of fly tarsi to first a negative control (deionized water only) and then a positive (30% sucrose solution) control solution (Shiraiwa and Carlson, 2007). Each control solution (ca. 1 ml) was pipetted onto a separate clean glass microscope slide and flies were manually manipulated so that the tarsi contacted the control solution. Flies exhibiting PER to the positive control solution were allowed to contact the positive control solution with their proboscis for up to 2 s before the fly was withdrawn. Flies exhibiting the appropriate PER response to each control solution were subsequently used in additional assays (as described below) after first rinsing the fly tarsi in deionized water and then dabbing the tarsi on clean tissue to remove any remaining liquid residue.

Flies were subsequently examined for PER to test solutions of 30% sucrose containing imidacloprid at either a low concentration (10 µg/ml) or a high concentration (4000 µg/ml). The low concentration was selected based on findings by Hubbard and Murillo (Hubbard and Murillo, 2022) that flies from the behaviorally resistant BRS strain readily feed and survive on granular sucrose containing imidacloprid at concentrations <100 µg/g. Thus, the low imidacloprid concentration was not anticipated to result in an aversive behavior in these resistant flies. The high concentration of 4000 µg/ml imidacloprid was used because this was the challenge concentration Hubbard and Gerry (2020) used for selecting and maintaining behavioral resistance in the BRS fly strain, and these flies show a significant reduction in feeding on imidacloprid at this concentration (Hubbard and Gerry, 2020; Hubbard and Murillo, 2022).

Flies were held so that the tarsi contacted the test solution and flies could also reach the test solution by extending their proboscis. Flies extending their proboscis immediately following tarsal contact to the test solution were allowed to maintain continuous proboscis contact with the test solution for up to 10 s. The presence or absence of PER was observed and recorded at 0, 2, and 10 s of continuous proboscis contact to the test solution. Any fly retracting the proboscis between these observation times was recorded as PER- at the next observation time, and testing was concluded for that individual fly. Five replicate cohorts of 30 flies each (n=150 flies) were tested for each house fly strain (UCR, BRS) and for each imidacloprid concentration (Low, High) for a total of 600 flies tested.

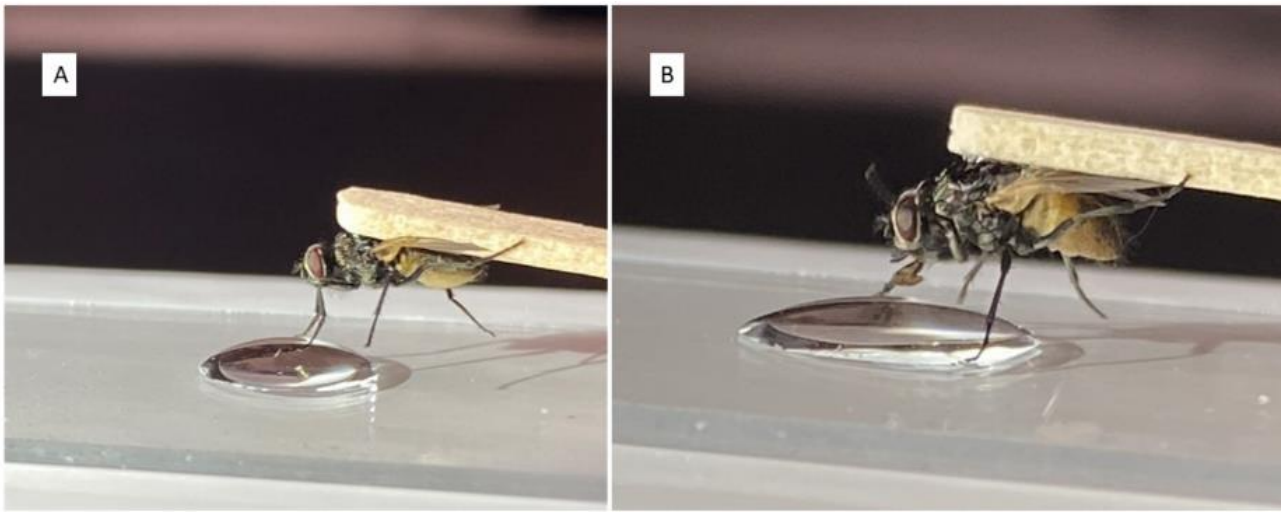

Figure 1 Houseflies glued on their dorsal thorax to the flat end of a wooden toothpick for PER assays. (**A**) A fly failing to exhibit PER following tarsal contact with a water control. (**B**) A fly demonstrating PER following tarsal contact with the sucrose control.

2.4.3 Proboscis Extension Response – Tarsal Contact

Adult house flies were aspirated from adult colony cages and handled as described for the proboscis contact assay above, except that flies were held in a position that allowed flies to contact control and test solutions with their tarsi only and prevented contact with their proboscis. As previously described in the proboscis assay, flies responding appropriately to both the negative and positive control solutions were examined for PER to test solutions formulated with 30% sucrose and imidacloprid at either a low concentration (10 µg/ml) or high concentration (4000 µg/ml). In this tarsal contact assay, flies were unable to reach the test solution with their extended proboscis. Flies extending their proboscis immediately following tarsal contact with the test solution were allowed to exhibit PER without proboscis contact with the test solution for up to 10 s. The presence or absence of PER was observed and recorded at 0, 2, and 10 s of continuous proboscis extension. Any fly retracting the proboscis between observation times was recorded as PER- at the next observation time, and testing was concluded for that individual fly. Five replicate cohorts of 30 flies each were tested for each house fly strain (UCR, BRS) and for each imidacloprid concentration (Low, High) for a total of 600 flies tested.

2.5 Statistical Analysis:

Data was analyzed separately for each assay (proboscis or tarsal contact) and fly strain (BRS, UCR). The number of flies exhibiting PER in each replicate group were rank ordered within each observation time (0, 2, 10 s) and then analyzed using Friedman's Test (non-parametric ranked ANOVA) to test for an overall difference in PER among the two imidacloprid concentrations and for significant interactions between observation time and imidacloprid concentration.

Rank values were further analyzed within each observation time by Wilcoxon Rank Sum Test for differences in the number of flies exhibiting PER for the two imidacloprid concentrations at the same observation timepoint with significance adjusted for multiple comparisons within each assay (α=0.016). All statistics were performed in R v 4.3.1 ("R Core Team.," 2021).

2.6 Results

The mean proboscis extension response (PER) is provided by fly strain and observation time in Table 1 (proboscis contact assay) and Table 2 (tarsal contact assay). The mean number of flies exhibiting PER predictably decreased across sequential observation times (0-2-10 s) for all cohort groups, regardless of assay method, fly strain, or imidacloprid concentration as flies that retracted their proboscis at any point in the assay were removed from the assay before the next observation time.

2.6.1 Proboscis Contact Assay

Immediately upon tarsal contact with sucrose containing imidacloprid at the 0 s observation time (prior to first contact by the proboscis), a similar mean number of flies extended their proboscis to both the low and high imidacloprid concentration for the imidacloprid-susceptible UCR flies (14.0 ± 1.18 and 15.6 ± 0.98, respectively) and the imidacloprid-resistant BRS flies (18.0 ± 0.71 and 18.4 ± 0.51, respectively) (Table 1).
Across all observation times, imidacloprid concentration had no effect on the number of UCR flies exhibiting PER ($F=0.28$; $df=1,29$; $p=0.60$) and there was no interaction between observation time and imidacloprid concentration ($F=0.80$; $df=2,29$; $p=0.37$). Similarly, within each observation time, there was no difference in the number of UCR flies exhibiting PER between imidacloprid concentrations at 0 s ($W=18$, $p=0.27$), 2 s ($W=13$, $p=0.98$), or 10 s ($W=10$, $p=0.66$) (Figure 2A). Thus, the proportion of UCR flies exhibiting PER decreased similarly across subsequent observation times for both the low and high imidacloprid concentrations with 8.8 ± 1.28 and 9.0 ± 1.38 of flies, respectively (ca. 30% for each group), continuing to exhibit PER at 2 s followed by 5.4 ± 0.75 (18%) and 4.6 ± 0.81 (15%) of flies, respectively, still exhibiting PER at 10 s. After adjusting PER for flies removed from the assay at each previous observation time (Figure 3A), ca. 50-60% of UCR flies are noted to have continued exhibiting PER from one observation time to the next, with no significant difference between the low or high imidacloprid concentrations ($W>9$, $p> 0.05$).

In contrast to the UCR flies, imidacloprid concentration had a significant effect on the number of BRS flies exhibiting PER ($F=14.79$; $df=1,29$; $p=0.0007$), and there was also a significant interaction between observation time and imidacloprid concentration ($F=5.81$; $df=2,29$; $p=0.008$). Within observation times, the number of flies exhibiting PER did not vary with imidacloprid concentration at 0s ($W=14.5$, $p=0.8$), but there was a significant difference at 2 s ($W=0$, $p=0.007$) and 10 s ($W=0$, $p=0.006$) (Figure 2B).

At the 2 s observation time, 14.2 ± 0.97 (45%) of BRS flies continued to exhibit PER to the low imidacloprid concentration while only 6.4 ± 0.68 (22.5%) of BRS flies continued to exhibit PER to the high imidacloprid concentration.

This difference carried over to the 10 s observation time, with 10.0 ± 1.3 (35%) of BRS flies exhibiting PER to the low imidacloprid concentration while only 3.2 ± 0.58 (12%) of BRS flies exhibiting PER to the high imidacloprid concentration. After adjusting PER for flies removed from the assay at each previous observation time, it was clear that the significant reduction in PER occurred only at the 2 s observation time (W=0, p=0.007) when PER continued for 79% of BRS flies exposed to the low imidacloprid concentration but only 35% of BRS flies exposed to the high imidacloprid concentration (Figure 3B). While the adjusted PER was also lower at 10 s for BRS flies exposed to the high imidacloprid concentration (49%) relative to the low imidacloprid concentration (70%), this difference was not significant (W=3, p=0.06).

2.6.2 Tarsal Contact Assay

Upon initial tarsal contact with sucrose containing imidacloprid at the 0 s observation time, a similar number of flies exhibited PER to both the low and high imidacloprid concentration for the imidacloprid-susceptible UCR flies (13.0 ± 0.77 and 13.8 ± 1.43, respectively) and the imidacloprid-resistant BRS flies (16.0 ± 1.18 and 12.4 ± 2.25, respectively) (Table 2). These mean PER values were similar to those observed for both fly strains in the proboscis contact assay at 0 s before flies were allowed to contact the test solutions using their proboscis.

Across all observation times in the tarsal contact assay, imidacloprid concentration had no effect on the number of UCR flies exhibiting PER (F=0.62; df=1,29; p=0.43) and there was no interaction between observation time and imidacloprid concentration (F=1.14; df=1,29; p=0.29). Within each observation time, there also was no difference in the number of UCR flies exhibiting PER between imidacloprid concentrations at 0s (W=13, p=0.95), 2 s (W=9, p=0.5), or 10 s (W=12.5, p=1) (Figure 4A). Although, there was an overall effect of imidacloprid concentration on the number of BRS flies exhibiting PER (F=12.89; df=1,29; p=0.001), there was no interaction between observation time and imidacloprid concentration (F=0.49; df=1,29; p=0.52) and there were no differences in PER by imidacloprid concentration within each observation time at 0s (W=6.5, p=0.26), 2 s (W=4, p=0.09), or 10 s (W=2.5, p=0.04) when p-values were adjusted for multiple comparisons (Figure 4B).

For both the UCR and BRS fly strains, the proportion of flies exhibiting PER in the tarsal contact assay decreased similarly across subsequent observation times for both the low and high imidacloprid concentrations. For UCR flies exposed to the low or high imidacloprid solution, 10.0 ± 1.45 (33%) or 9.4 ± 0.81 (31%) of flies, respectively, continued to exhibit PER at 2 s followed by 5.4 ± 1.72 (18%) or 5.6 ± 1.36 (19%) still exhibiting PER at 10 s (Figure 4A).

For BRS flies exposed to the low or high imidacloprid solution, 10.0 ± 1.30 (33%) or 6.4 ± 1.21 (21%) of flies, respectively, continued to exhibit PER at 2 s followed by 7.2 ± 0.73 (24%) or 4.0 ± 0.89 (13%) still exhibiting PER at 10 s (Figure 4B).

After adjusting PER for flies removed from the assay at each previous observation time, a similar proportion of UCR flies and BRS flies continued to exhibit PER at 2 s (70-75% and 52-62%, respectively) and at 10 s (50-60% and 65-74%, respectively) (Figure 5A, 5B) with no differences between imidacloprid concentration at any observation time ($W > 2.5$, $p > 0.016$).

2.7 Discussion

Houseflies from both fly strains exhibited PER when placed in tarsal contact with sucrose solutions as would be expected given previous studies demonstrating detection of sugars by the tarsi (Edgecomb et al., 1987; Smith et al., 1983; Stoffolano et al., 1990; Loy et al., 2016) . To understand taste detection and the associated behaviors, it is important to differentiate between detection and discrimination. Detection refers to identifying the presence of a substance, while discrimination refers to the ability to distinguish between different concentrations of the substance. According to our results, there was no evidence for detection of imidacloprid or at least discrimination among the low (non-lethal) or high (lethal) imidacloprid concentrations by the resistant BRS flies when flies contacted the sucrose solutions using their tarsi alone. Rather, the BRS flies exhibited a variable response to the solutions with low or high imidacloprid concentration only following proboscis contact with the solutions. Thus, the behavioral resistance to imidacloprid (reduced feeding) previously reported for these flies (Hubbard and Gerry, 2020) most likely results from activation of gustatory receptors (GRs) associated with the labellum or other mouthparts following contact with imidacloprid. This is supported by the rapidity of the response, with substantial numbers of resistant BRS flies quickly retracting their proboscis within 2 s of initial proboscis contact to the high imidacloprid solution. A rapid retraction of the proboscis would limit exposure to and especially uptake (by feeding) of the toxic food, an important consideration given that the behaviorally-resistant BRS flies lack substantial physiological resistance to imidacloprid (Hubbard and Gerry, 2020) and thus would die if a high dose of imidacloprid was consumed in more than trace amounts. In contrast, the imidacloprid-susceptible UCR flies continued to exhibit PER similarly to both the low and high imidacloprid solutions during the proboscis contact assay when flies were allowed to contact the solutions with their proboscis for up to 10 s.

The BRS house fly strain used in this study was previously selected for a high level of behavioral resistance to imidacloprid which resulted in BRS flies significantly reducing their contact time with sucrose treated with imidacloprid relative to sucrose alone (Hubbard and Gerry, 2020, 2021). The similar landing rate of BRS house flies on sucrose with or without imidacloprid (Hubbard and Gerry, 2020) suggests that these behaviorally resistant flies cannot detect imidacloprid prior to physical contact of their proboscis with an imidacloprid-treated sugar source. Furthermore, the selected resistance was specific to imidacloprid as BRS flies readily

consumed sugar baits containing another related neonicotinoid insecticide (dinotefuran) (Hubbard and Gerry, 2020).

The gustatory system is responsible for detecting non-volatile cues in the environment and is primarily involved in feeding behavior, allowing animals to detect and discriminate between nutritious and noxious foods (Wang et al., 2004). Insects have gustatory receptor neurons (GRNs) that are widely distributed over the body surface and activation of GRNs in different peripheral tissues will mediate distinctive behaviors (Dethier, 1976; Wang et al., 2004).

A highly conserved clade of GRs plays a critical role in the detection of and response to chemical compounds as part of the insect taste system (e.g., (Isono, 2010; Marella et al., 2006)). In Diptera, taste detection is mediated by sensory bristles on the proboscis, internal mouthparts, legs, wings, and ovipositor (Stocker, 1994; Wang et al., 2004), with activation of gustatory neurons on tarsal leg segments following contact with sugars resulting in proboscis extension and feeding initiation (Dethier, 1976; Wang et al., 2004). The taste organs of flies are predominantly located on the labellum at the tip of the proboscis and on the tarsi (Falk and Atidia, 1975; Shiraiwa and Carlson, 2007). Taste receptors on the legs are common among many insects, including Lepidoptera (Calas et al., 2006), Hymenoptera (Jaleel et al., 2021), Orthoptera (Gaaboub et al., 2005), Coleoptera (Yosano et al., 2020), and Blattodea (King and Gunathunga, 2023; Rajashekar et al., 2012). Georghiou (1972) categorized behavioral resistance as either stimulus-independent or stimulus-dependent. Stimulus-independent behavioral resistance is a result of the insect's natural avoidance of an environment or situation where it might be exposed to an insecticide (Georghiou, 1972; Hubbard and Gerry, 2020). Stimulus-dependent behavioral resistance refers to an insect's increased ability to detect and limit contact with a toxic substance, possibly due to the substance's repellent or irritant properties, formulation, or presentation, resulting in an aversive response (Georghiou, 1972; Hubbard and Gerry, 2020). In our study, flies were unable to avoid tarsal contact with the sucrose solution containing imidacloprid due to the design of the assay, but they could avoid extending their proboscis or they could retract their proboscis during the trial period making their proboscis extension response (PER) dependent on the detection and discrimination of imidacloprid concentration.

In general, insects can use multiple mechanisms to avoid consuming a toxicant present in a sugar food bait including 1) activation of bitter-sensing GRNs by a bait component resulting in feeding cessation or 2) inhibition of sugar-sensing GRNs reducing recognition of the sugar bait as a suitable food source (French et al., 2015; Rimal and Lee, 2019). *Drosophila spp*. discriminate among sugar concentrations using GRNs on the tarsi and can be trained to avoid sugar concentrations when these are associated with a negative stimulus (Masek and Scott, 2010).

An unusual gain-of-function adaptation in some populations of the German cockroach (*Blatella germanica*) provides resistant cockroaches with protection from toxic baits containing glucose as the result of an acquired sensitivity to glucose with both activation of bitter-sensing GRNs and suppression of sugar-sensing GRNs following contact with glucose (Wada-Katsumata et al., 2011, 2013, 2018).
A study found that bait-resistant cockroach strains also discriminate between different doses of glucose, with an inverse relationship between glucose dose and feeding response when aqueous solutions of glucose were presented to the cockroach paraglossae following ablation of the maxillary and labial palps (Wada-Katsumata et al., 2011).

According to our results, discrimination between the low and high concentrations of imidacloprid by the resistant BRS fly strain occurred after proboscis contact with the test solutions, however, the specific location of the GRNs associated with the house fly mouthparts and the specific GRs responsible for the detection of imidacloprid by these house flies are not known. In *Drosophila*, bitter-sensing GRNs that detect aversive tastants, including noxious substances, are characterized by subsets of GRs that do not overlap with those expressed in sweet-sensing GRNs (Montell, 2009; Scott et al., 2001). The rapid proboscis retraction of BRS flies after proboscis contact with the high imidacloprid solution suggests a strong aversive behavior, perhaps due to selection in these flies for dose-dependent activation of bitter-sensing GRNs by imidacloprid, allowing for greater discrimination of imidacloprid concentration to avoid a lethal exposure to this toxicant.

Investigating insecticide resistance in field populations can provide insights into evolutionary processes. Strong selective agents and pressure can lead to rapid evolution of resistance. In some cases, behavioral resistance to an insecticide can provide greater protection than physiological resistance since resistance cannot be overcome by increasing the insecticide concentration (Hubbard and Gerry, 2021). Additionally, behavioral resistance has been shown to be stable over time even in the absence of exposure to imidacloprid, suggesting that implementing traditional insecticide resistance management approaches, such as rotating or temporarily halting the use of an insecticide, may not be effective in reducing behavioral resistance (Hubbard et al., 2023). House fly susceptibility to imidacloprid was high soon after release of the first commercial fly bait containing this insecticide (Butler et al., 2007) but bait effectiveness quickly deteriorated likely due to rapid selection for behavioral resistance to imidacloprid in house fly populations under intense selection pressure (Mullens et al., 2010; Murillo et al., 2015). Given the specificity of BRS flies for behavioral resistance to imidacloprid relative to the related neonicotinoid dinotefuran (Hubbard and Gerry, 2020), it seems that either GRs specifically detect imidacloprid, or if dinotefuran is detected by fly mouthparts- associated GRs, these flies are unable to discriminate a lethal concentration of this toxicant.

Future studies to characterize the specific GRs involved in imidacloprid detection could guide structural modification of imidacloprid to avoid detection or discrimination by resistant flies, thereby rescuing the imidacloprid compound as a useful toxicant for fly control.

The PER assay as described in this study would be a useful way to evaluate the progression of behavioral resistance resulting from selection for taste aversion. These assays also provide greater detail on mechanisms of resistance than typical insecticide exposure and mortality assays.

For example, the rate of proboscis retraction could provide clues as to how the toxicant is detected, and the presence or absence of PER to different concentrations of a toxicant could provide insight into dose discrimination or substance detection thresholds.

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Table 1. Proboscis extension response (PER) of susceptible and resistant house flies (n=30 flies/replicate; 5 replicates) during the proboscis contact assay observed at three times (0, 2, 10 s) following initial tarsal contact with a sucrose solution containing imidacloprid at either low (10 μg/ml) or high (4000 μg/ml) concentration. Bold values indicate significant differences in PER between high and low imidacloprid concentrations determined using the Wilcoxon Rank Sum test with p-value adjusted for multiple comparisons within each assay (α=0.016).

Trial	Strain	Imidacloprid Concentration	Observation Time (s)	PER(Mean+SE)	p value	W
Proboscis assay	UCR (susceptible)	High	0	15.60±0.98	0.27	18
		Low		14.00±1.18		
		High	2	9.00±1.38	0.98	13
		Low		8.80±1.28		
		High	10	4.60±0.81	0.66	10
		Low		5.40±0.75		
	BRS (resistant)	High	0	18.40±0.51	0.8	14.5
		Low		18.00±0.71		
		High	2	**6.40±0.68**	**0.007**	**0**
		Low		**14.20±0.97**		
		High	10	**3.20±0.58**	**0.006**	**0**
		Low		**10.00±1.30**		

Table 2 Proboscis extension response (PER) of susceptible and resistant house flies (n=30 flies/replicate; 5 replicates) during tarsal contact assay observed at three times (0, 2, 10 s) following initial tarsal contact with a sucrose solution containing imidacloprid at either low (10 μg/ml) or high (4000 μg/ml) concentration. The analyses were performed using the Wilcoxon Rank Sum test with p-value adjusted for multiple comparisons within each assay (α=0.016).

Trial	Strain	Imidacloprid Concentration	Observation Time (s)	PER(Mean+SE)	p value	W
Tarsal assay	UCR (susceptible)	High	0	13.8±1.43	0.95	13
		Low		13.0±0.77		
		High	2	9.4±0.81	0.5	9
		Low		10.0±1.45		
		High	10	5.6±1.36	1	12.5
		Low		5.4±1.72		
	BRS (resistant)	High	0	12.4±2.25	0.26	6.5
		Low		16.0±1.18		
		High	2	6.4±1.21	0.09	4
		Low		10.0±1.30		
		High	10	4.0±0.89	0.04	2.5
		Low		7.2±0.73		

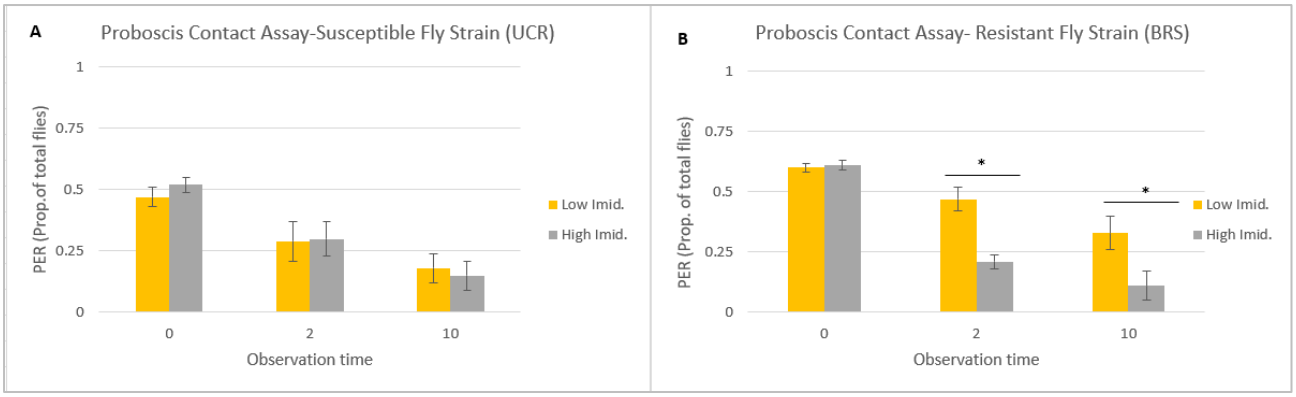

Figure 2. Proboscis contact assay. Flies were allowed to contact the solution with both the tarsi and proboscis. Columns show the mean proportion of UCR-susceptible (A) or BRS-resistant (B) strain house flies (5 replicate groups of 30 flies = 150 flies per fly strain) that exhibited a continuous proboscis extension response (PER) at 0, 2 and 10 s following start of tarsal contact with a sucrose solution containing imidacloprid at either low (10 µg/ml) or high (4000 µg/ml) concentration (error bars indicate the standard error of the mean). The number of flies exhibiting PER was analyzed using Wilcoxon Rank Sum Test with p-value modified for multiple comparisons ($p<0.016$) with differences among imidacloprid concentrations indicated by an asterisk (*).

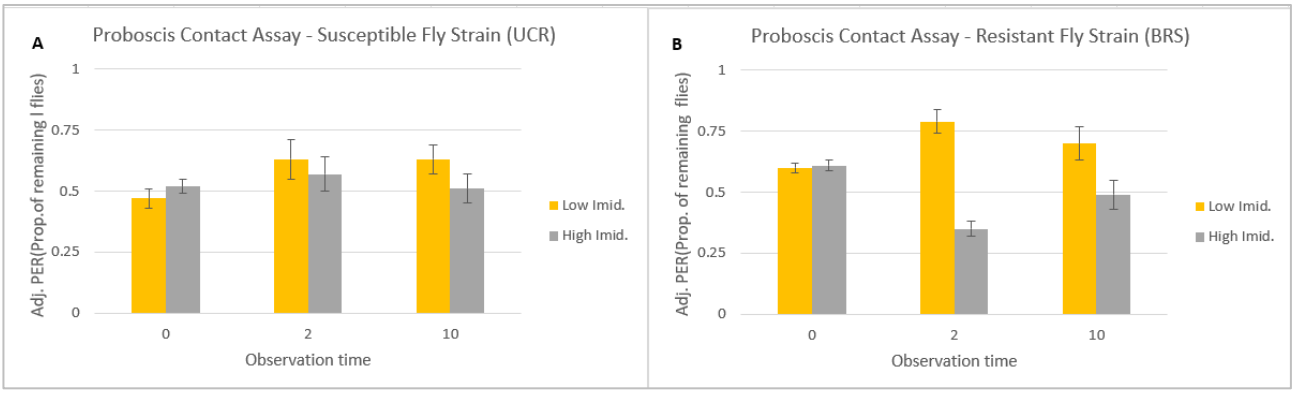

Figure 3. Proboscis contact assay - Adjusted PER. Flies were allowed to contact the solution with both the tarsi and proboscis. Columns show the proportion of flies remaining from the previous observation time that continued to exhibit PER. Flies removed at each timepoint are thus not included in the PER calculation for the next timepoint. Columns show adjusted PER for UCR-susceptible (A) or BRS-resistant (B) house flies following contact with a sucrose solution containing imidacloprid at either low (10 µg/ml) or high (4000 µg/ml) concentration (error bars indicate the standard error of the mean). The proportion of remaining flies exhibiting PER was analyzed using Wilcoxon Rank Sum Test with p-value modified for multiple comparisons ($p<0.016$) with differences among imidacloprid concentrations indicated by an asterisk (*).

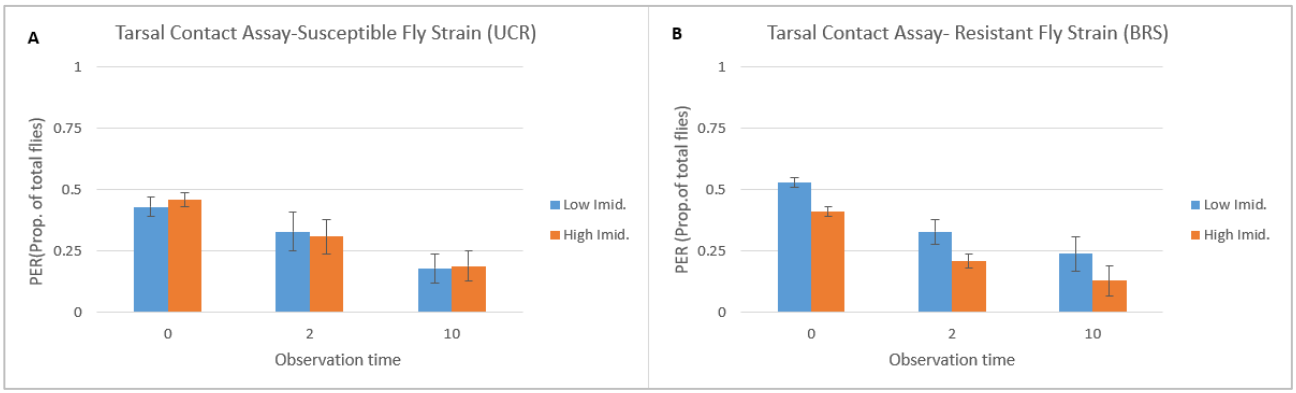

Figure 4. Tarsal contact assay. Flies were allowed to contact the solution with the tarsi only. Columns show the mean proportion of UCR-susceptible (A) or BRS-resistant (B) strain house flies (5 replicate groups of 30 flies = 150 flies per fly strain) that exhibited a continuous proboscis extension response (PER) at 0, 2 and 10 s following start of tarsal contact with a sucrose solution containing imidacloprid at either low (10 µg/ml) or high (4000 µg/ml) concentration (error bars indicate the standard error of the mean). The number of flies exhibiting PER was analyzed using Wilcoxon Rank Sum Test with p-value modified for multiple comparisons ($p<0.016$). There were no significant differences among imidacloprid concentrations within observation times.

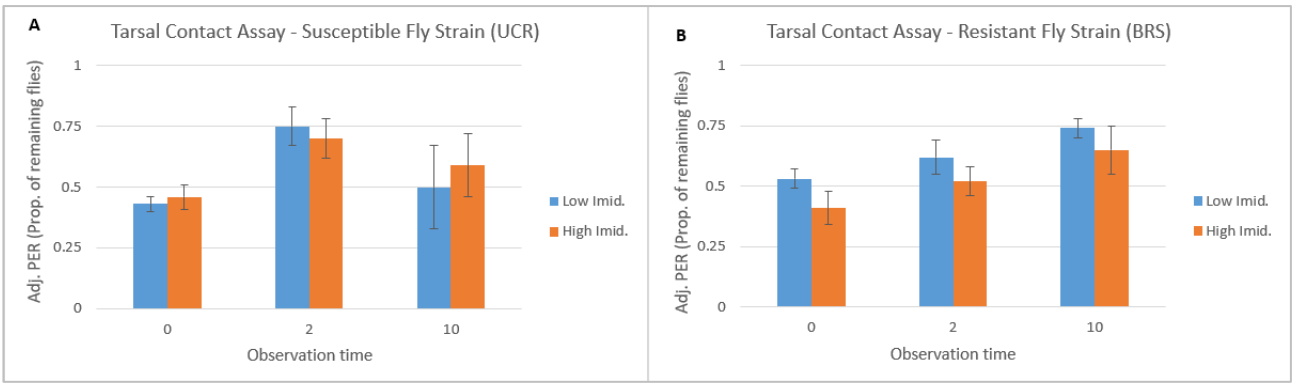

Figure 5. Tarsal contact assay - Adjusted PER. Flies were allowed to contact the solution with the tarsi only. Columns show the proportion of flies remaining from the previous observation time that continued to exhibit PER. Flies removed at each timepoint are thus not included in the PER calculation for the next timepoint. Columns show adjusted PER for UCR-susceptible (A) or BRS-resistant (B) house flies following contact with a sucrose solution containing imidacloprid at either low (10 µg/ml) or high (4000 µg/ml) concentration (error bars indicate the standard error of the mean). The proportion of remaining flies exhibiting PER was analyzed using Wilcoxon Rank Sum Test with p-value modified for multiple comparisons ($p<0.016$). There were no significant differences among imidacloprid concentrations within observation times.

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Chapter 3. Field monitoring of houseflies and related natural antagonists in dairy farms, in Reggio Emilia, Italy

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3.1 Abstract

The housefly is a common insect found in livestock farms. A monitoring activity was conducted on six dairy cattle farms in Reggio Emilia, Italy, from June to September 2021-2023. The farms were divided based on their fly management practices, either organic (release of pupal parasitoids) or conventional (chemical treatment). The objective of the monitoring activities was to assess the activity and relative abundance of houseflies and their pupal parasitoids in two zones of each farm: the calves' area and the dunghill area. Additionally, the aim was to compare the differences in terms of species abundance between farms with different management. To monitor flies, a rolling sticky trap was placed in each farm and zone. To determine the activity and abundance of pupal parasitoids, a minimum of 40 pupae were collected from the designated zones. No differences were found in the number of captured flies and the proportion of specimens (houseflies and pupal parasitoids) between the farms in terms of the kind of management. However, differences were observed for individual farms. The presence of the pupal parasitoid *Muscidifurax* sp. was recorded especially in organic farms where species of this genus were released. *Spalangia* sp. was most abundant in conventional farms except for the organic farm B3 where this was found more than the others with the same management. Based on the monitoring results, it is suggested that in order to reduce housefly infestation to an acceptable level, it is important to consider environmental parameters, cleaning practices, and the management of different zones on single farms, as well as the use of mass traps.

3.2 Introduction

The housefly, *Musca domestica* L. breeds in decomposing manure and is a common pest on livestock farms (Birkemoe et al., 2009), where it is mainly controlled by means of chemical pesticides. This method of control is continuously challenged by the rapidly evolving resistance of houseflies to insecticide (Keiding, 1999; Birkemoe et al., 2009). A study found that in intensive chicken farms in North Italy houseflies exhibited high levels of resistance to four class of pesticides: organophosphates, pyrethroids, Spinosad and neonicotinoids (Pezzi et al., 2011). In combination with resistance problems, increased interest in organic farming and a general acknowledgement of the negative effects of pesticides have encouraged the use of other control methods. Mechanical control practices can reduce fly abundance and reduce oviposition sites.

A dry and well-ventilated manure storage area is essential for fly management, as manure moisture promotes larval development (Watson et al., 1998; Machtinger et al., 2015). Proper composting of manure increases the internal temperature of the waste and lowers the moisture content, making the substrate unsuitable for larval development (Abu-Rayyan et al., 2010). Alternatively, manure is spread in a thin layer on agricultural fields in order to promote drying and reduce fly development (Machtinger et al., 2016). The effectiveness of light-traps and colored glue-boards in trapping and controlling houseflies in swine farms was investigated by Colacci et al. (2021), while Ruiu et al. (2011) explored the potential use of two bioinsecticidal formulations containing *Brevibacillus laterosporus* spores and azadirachtin in a dairy farm in Sardinia.

Several biological control agents, such as parasitoids and predators, are being mass reared and commercialized to farmers (Geden, 2005; Birkemoe et al., 2009). Many of these parasitoids are cosmopolitan and have been introduced into many areas of the world. Filth fly parasitoids are present in all habitats where suitable hosts can be found, including those associated with poultry, cattle, equine, swine, and other animal operations, as well as refuse and forensic situations (Machtinger and Geden, 2018). Several studies have been conducted on the release of pupal parasitoids, especially for *Spalangia* sp. against houseflies and their effectiveness in controlling these pests in Danish dairy and pig farms (Skovgård and Jespersen, 1999; Skovgård and Nachman, 2004; Skovgård, 2004). A study on parasitoids in two dairies farms in southern California found that *Spalangia* sp. were the predominant parasitoids recovered in field, while *Muscidifurax sp.* were predominant in sentinel housefly pupae (Meyer et al., 1990).

Normally, naturally occurring parasitoid populations are not sufficient to control fly populations due to the shorter development time and higher fecundity of the flies. Augmenting natural parasitoid populations by releasing commercially-produced parasitoids can increase fly control (Machtinger and Geden, 2018). The effectiveness of biological control with pupal parasitoids may depend on the suitability of the released species to the climate and habitat of the release site, with endemic species usually being the most suitable (Quarles, 2006).

A monitoring program should be established to assess fluctuations in fly populations, to determine when to implement additional pest management strategies and to evaluate the effectiveness of the pest management program. The monitoring of flies has been extensively studied in several environments, particularly in poultry farms (Lysyk and Axtell, 1986; Aggarwal and Dogra, 2010), sheep and camel facilities (Albarrak, 2009), horse facilities (Machtinger et al., 2016), swine housing (Skovgård and Nachman, 2004; Birkemoe et al., 2009) and dairy farms (Kaufman et al., 2001; Skovgård and Nachman, 2004; Gerry et al., 2011).

In the production areas of Parmigiano Reggiano in Italy, there are many flies that cause significant distress to the cows, resulting in reduced milk production (Arsenopoulos et al., 2018).

Research conducted on cattle farms has shown that the housefly is the primary bacterial vector for *S. aureus* and *E. coli*, and a less significant carrier of *Enterobacter*, *Proteus*, and *Pasteurella ssp*. These bacteria are responsible for causing transmissible mastitis, an infection that spreads eight times more easily when transmitted by flies (Anderson et al., 2012; Castro et al., 2016; Arsenopoulos et al., 2018). Mitigating this issue could be achieved by reducing the role of flies in transmitting these pathogens (Arsenopoulos et al., 2018). However, there are no studies available on the distribution and abundance of this pest and their natural enemies, pupal parasitoids in livestock farms in this region. The objectives of this work were to monitor the trend of the populations of *M. domestica* over time, to monitor the species of parasitoids present in local farms, and to make comparisons on the densities of pest and parasitoids between farms with different types of management (conventional and biological).

3.3 Material and methods

The monitoring of the populations of *M. domestica* and their pupal parasitoids in six selected livestock farms in Emilia, specialized in the production of cow's milk intended for transformation into Parmigiano Reggiano, was carried out for three years (2021-2023) from June to September except for one farm C2 (conventional) only for 2 years. The selected farms have some similar characteristics: the same breed of animals, the same type of bedding and the similar subdivision of the different environments; three of them are organic (B) and the others are conventionally managed (C) (i.e. with the use of synthetic pesticides for pest control). The Arp Emilia Romagna daily climate dataset was used to determine the daily mean temperature (T_med). This dataset was created through spatial interpolation on a regular grid using values recorded by a network of historical weather stations (Antolini et al., 2016).

To monitor *M. domestica*, two Silvalure FLY ROLL MAXI roller sticky traps (Figure 10) were used per farm. One trap was placed in the calf area (Zone C) (Figure 6-7), 1.5-2m above the ground, away from direct sources of heat or drafts, as directed by the manufacturer. The other trap was placed in the manure zone (dunghill- Zone D) (Figure 8-9), also using the same precautions. The exposed roller sheets were replaced once a week. The traps were labelled for each farm, area, and monitoring day and kept separately. The catches on the sticky traps were then counted and/or estimated at the laboratory of entomology UNIMORE in Reggio Emilia, Italy. To monitor the parasitoids, a minimum of 40 pupae was sampled each week in the predetermined locations (Zone C and D) (Figure 11). The pupae were collected using entomological tweezers, primarily from the outer perimeter of the calf boxes or fences and the inner margins of the dunghill, where more pupae had been found during previous investigations. The collected pupae were introduced in 50 ml plastic tubes closed with a piece of panty hose tightened at the edge with an elastic band and placed in a climatic chamber at 25± 2 °C and 70 % RH for 60 days. The emergence of the flies and/or their parasitoids was checked daily.

The variables were calculated for each area, year and farm as follows:

- number of flies caught with roller sticky trap;

- proportion of emerged houseflies: number of emerged flies/totals collected pupae

- proportion of emerged pupal parasitoids for each parasitoid genus (*Muscidifurax* sp. and *Spalangia* sp): number of emerged parasitoids/totals collected pupae

3.4 Statistics

Data were analyzed separately for each dependent variable: number of flies captured, percentage of house fly emergence, percentage of *Muscidifurax* sp. emergence, percentage of *Spalangia* sp. emergence from collected pupae. All statistics were performed in R, v 4.3.1. The GLMM model with negative binomial distribution was run for the number of flies collected, considering the model predictors: Farm, Area, Year, Months, daily Temperature, Management. Model comparison was evaluated using ANOVA to determine the effect of the predictors on the final model. Marginal means were calculated using the emmeans package of R, v 4.3.1 and then the comparison for each farm, year and zone was evaluated using the contrast function. Non-parametric tests were used to compare the proportions of species emerged from collected pupae due to the violation of assumptions of several models. Kruskal-Wallis's test was performed to compare the farms in each zone and year considered, and post hoc Dunn's test was performed in case of significant statistical differences ($p<0.05$). In this non-parametric analysis, we did not consider the daily temperature as it may have affected the development of fly pupae or parasitoids, which were not monitored. However, the field temperature did not affect the emergence of specimens that occurred in the laboratory under controlled temperature conditions.

3.5 Results

3.5.1 Flies captured on sticky traps

The quantity of flies caught on the sticky trap varied from 2021 to 2023 (Figure 12). The farm, zone, and year are all factors that affected this parameter. The statistical analysis revealed that the percentage of houseflies that emerged was significantly impacted by the farm ($P=0.00028$), year of monitoring ($P<0.0001$), and zone ($P<0.0001$) but was not affected by the type of management ($P=0.24$) and T_med ($P=0.65$). In the first year, B3 farm had the highest number of captured flies in the calves' zone with a median value of 70 flies, while C2 farm had the lowest with a median value of 1 in the same zone ($E= 3.47$, $P<0.0001$) (Table 4). Overall, in the first year, the organic farms (B) were statistically different from the conventional (C) ($P<0.05$), except for B2 and B3, with 14 and 70 captured flies, respectively ($E=-1.30$, $P=0.006$), and C2 and C3, with values of 1 and 16 flies, respectively ($E= -2.05$, $P=0.002$) (Table 4).

The D zone had the lowest value for all farms, with a range of 3-6 flies captured with a sticky trap with no statistically significant differences among the farm for this zone (P>0.05) (Table 3-4 and Figure 12).

In the second year, B2 farm had the highest median number of captured flies in the calves' zone with a median value of 536, followed by C3 farm with 210 flies (E=0.46, P=0.97) (Table 5). Meanwhile, C2 farm had the lowest median value of 1.5 in the same zone than B2 (E=4.83, P<0.0001) and C3 (E=-4.36, P<0.0001) (Table 3-5). Significant statical difference was recorded also between B1 (32 flies) and B3 (14 flies) (E=1.35, P=0.06), B1 (32 flies) and C1(9 flies) (E=2.65, P=0.008), B2(536 flies) and B3(14 flies) (E=2.49, P<0.0001), B3(14 flies) and C2(1.5 flies) (E=2.34, P=0.004), B3(14 flies) and C3(210 flies) (E=-2.02, P=0.007) (Table 5). The D zone had the lowest value for all farms compared to the C zone, with the highest median value for C3 in the D zone of 60.5 flies, compared to the range of 1.5-5.5 for the other farms (P<0.05) (Table 3-5, Figure 12).

In the third year, the highest number of flies captured was recorded in B2 farm C zone with a value of 431, followed by C3 with a median value of 189.5 flies captured. The lowest value in this zone for this parameter was recorded for B3 with a median value of 25.5 (Table 3). The statistically significant difference were recorded for B1(48 flies) and B2 (431 flies) (E=-1.53, P=0.0008), B1 (48 flies) and B3 (25.5) (E=1.51, P=0.005), B2(431 flies) and B3(25.5 flies) (E= 3.05, P<0.0001), B2 (431 flies) and C1(40 flies) (E=2.54, P<0.0001), B3 (25.5 flies) and C3 (189.5) (E=-2.49, P=0.0001), C1 (40 flies) and C3(189.5 flies) (E=-1.98, P=0.009) (Table 6) . In the D zone, the lowest value was recorded for B2 with a median of 3 flies captured, followed by B3 farm with 4.5 (Table 3). Statistically significant differences were recorded between farm B1 (11 flies) and B2 (3 flies) (E=1.45, P=0.02) (Table 6). The other farms had a value of 11-15 flies captured in sticky traps with no statistically significant differences recorded (P>0.05) (Table 3-6 and Figure 12).

3.5.2 Proportion of emerged houseflies from collected pupae

Significant differences were observed in the emergence of houseflies from the collected pupae between 2021 and 2023. In the first year, statistically significant differences were recorded among the farms in Zone C (χ^2= 14.19, df=5, P=0.01). The median proportion of flies in C zone was highest in the C3 Farm at 0.68 (68%), followed by 0.4 (40%) in C2 and 0.24 (24%) in C1 (Table 3). The proportion in the organic farm was lower than that in the conventional farm (Figure 13). Statistically significant differences were found between organic and conventional farms (P<0.05) (Table 7). In the dunghill area at C3, the highest median proportion was 12%, which was significantly higher than the value of 0% in B3 (Z=-1.87, P=0.03) and the value of 0% in C2 (Z=-2.09, P=0.01). However, there were no statistically significant differences between B1 (median value of 5%) and C3 (12%) (Z=-0.58, P=0.28) for high variability in each farm (Figure 13 and Table 3-7). In the second year, the emergence of houseflies was higher in both areas of B2 farm compared to the others (Figure 13), with a median proportion of 0.86 (86%) in the calves' area and 0.61 (61%) in the dunghill area. Statistical significance differences among the farms in Zona C were recorded for this year (χ^2= 18.53, df = 5, P = 0.002).

The farm with the lowest proportion of housefly emergences was B1, specifically in the calves' area, with a value of 0.12 (12%). No houseflies were recorded in the dunghill area due to the absence of manure during the monitoring period (Figure 13).

Significant statistical differences were observed in Zone C between B1 (12%) and B2 (86%) (Z=-3.4, P=0.0003). B2 had the highest proportion of recorded flies among all farms (P<0.05), with C2 (18%) and C3 (54%) (Z=-2.48, P=0.006). Statistically significant differences were found among the farms in Zona D (χ^2=11.4, df=4, 0.02, P=0.02). Specifically, farm B2 (61%) showed a statistically significant difference compared to C1 (0%) (Z=3.3, P=0.0004), as did B3 (18%) compared to C1 (0%) (Z=1.74, P=0.04), C2 (53%) compared to C1 (0%) (Z=-2.17, P=0.01), and B2 (61%) compared to C3 (44%) (Z=1.93, P=0.02) (refer to Table 8). The captured flies' highest median value in Zone D this year was recorded for B2 (61%), followed by C2 (53%) and C3 (44%), with the lowest being B3 (18%) (Table 3 and Figure 13).

In the third year, marginal statistical differences were observed in Zone C among the farms (χ^2= 7.86, df=4, P= 0.09). The emergence of houseflies was higher in C3 farm with a median value of 0.73 (73%) in this zone, followed by B2 farm with a median value of 0.53 (53%), with no statistically significant differences recorded (Z=-0.31, P=0.37) (Table 3-9). The B3 farm had the lowest median value for this zone, with a median value of 0.10 (10%), which was statistically significantly different from B2 (53%) (E=2.06, P=0.01), C1 (35%) (Z=-1.96, P=0.02), and C3 (73%) (Z=-2.31, P=0.01). There were no significant statistical differences between B1 (18%) and B3 (10%) (Z=0.83, P=0.2) (Table 3-9, Figure 12). In zone D, the highest median value was recorded in the B3 farm with a value of 0.35 (35%), followed by 0.25 (25%) recorded in the C3 farm. In Table 3, the lowest median value of 0.01 (1%) was recorded in B1 farm, followed by C1 farm with 0.04 (4%) and B2 farm with 0.07 (7%). There were no statistically significant differences in this zone among the farms for the high variability (χ^2=2.72, df=4, P=0.6) (Table 9 and Figure 13).

3.5.3 Proportion of Muscidifurax sp *emerged from collected pupae*

In the first year, statistically significant differences were observed among the farms in Zone C (χ^2=17.82, df=5, P=0.03). The maximum value of 0.15 (15%) was recorded in this zone for B1 farm compared to the other farms (P<0.05) (Figure 14, Table 3-10). For this year in Zone D, all farms were statistically different from each other (χ^2=29.52, df=5, P<0.05) (Table 10). The highest median value was recorded for B2 (13%) followed by B1 (6%). The lowest median value was recorded for farm C1 (0-3%) with no record in B3 farm (Table 3). The emergence of this parasitoid was higher in zone D compared to zone C across all farms and years. The median values for B2 farm were 0.13 (13%) in the first year, followed by B1 with a value of 0.12 (12%), and C3 farm with median values of 0.11 (11 %) in this year (Table 3, Figure 14).

No statistically significant differences were found among the farms in Zone C during the second year (χ^2=6.04, df=5, P=0.302). The highest median value was recorded in farm C3 (1%) compared to the other farms, which had values ranging from 0-5% (Table 3). In Zone D, no statistically significant differences were observed among the farms (χ^2=6.07, df=5, P=0.19). The highest median value was recorded in farm C3 (2%) compared to the other farms, which had values ranging from 0-2% (Table 3).

Statistically significant differences were found among the farms in Zone C in the third year (χ^2=19.72, df=4, P=0.0005). The highest median value was recorded in B1 (15%) compared to a range of 0-12% in B2 (Z=2.72, P<0.0001), a range of 0-4% in B3 (Z=3.43, P<0.0001), a range of 0-1% in C1 (Z=3.46, P<0.0001), and a range of 0-1% in C3 (Z=3.76, P<0.0001) (Table 3-11). Statistically significant differences were recorded between farm B2 (0-12%) and farm B3 (0-4%) (Z=0.99, P=0.01), as well as between farm B3 (0-4%) and farms C1 (0-1%) (Z=-0.33, P=0.03) and C3 (0-1%) (Z=-0.06, P=0.04). No statistically significant differences were found in Zone D among the farms for this year (χ^2= 7.03, df=4, P=0.13). The parameter with the highest median value was recorded for B1 (12%), followed by C3 (11%), and C1 (range of 0-10%) and B2 (range of 0-16%), with no statistically significant differences (P>0.05) (Table 3 and Figure 14).

3.5.4 Proportion of Spalangia sp *emerged from collected pupae*

In the first year, there were significant statistical differences among the farms in zone C (χ^2=14.19, df=5, P=0.01). The farm with the highest median value of *Spalangia* sp emergences was C1, with a range of 0-5%, followed by C2 with a range of 0-8% (Z=-1.39, P=0.05). This species did not emerge in farms B1, B2, B3, and C3 in this zone for this year. In Zone D, no statistical differences were registered among the farms (χ^2= 8.91, df=5, P=0.11). In this Zone and year, Farm C2 had the highest range of emergence at 0-8%, followed by B1 at 0-4% and C3 at 0-3 % of this parasitoid emergence. There were no records for B2, B3, C2 farm in this zone and year (Table 3, Figure 15).

No statistically significant difference was observed among the farms in Zone C during the second year (χ^2= 5.18, df=5, P=0.39). The highest median emergence value was recorded in C1 farm at 0.18 (18%), followed by 3% of C2, 2% of B3, and 1% of C3 (Table 3 and Figure 15). The range of emergence values for B1 was 0-3% and for B2 was 0-1%, resulting in the lowest recorded value. No statistically significant difference was found among the farms in Zone D (χ^2= 2.65, df=4, P=0.62). The highest number of emergences of this parasitoid species was recorded at C2 (6-20%) followed by B3 farm (0.5-12%) (Table 3 and Figure 15). The lowest values for this species in this year and Zone were recorded at Farm B2 (0-4%) and C3 (0-1%) (Table 3 and Figure 15).

In the third year, Zone C, there were statistically significant differences recorded among the farms (χ^2= 13.94, df=4, P=0.007). The highest median emergence value was recorded in C1 farm at 6%, followed by 4% of B3 (Z=0.97, P=0.16) (Table 3-12 and Figure 15).

Statistically significant differences were found between B1 (0%) and B3 (4%) (Z=-2.73, P=0.003), B1 (0%) and C1 (6%) (Z=-1.96, P=0.02), B2 (0-9%) and C3 (0%) (Z=2.009, P=0.02), B3 (0-9%) and C3 (0%) (Z=3.07, P=0.001), and C1 (6%) and C3 (0%) (Z=2.34, P=0.019). No record of this species was found in Farm C3 and Farm B1 in this year and zone (Figure 15). In Zone D, no statistically significant differences were observed among farms (χ^2= 1.94, df=4, P=0.75). The proportion range was highest in Farm C1 (7-14%), followed by B3 at 2-7%, B1 at 0-2%, B2 at 0-2%, and there was no record for Farm C3.

3.6 Discussion

In the Parmigiano production area, conventional farms are managed through chemical treatments (mostly with spray), which are typically administered every two weeks from April to October. In organic farms, parasitoid releases are made on a weekly basis during the same period. This study suggests that neither the release of parasitoids nor the use of chemical treatments had a great impact on the abundance of houseflies and parasitoids in the monitored areas. These results are in agreement with Meyer et al., (1990) who found that the density of housefly populations was not influenced by the presence of parasitoids. As noted by Birkemoe et al., (2009), the immigration of flies from neighboring areas may have obscured the effects of local biological control of fly pupae. All farms were in open areas, and other factors could have influenced the results, beside the considered parameters. The variability within the same farm throughout the year, particularly in the dunghill zone, and the lower number of pupae found may be attributed to the implementation of a separation system for liquid and solid fractions of manure in almost all farms after the first year of experiment. This separation of components has reduced fly infestations in this area, resulting in an increase in adjacent areas such as the calves' zone. The number of flies caught on the traps is only indicative in our study, as the rollers were sometimes placed in sub-optimal areas according to management practices and farmers' needs. Housefly densities are known to depend strongly on temperature (Birkemoe et al., 2009) but no differences were recorded for mean temperature and summer months in the number of houseflies captured in traps. The zone has affected the number of captured flies, which was very low in the dunghill area. The traps were positioned in open areas where houseflies had the opportunity to fly freely without barriers, unlike confined areas. Therefore, the efficiency of the traps in this area was very low. Comparing the number of flies captured in calves' zone, the highest values was found in B1 and B2 farm and the C3 farm indicating that the management of flies did not influence this parameter. The differences could be related to change of beddings and the manure quantities present inside this units. The cow manure is the most suitable substrate for fly oviposition (Shah et al., 2016) and a high quantity of it in a confined area resulted more attractive for the flies, resulting in higher catches in the traps.

As found by Birkemoe et al., (2009) in the Norwegian pig farms, differences in manure quantities present inside the production units may partly explain the variation in success of biological control between the farms.

The emergence of houseflies was found to be unrelated to temperature, which contrasts with the findings of (Birkemoe et al., 2009) who identified August and September as the most suitable months. In our experiment, we collected pupae and placed them in controlled laboratory conditions. This difference in methodology may have contributed to different experimental outcomes. While temperature may have affected the time of pupation in the field, we did not monitor the developmental stage of the flies.

In Scandinavian dairy farms, an increase in temperature led to an increase in parasitization. However, a larger number of parasitoids needed to be released in relation to the higher density of houseflies (Skovgård and Nachman, 2004; Birkemoe et al., 2009). No increase in parasitization was observed with the warming temperature in our case.

The proportion of emerged *Muscidifurax* sp. was higher in organic farms where this genus was officially released in higher proportion than *Spalangia* sp,as declared by private company of pest control. However, at B3 farm, which is also organic, the proportion of this pupal parasitoid was lower for all three years. This difference may be attributed to the farm's cleaning practices, which differed slightly from those of the other farms. Scrupulous and periodic cleaning sessions were carried out every week in stable B3 to completely remove manure residues. This practice prevents parasitization and makes it difficult to find parasitized pupae. In general, these parasitoids were found in all the farms and zones. *Muscidifurax* species may disperse and parasitize at distances of 8–100 m from the release point (Floate et al., 2000). It is relevant to consider the position of the studied farms in relation to other farms. The proportion of *Muscidifurax sp*. was similar in both the calves' zone and the dunghill zone of B1 and dunghill zone of C3, suggesting that quantities of manure in beddings and in dunghill had a positive influence on the parasitization of this pupal parasitoid. This is clearly demonstrated in Skovgård and Nachman (2004), where the removal of manure increased the number of flies and reduced the parasitism. The other farms with lower proportion of this parasitoid had the calves in the boxes area and not in fences and this could have affected the parasitization. It should be noted that the C3 stable has two areas with a high concentration of manure, even though only one was selected for sampling. These areas were rarely emptied during the sampling period, resulting in a high number of collected pupae. In the study of Scott et al., (1991) the toxicity of seven insecticides was evaluated against unparasitized houseflies' pupae and those parasitized by *Muscidifurax raptor* and *Spalangia cameroni*. Pyrenone was found to be less toxic to *M. raptor* than houseflies, while all insecticides were less toxic to *S. cameroni*. In this study, *Muscidifurax sp*. was found in chemically treated stables, particularly in the C3 farm. The high level of infestation and abundance of pupae allowed the parasitoid species to survive independently despite the treatment, due to the high number of hosts available.

The proportion of *Spalangia* sp. was comparable across all farms and zones throughout the years, suggesting that this species is not affected by insecticide treatment (in conventional farms) or the release of parasitoids (organic farm). Although private pest control companies have confirmed that the release of parasitoids was generally shifted towards *Muscidifurax sp*., it is important to note that *Spalangia* sp. was the most abundant in B3 farm, where it was released with a lower proportion than *Muscidifurax* sp. Additionally, C1 and C2 did not have any parasitoids released. It was demonstrated in several studies that adults of *Spalangia* sp. have a different foraging behavior than adults of *Muscidifurax* sp (Floate, 2002; Geden, 2002). This can explain that *Spalangia* sp. can coexist with other species.

Spalangia sp. is the most common naturally occurring parasitoid wasp of housefly on Norway pig farms (Birkemoe et al., 2009). This was also found by the study of Skovgård and Nachman, (2004) in Danish dairy farms. In our study, the percentage of parasitization for these pupal parasitoids was about 15 %, which is in contrast to the 50 % recorded in previous studies (Skovgård and Nachman, 2004; Birkemoe et al., 2009). The issue related to the farms that may have influenced the proportion of emergence of *Muscidifurax* sp. seems not to have influenced the emergence of *Spalangia* sp. suggesting that this species is more adapted to these environments. The success of a management program with pupal parasitoids may be influenced by specific environmental factors, such as sensitivity to insecticides, the use of low-quality commercial colonies, microhabitat preferences, host availability and lack of optimal timing and release methods (Petersen and Meyer., 1985). To develop a biocontrol managing strategy, a control agent should be chosen that is highly efficient at suppressing the pest population growth (Kruitwagen et al., 2018).

3.7 Conclusion

This is the first study conducted on the monitoring of houseflies' populations and their parasitoids in dairy farms in Northern Italy. Fly and parasitoid abundance was similar on conventionally managed and organic farms. Therefore, it is necessary to modify and improve housefly management programs based on the characteristics of individual farms in this area. When considering population dynamics, it is important to consider parameters beyond the farm itself, as these areas are often developed in open spaces where field activities may have an impact. Additionally, it is important to note that some stables are in close proximity to one another, allowing flies and their natural enemies to disperse and move between areas. Another crucial factor to consider is the cleanliness of the environment. Cleaning severely and for extended periods reduces the number of pupae available to parasitoids but may increase the fluctuation in fly population density, as shown in some studies. Sporadic cleaning practices increase the level of parasitization but also increase the number of flies, requiring a corresponding increase in released parasitoids per area.

To ensure effective management of fly populations, it is recommended to implement targeted cleaning and environmental practices. This includes regularly removing old materials from the edges of fences to create suitable habitats for parasitoid populations. Additionally, the use of traps to monitor population density fluctuations is advised to enable timely intervention. It is important to consider that the life cycles of flies are typically faster than those of parasitoids. However, the use of traps and the targeted release of an appropriate number of pupal parasitoids can effectively reduce the number of flies to acceptable levels. Trapping adult flies is complementary to biological control and is recommended to improve the effectiveness of pest management.

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Figure 6. Calves' zone (Zone C) for C1 farm

Figure 7. Calves' zone (Zone C) for B1 farm.

Figure 8. Dunghill zone (Zone D) for C2 Farm.

Figure 9. Dunghill zone (Zone D) for B1 Farm.

Figure 10. Silvalure FLY ROLL MAXI roller sticky traps in each farm and zone.

Figure 11. Sampling of pupae in dunghill's zone (Zone D)

Table 3 Median value and interquartile range in square, for all variables across the farm and the year divided for the zone C (Calves) and D (dunghill).

Farm	Variable	N- captured flies		Prop. *Muscidifurax sp*		Prop. *Spalangia sp*		Prop. Houseflies	
	Year	C	D	C	D	C	D	C	D
B1	1	43[9-52]	6[4-7]	0[0-0.03]	0.06[0-0.11]	0	0[0-0.04]	0[0-0.42]	0.05[0-0.18]
	2	32 [8-182]	/	0[0-0.005]	/	0[0-0.03]	/	0.12[0.08-0.29]	/
	3	48[37-94]	11[3.5-28]	0.15[0.11-0.21]	0.12[0.03-0.2]	0	0.008[0-0.02]	0.18[0.08-0.23]	0.01[0-0.17]
B2	1	14[8-34]	5[4-11]	0	0.13[0.02-0.25]	0	0	0[0-0.09]	0[0-0-07]
	2	536[128-569]	4[3-6.5]	0	0[0-0.02]	0[0-0.01]	0[0-0.04]	0.86[0.83-0.90]	0.61[0.42-0.87]
	3	431[321-523]	3[0-7]	0[0-0.12]	0[0-0.16]	0[0-0.09]	0[0-0.02]	0.53[0.09-0.74]	0.07[0-0.30]
B3	1	70[53-105]	4[2-7]	0	0	0	0	0	0
	2	14[7.5-27.5]	5.5[2.5-7]	0	0	0.02[0.01-0.11]	0.06[0.005-0.12]	0.25[0.13-0.37]	0.18[0.08-0.53]
	3	25.5[16-35]	4.5[1.5-8]	0[0-0.004]	0	0.04[0.009-0.06]	0.02[0-0.07]	0.10[0.05-0.20]	0.35[0-0.71]
C1	1	3[1-8.50]	6 [2.75-9.25]	0	0[0-0.03]	0.01[0-0.05]	0[0-0.01]	0.24[0.12-0.35]	0
	2	8.5[7.25-9.75]	1.5[0.75-2.25]	0	0	0.18[0.09-0.27]	0.13[0.06-0.20]]	0.12[0.06-0.18]	0
	3	40[34-40.75]	14.5 [5-23.25]	0[0-0.1]	0.02[0-0.10]	0.06[0.004-0.13]	0.07[0-0.14]	0.35[0.21-0.57]	0.04[0-0.11]
C2	1	1[1-3.4]	3[1-4.50]	0[0-0.01]	0	0[0-0.08]	0	0.4[0.25-0.51]	0
	2	1.5[0-4.5]	1[0-8.75]	0[0-0.005]	0	0.03[0.01-0.06]	0.04[0-0.121]	0.18[0.14-0.21]	0.53[0.30-0.75]
	3	/	/	/	/	/	/	/	/
C3	1	16[11.5-18.5]	6[5-6.50	0	0[0-0.09]	0	0[0-0.03]	0.68[0.06-0.92]	0.12[0-0.16]
	2	210[196-250]	60.5[42-94.75]	0.01[0-0.03]	0.02[0-0.04]	0.01[0-0.02]	0[0-0.01]	0.54[0.43-0.72]	0.44[0.26-0.63]
	3	189.5[135.5-345]	15[3-15.00]	0[0-0.01]	0.11[0.1-0.25]	0	0	0.73[0.65-0.81]	0.25[0.18-0.27]

Table 4 Comparison made between the number of captured flies among the farms and in each zone in the first year. A GLMM model was performed, and mean values were obtained using the function contrast with E representing the estimate model value and P rep resenting the p-value from the contrasts. Only statistically significant differences (P<0.05) are highlighted in bold.

1st year- N captured flies												
Zone C												
Farms	B1		B2		B3		C1		C2		C3	
	E	P	E	P	E	P	E	P	E	P	E	P
B2	0.38	0.9	0	1	-1.3	**0.006**	1.29	0.2	2.16	**0.0002**	0.11	0.99
B3	-0.91	0.13	-1.3	**0.006**	0	1	2.6	**<0.0001**	3.47	**<0.0001**	1.42	**0.01**
C1	1.68	0.03	1.29	0.2	2.6	**<0.0001**	0	1	0.87	0.77	-1.17	0.38
C2	2.55	**<0.0001**	2.16	**0.0002**	3.47	**<0.0001**	0.87	0.77	0	1	-2.05	**0.002**
C3	0.5	0.84	0.11	0.99	1.42	**0.01**	-1.17	0.38	-2.05	**0.002**	0	1
Zone D												
Farms	B1		B2		B3		C1		C2		C3	
	E	P	E	P	E	P	E	P	E	P	E	P
B2	-0.19	0.99	0	1	0.98	0.13	0.66	0.85	1.31	0.08	-0.06	1
B3	0.78	0.36	0.98	0.13	0	1	-0.31	0.99	0.33	0.98	-1.05	0.18
C1	0.47	0.96	0.66	0.85	-0.31	0.99	0	1	0.64	0.92	-0.73	0.84
C2	1.11	0.21	1.31	0.08	0.33	0.98	0.64	0.92	0	1	-1.3	0.1
C3	-0.26	0.99	-0.06	1	-1.05	0.18	-0.73	0.84	-1.3	0.1	0	1

Table 5 Comparison made between the number of captured flies among the farms and each zone in the second year. A GLMM model was performed, and mean values were obtained using the function contrast with E representing the estimate model value and P representing the p-value from the contrasts. The symbol "\" indicates the absence of record values. Only statistically significant differences (P<0.05) are highlighted in bold.

2nd year- N captured flies												
Zone C												
Farms	B1		B2		B3		C1		C2		C3	
	E	P	E	P	E	P	E	P	E	P	E	P
B2	-1.13	0.21	0	1	2.49	**<0.0001**	3.79	**<0.0001**	4.83	**<0.0001**	0.46	0.97
B3	1.35	0.06	2.49	**<0.0001**	0	1	1.3	0.56	2.3	**0.004**	-2.02	**0.007**
C1	2.65	**0.008**	3.79	**<0.0001**	1.3	0.56	0	1	1.04	0.85	-3.32	**0.0011**
C2	3.69	**<0.0001**	4.83	**<0.0001**	2.3	**0.004**	1.04	0.85	0	1	-4.36	**<0.0001**
C3	-0.67	0.85	0.46	0.97	-2.02	**0.007**	-3.32	**0.0011**	-4.36	**<0.0001**	0	1
Zone D												
Farms	B1		B2		B3		C1		C2		C3	
	E	P	E	P	E	P	E	P	E	P	E	P
B2	\	\	0	1	-0.86	0.53	1.2	0.71	-0.43	0.95	-2.72	**0.0001**
B3	\	\	-0.86	0.53	0	1	2.06	0.2	0.42	0.96	-1.86	**0.02**
C1	\	\	1.2	0.71	2.06	0.2	0	1	-1.64	0.48	-3.92	**0.0008**
C2	\	\	-0.43	0.95	0.42	0.96	-1.64	0.48	0	1	-2.2	**0.0077**
C3	\	\	-2.72	**0.0001**	-1.86	**0.02**	-3.92	**0.0008**	-2.2	**0.0077**	0	1

Table 6 Comparison made between the number of captured flies among the farm and each zone in the third year. A GLMM model was performed, and mean values were obtained using the function contrast with E representing the estimate model value and P representing the p-value from the contrasts. The "\" symbol indicates the absence of recorded values. Only statistically significant differences (P<0.05) are highlighted in bold.

3rd year- N captured flies												
Zone C												
Farms	B1		B2		B3		C1		C2		C3	
	E	P	E	P	E	P	E	P	E	P	E	P
B2	-1.53	**0.0008**	0	1	3.05	**<0.0001**	2.55	**<0.0001**	\	\	0.55	0.83
B3	1.51	**0.005**	3.05	**<0.0001**	0	1	-0.51	0.85	\	\	-2.49	**0.0001**
C1	1	0.21	2.55	**<0.0001**	-0.51	0.85	0	1	\	\	-1.98	**0.009**
C2	\	\	\	\	\	\	\	\	\	\	\	\
C3	-0.97	0.38	0.55	0.83	-2.49	**0.0001**	-1.98	**0.009**	\	\	0	1
Zone D												
Farms	B1		B2		B3		C1		C2		C3	
	E	P	E	P	E	P	E	P	E	P	E	P
B2	1.45	**0.02**	0	1	0	1	-1.1	0.43	\	\	-0.84	0.55
B3	1.45	0.12	0	1	0	1	-1	0.43	\	\	-0.84	0.72
C1	0.44	0.94	-1.1	0.43	-1	0.43	0	1	\	\	0.17	0.99
C2	\	\	\	\	\	\	\	\	\	\	\	\
C3	0.61	0.8	-0.84	0.55	-0.84	0.72	0.17	0.99	\	\	0	1

Table 7 Proportion of emerged houseflies among farms and zones in the first year. A Kruskal-Wallis test was performed, followed by a post-hoc Dunn test for P<0.05. Only statistically significant differences (P<0.05) are highlighted in bold.

1st year- Proportion emergence flies												
Zone C												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	0.01	0.49	0	1	0.87	0.19	-1.85	**0.03**	-2.21	**0.01**	-1.86	**0.03**
B3	0.89	0.18	0.87	0.19	0	1	-2.73	**0.003**	-3.09	**0.0009**	-2.74	**0.003**
C1	-1.84	**0.03**	-1.85	**0.03**	-2.73	**0.003**	0	1	-0.35	0.36	-0.004	0.49
C2	-2.19	**0.01**	-2.21	**0.01**	-3.09	**0.0009**	-0.35	0.36	0	1	0.35	0.36
C3	-1.84	**0.03**	-1.86	**0.03**	-2.74	**0.003**	-0.004	0.49	0.35	0.36	0	1
Zone D												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	1.05	0.14	0	1	0.23	0.4	-0.43	0.33	0.45	0.32	-1.64	0.05
B3	1.29	0.09	0.23	0.4	0	1	-0.66	0.25	0.21	0.41	-1.87	**0.03**
C1	0.62	0.26	-0.43	0.33	-0.66	0.25	0	1	0.88	0.18	-1.2	0.11
C2	1.5	0.06	0.45	0.32	0.21	0.41	0.88	0.18	0	1	-2.09	**0.01**
C3	-0.58	0.28	-1.64	0.05	-1.87	**0.03**	-1.2	0.11	-2.09	**0.01**	0	1

Table 8 Proportion of emerged houseflies among farms and zones in the second year. A Kruskal-Wallis test was performed, followed by a post-hoc Dunn test for P<0.05. The symbol "\" indicates the absence of record values. Only statistically significant differences (P<0.05) are highlighted in bold.

2nd year- Proportion emergence flies												
Zone C												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	-3.4	**0.0003**	0	1	2.42	**0.007**	-0.9	0.18	0.5	0.3	-2.13	0.09
B3	-0.94	0.17	2.42	**0.007**	0	1	-0.04	0.48	1.36	0.08	-1.21	0.07
C1	-0.9	0.18	2.18	**0.01**	-0.04	0.48	0	1	1.29	0.09	-0.97	0.16
C2	0.5	0.3	3.65	**0.0001**	1.36	0.08	1.29	0.09	0	1	-2.48	**0.006**
C3	-2.13	0.09	-1.42	**0.01**	-1.21	0.07	-0.97	0.16	-2.48	**0.006**	0	1
Zone D												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	\	\	0	1	1.57	0.05	3.3	**0.0004**	1.11	0.13	1.93	**0.02**
B3	\	\	1.57	0.05	0	1	1.74	**0.04**	-0.44	0.32	0.22	0.41
C1	\	\	3.3	**0.0004**	1.74	**0.04**	0	1	-2.17	**0.01**	-1.64	0.05
C2	\	\	1.11	0.13	-0.44	0.32	-2.17	**0.01**	0	1	0.7	0.23
C3	\	\	1.93	**0.02**	0.22	0.41	-1.64	0.05	0.7	0.23	0	1

Table 9 Proportion of emerged houseflies among farms and zones in the third year. A Kruskal-Wallis test was performed, followed by a post-hoc Dunn test for P<0.05. The symbol "\" indicates the absence of record values. Only statistically significant differences (P<0.05) are highlighted in bold.

3rd year- Proportion emergence flies												
Zone C												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	-1.37	0.08	0	1	2.06	**0.01**	0.1	0.45	\	\	-0.31	0.37
B3	0.83	0.2	2.06	**0.01**	0	1	-1.96	**0.02**	\	\	-2.31	**0.01**
C1	-1.26	0.1	0.1	0.45	-1.96	**0.02**	0	1	\	\	-0.24	0.33
C2	\	\	\	\	\	\	\	\	\	\	\	\
C3	-1.66	**0.04**	-0.31	0.37	-2.31	**0.01**	-0.24	0.33	\	\	0	1
Zone D												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	-0.04	0.48	0	1	-0.67	0.25	0.21	0.41	\	\	-1.16	0.12
B3	-0.7	0.24	-0.67	0.25	0	1	0.81	0.2	\	\	-0.2	0.41
C1	0.17	0.42	0.21	0.41	0.81	0.2	0	1	\	\	-1.31	0.09
C2	\	\	\	\	\	\	\	\	\	\	\	\
C3	-1.21	0.11	-1.16	0.12	-0.2	0.41	-1.31	0.09	\	\	0	1

Table 10 Proportion of *Muscidifurax* sp emerged among farms and zones in the first year. A Kruskal-Wallis test was performed, followed by a post-hoc Dunn test for P<0.05. Only statistically significant differences (P<0.05) are highlighted in bold.

1st year- *Muscidifurax sp* emergence												
Zone C												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	3.34	**0.0004**	0	1	-1.17	0.12	0	0.5	-1.61	0.05	0	0.5
B3	2.17	**0.01**	-1.17	0.12	0	1	1.17	0.12	-0.44	0.32	1.17	0.5
C1	3.34	**0.0004**	0	0.5	1.17	0.12	0	1	-1.61	0.05	0.12	0.5
C2	1.73	**0.04**	-1.61	0.05	-0.44	0.32	-1.61	0.05	0	1	1.61	0.05
C3	3.34	0.05	0	0.5	1.17	0.5	0.12	0.5	1.61	0.05	0	1
Zone D												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	-1.24	**<0.0001**	0	1	4.05	**<0.0001**	4.17	**<0.0001**	3.77	**<0.0001**	3.12	**<0.0001**
B3	2.81	**<0.0001**	4.05	**<0.0001**	0	1	0.11	**<0.0001**	-0.28	**<0.0001**	-0.93	**<0.0001**
C1	2.93	**<0.0001**	4.17	**<0.0001**	0.11	**<0.0001**	0	1	-0.4	**<0.0001**	-1.05	**<0.0001**
C2	2.5	**<0.0001**	3.77	**<0.0001**	-0.28	**<0.0001**	-0.4	**<0.0001**	0	1	-0.65	**<0.0001**
C3	1.88	**<0.0001**	3.12	**<0.0001**	-0.93	**<0.0001**	-1.05	**<0.0001**	-0.65	**<0.0001**	0	1

Table 11 Proportion of *Muscidifurax sp* emerged among farms and zones in the third year. A Kruskal-Wallis test was performed, followed by a post-hoc Dunn test for P<0.05. With symbol \ it was indicated the absence of record value. Only statistically significant differences (P<0.05) are highlighted in bold.

3rd year- *Muscidifurax sp* emergence												
Zone C												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	2.72	**<0.0001**	0	1	0.99	**0.01**	0.74	**0.02**	\	\	1.03	**0.01**
B3	3.43	**<0.0001**	0.99	**0.01**	0	1	-0.33	**0.03**	\	\	-0.06	**0.04**
C1	3.46	**<0.0001**	0.74	**0.02**	-0.33	**0.03**	0	1	\		0.29	**0.03**
C2	\	\	\	\	\	\	\	\	\	\	\	\
C3	3.76	**<0.0001**	1.03	**0.01**	-0.06	**0.04**	0.29	**0.03**	\	\	0	**0.01**

Table 12 Proportion of *Spalangia* sp emerged among farms and zones in the first year. A Kruskal-Wallis test was performed, followed by a post-hoc Dunn test for P<0.05. Only statistically significant differences (P<0.05) are highlighted in bold.

1st year- *Spalangia sp* emergence												
Zone C												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	0.3	0.38	0	1	-0.6	0.27	-1.62	0.05	-3.02	**0.001**	-0.01	0.49
B3	-0.3	0.38	-0.6	0.27	0	1	-1.02	0.15	-2.41	**0.007**	0.59	0.27
C1	-1.31	0.09	-1.62	0.05	-1.02	0.15	0	1	-1.39	0.08	-1.61	0.05
C2	-2.72	**0.003**	-3.02	**0.001**	-2.41	**0.007**	-1.39	0.08	0	1	3.01	**0.001**
C3	0.29	0.38	-0.01	0.49	0.59	0.27	-1.61	0.05	3.01	0.001	0	1

Table 13 Proportion of *Spalangia* sp emerged among farms and zones in the third year. A Kruskal-Wallis test was performed, followed by a post-hoc Dunn test for P<0.05. The symbol "\" indicates the absence of record values. Only statistically significant differences (P<0.05) are highlighted in bold.

3rd year- *Spalangia sp* emergence												
Zone C												
Farms	B1		B2		B3		C1		C2		C3	
	Z	P	Z	P	Z	P	Z	P	Z	P	Z	P
B2	-1.62	0.05	0	1	-1.27	0.1	-0.33	0.36	\	\	2.009	**0.02**
B3	-2.73	**0.003**	-1.27	0.1	0	1	0.97	0.16	\	\	3.07	**0.001**
C1	-1.96	**0.02**	-0.33	0.36	0.97	0.16	1		\	\	2.34	**0.009**
C2	\	\	\	\	\	\	\	\	\	\	\	\
C3	0.38	0.35	2.009	**0.02**	3.07	**0.001**	2.34	**0.009**	\	\	0	1

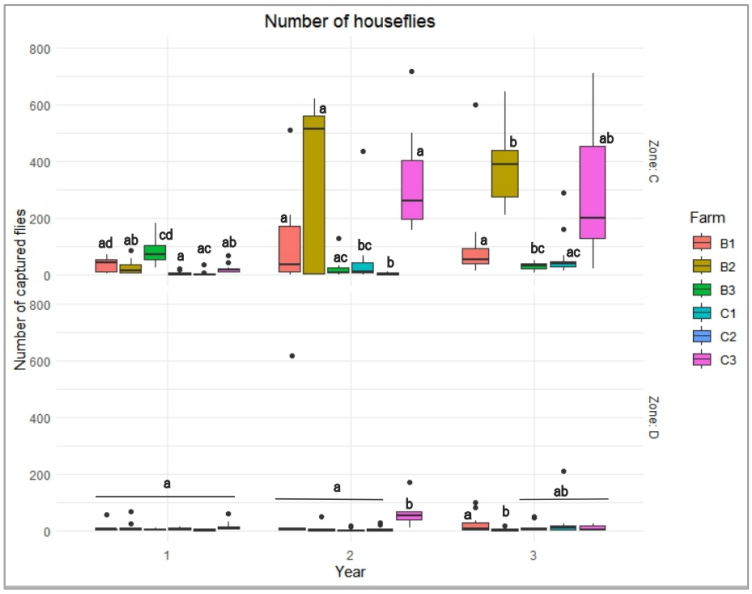

Figure 12. Number of houseflies captured on sticky traps in relation to the year and farm (different colors) and different Zone: C (calves zone) and D (dunghill zone). B indicates organic farms and C conventional ones. Statistically significant differences among the farms are summarized in Tables 4-5-6 and with different letters in case of P<0.05, comparing each farm in each zone for each year.

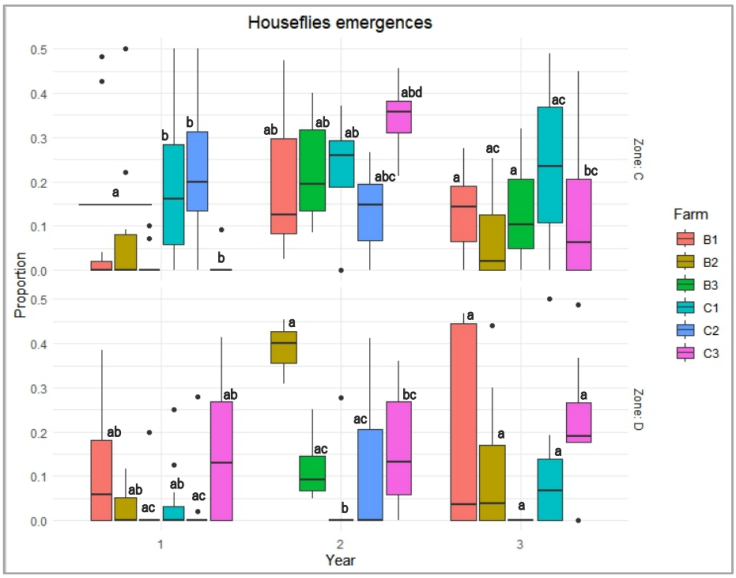

Figure 13. Proportion of houseflies emerged from collecting pupae in Zone C (calves' zone) and D (dunghill zone) for each farm (different color). B indicates organic farms and C conventional ones. Statistically significant differences among the farms are summarized in Tables 7-8-9 and with different letters in case of P<0.05, comparing each farm in each zone for each year.

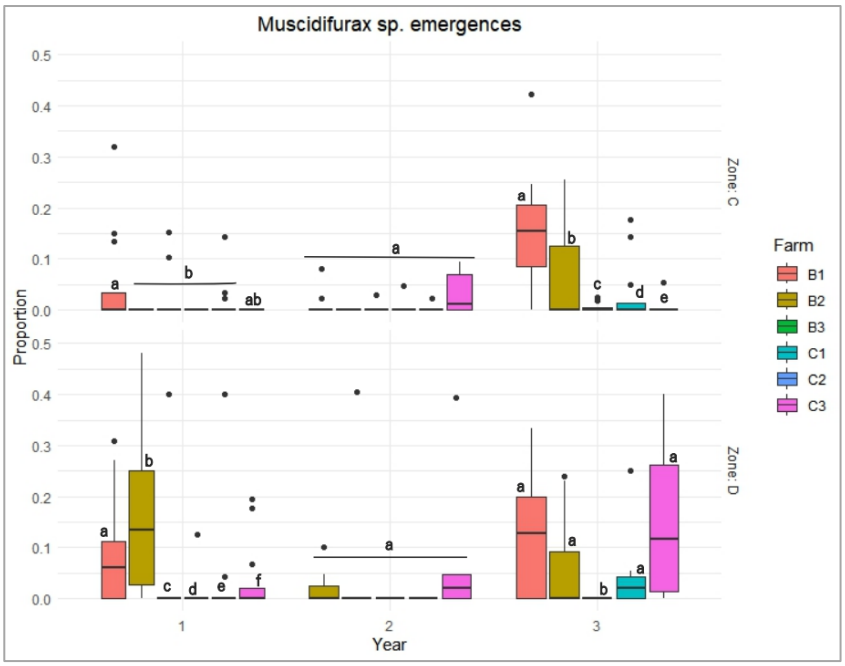

Figure 14. Proportion of *Muscidifurax sp* emerged from collected pupae in Zone C (calves' zone) and D (dunghill zone) for each farm (different color). B indicates organic farms and C conventional ones. Statistically significant differences among the farms are summarized in Table 10 for the first year and table 11 for the third year Zone C. Different letters indicate statistically significant differences for each farm and zone with P<0.05.

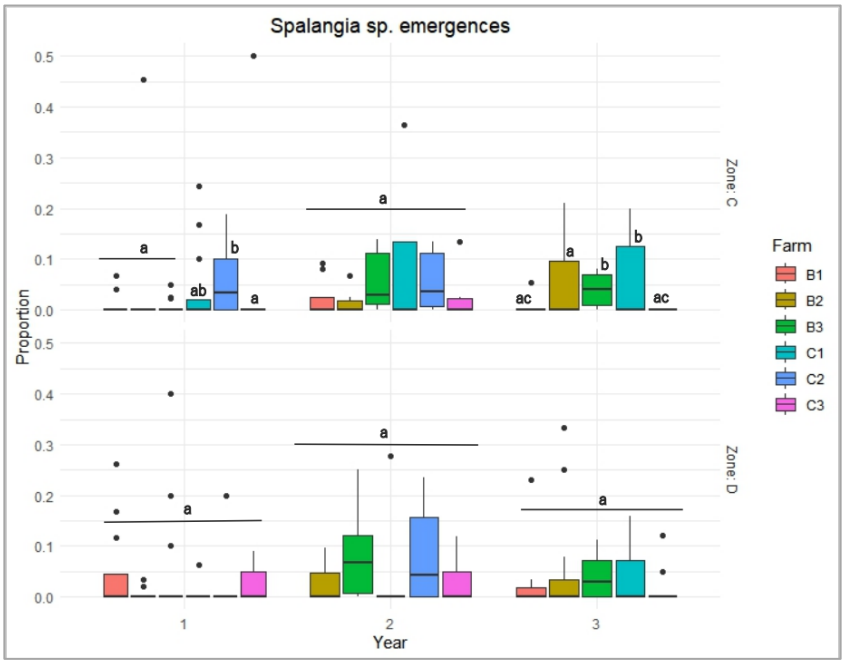

Figure 15. Proportion of *Spalangia sp* emerged from collected pupae in Zone C (calves' zone) and D (dunghill zone) for each farm (different color). B indicates organic farms and C conventional ones. Statistical significative differences among the farms were summarized in Table 12-13 for the first and third year in Zone C. Different letters indicate statistically significant differences for each farm and zone with P<0.05.

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Chapter 4. A comparison of commercialized and wild populations of housefly parasitoids using molecular and behavioral approaches

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4.1 Abstract

Spalangia sp., *Muscidifurax* sp., *Nasonia* sp. are common pupal parasitoids of *Musca domestica* used worldwide for its biocontrol. However, in Italy there is a lack of knowledge regarding the molecular characterization and distribution of natural populations of these parasitoids and dairy farms mostly rely on commercial populations for biocontrol purposes. This study aimed to investigate and characterize the populations of housefly pupal parasitoids present in conventional and organic dairy farms in Emilia Romagna, Italy and purchased from Italian and USA private companies using molecular and taxonomic approaches. Additionally, the objective was to compare the parasitization behaviour among wild and commercial species. The COI gene was analyzed for each species and haplotype based on provenience. Four species of *Muscidifurax* sp. were identified in the USA commercial bag, while the Italian commercial bags were characterized by the presence of *M. zaraptor* haplotype "a" and *M. raptorellus*. *Nasonia vitripennis* was found in the Italian commercial bag, which was identified only by taxonomic approach. Two different population were identified in the organic farms, and in the conventional farms, *M. zaraptor* haplotype "a" and haplotype "b". *Spalangia cameroni* was identified in one conventional farm analyzed, and *S. nigroaenea* was also identified in conventional farm using molecular and taxonomical approaches. The tested species and haplotypes were subjected to a behavioral assay. For each species and population (*M. raptor, M. zaraptor* haplotype "a"-"b", *S. cameroni* and *N. vitrippenis*), single mated females were provided with 10 housefly pupae each, and their parasitization activities were recorded for 4 hours. Statistically significant differences were found between species and populations for acceptance latency, drumming activity on single pupa, and number of unsuccessful parasitization. Identifying the species and populations present in the Emilia Romagna region of Northern Italy could contribute to the development of specific and targeted biological programs for the farms, using parasitoid species that do not compete with each other in terms of population and species, thus maximizing their effectiveness.

4.2 Introduction

Pteromalid genera *Muscidifurax, Spalangia*, and *Nasonia* are parasitoids known for their ability to attack the pupae of house flies, stable flies, and other Diptera (Whiting, 1967; Geden et al., 1998). Augmentative releases of these parasitoids have been an effective component of integrated pest management for the control of flies on livestock farms (Geden et al., 1992; Petersen and Cawthra, 1995). The use of parasitoids to control flies has increased significantly, with many commercial insectaries now producing pteromalids for release in animal production facilities (Geden et al., 1998). Research into the biological control of fly species has prompted investigations into their natural enemies across different regions worldwide. The work of Kogan and Legner (1970) documents this research. *Muscidifurax raptor* was collected from various geographical areas in the 1960s and 1970s, including the southeastern United States, southern Mexico, Costa Rica, Puerto Rico, Uruguay, Chile, Denmark, Israel, and South Africa (Legner, 1969; Kogan and Legner, 1970). In 1990, Meyer et al. evaluated the effectiveness of commercial and naturally occurring fly parasitoids in controlling stable flies and house flies on California dairies. It is worth noting that Muscidifurax *spp.* accounted for 73.2% of the parasitoids recovered on treated dairy farms, while *Spalangia* sp. accounted for only 20.6% (Meyer et al., 1990). Genetic and taxonomical approaches were used to identify new species of *Muscidifurax sp* in China. This highlights a gap in knowledge regarding the distribution and identification of some species of housefly pupal parasitoids (Xiao et al., 2018). In 2019, a study was conducted in Canada to identify and sequence the mitochondrial genes of Canadian invertebrates. The study specifically focused on pupal parasitoids of houseflies, particularly *Spalangia* sp. in the Pteromalidae family (deWaard et al., 2019). A previous study conducted by Taylor et al. in Nebraska in 1997 involved sequencing partial mitochondrial genes (COI and COII) related to *Muscidifurax sp*. (Taylor et al., 1997). There is currently a lack of studies in Italy on the distribution, molecular and taxonomic identification of pupal parasitoids of house flies. This is despite the fact that the Emilia Romagna region, which includes the area of Parmigiano Reggiano cheese production, has a high number of dairy cow farms with a serious problem of house fly infestation. Considering the behavior of pupal parasitoids in terms of parasitization performance, a study was performed to investigate the effect of host species on the oviposition behavior of *Nasonia vitripennis* and *Muscidifurax zaraptor*, focusing on host recognition, handling time, and aggression (Rivers, 1996). The study discovered that the duration of behavioral events leading up to puparium drilling was influenced by the host species, with *N. vitripennis* exhibiting aggressive behavior and disrupting *M. zaraptor* oviposition attempts at low densities. Furthermore, *Spalangia endius* Walker altered drilling activity and parasitization sites in housefly pupae. Young hosts had equal chances of successful drilling attempts, while older hosts had earlier and more frequent attempts (King, 2001). These studies demonstrate how parasitization performance can vary with changes in the host, and how using different pupae ages of the same host species can result in changes in parasitization. Currently, there are no studies that test individual pupal parasitoids species with different origins, whether commercial or wild populations.

The objective of this study was to characterize the pupal parasitoid populations of house flies on dairy farms in Emilia Romagna (Northern Italy) using molecular and behavioral approaches. Additionally, the study compared the parasitization rates and performance of commercialized and wild populations/species.

4.3 Material and Method

4.3.1 Rearing of houseflies and parasitoids

The study was conducted at the Applied Entomology Laboratory of the Interdepartmental BIOGEST-SITEIA Center, University of Modena and Reggio Emilia, Italy. During the summer of 2023, pupae were collected from 2 organic livestock farms (Farm B1, Farm B2) and one conventional farm (Farm C3) in the Reggio Emilia area to establish colonies of houseflies (*M. domestica*), *Spalangia* sp., *Muscidifurax* sp. in the laboratory. A colony of *Nasonia* sp. was established from the specimens emerged from parasitized pupae (BC), supplied by a private company specializing in their production. Adult houseflies were housed in polyester mesh cages measuring 32.5 x 32.5 x 32.5 cm, situated in a climatic chamber at a temperature of 27 ± 1°C, relative humidity of 60 ± 1%, and a 16-hour light and 8-hour dark photoperiod. They were given water, honey droplets, and a standard diet (described below) to facilitate oviposition. Every 2-3 days, the eggs were transferred to closed containers, and after hatching, more food was added consisting of 60 g of wheat bran, 40 g of alfalfa pellets, and 3 g of milk powder and water mixture. The diet provided was modified to match that of Bell et al., (2010).

The parasitoid species were reared separately, according to their origin, in a container measuring d12 x h 8cm, located inside a climate-controlled room with a photoperiod of 16:8 L:D, a relative humidity of 60 ± 1%, and a temperature of 25 ± 1°C. They were provided with water, honey droplets, and fresh or frozen *M. domestica* pupae for 24-48 hours of oviposition. Parasitized pupae were replaced every 3 days, and the newly emerged specimens were captured and returned to the container after 25 days for *Spalangia* sp. and 17-22 days for *Muscidifurax* sp. and approximately two weeks for *Nasonia* sp.

4.3.2 Iso-female lines

Three couples of parasitoids from the same population and species were deliberately separated to establish three different lines within the group based on the genus *Muscidifurax* sp. (farm O_B1 L 1,2,3; farm B2 L 1,2,3; R colony L 1,2, farm C L 1,2,3) *Spalangia* sp. (R L1) and *Nasonia* sp. (BC L1). Under controlled conditions, the separated couples were allowed to mate for a period of 24 to 48 hours at a temperature of 26°C and a relative humidity of 60% to 70%. To maintain their nutritional needs, the parasitoids were provided with droplets of a honey-water solution twice a week. After the mating period, single females from each couple were isolated and placed in a climatic chamber under the same conditions described above.

Each isolated female was then provided with pupae to lay eggs. The resulting offspring from each isolated female, referred to as the F1 generation, were monitored. These F1 individuals were placed in small containers and provided with pupae as their developmental substrate. The generations of the same iso-female line were then identified with molecular and taxonomical approaches.

4.3.3 Molecular protocol

The molecular analysis was conducted at the Laboratory of Evolutionary Zoology in the Department of Life Sciences at the University of Modena and Reggio Emilia, UNIMORE, Italy. The analysis was performed on different pupal parasitoids *of Musca domestica* emerged from a USA commercial bag (BC USA), an Italian Commercial bag (BC ITA), from Rearing UNIMORE 2021 (R21), from rearing iso female lines (L) of *Spalangia* sp., *Muscidifurax* sp. emerged from pupae collected in two organic managed farms (B1L and B2L), in one conventional (C3L) managed farm, and in the rearing colony (RL). The adult parasitoid samples were separated with labels from field and private companies, and then stored in 90% ethanol and at -20°C at the Department of Life Sciences of the University of Modena and Reggio Emilia until used for molecular analysis. Single specimens from the available samples were photographed before being used for nucleic acid isolation (Table 14). For *Muscidifurax* sp., total genomic DNA was extracted from individual adult parasitoids with the QuickExtract™ DNA Extraction Solution kit (Lucigen), following the manufacturer's protocol. This method allowed to recover the specimen, which was then stored at -20°C as a voucher. For *Spalangia* sp., the head was removed from adult specimens and genomic DNA was isolated using the Epicentre® MasterPure™ kit (Lucigen), while the remaining specimen was retained as a voucher and stored at -20°C. A 672 bp DNA fragment of the mitochondrial gene cytochrome oxidase subunit I (COI), was amplified in *Muscidifurax* specimens using the primers LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and 9 (5'-CCC GGT AAAB ATT AAA ATA TAA ACT TC-3'), while a 442 bp fragment of the COI gene was amplified in *Spalangia* specimens, using the primers 6 (5'-GGA GGA TTT GGA AAT TGA TTA GTT CC-3') and 9. Both fragments were amplified using the protocols described in Cesari et al., (2009). The amplified products were then gel purified using the Wizard Gel and PCR cleaning (Promega) kit, while sequencing reactions were performed using the ABIPRISM® BigDye™ Terminator Version 1.1 Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on purified amplicons. Each sequencing reaction contained 0.2 µM of a single PCR primer to initiate the sequencing reaction, 2 µl of BigDye™, 70 ng of purified products, 4 µl of 5x BigDye™ Terminator Version 1.1 Sequencing Buffer (Applied Biosystems, Foster City, CA, USA) and bidistilled H2O for a final volume of 20 µl. Cycling conditions for sequencing reactions consisted of 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Both strands were sequenced using an ABI Prism 3100 (Applied Biosystems, Foster City, CA, USA) in the LABGEN Laboratory of the Department of Life Sciences, University of Modena, and Reggio Emilia, UNIMORE, Italy. Obtained chromatograms were read and checked with FinchTV software (Geospiza Inc.).

Each electropherogram were checked for presence of ambiguous bases, as sequences were translated to amino acids using the invertebrate mitochondrial code implemented in MEGA11 (Tamura et al., 2021) in order to check for the presence of stop codons and therefore of pseudogenes.

4.3.4 Taxonomic protocol

The taxonomic identification was carried out at the Faculty of Biology, Alexandru Ioan Cuza University Iasi, Romania, by the taxonomy specialist Dr. Habil. Mircea-Dan Mitroiu. A sample of each iso-female line of *M. domestica* pupal parasitoids, separated by provenance as described above, was collected and preserved for the shipment in labelled vials containing 1 mL of 70% alcohol. Later the specimens were glued to the end of triangular cards so that all the identifying characteristics of the species were visible. The specimens were then dried with hexamethyldisilazane (HMDS) and photographed using a Leica DFC500 camera attached to a Leica M205 stereomicroscope. Specific images were taken of the whole specimen (habitus), details of the antennae (pedicel), mesopleuron, propodeum, and petiole.

4.3.5 Design of experiment

1. **Mating and initial conditions:**

Newly emerged parasitoids, segregated by species and haplotype (based on the results of taxonomic and molecular analyses), were paired, and allowed to mate for a period of 24 hours for *S. cameroni* and *N. vitripennis* and 48 h for *M. zaraptor* and *M. raptor*. Mating took place in a controlled environment at 26°C with a relative humidity ranging between 60% and 70%. To support the nutritional needs of the mating pairs, droplets of a honey-water solution were provided as a food source.

2. **Isolation of females and oviposition:**

After the 24/48-hour mating period, each mating couple was disassembled by removing the male. Individual females were then isolated and exposed to a set of 10 housefly pupae in a Petri dish, maintaining the same controlled environmental conditions as before. A total of 65 replicates of *Muscidifurax zaraptor* (haplotype a) from 7 different cohorts, 20 replicates of *M. zaraptor* (haplotype b) from 2 different cohorts, 20 replicates of *M. raptor* from one cohort, 25 replicates of *Nasonia vitripennis* from 3 different cohorts, and 20 replicates of *Spalangia cameroni* from 3 different cohorts were recorded.

3. **Behavioral observations:**

The behavior of each isolated female, specifically in response to the presence of 10 housefly pupae, was recorded. The video recording was made with a digital microscope for a duration of 4 hours, capturing various aspects of the female's interactions with the pupae. The video than was analyzed using Solomon Coder beta 17.03.22 software.

4. **Data collection:**

Several key parameters were recorded:

1. Acceptance latency= the time elapsed from the introduction of the female on Petri dishes to the first contact with pupae.
2. Drumming time = the time during which the female rhythmically taps her antennae on each pupa.
3. Number of parasitized pupae= total number of parasitized pupae, which is measure of the success of the oviposition process by the female (based on behaviour of females).
4. Time to complete parasitization refers to the moment from the start of drilling until the ovipositor is fully inserted into the pupa. The female then lays eggs inside the pupa (host feeding was not considered in the recording time).
5. Time of unsuccessful parasitization events refers to the moment when the female has partially drilled into the pupae or flexed her abdomen in an attempt to parasitize but has not yet fully inserted her ovipositor into the pupa (excluded the drilling before the host feeding).
6. Number of unsuccessful parasitization events.

4.4 Statistical analysis

Nucleotide sequences were aligned with the Clustal algorithm implemented in MEGA11 (pairwise and multiple alignment parameters: Gap opening penalty: 15, Gap extension penalty: 6.66) (Tamura et al., 2021) and checked by visual inspection. For appropriate molecular comparisons, we included in our analysis COI sequences from GenBank. Uncorrected p-distances between scored haplotypes were determined using MEGA11, after checking that scored COI sequences were not subject to saturation, using the program DAMBE (Xia, 2000). A parsimony cladogram network analysis between haplotypes was performed using TCS 1.21 (Clement et al., 2000). Both newly analyzed and GenBank sequences were included in the analysis, to provide a better qualitative information for pathways of presence of these species.

Behavioral statistical analyses were performed using R software version 4.3.1. A Generalized Linear Model (GLM) Gamma distribution family, was used to model each response variable expressed in minutes, including acceptance latency, time of drumming, time to complete parasitization, and time of unsuccessful parasitization, considering both species and haplotype as factors. This approach was chosen to account for response variables that may deviate from a normal distribution. After fitting the GLM for each response variable, pairwise comparisons were made between different species and haplotypes. The Tukey method was used for these comparisons, providing a reliable means of identifying specific species and haplotypes with statistically significant differences. This method is particularly effective in handling multiple comparisons ($P < 0.05$).

A Generalized Linear Mixed Model (GLMM), Poisson distribution family, was fitted to response variables expressed as counts, including both species and haplotype for number of parasitized pupae and number of unsuccessful parasitization events. Pairwise comparisons were made between different species and haplotypes for each count-based variable after GLMM fitting.

The Tukey method was used to compare specific species and haplotypes, identifying statistically significant differences even when considering multiple comparisons ($P < 0.05$).

4.5 Results

4.5.1 Molecular and taxonomic analyses

The molecular analysis confirmed the taxonomic identification of the four species of pupal parasitoids: *Spalangia cameroni* (Figure 16 A), *Muscidifurax raptor* (Figure 17 A), *Muscidifurax zaraptor* (Figure 18 A) and *S. nigroaenea. Nasonia vitripennis* was only identified with a morphological approach (Figure 19 A). In *S. cameroni* the mesopleuron has the longitudinal carinae of the subalar area extending onto upper mesepimeron only dorsally, the subalar scrobe is usually angled anteroventrally, the episternal scrobe and precoxal scrobes are connected by a distinct punctate line to form an anteriorly directed V-like groove (Figure 16 B), and the petiole is approximately 2.2-2.7 times longer than its medial width and has no setae (Figure 16 C). In *M. raptor* the median area of the propodeum is closed behind (Figure 17B) and antennal pedicel is slender in the median zone (Figure 17 C). In *M. zaraptor*, the proximal part of the pedicel of the antennae is noticeably slimmer (Figure 18 C) and the median area of the propodeum is closed behind due to the fusion of the lateral and median plicae (Figure 18 B) (Kogan and Legner, 1970). The most obvious morphological differences between the species of *Nasonia* sp. concern the structure of the forewing and antennae (Darling and Werren, 1990). The morphological differences concern the antenna as shape of scape, size of pedicel and forewing as size, setation, length and shape of stigma that would affect courtship in subtle ways that could result in female unreceptivity (Darling and Werren, 1990). The males have triangular wings intermediate than other species of the same genus (Figure 19 C). The ratio of wing length to wing width, which is diagnostic for males of *N. vitripennis*, is 3.31-3.86. Females of *N. vitripennis* have apical setae on the forewing, and the stigma vein of females is irregular in outline and long (Figure 18 B)(Darling and Werren, 1990).

For *Muscidifurax* specimens, six different haplotype networks were scored (Figure 20). Eight different haplotypes were identified for *M. zaraptor*, with p-distances ranging from 0.2% to 1.3% and were all connected within the same network (Table 15, Figure 20). Two haplotypes had different origins: one haplotype (a) was scored in R21, B1 and in different isofemale lines (B1 (L1,2,3), B2 (L 2,3), C (L3), R (L 1,2)) (Table 15, Figure 20), whereas haplotype (b) was found in BC USA, R21, BC ITA, and C3 (L 1,2). The single *M. zaraptor* specimen emerged from the USA commercial bag (Mr BC USA F5) presented a private haplotype that was not encompassed in the main *M. zaraptor* network, presenting higher p-distances values (4.1-5.5%).

Seven totally different haplotypes were found for *M. raptorellus* (commercial samples), with p-distances ranging from 0.4% to 1.8% and were all connected within the same network (Table 15, Figure 20). Most haplotypes were found only in one locality, with the lone exception of a haplotype that was identified in BC USA, in BC ITA and in the GenBank specimen.

Two haplotypes connected within the same network were found for *M. raptor*, differing for a p-distance of 1.2% (Table 15, Figure 20). One of these haplotypes was found both in the B2 Farm (Mr B2 L1) and in the GenBank specimen. Moreover, three *M. raptor* specimens showed haplotypes that were not included in the main *M. raptor* network, presenting also in this case higher p-distance values. Two haplotypes found in specimens from the C3 farm formed a private network (p-distance with respect of closer M. *raptor* haplotype: 5.0-5.8%), whereas the other one was completely disjointed from all other networks (p-distance with respect of closer M. *raptor* haplotype: 2.3%).

For *Spalangia* specimens, five different haplotype networks were identified (Figure 21). Three networks comprised haplotypes found in *Spalangia* sp. specimens from GenBank, hailing from Russia, from Manitoba in Canada, and from British Columbia and Quebec in Canada, respectively. The fourth haplotype network grouped haplotypes identified in specimens from BC USA, R21, and Sc C3 L3, with intra-network p-distances ranging from 0.2% to 0.6% (Table 16 and Figure 21). These specimens were morphologically confirmed as *S. cameroni*. The fifth haplotype network encompassed two haplotypes identified only in specimens from C3, with a p-distance between them of 0.2% (Table 16 and Figure 21). These specimens were morphologically confirmed as *S. nigroaenea*. *S. cameroni* and *S. nigroaenea* were highly differentiated both between them (p-distance: 20.4-20.6%) and when compared with the other three *Spalangia* taxa (21.6-24.1% for *S. cameroni*; 18.1-19.5% for *S. nigroaenea*).

4.5.2 Behaviour analysis

After identifying the haplotypes, it was decided to compare the species and haplotypes not on the basis of farm and commercial origin, but on the basis of COI gene sequencing and taxonomical identification. For the parameter of acceptance latency, a high variability in response time was observed for all species and haplotypes (Figure 22). On average, the mean acceptance time was less than 10 minutes for each species and haplotype. When comparing *M. zaraptor* for both haplotypes (a-b), there was a statistical difference that was borderline acceptable (E=1.55, P=0.06), although the median response time was 0.3 minutes for haplotype "a" and 0.81 minutes for haplotype "b" (Table 17). Comparing the other species, the statistical difference was recorded for *S. cameroni* than *M. zaraptor* haplotype "b" (E=-2.25, P=0.01) and then *N. vitripennis* (E=-2.12, P=0.017) (Figure 21, Table 18). No statistically significant differences were observed between *M. raptor* and two haplotypes of *M. zaraptor* (haplotype a: E=0.3, P=0.98; haplotype b: E=1.86, P=0.08), *M. raptor* and *S. cameroni* (E=-0.38, P=0.98) and *M. raptor* with *N. vitripennis* (E=1.73, P=0.09).

Borderline statistical differences were recorded for *N. vitripennis* and *M. zaraptor* haplotype "a" (E=1.42; P=0.06) but not significant for *N. vitripennis* and *M. zaraptor* haplotype "b" (E=-0.12, P=0.99) (Table 18).

For the parameter 'drumming on single pupa', the response did not exceed 50 min for each pupa (Table 17). No statistically significant differences were found for the genus *Muscidifurax* sp. (*M. zaraptor "a"- "b"* E=0.1 P=0.9; *M. zaraptor "a"-M raptor* E= 0.02 P=0.99; *M. zaraptor "b"- M. raptor* E=0.12 P=0.9) (Table 18 and Figure 23). The median time spent drumming on each pupa for these species was approximately 7 minutes (Table 17). The *species N. vitripennis* had the highest response for this parameter, with a median pupal drumming time of 24.94 min, while *S. cameroni* had the lowest, with a median of 3.93 min (Table 18 and Figure 23).

The median number of parasitized pupae was approximately 1-2 for each species and haplotype, with no statistically significant differences (P>0.05) (Table 17 and Figure 24). Similarly, there were no statistically significant differences in the time of parasitization of each pupa between the species and haplotype (P> 0.05), with a range of 17-27 minutes (Table 18 and Figure 25). On average, successful parasitization occurred within 23 min for *M. zaraptor* haplotype "a", 16 min for *M. zaraptor* haplotype "b", *M. raptor* 21 min, *N. vitripennis* 20 min, *S. cameroni* 27 min (Table 17).

The number of unsuccessful parasitization events varied between species and haplotypes (Figure 25). The highest number of unsuccessful parasitization events was recorded for *M. zaraptor* haplotype "a," with a median value of 13, while the lowest was recorded for *S. cameroni*, with only one event (Figure 26 and Table 17). Statistically significant differences were observed for all species and haplotypes, except for the *M. zaraptor* haplotype, which was similar (E=0.04, P=0.94) (Table 18). During the period of unsuccessful parasitization, no statistically significant differences were observed among all species and haplotypes (Table 18). The median duration for each event was approximately 2 minutes (Table 18 and Figure 27).

4.6 Discussion

The *Muscidifurax zaraptor* haplotype "a", which was found in each farm (B1L , B2L, R, C3L), is not a commercial strain that has been released in Italy as part of the biological control program for house flies. It is noteworthy that *M. zaraptor* haplotype "b" was found only in the conventional farm (C L1, L2), but it is common with the BC USA and BC ITA species, which are generally released in organic farms but not in conventional ones.
Muscidifurax raptorellus, which is normally present in both commercial bags, was not found in the farms. *M. raptor* was found only in Mr R21 and B2(L1), where this species had never been released. This suggests that wild and commercial species can coexist in the same area. Although the sequences of the mitochondrial gene of COI in GenBank coincide with two other species found in China, *M. simildanacus* and *M. sinsensilla* (Xiao et al., 2018), taxonomic analyses revealed that they are American species, based on the characters marked in the results (antennal pedicel and propodeum).

Spalangia cameroni and *S. nigroanea*, which were found in a conventional farm (C3) for COI mitochondrial sequences, are not closely related to the species found in GenBank (Manitoba in Canada, British Columbia and Quebec in Canada) (deWaard et al., 2019).

It is noteworthy that the *S. cameroni* species found in Italy has the same haplotype as the commercial bag from the USA, which is usually released in northern Italy for the biocontrol of houseflies in organic farms but not in conventional ones. The ability of these parasitoids to spread over long distances is demonstrated by *S. cameroni* (Machtinger et al., 2016). *Nasonia vitripennis* was identified only from pupae purchased by an Italian private company and was not found in livestock farms.

The behavior of the species analyzed varied according to the parameters considered. Female parasitoids of *Spalangia sp.* are ready to mate and oviposit immediately after emerging from the host puparium. Females obtain their food by ingesting the host's haemolymph as it exudes from the oviposition site (Gerling and Legner, 1968). The typical sequence of oviposition has 4 phases - finding the host area, locating the host pupae (acceptance latency), drumming, drilling and feeding (Morgan, 1981). All species discriminate between parasitized and and non-parasitized pupae. They usually discriminate after drilling the puparium, but sometimes only after drumming with the antennae (Murphy, 1980). In general, acceptance latency of pupae can be influenced by (1) the female's physical interaction with the male, (2) physical interaction with other females, and (3) a reduction in the physiological response at the neurophysiological level or (4) an adaptation of the process due to chemical receptors (Jones, 1982). In the present study, females were tested individually, so there was no influence from other individuals of the same or opposite sex. Jones (1982) suggested that *S. endius* first seeks the host's habitat and then the host itself. This sequential attraction is exemplified by the behavior of some braconid parasitoids that parasitize tephritid larvae (Jones, 1982). In general, after mating, *N. vitripennis* females become restless and begin to search for host pupae to parasitize (King et al., 2000; Ruther and Hammerl, 2014; Mair and Ruther, 2019). For orientation over medium and short distances, females use host odors (Whiting, 1967; Mair and Ruther, 2019). This is not consistent with our study, where the highest acceptance latency was recorded for *N. vitripennis* species under controlled laboratory conditions. In accordance with Rivers, (1996) for *N.vitrippenis* the behavior of ovideposition was not immediately initiated by a female wasp once isolated with a fly pupa. Often, before contact with a host, the wasp prepared itself for several minutes or searched the container. After meeting a pupa fly, the female parasitoid touched the puparium with both antennae, mounted the puparium, and began to walk the length of it. While the female was walking, she also beat or drummed puparium with its antennae (Rivers, 1996). This behavoir has been observed more for *N.vitrippenis* justifying higher acceptance latency for this species than others of our study. Although each female was placed separately in a Petri dish under the same conditions, differences were observed not only between species but also between haplotypes of the same species. This suggests that acceptance latency in this study was influenced by physiological and genetic factors.

This aspect was confirmed for the parasitoids *Trissolcus euschisti* and *Telenomus podisi* (Hymenoptera: Scelionidae) of *Halyomorpha halys*, where the acceptance latency varied among different isofemale lines (Costi et al., 2020). Differences between species were also found for the drumming parameter, meant as the tapping of the parasitoid antennae on the puparium (Frederickx et al., 2014).

The average drumming times per pupa of the studied species and populations differ in relation to the number of pupae drummed. When *N. vitripennis* females encounter a host pupa, they must decide whether to lay eggs, how many eggs to lay, and the sex ratio of the offspring (Mair and Ruther, 2019). In our study, *N. vitripennis* females identified one or at most two pupae that they considered suitable for oviposition. The species *M. raptor, M. zaraptor* and *S.cameroni* explored and then drummed more pupae than *N. vitripennis*. In the study of McKay and Broce, (2004), *M. zaraptor* showed a period of 3.48 min of drumming activity compared to our study with a median of 7.27 min. This discrepancy was due to the fact that in McKay and Broce, (2004) drumming time was considered as the time of drumming before the drilling activity and then parasitization for each pupa, whereas in our study drumming activity was considered for each pupa, from the introduction of the female into the Petri dish until the end of the recording. In our study, drumming activity is considered to be the exploration of pupae by females with or without drilling and/or parasitising activity. In our investigation both species of the genus *Muscidifurax* had a similar median drumming time for each pupa. Gerling and Legner's study reported that *N. vitripennis* can drum with its antennae on the pupae for an average of 1-2 minutes (Gerling and Legner, 1968). This differs from our study where this species drummed the pupae for a median of 24 minutes in relation to the number of pupae drummed. However, the same study reported that the drumming activities of *S. cameroni* lasted up to 10 minutes (Gerling and Legner, 1968), which is in contrast with our findings where this species drums the pupae for a maximum of 5.14 minutes. The number of parasitized pupae is similar for each species and population, ranging from 1 to a maximum of 2 parasitized pupae. Oviposition occurs when the parasitoid female drills a hole in the puparium, inserts her ovipositor, and lays an egg inside it. Most eggs are deposited on the dorsum of the abdomen but occasionally an egg may be found on almost any part of the pupa (Gerling and Legner, 1968). Regarding the time of parasitization, including also the drilling activity, our results on *M. zaraptor* for both haplotypes are in agreement with McKay and Broce, (2004) with a range of 18-20 min. In *N. vitripennis*, the parasitization process can be completed in as little as 60 minutes (Gerling and Legner, 1968). In our case, however, the process takes a similar amount of time as in other species, which is a maximum of 30 minutes. In *S. cameroni*, complete parasitization generally occurs within 10 to 120 minutes (Gerling and Legner, 1968). However, in our study, it took a maximum of 30 minutes, which is similar to other tested species. Unsuccessful parasitization in this study refers to the tapping and drilling activities of female parasitoids without oviposition. The process of host selection in a microenvironment was described by Edwards (1955) for *N. vitripennis* (Edwards, 1955).

Following this study, (Gerling and Legner, 1968) found that the *Spalangia* sp. female drums the host puparium, taps it with the tip of her abdomen, and finally drills into it at a specific point. This process of tapping can also occur after complete parasitization (McKay and Broce, 2004) as a possibile deposition of a marking pherormone, as demostrated for *M. raptor,* which uses an external marking pheromone to deter superparasitism and competiton with other conspecific females (Podoler and Mendel, 1977).

However, Wylie (1971) proposed that a female *M. zaraptor* discriminates between parasitized hosts only after inserting her ovipositor into the puparium and detecting the venom injected by the previous female. According to McKay and Broce (2004), *M. zaraptor* females prefer to oviposit on unparasitized housefly pupae rather than on those attacked by other *M. zaraptor* females, by *N. vitripennis*, or *S. cameroni*. In our study, tapping and drilling activities (unsuccessful parasitization) occurred before the oviposition, suggesting that females first explored the pupae in order to select them. The duration of this process was similar in all species and haplotypes analyzed, as was the number of occurrences. The genus *Muscidifurax* sp. had the highest recorded values for this parameter, followed by *N. vitripennis* and *S. cameroni*. In our study, the unsuccessful parasitization is related to the tapping and drilling as described before and then host feedding. The female of *M. raptor* (Tucker and Kaufman, 2016) and *M. zaraptor* (McKay et al., 2007) can feed on fly pupae by using their ovipositor (drilling) to attract the haemolymph of the fly to the surface of the puparium . After feed, they can deposit a single egg in the outer surface of the pupa or move to another puparium for oviposition (unsuccesful parasitization) (McKay, 2002) .The ovipositor tip probes the house fly pupa several times and then is held stationary to allow the liquid secretion (McKay, 2002). The host feeding is crucial for these species, allowing the females to mature her eggs after the mating (Coats, 1976). As demonstrated in Legner and Gerling's study, host feeding is essential for *M. raptor* during the first three days. A higher rate of longevity was observed with a higher proportion of killed pupae for host feeding compared to *S. cameroni* and *N. vitrippenis* (Legner and Gerling, 1967). This may explain why the number of attempts in *Muscidifurax* sp is higher than in other species.

4.7 Conclusions

This is the first study on the housefly parasitoid species found in Italy and the first to compare the parasitization process between different haplotypes and commercially available species found in dairy farms. The wild species considered are those found in conventional farms, where no parasitoids had ever been released. However, one of the haplotypes found for *M. zaraptor* is common to all farms, whether organic or conventional. This suggests a wide dispersal of parasitoids or a random placement of the same haplotype on the conventional farm. It can be concluded that among the considered parameters, only acceptance latency was found to be different between the haplotypes of *M. zaraptor.* Furthermore, there seems to be a consistent behavioral pattern within species of the same genus, as opposed to those of a different genus.

Muscidfurax raptor was discovered on an organic farm where it had never been intentionally introduced. This suggests that different species of parasitoids can coexist in the same environment, whether it is commercial or wild. The different rates of unsuccessful parasitization suggest the existence of distinct chemical and exploratory marking patterns, which require further investigation to understand how these species behave in the presence of conspecifics and other species.

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Table 14 Geographical information, Year of sampling, genus of pupal parasitoids species analyzed, acronym, subsamples utilized for mitochondrial analyses, provenience (Commercial bag, Rearing, Farm) and management referred to the farms.

Country	Region	Locality	Year	Genus	Acronym	Subsamples	Provenience	Management
USA	\	\	2021	*Muscidifurax*	Mr BC USA	F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F20 F21	Commercial bag	\
Italy	Emilia Romagna	Reggio Emilia	2021	*Muscidifurax*	Mr R21	F1 M2 F3 F4 F5 F6 F7 M8 F9 M10	Laboratory UNIMORE	\
Italy	\	\	2021	*Muscidifurax*	MR BC ITA	M1 F3 M4 F5 M6 M9 F10	Commercial bag	\
Italy	Emilia Romagna	Reggio Emilia	2021	*Muscidifurax*	Mr C3	F1 F3 F4 F5 F6	Farm	Conventional
Italy	Emilia Romagna	Reggio Emilia	20.21	*Muscidifurax*	Mr B1	M1 M4	Farm	Organic
Italy	Emilia Romagna	Reggio Emilia	2023	*Muscidifurax* (Isofemale)	Mr C3 L	L1M1 L2M1 L3F1	Farm	Conventional
Italy	Emilia Romagna	Reggio Emilia	2023	*Muscidifurax (isofemale)*	Mr B1L	L1F1 L2F1 L3F1	Farm	Organic
Italy	Emilia Romagna	Reggio Emilia	2023	*Muscidifurax* (Isofemale)	Mr B2 L	L1M1 L2F1 L3F1	Farm	Organic
Italy	Emilia Romagna	Reggio Emilia	2023	*Muscidifurax* (Isofemale)	Mr R L	L1F1 L2F1	Laboratory UNIMORE	\
USA	\	\	2021	*Spalangia*	SC BC USA	F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 F11 F12	Commercial bag	\
Italy	Emilia Romagna	Reggio Emilia	2021	*Spalangia*	SC R21	F1 M2 F4 F9 M11 F15 M16	Laboratory UNIMORE	\
Italy	Emilia Romagna	Reggio Emilia	2021	*Spalangia*	SC C3	M1 F2 M3 M4	Farm	Conventional
Italy	Emilia Romagna	Reggio Emilia	2023	*Spalangia* (isofemale)	SC C3 L	L3M2 L3M3	Farm	Conventional

Figure 16. *Spalangia cameroni* with main morphological characteristics identifying the species. A. The habitus of the species, B. Details of mesopleuron, C. Details of petiole. Photo by Dr. Habil. Mircea-Dan Mitroiu.

Figure 17. *Muscidifurax raptor* with main morphological characteristics identifying the species. A. The habitus of the species, B. Details of propodeum, C. Details of antennal pedicel. Photo by Dr. Habil. Mircea-Dan Mitroiu

Figure 18. *Muscidifurax zaraptor* with main morphological characteristics identifying the species. A. The habitus of the species, B. Details of propodeum, C. Details of antennal pedicel. Photo by Dr. Habil. Mircea-Dan Mitroiu

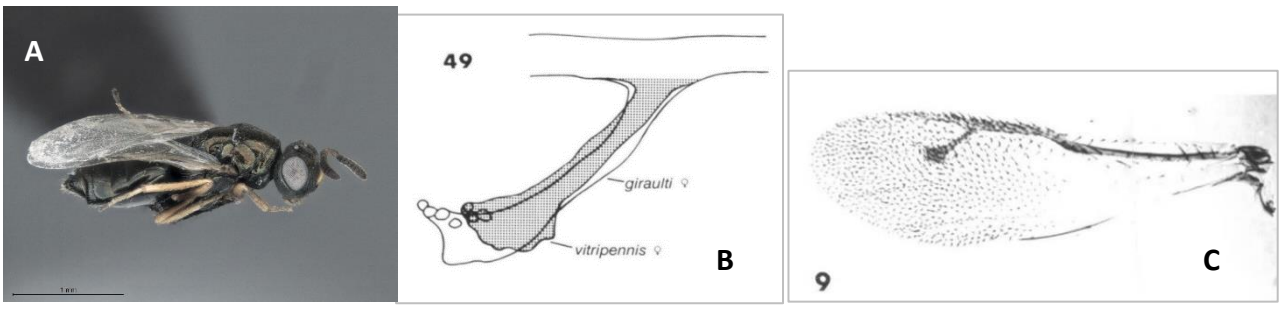

Figure 19. *Nasonia vitripennis* with main morphological characteristics identifying the species. A. the habitus of female. Photo by Dr. Habil Mircea-Dan Mitroiu; B. stigma of females wing than *N. girault.* Illustration by Darling and Wenner 1990; C. Wings of male of *N. vitripennis*. Photo by Darling and Wenner 1990.

Table 15 Genetic distances (p-distance) computed on 672 bp the COI gene among *Muscidifurax* specimens (Mr). B,C: conventional farms; R: rearing colony in the UNIMORE lab; BCUSA: commercial bag from the United States; BCITA: commercial bag from Italy. L: specific iso-female of the farms.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
MT985329 *Muscidifurax raptorellus* Chile mtDNA																																																
MT712139 *Muscidifurax similadanacus (zaraptor)* mtDN	0.118																																															
MT712140 *Muscidifurax sinesensilla (raptor)* mtDNA	0.095	0.116																																														
Mr R21 F1	0.117	**0.011**	0.122																																													
Mr R21 M2	0.092	0.112	**0.000**	0.114																																												
Mr R21 F3	0.118	**0.011**	0.124	0.000	0.118																																											
Mr R21 F4	0.124	**0.008**	0.124	0.013	0.121	0.013																																										
Mr R21 F5	0.119	**0.011**	0.126	0.000	0.120	0.000	0.013																																									
Mr R21 F6	0.112	**0.009**	0.112	0.002	0.112	0.000	0.007	0.000																																								
Mr R21 F7	0.094	0.106	**0.012**	0.109	0.011	0.112	0.113	0.114	0.106																																							
Mr R21 M8	0.117	**0.011**	0.123	0.000	0.116	0.000	0.013	0.000	0.000	0.111																																						
Mr R21 F9	0.114	**0.006**	0.114	0.000	0.114	0.000	0.007	0.000	0.002	0.108	0.000																																					
Mr R21 M10	0.117	**0.011**	0.120	0.000	0.114	0.000	0.013	0.000	0.002	0.109	0.000	0.000																																				
Mr BCUSA F1	**0.004**	0.109	0.090	0.109	0.090	0.111	0.114	0.113	0.107	0.091	0.109	0.109	0.109																																			
Mr BCUSA F2	0.106	**0.004**	0.103	0.006	0.103	0.007	0.014	0.007	0.009	0.097	0.007	0.006	0.006	0.101																																		
Mr BCUSA F3	**0.004**	0.109	0.090	0.109	0.090	0.111	0.114	0.113	0.107	0.091	0.109	0.109	0.109	0.000	0.101																																	
Mr BCUSA F4	**0.000**	0.114	0.092	0.114	0.092	0.115	0.119	0.118	0.112	0.093	0.114	0.114	0.114	0.004	0.106	0.004																																
Mr BCUSA F5	0.080	**0.050**	0.091	0.047	0.091	0.050	0.053	0.051	0.050	0.080	0.049	0.047	0.047	0.080	0.041	0.080	0.080																																
Mr BCUSA F6	**0.006**	0.109	0.092	0.109	0.092	0.111	0.114	0.113	0.107	0.093	0.109	0.109	0.109	0.011	0.101	0.011	0.006	0.075																																
Mr BCUSA F8	**0.004**	0.109	0.092	0.109	0.092	0.111	0.114	0.113	0.107	0.093	0.109	0.109	0.109	0.009	0.101	0.009	0.004	0.075	0.002																																
Mr BCUSA F9	0.116	**0.002**	0.112	0.009	0.112	0.009	0.011	0.009	0.011	0.106	0.009	0.009	0.009	0.112	0.006	0.112	0.116	0.052	0.112	0.112																																
Mr BCUSA F10	0.112	**0.004**	0.110	0.002	0.110	0.002	0.009	0.002	0.004	0.104	0.002	0.002	0.002	0.108	0.002	0.108	0.112	0.048	0.108	0.108	0.006																														
Mr BCUSA F20	0.114	**0.006**	0.114	0.000	0.114	0.000	0.007	0.000	0.002	0.108	0.000	0.000	0.000	0.109	0.006	0.109	0.114	0.047	0.109	0.109	0.009	0.002																													
Mr BCUSA F21	0.114	**0.006**	0.114	0.000	0.114	0.000	0.007	0.000	0.002	0.108	0.000	0.000	0.000	0.109	0.006	0.109	0.114	0.047	0.109	0.109	0.009	0.002	0.000																											
Mr BCITA M1	**0.018**	0.101	0.090	0.101	0.090	0.103	0.107	0.106	0.099	0.086	0.102	0.101	0.101	0.018	0.093	0.018	0.018	0.077	0.015	0.013	0.103	0.099	0.101	0.101																										
Mr BCITA F3	**0.000**	0.114	0.092	0.114	0.092	0.115	0.119	0.118	0.112	0.093	0.114	0.114	0.114	0.004	0.106	0.004	0.000	0.080	0.006	0.004	0.116	0.112	0.114	0.114	0.018																									
Mr BCITA M4	**0.009**	0.105	0.092	0.105	0.092	0.106	0.110	0.109	0.103	0.093	0.105	0.105	0.105	0.013	0.097	0.013	0.009	0.071	0.006	0.004	0.107	0.103	0.105	0.105	0.013	0.009																								
Mr BCITA F5	**0.006**	0.107	0.094	0.107	0.094	0.109	0.112	0.111	0.105	0.095	0.107	0.107	0.107	0.011	0.099	0.011	0.006	0.073	0.004	0.002	0.109	0.105	0.107	0.107	0.011	0.006	0.002																							
Mr BCITA M6	**0.006**	0.107	0.094	0.107	0.094	0.109	0.112	0.111	0.105	0.095	0.107	0.107	0.107	0.011	0.099	0.011	0.006	0.073	0.004	0.002	0.109	0.105	0.107	0.107	0.011	0.006	0.002	0.000																						
Mr BCITA M9	**0.007**	0.112	0.100	0.112	0.100	0.112	0.114	0.112	0.112	0.100	0.112	0.112	0.112	0.012	0.103	0.012	0.007	0.075	0.005	0.002	0.114	0.110	0.112	0.112	0.012	0.007	0.002	0.000	0.000																				
Mr BCITA F10	0.112	**0.006**	0.112	0.000	0.112	0.000	0.007	0.000	0.002	0.106	0.000	0.000	0.000	0.108	0.006	0.108	0.112	0.045	0.108	0.108	0.009	0.002	0.000	0.000	0.099	0.112	0.103	0.105	0.105	0.110																		
Mr C3 F1	0.092	0.103	**0.050**	0.099	0.058	0.102	0.105	0.104	0.097	0.043	0.100	0.099	0.099	0.092	0.099	0.092	0.092	0.075	0.094	0.094	0.105	0.099	0.099	0.099	0.088	0.092	0.094	0.097	0.097	0.102	0.099																	
Mr C3 F3	0.084	0.102	**0.050**	0.097	0.050	0.102	0.106	0.105	0.097	0.035	0.101	0.097	0.097	0.084	0.097	0.084	0.084	0.071	0.086	0.086	0.104	0.097	0.097	0.097	0.082	0.084	0.086	0.089	0.089	0.093	0.097	0.004																
Mr C3 F4	0.114	**0.006**	0.114	0.000	0.114	0.000	0.007	0.000	0.002	0.108	0.000	0.000	0.000	0.109	0.006	0.109	0.114	0.047	0.109	0.109	0.009	0.002	0.000	0.000	0.101	0.114	0.105	0.107	0.107	0.112	0.000	0.099	0.097															
Mr C3 F5	0.114	**0.006**	0.114	0.000	0.114	0.000	0.007	0.000	0.002	0.108	0.000	0.000	0.000	0.109	0.006	0.109	0.114	0.047	0.109	0.109	0.009	0.002	0.000	0.000	0.101	0.114	0.105	0.107	0.107	0.112	0.000	0.099	0.097	0.000														
Mr C3 F6	0.116	**0.005**	0.121	0.000	0.121	0.000	0.005	0.000	0.000	0.114	0.000	0.000	0.000	0.111	0.005	0.111	0.116	0.053	0.116	0.116	0.008	0.000	0.000	0.000	0.107	0.116	0.111	0.113	0.113	0.113	0.000	0.103	0.103	0.000	0.000												
Mr C3 L1M1	0.116	**0.010**	0.121	0.000	0.114	0.000	0.013	0.000	0.002	0.109	0.000	0.000	0.000	0.109	0.006	0.109	0.114	0.047	0.109	0.109	0.009	0.002	0.000	0.000	0.101	0.114	0.105	0.107	0.107	0.112	0.000	0.099	0.097	0.000	0.000	0.000												
Mr C3 L2M1	0.116	**0.011**	0.122	0.000	0.114	0.000	0.013	0.000	0.002	0.109	0.000	0.000	0.000	0.109	0.006	0.109	0.114	0.047	0.109	0.109	0.009	0.002	0.000	0.000	0.101	0.114	0.105	0.107	0.107	0.112	0.000	0.099	0.097	0.000	0.000	0.000	0.000											
Mr C3 L3F1	0.122	**0.008**	0.122	0.013	0.116	0.013	0.000	0.013	0.007	0.109	0.013	0.007	0.013	0.110	0.013	0.110	0.114	0.051	0.110	0.110	0.011	0.009	0.007	0.007	0.102	0.114	0.105	0.107	0.107	0.114	0.007	0.101	0.101	0.007	0.007	0.005	0.013	0.013										
Mr B2 L1M1	0.099	0.118	**0.028**	0.120	0.024	0.122	0.122	0.123	0.107	0.023	0.121	0.109	0.120	0.092	0.103	0.092	0.092	0.084	0.092	0.092	0.112	0.105	0.109	0.109	0.088	0.092	0.092	0.094	0.094	0.098	0.108	0.052	0.043	0.109	0.109	0.113	0.119	0.121	0.122								
Mr B2 L2F1	0.121	**0.008**	0.121	0.012	0.118	0.012	0.000	0.013	0.007	0.111	0.012	0.007	0.013	0.112	0.013	0.112	0.116	0.052	0.112	0.112	0.011	0.009	0.007	0.007	0.104	0.116	0.107	0.109	0.109	0.114	0.007	0.103	0.103	0.007	0.007	0.005	0.012	0.012	0.000	0.120								
Mr B2 L3M1	0.126	**0.006**	0.126	0.011	0.126	0.011	0.000	0.011	0.005	0.116	0.011	0.005	0.011	0.118	0.012	0.118	0.123	0.055	0.118	0.118	0.009	0.007	0.005	0.005	0.111	0.123	0.114	0.116	0.116	0.116	0.005	0.109	0.110	0.005	0.005	0.005	0.011	0.011	0.000	0.123	0.000							
Mr B1 L1F1	0.125	**0.008**	0.125	0.013	0.124	0.013	0.000	0.013	0.007	0.115	0.013	0.007	0.013	0.117	0.014	0.117	0.121	0.054	0.117	0.117	0.012	0.009	0.007	0.007	0.109	0.121	0.112	0.114	0.114	0.114	0.007	0.107	0.108	0.007	0.007	0.005	0.013	0.013	0.000	0.122	0.000	0.000						
Mr B1 L2F1	0.121	**0.007**	0.119	0.012	0.116	0.012	0.000	0.013	0.009	0.109	0.012	0.006	0.012	0.112	0.013	0.112	0.116	0.050	0.112	0.112	0.011	0.009	0.006	0.006	0.103	0.116	0.107	0.109	0.109	0.114	0.006	0.101	0.099	0.006	0.006	0.005	0.012	0.012	0.000	0.118	0.000	0.000	0.000					
Mr B1 L3F1	0.121	**0.007**	0.119	0.012	0.116	0.012	0.000	0.013	0.009	0.109	0.012	0.006	0.012	0.112	0.013	0.112	0.116	0.050	0.112	0.112	0.011	0.009	0.006	0.006	0.103	0.116	0.107	0.109	0.109	0.114	0.006	0.101	0.099	0.006	0.006	0.005	0.012	0.012	0.000	0.118	0.000	0.000	0.000	0.000				
Mr B1 M1 9	0.116	**0.005**	0.124	0.005	0.120	0.005	0.007	0.005	0.002	0.112	0.005	0.002	0.005	0.110	0.007	0.110	0.115	0.054	0.115	0.115	0.005	0.002	0.002	0.002	0.107	0.115	0.110	0.113	0.113	0.113	0.002	0.103	0.103	0.002	0.002	0.003	0.005	0.005	0.007	0.121	0.007	0.007	0.007	0.007	0.007			
Mr B1 M4	0.114	**0.002**	0.116	0.006	0.114	0.006	0.006	0.006	0.006	0.109	0.006	0.004	0.006	0.112	0.006	0.112	0.116	0.052	0.112	0.112	0.004	0.002	0.004	0.004	0.103	0.116	0.107	0.109	0.109	0.114	0.004	0.103	0.102	0.004	0.004	0.003	0.006	0.006	0.006	0.112	0.006	0.004	0.006	0.006	0.006	0.000		
Mr R L1F1	0.121	**0.008**	0.121	0.012	0.119	0.012	0.000	0.013	0.007	0.111	0.012	0.007	0.013	0.112	0.013	0.112	0.116	0.052	0.112	0.112	0.011	0.009	0.007	0.007	0.105	0.116	0.107	0.110	0.110	0.114	0.007	0.103	0.103	0.007	0.007	0.005	0.012	0.012	0.000	0.120	0.000	0.000	0.000	0.000	0.000	0.007	0.006	
Mr R L2F1	0.121	**0.007**	0.119	0.012	0.116	0.012	0.000	0.013	0.009	0.109	0.012	0.006	0.012	0.112	0.013	0.112	0.116	0.050	0.112	0.112	0.011	0.009	0.006	0.006	0.103	0.116	0.107	0.109	0.109	0.114	0.006	0.101	0.099	0.006	0.006	0.005	0.012	0.012	0.000	0.118	0.000	0.000	0.000	0.000	0.000	0.007	0.006	0.000

Table 16 Genetic distances (p-distance) computed on 442 bp the COI gene among *Spalangia* specimens (Sc). C3: conventional farm; R: rearing colony in the UNIMORE lab; BC USA: commercial bag from the United States.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
. Canada Manitoba																																							
. Canada Manitoba	**0.000**																																						
. Canada Manitoba	**0.000**	**0.000**																																					
. Canada Manitoba	**0.000**	**0.000**	**0.000**																																				
. Canada Manitoba	**0.000**	**0.000**	**0.000**	**0.000**																																			
. Canada Manitoba	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**																																		
. Canada Manitoba	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**																																	
. Canada Manitoba	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**																																
. Canada Manitoba	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**																															
. Canada Manitoba	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**																														
. Canada Manitoba	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**																													
. Canada Manitoba	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**																												
. Canada Manitoba	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**																											
BGB1936 15 Canada Quebec	0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151																										
MG373820 *Spalangia* sp. POSPD830 15 Canada British Columb	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	0.152	**0.000**																									
6 Russia	0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.165	0.151	0.152																								
Sc R21 F1	0.239	0.241	0.240	0.240	0.241	0.241	0.239	0.241	0.239	0.241	0.241	0.241	0.241	0.218	0.219	0.225																							
Sc R21 M2	0.239	0.241	0.240	0.240	0.241	0.241	0.239	0.241	0.239	0.241	0.241	0.241	0.241	0.218	0.219	0.225	**0.000**																						
Sc R21 F4	0.239	0.241	0.240	0.240	0.241	0.241	0.239	0.241	0.239	0.241	0.241	0.241	0.241	0.218	0.219	0.227	**0.002**	**0.002**																					
Sc R21 F9	0.239	0.241	0.240	0.240	0.241	0.241	0.239	0.241	0.239	0.241	0.241	0.241	0.241	0.218	0.219	0.225	**0.000**	**0.000**	**0.002**																				
Sc R21 M11	0.239	0.241	0.240	0.240	0.241	0.241	0.239	0.241	0.239	0.241	0.241	0.241	0.241	0.218	0.219	0.225	**0.000**	**0.000**	**0.002**	**0.000**																			
Sc R21 F15	0.239	0.241	0.240	0.240	0.241	0.241	0.239	0.241	0.239	0.241	0.241	0.241	0.241	0.218	0.219	0.225	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**																		
Sc R21 M16	0.239	0.241	0.240	0.240	0.241	0.241	0.239	0.241	0.239	0.241	0.241	0.241	0.241	0.218	0.219	0.225	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**	**0.000**																	
Sc BCUSA F1	0.237	0.238	0.238	0.238	0.239	0.239	0.237	0.238	0.237	0.239	0.238	0.238	0.238	0.216	0.217	0.222	**0.004**	**0.004**	**0.006**	**0.004**	**0.004**	**0.004**	**0.004**																
Sc BCUSA F2	0.237	0.238	0.238	0.238	0.239	0.239	0.237	0.238	0.237	0.239	0.238	0.238	0.238	0.216	0.217	0.222	**0.004**	**0.004**	**0.006**	**0.004**	**0.004**	**0.004**	**0.004**	**0.000**															
Sc BCUSA F3	0.237	0.238	0.238	0.238	0.239	0.239	0.237	0.238	0.237	0.239	0.238	0.238	0.238	0.216	0.217	0.222	**0.004**	**0.004**	**0.006**	**0.004**	**0.004**	**0.004**	**0.004**	**0.000**	**0.000**														
Sc BCUSA F4	0.237	0.238	0.238	0.238	0.239	0.239	0.237	0.238	0.237	0.239	0.238	0.238	0.238	0.216	0.217	0.222	**0.004**	**0.004**	**0.006**	**0.004**	**0.004**	**0.004**	**0.004**	**0.000**	**0.000**	**0.000**													
Sc BCUSA F5	0.237	0.238	0.238	0.238	0.239	0.239	0.237	0.238	0.237	0.239	0.238	0.238	0.238	0.216	0.217	0.222	**0.004**	**0.004**	**0.006**	**0.004**	**0.004**	**0.004**	**0.004**	**0.000**	**0.000**	**0.000**	**0.000**												
Sc BCUSA F6	0.237	0.238	0.238	0.238	0.239	0.239	0.237	0.238	0.237	0.239	0.238	0.238	0.238	0.216	0.217	0.222	**0.004**	**0.004**	**0.006**	**0.004**	**0.004**	**0.004**	**0.004**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**											
Sc BCUSA F9	0.237	0.238	0.238	0.238	0.239	0.239	0.237	0.238	0.237	0.239	0.238	0.238	0.238	0.216	0.217	0.222	**0.004**	**0.004**	**0.006**	**0.004**	**0.004**	**0.004**	**0.004**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**										
Sc BCUSA F10	0.237	0.238	0.238	0.238	0.239	0.239	0.237	0.238	0.237	0.239	0.238	0.238	0.238	0.216	0.217	0.222	**0.004**	**0.004**	**0.006**	**0.004**	**0.004**	**0.004**	**0.004**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**									
Sc BCUSA F11	0.237	0.238	0.238	0.238	0.239	0.239	0.237	0.238	0.237	0.239	0.238	0.238	0.238	0.216	0.217	0.222	**0.004**	**0.004**	**0.006**	**0.004**	**0.004**	**0.004**	**0.004**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**								
Sc BCUSA F12	0.237	0.238	0.238	0.238	0.239	0.239	0.237	0.238	0.237	0.239	0.238	0.238	0.238	0.216	0.217	0.222	**0.004**	**0.004**	**0.006**	**0.004**	**0.004**	**0.004**	**0.004**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**							
Sc C3 L3 M3	0.239	0.241	0.240	0.240	0.241	0.241	0.239	0.241	0.239	0.241	0.241	0.241	0.241	0.218	0.219	0.225	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**	**0.000**	**0.000**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**						
Sc C3 L3 M2	0.239	0.241	0.240	0.240	0.241	0.241	0.239	0.241	0.239	0.241	0.241	0.241	0.241	0.218	0.219	0.225	**0.000**	**0.000**	**0.002**	**0.000**	**0.000**	**0.000**	**0.000**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.004**	**0.000**					
Sc C3 M1	0.183	0.184	0.184	0.184	0.185	0.185	0.183	0.184	0.183	0.185	0.185	0.184	0.185	0.192	0.193	0.187	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206				
Sc C3 F2	0.183	0.184	0.184	0.184	0.185	0.185	0.183	0.184	0.183	0.185	0.185	0.184	0.185	0.192	0.193	0.187	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	**0.000**				
Sc C3 M3	0.183	0.184	0.184	0.184	0.185	0.185	0.183	0.184	0.183	0.185	0.185	0.184	0.185	0.192	0.193	0.187	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	0.206	**0.000**	**0.000**			
Sc C3 M4	0.181	0.182	0.182	0.182	0.183	0.183	0.181	0.182	0.181	0.183	0.182	0.182	0.182	0.194	0.195	0.184	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	0.204	**0.002**	**0.002**	**0.002**		

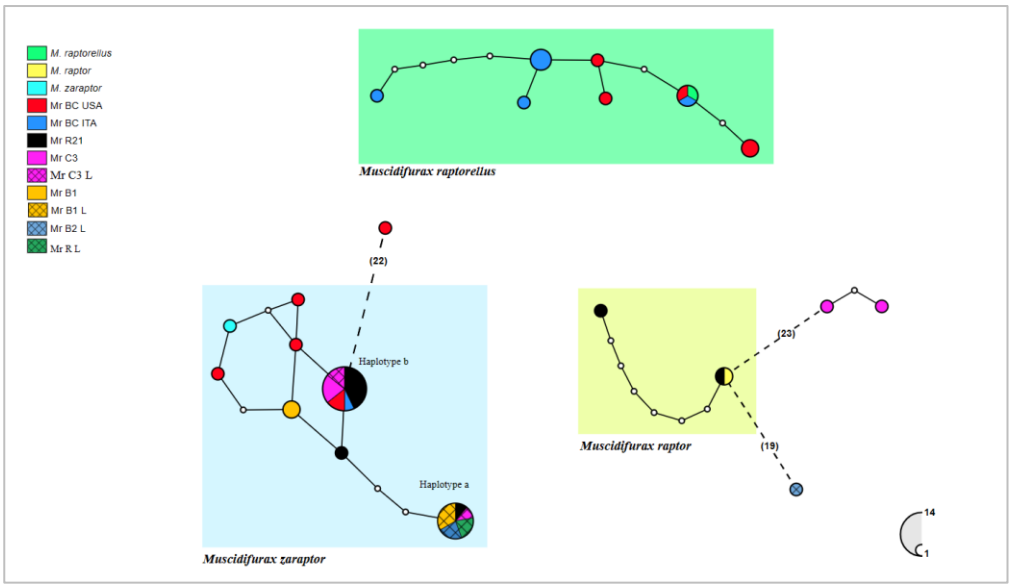

Figure 20. Haplotype network analysis based on 672 bp of the COI gene of *Muscidifurax* specimens. The circles represent haplotypes, and the circle surface indicates haplotype frequency. White circles indicate putative/missing haplotypes. Networks that fall below the 95% connection limit are disconnected. Mr BC USA: specimens from a commercial bag from the USA; Mr BC ITA: specimens from an Italian commercial bag; MR R UNIMORE: specimens from the UNIMORE Rearing colony. The isofemale lines from C3 farm (subsamples Mr C3 and Line - Mr C3 L), B1 farm (Mr B1 L), B2 farm (Mr B2 L), and the rearing colony Unimore (Mr R L) are also included.

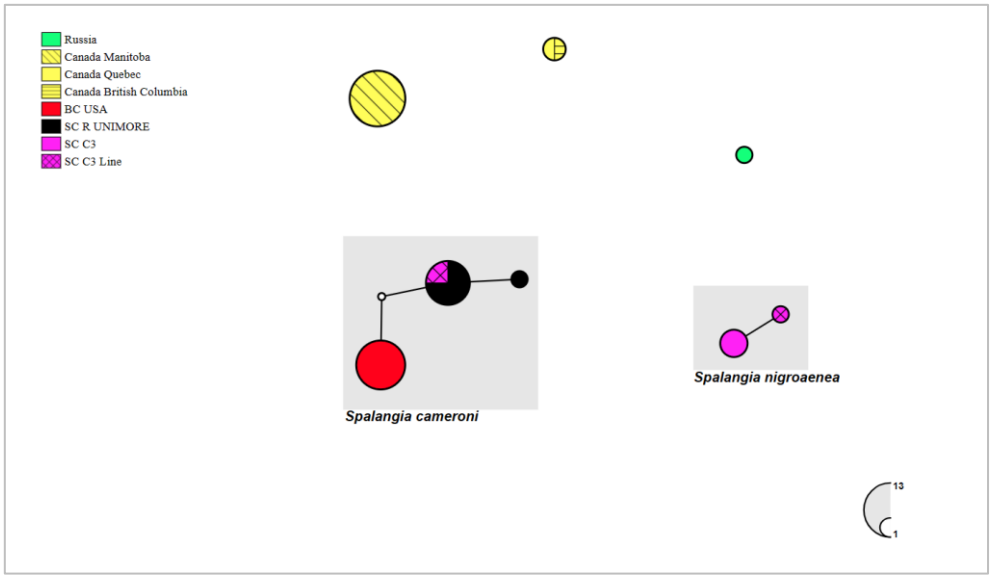

Figure 21. Haplotype network analysis based on 442 bp of the COI gene of *Spalangia* specimens. Circles denote haplotypes, while circle surface represents haplotype frequency. White circles show putative/missing haplotypes. Networks falling below the value of the 95% connection limit are disconnected. SC R UNIMORE: UNIMORE Rearing colony of S. cameroni based on mix of pupal parasitoids of field in 2021; SC BC USA: S. cameroni from USA commercial bags; SC C3: *Spalangia* sp. specimens found in the commercial farm C3.

Table 17 Behavioral parameters observed for the different species/haplotypes. The median value and, in the square, interquartile range, are reported for each parameter.

Species	Acceptance latency	Drumming activities on single pupa	N parasitized pupae	Time of parasitization	N unsuccessful parasitization event	N unsuccessful parasitization time
M. zaraptor a	0.3 [0.14-2.13]	7.27 [4.72-9.77]	2 [1-2]	20.04 [15.40-26.73]	13 [7-22]	2.6 [1.65-3.8]
M.zaraptor b	0.81 [0.26-1.7]	7.95 [6.2-10.15]	1 [1-2.25]	17.64 [11.90-25.13]	16.5 [7-22.25]	2.76 [2.09-3.69]
M.raptor	0.22 [0.16-0.32]	6.84 [5.68-8.9]	1.5 [1-3]	20.42 [17.71-27.39]	10.5 [2.5-17.25]	2.92 [1.53-3.43]
N.vitripennis	1.22 [0.27- 3.07]	24.94[19.2-30.32]	1 [1-2]	20.61 [17.92-29.89]	3 [0-7]	1.71 [0-5.08]
S.cameroni	0.32 [0.38-1.50]	3.93 [3.29-5.14]	1 [1-2]	27.56 [15.38-34.91]	1 [0-3]	1.57 [0-3.45]

Table 18 Comparison among species and haplotype for all analyzed behavioral parameters. E is the estimate value of the model and P is p value marked in bold in the case of significative statistically differences (P<0.05). E_a is referred to the estimate of GLM model and E_b is referred to the estimated value of GLMM model, performed separately for each parameter.

Comparison among species		Acceptance latency		Drumming activities on single pupa		N parasitized pupae		Time of parasitization		N unsuccessful parasitization event		Unsuccessful parasitization time	
		E_a	P	E_a	P	E_b	P	E_a	P	E_b	P	E_a	P
M.zaraptor a	*M.zaraptor b*	1.55	**0.06**	0.1	0.9	-0.17	0.91	-0.15	0.87	0.04	0.947	0.11	0.96
	M.raptor	0.3	0.98	0.02	0.99	-0.03	1	-0.04	0.99	0.43	**<0.01**	0.1	0.97
	N.vitripennis	1.42	**0.06**	1.19	**<0.01**	-0.33	0.44	-0.03	0.99	-0.94	**<0.01**	0.27	0.58
	S.cameroni	-0.69	0.75	-0.53	**<0.01**	-0.15	0.97	0.22	0.57	-1.81	**<0.01**	0.07	0.99
M.zaraptor b	*M.raptor*	1.86	0.08	0.12	0.9	-0.2	0.91	-0.19	0.86	0.48	**<0.01**	0.22	0.86
	N.vitripennis	-0.12	0.99	1.08	**<0.01**	-0.15	0.97	0.11	0.96	-0.99	**<0.01**	0.16	0.96
	S.cameroni	-2.25	**0.01**	-0.64	**<0.01**	0.26	0.81	0.38	0.3	-1.86	**<0.01**	-0.04	1
M.raptor	*N.vitripennis*	1.73	0.09	1.21	**<0.01**	-0.36	0.53	-0.07	0.99	-0.51	**<0.01**	0.38	0.47
	S.cameroni	-0.38	0.98	-0.51	**0.004**	0.05	0.99	0.18	0.87	-1.38	**<0.01**	0.18	0.95
S.cameroni	*N.vitripennis*	-2.12	**0.01**	-1.71	**<0.01**	0.42	0.38	0.26	0.57	-0.86	**<0.01**	-0.2	0.93

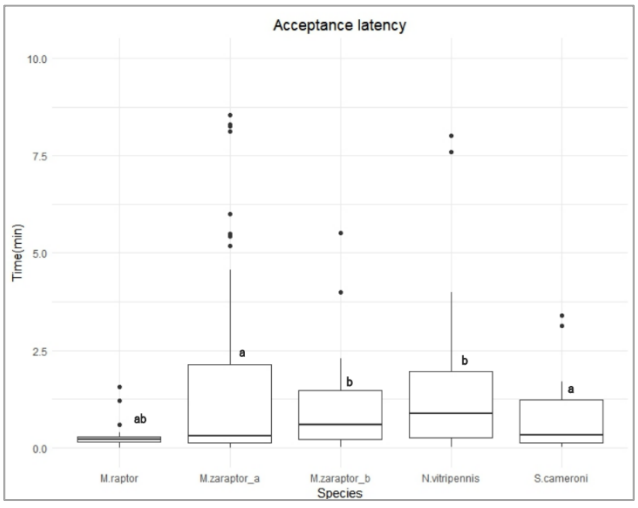

Figure 22. Response for the acceptance latency parameter across different species and haplotypes. The comparison between these parameters was analyzed using the Tukey method after fitting the GLM model, Gamma family. Different letters indicate statistically significant differences (P<0.05).

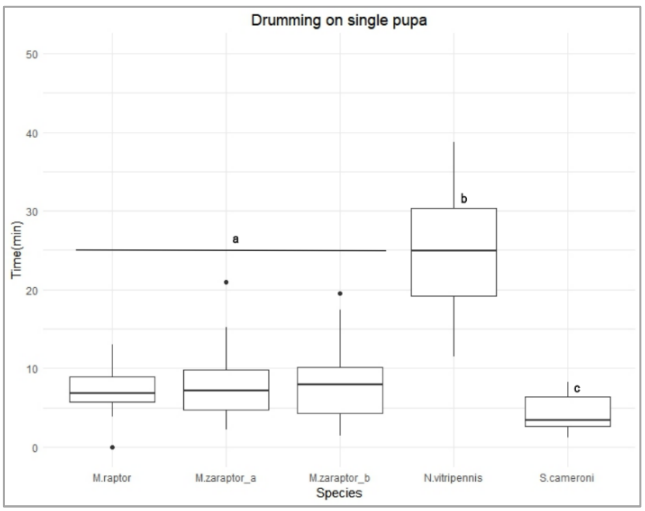

Figure 23. Response for the drumming on single pupa across different species and haplotypes. The comparison between these parameters was analyzed using the Tukey method after fitting the GLM model, Gamma family. Different letters indicate statistically significant differences (P<0.05).

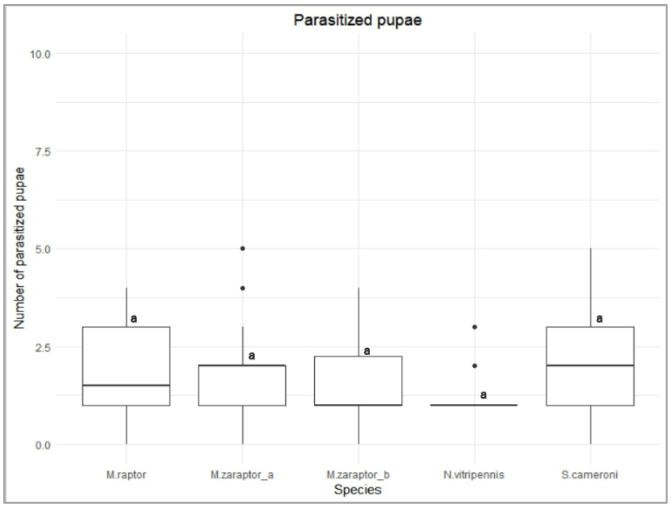

Figure 24. Response for the number of parasitized pupae across different species and haplotypes. The comparison between these parameters was analyzed using the Tukey method after fitting the GLMM model, distribution Poisson family. No statistically significant differences were recorded.

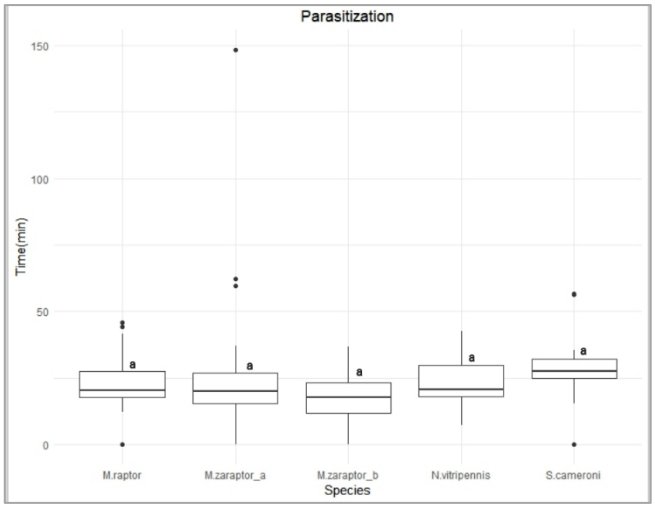

Figure 25. Response for parasitization time on single pupa across different species and haplotypes. The comparison between these parameters was analyzed using the Tukey method after fitting the GLM model, Gamma family. No statistically significant differences were recorded.

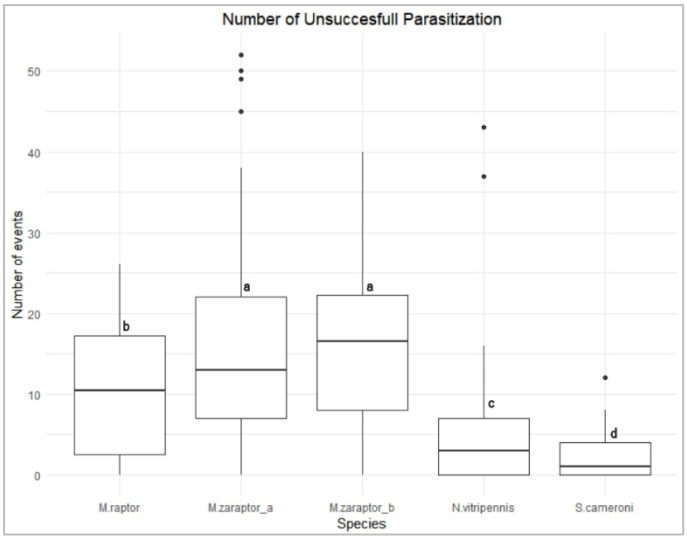

Figure 26. Response for the number of unsuccessful Parasitization event across different species and haplotypes. The comparison between these parameters was analyzed using the Tukey method after fitting the GLMM model, distribution Poisson family. Different letters indicate statically significant difference (P<0.05).

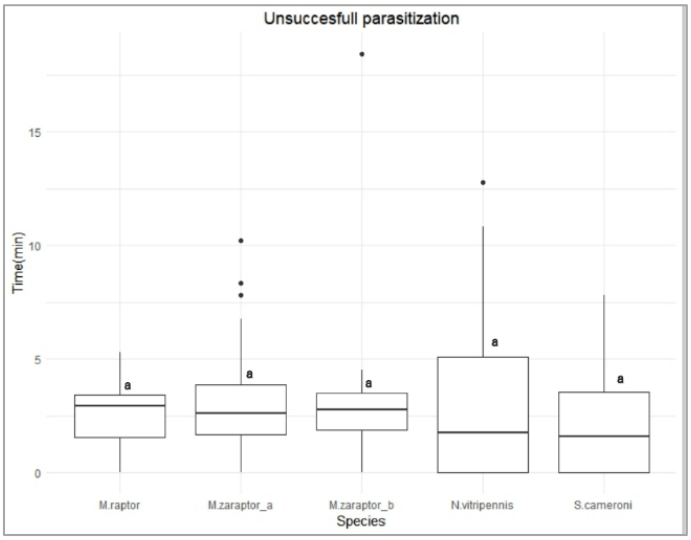

Figure 27. Response for Unsuccessful parasitization time across different species and haplotypes. The comparison between these parameters was analyzed using the Tukey method after fitting the GLM model, Gamma family. No statistically significant differences were recorded.

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Research article

Chapter 5. Parasitization activity of *Spalangia cameroni* and *Muscidifurax zaraptor* (Hymenoptera, Pteromalidae), pupal parasitoids of *Musca domestica* (Diptera, Muscidae)

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5.1 Abstract

Musca domestica Linnaeus (Diptera, Muscidae) is a significant pest in livestock farms and a major concern for both humans and farm animals due to its ability to transmit over 200 pathogens. The use of pupal parasitoids is a sustainable strategy for controlling this pest. *Spalangia cameroni* Perkins and *Muscidifurax zaraptor* Girault & Sanders (Hymenoptera, Pteromalidae) are commonly used as biocontrol agents for *M. domestica*. The objective of this study was to determine the oviposition peak of the female parasitoids in relation to their age and the sex ratio of adult progeny. For both species, 20 fresh *M. domestica* pupae (24-48h) were provided daily to each fertilised female for 14 days, after which the pupae were checked for parasitoid emergence. A control group of 20 pupae without female parasitoids was maintained. The results showed that *S. cameroni* had a higher overall percentage of parasitisation (57.71%) compared to *M. zaraptor* (32.41%). The parasitisation ratio of *S. cameroni* remained almost constant throughout the 14-day period, while that of *M. zaraptor* decreased drastically after the 11th day. Peak oviposition for *S. cameroni* was on day 5 with 13 parasitised pupae per female, while *M. zaraptor* parasitised 8 pupae/day in 4 days during its peak oviposition period (between days 3 and 8). The newly emerged parasitoids had a skewed sex ratio towards females: 81% for *S. cameroni* and 66% for *M. zaraptor*. The presence of these parasitoid species resulted in fewer new housefly emergences than in the control group, where natural pupal mortality was lower in the absence of parasitoids. These findings may be useful for optimising the mass production and time-use of the two parasitoid species for management of houseflies in livestock farms.

Key words: houseflies, biocontrol, wasp, oviposition, mortality

5.2 Introduction

The housefly (*Musca domestica* L.) is a widespread pest closely tied to human environments. It is commonly located in the vicinity of poultry, cattle, horse, pig, and dairy farms. The consistent presence of this pest in proximity of humans has facilitated its thriving in diverse ecosystems and the exploitation of various food sources. Moreover, its adaptability to several environments confer to this pest the credit of formidable adversary, able to evade, adapt to, and even develop resistance against the most steadfast control measures (Gogarten et al., 2019; Geden et al., 2021). The widespread use of insecticides (Wang et al., 2019) has led to the emergence of resistance in houseflies, emphasizing the imperative for safer and more sustainable alternatives, such as biological control (Ardburi and Tangkawanit, 2022). Sustainable management of houseflies might include various biological control methods such as application of microorganism , augmentation or release of natural and exotic predators and parasitoids (Geden et al., 2021).

For almost 50 years, naturally occurring pupal parasitoids mainly from the Pteromalidae family have been extensively used in augmentative biocontrol in animal facilities. However, it is often insufficient to depend exclusively on natural parasitoid populations to keep housefly populations within acceptable limits. This is mainly because development of parasitoids takes more time compare to that of their hosts (Geden et al., 2021). Nonetheless, augmentative releases of parasitoids could be successfully improved if integrated with other pest management methods as also highlighted by Geden et al., 1995, Skovgård and Nachman, 2004, and McKay et al., 2007. Among the diverse number of species capable of parasitizing house fly pupae, the most commonly commercialized are *Muscidifurax raptor* Girault and Sanders, *Muscifurax zaraptor* Kogan and Legner, *Muscidurax raptorellus* Kogan and Legner, *Spalangia cameroni* Perkins, *Spalangia endius* Walker, *Nasonia vitripennis* (Walker), and *Trichomalopsis sarcophagiae* (Gahan). Most of these species have similar life histories and reproduction. Female parasitoid locates a suitable fly puparium, drills through it, and lays one egg in solitary species or multiple eggs if gregarious (Gerling and Legner, 1968; Geden et al., 2021). The resulting parasitoid larva or larvae subsequently feed on the pupa, emerging as adults within two to four weeks. The developmental duration of the parasitoid, from egg to adult stage, varies from 14 to 30 days at warm temperatures and is influenced by several factors, including temperature, biotypes, host, species, sex, and environment (Birkemoe et al., 2012; Geden et al., 2021). One of the most effective parasitoid wasps that targets house and stable flies in livestock is *S. cameroni* Perkins (Birkemoe et al., 2012). The species is drawn to the scent of substrate that holds housefly larvae and is capable of parasitizing pupae at depths of up to 10cm (Machtinger et al., 2015). Though a solitary species, under laboratory conditions *S. cameroni* lays more than one egg in a single pupa(Gerling and Legner, 1968). Machtinger et al. (2015) indicated that *S. cameroni,* demonstrates a preference for young and fresh pupae.

Moreover, *S. cameroni* displays superior puparia locating abilities, resulting in a higher parasitization rate compared to *M. raptor*, as indicated by Legner's study (1967).

Another species of pupal parasitoid wasp, *M. zaraptor* Kogan and Legner, is used in programs aimed at managing housefly populations (Weinzierl and Jones, 1998). Similar to *M. raptor*, this species does not distinguish between fresh and frozen hosts (Floate, 2002), a positive characteristic for biocontrol purposes in term of mass production. Female *M. zaraptor* can identify parasitized hosts by inserting its ovipositor into the pupa to detect the venom that was injected by previous females. However, *M. zaraptor* females exhibit a preference for ovipositing on unparasitized pupae, rather than those that have been attacked by other females or species, including *N. vitripennis* and *S. cameroni* Perkins in laboratory condition (McKay and Broce, 2004). In contrast to *S. cameroni*, *M. zaraptor* females appear to solely respond to the odors emanating from housefly pupae and possibly utilize olfactory cues while seeking hosts (McKay and Broce, 2004).

The study had two primary aims: firstly, to evaluate the effectiveness of wasp parasitization over a defined two-week period, and secondly to explore the relationship between the age of female wasps and the production of parasites, with a specific focus on the gender of newly formed adult wasps. The research also aimed to assess the overall impact of the wasps in reducing housefly emergence. The research was conducted to examine the crucial role of wasps in the effective management of the housefly population through rigorous experimentation. The outcomes not only contribute to our understanding of the complex dynamics between wasps and houseflies, but also underline the practical importance of integrating wasps into pest management strategies.

5.3 Material and methods

5.3.1 Rearing of houseflies and parasitoids

The study was conducted at the Applied Entomology Laboratory of the Interdepartmental BIOGEST-SITEIA Center, University of Modena and Reggio Emilia. During the summer of 2020, pupae were collected from livestock farms in the Reggio Emilia area to establish colonies of houseflies (*M. domestica*), *S. cameroni*, and *M. zaraptor*. The adult houseflies were kept in polyester mesh cages measuring 32.5 x 32.5 x 32.5 cm, situated in a climatic chamber with a temperature of 27 ± 1°C, relative humidity of 60 ± 1%, and a 16-hour light and 8-hour dark photoperiod. They were given water, honey droplets, and a standard diet (described below) to facilitate egg-laying. Every 2-3 days, eggs were moved to enclosed containers, and after hatching, more diet was added consisting in 60 g of wheat *bran*, 40 g of alfalfa pellets, and 3 g of milk powder and water mixture. The diet provided was altered to conform to that of Bell et al., (2010).

The parasitoid species were reared in polyester mesh cages measuring 17.5 x 17.5 x 17.5 cm, located inside a climate-controlled room, with a relative humidity of 60 ± 1%, and a temperature of 25 ± 1°C.

They were supplied with water, honey droplets, and fresh or frozen *M. domestica* pupae for oviposition lasting 24-48 hours. Parasitized pupae were substituted every 3 days, and the newly emerged specimens were captured and returned to the cages after 25-27 days for *S. cameroni* and 17-22 days for *M. zaraptor*.

5.3.2 Design of the experiment

A male and female from each parasitoid species (aged no more than 24 hours) were paired in a containing honey drops for 48 hours to mate. The female was subsequently transferred to a sealed petri dish with 20 fresh pupae and honey drops, placed in a climatic chamber. Every 24 hours for a period of 14 days, the female parasitoid was removed and placed in a new dish with 20 fresh pupae. This procedure was repeated for 30 replicates of each species. A daily control group was established comprising of 20 housefly pupae in absence of the parasitoid. Neo-emergences of *M. domestica, S. cameroni* or *M. zaraptor* were counted every day, and the intact pupae subjected to dissection after 50 days.

5.3.3 Data collection

The trend of parasitization was calculated by counting the number of parasitoids emerged in each day for each species and then by calculating the rate of parasitization using the following equation:

Parasitization rate= (number of parasitized pupae) / (total number of pupae offered).

The sex ratio of F1 for each parasitoid species was determined by counting the daily numbers of females and males. The percentage of female-male emergence for each day and species was also calculated by using the following equation:

Percentage female emergence = (number of female emerged) / (total number of pupae) * 100.

The number of houseflies emerged in presence of parasitoids was also determined for each day and species. The percentage of housefly emergence was calculated dividing the number of emerged flies by the total number of pupae.

The mortality rate of female parasitoids for each species was calculated as a binary event, with mortality being considered as 1 and survival as 0. Similarly, the number of oviposition events was indicated as binary event 1, and 0 in the case of no oviposition. In the case of female mortality, the number of oviposition events was considered as NULL, rather than 0.

5.4 Data analysis

The statistical analysis was performed using R version 4.3.1 software. A significance level of $\alpha = 0.05$ was used for each test. After conducting a descriptive data analysis, we selected models with lower AIC values to fit the data. A generalized linear mixed model (GLMM) with a negative binomial distribution was fitted for the dependent variable, parasitization rate, and the independent variables, days and species. The variable sex ratio of F1 was considered for each species separately in relation to the days. The emergence of flies was dependent on the interaction between the independent variables' day and treatment (presence or absence of parasitoids). The models were fitted using the glmmTMB package, and the marginal means were calculated using the emmeans package. The mortality of the examined parasitoid females and the number of oviposition (successful parasitism resulting in F1 adults) for each species during the testing period were analyzed using a logistic regression model to determine the effects of species and time.

5.5 Results

5.5.1 Parasitization ratio

The proportion of parasitized pupae varied between the two species, as shown in Figure 27. For *M. zaraptor*, significant statistical differences were observed between days, particularly between day 2 (median value of 4.5 parasitized pupae, 18%) and days 3, 4, 5, 6 and 7 (median value of 7-10.5 parasitized pupae, 23-45 %) ($P < 0.05$). Additional differences were observed between day 2 and day 12 (median of 2.5 parasitized pupae, 13%) ($P = 0.01$), day 13 (median of 1.5 emerged parasitoid, 7.5%) ($P = 0.01$) and day 14 (range of 0-2 emerged parasitoids, 5%) ($P < 0.0001$) (Table 19, Figure 27). The parasitization trend of *M. zaraptor*, after day 2 (median of 18 % parasitized pupae), increased until day 7 (median of 10 parasitized pupae, 23%), but showed a decrease after day 11 (median of 5 parasitized pupae, 20%) (Figure 28, Table 19).

Throughout the experimental period, the parasitization ratio in *S. cameroni* showed relative stability, with a peak on day 5, when an average of 13.5 pupae were parasitized (median of 65%), compared to other days with median values between 40% and 55% ($P=0.03$) (Figure 28, Table 20).

Statistically significant differences in parasitization were observed between the two species ($P<0.0001$) (Table 21). Specifically the 5th day (median of 13.5 parasitized pupae, 65%) was the peak of *S. cameroni* than *M. zaraptor* (median of 7 parasitized pupae, 23%) ($P =0.02$) (Figure 28, Table 21). However, the differences between the two species were recorded also in 14th day corresponded as the lowest value of parasitization ratio for *M. zaraptor* (0-2 parasitized pupae, 0-10%) than *S. cameroni* (median value of 8 parasitized pupae, 40%) ($P<0.0009$) (Table 21, Figure 28).

5.5.2 Sex ratio of F1

The sex ratio in the F1 generation of new emergences for both parasitoid species was shifted towards females compared to males, as shown in Figure 29-30.

In the case of *M. zaraptor*, statistically significant differences between female and male progeny were observed for each day ($P > 0.05$), except for day 14 where no statistical difference was recorded, showing a range of 0-1 emerged parasitoids for both genera (0-5%) (Table 22). The number of emerged males remained relatively constant throughout the experiment, with only 1-2 males emerging ($P > 0.05$). In contrast, the trend for females changed significantly from day 12 to the end of the experiment (Figure 29). Differences in the number of emerged females were observed between day 2 (median of 2 emerged females-10%) and days 3-7 (median of 5-5.5 emerged females, 22-40%), as well as between day 12 (median of 2 emerged females-10%) and day 14 (range of 0-1 emerged females 0-5 %) ($P < 0.05$) (Figure 29, Table 22).

Throughout the experimental period, the rate of female emergence in *S. cameroni* remained relatively stable, reaching a peak on day 5 with a median of 10 [8.25-13] (50%) emerged females compared to other days (Figure 30). The trend of male emergence in *S. cameroni* remained constant throughout the experiment, with a median of 1-2 males emerging (5-10%) (Figure 29). Significant statically differences were recorded between females and males for each day of experiment ($P<0.0001$) (Table 23).

5.5.2 Dissection of pupae

In the dissection of pupae, immature stages of the parasitoids were not found for both species. The mortality of pupae might not be related to the parasitoids but by causal effect. No statical analysis was performed as no differences were found between the two species for each day of the experiment.

5.5.3 Emergences of houseflies

A decrease in the number of emerging houseflies in the presence of *M. zaraptor* was observed (Table 25). The median value was 5 emerged flies on day 5 and day 9 (25%) the lowest median value, while the increase of emergence occurred after day 11, with a median value of 12-13 houseflies (60-65%) (Figure 31, Table 25). Although the number of emerged flies increased from day 11 (median of 10.5) (52.5%) to day 14 (median of 13) (65%), statistically significant differences were observed compared to the control group (no parasitoid), which exhibited a median of 18 emerged flies for each day (90%) ($P < 0.05$) (Table 24).

For *S. cameroni*, the trend of housefly emergence remained relatively constant, with a minimum median of 4.5 emerged flies (22.5%) on day 6 and a maximum median value of 8 emerged flies (40%) on day 14 (Figure 31, Table 25). Statistically significant differences were found for each day when comparing the number of emerged flies in the presence of *S. cameroni* to the control group ($P < 0.05$) (Table 24).

Additionally, a comparative analysis between parasitoid species showed statistically significant differences in the number of emerged flies. On the second day, there were 9 emerged flies (45%) in the presence of *M. zaraptor* compared to 6 emerged flies (30%) in *S. cameroni* (P = 0.0001) (Table 25). On the 11th day there were 10.5 emerged flies (52.5%) in presence of *M. zaraptor* compared to 7 emerged flies (35%) in *S. cameroni* (P = 0.02) (Table 25). On day 12, there were 12 emerged flies (60%) in *M. zaraptor* compared to 7 emerged flies (35%) in *S. cameroni* (P = 0.008). On day 13, there were 12 emerged flies (20%) in *M. zaraptor* compared to 6 emerged flies (30%) in *S. cameroni* (P < 0.0001). On day 14, there were 13 emerged flies (65%) in *M. zaraptor* compared to 6 emerged flies (30%) in *S. cameroni* (P = 0.05) (Table 25).

5.5.4 Mortality rate of tested parasitoid female

The mortality rate was calculated separately for both species, with a focus on day 2 compared to the other days. This variable was also computed as a species fixed factor, with a significant statistical difference between species (P<0.0001). In *M. zaraptor*, mortality was significantly different between day 2 (no dead females) and day 11 (8 dead females) (P=0.05), with day 12 (8 dead females) (P=0.05) and with day 13 (11 dead females) (P=0.01). Additionally, a significant difference was observed between day 2 and day 14 (13 dead females) (P=0.003), which was the highest day of mortality for this specie (Table 26). A total of 13 female specimens died during the experiment, which had a statistically significant impact on the mortality rate of this species.

In *S. cameroni*, there was no statistically significant difference in mortality rates among the observed days (P>0.05). One female died on the 5th day and three females died on the 14th day. A total of 3 female specimens died during the experiment, which had no statistically significant impact on the mortality rate of this species.

5.5.5 Number of the oviposition events

The frequency of oviposition was considered as a binary event than the days comparing the two species, with a focus on day 2 compared to the other days. The frequency ovipositions was expressed as a binary event 1 in the case of ovipositions and 0 no ovipositions.

Statistically significant differences were observed in *M. zaraptor* between day 2 (27 oviposition events) and day 12 (16 oviposition events) (P=0.02). Additionally, statistically significant differences were observed between day 2 and day 13 (14 oviposition events) (P=0.03). Furthermore, day 14 (9 oviposition) was statistically different from day 2 (P=0.004) (Table 26).

Statistically significant differences were found between species comparing a total of frequency oviposition in *M. zaraptor* (295 oviposition events) to *S. cameroni* (325 oviposition events) (P=0.003). No statistically significant differences were reported for *S. cameroni* for this parameter among the days.

5.6 Discussion

During the experiments, single mated females were tested to avoid intraspecific competition. *M. zaraptor* demonstrated an increased tendency to parasitize in the first day few days after emergences, whereas *S. cameroni* showed almost constant parasitism. In the research conducted by Legner and Gerling, (1967) *S. cameroni* displayed a peak in oviposition on the fourth day, which differs from the five-day peak observed in our study. Furthermore, the trend of parasitization showed a decrease after the tenth day, in contrast to our study where the decrease was observed on the final days of the experiment. This variation may be attributed to differences in the population tested with different origins. As shown for the scelionid parasitoids of *Halyomorpha halys*, *Trissolcus euschisti*, and *Telenomus podisi*, different populations can exhibit different parasitization behaviour (Costi et al., 2020). In Legner and Gerling's study, the parasitization trend of *M. raptor* was similar to that of *M. zaraptor* in our study. Both species showed a decrease in parasitization after day 11 of the test. The level of parasitism recorded for *S. cameroni* was higher than for *M. zaraptor* throughout the experiment, in contrast to the results of Mann et al., (1990), where *M. zaraptor* had a higher number of parasitized pupae than *S. cameroni*. Indeed, at the peak of parasitism, the average number of parasitized pupae in our study was 8 for *M. zaraptor* and 11 for *S. cameroni*, compared to 9.3 and 5 respectively in Mann's study. The divergence in results may be related to competition between females. Although the ratio of parasitoids to pupae was the same as in the Mann study (1:20), in our tests two females simultaneously instead of one. In another study Morgan et al., (1989) found that the trend of parasitization in *S. cameroni* was influenced by time. In contrast to our results, in their study the trend of parasitization in this species decreased after day 6, with a peak on day 4 (Morgan et al., 1989). For the parameter sex ratio, in both species the ratio shifted towards females compared to males, but with different proportions depending on the number of parasitized pupae, as in the study by Floate in 2002. Analyzing their results, in *M. zaraptor* the number of F1 females was about 20.3 and 5.3 males on fresh pupae, while in *S. cameroni* it was 4.3 females and 2.0 males (Floate, 2002). In contrast to Floate's study, a higher number of newly emerged F1 parasitoids was observed in *S. cameroni* compared to *M. zaraptor*. Specifically, during the first two days of the experiment (48 hours, day 3), the average number of F1 females for *S. cameroni* was double that of females and the same for males, while for *M. zaraptor* there were 5 times fewer females and 4 times fewer males than in Floate's study. This could also be related to competition between females. In Floate's 2002 study, they tested 2 females rather than individuals, with a parasitoid-host ratio of 1:15. *M. zaraptor* produced more offspring in conspecific competition than *M. raptor* (King and Seidl, 1993) and *S. cameroni* (King, 1996). This suggests a discrepancy between our results and those of other studies. The proportion of females for *S. cameroni* was similar to *M. raptor* in fresh pupae (Geden and Kaufman, 2007) with a percentage of 63.8 in 24 h at a parasitoid-host ratio of 1:10, lower than our study for *S. cameroni* but similar to *M. zaraptor*. Another parameter that influences the sex ratio is the number of pupae per female parasitoid.

In *Spalangia endius,* higher host densities increase the number of parasitized pupae, whereas in *Muscidifurax raptor* parasitization decreases with higher host densities (Ables and Shepard, 1974). This is in agreement with our study and explains that in *M. zaraptor* the emergence of parasitoids in the ratio 1:20 parasitoid-host (our study) was lower than the 1:15 parasitoid-host in Floate's study, suggesting that a higher density in pupae reduces the number of parasitized pupae. It is noteworthy that *M. zaraptor* did not have a single peak, but a range of different oviposition peaks than *S. cameroni.* The production of two or more female peaks by *M. zaraptor* instead of one peak increases the likelihood that some offspring will find hosts of optimal age, while reducing the number of females searching for hosts at any given time (Coats, 1976). An alternative explanation for this production, which may depend on the cyclic maturation of ovarioles, does not fully explain why males were not produced in peaks. It is possible that the cyclic production of females, but not males, occurs to limit the competition between females competing for hosts (Coats, 1976). Competitive abilities are more important for this species than a high reproductive rate (Coats, 1976). During the last days of the experiment, the mortality rate of female *M. zaraptor* was higher than that of *S. cameroni*, where the mortality was only 10 % after 14 days. The time of mortality *of S. cameroni* in Morgan et al., (1989) was 3.0, 8.77, and 11.4 days for 50%, 90%, and 95% of the parasitoids, respectively. This is in contrast to our study, where the mortality of *S. cameroni* was not affected by the number of days. For *M. zaraptor*, mortality was found to be influenced by the age of the females during days 13 and 14 of the experiment. This is partially consistent with Coats' study where adult survival decreased after day 11 (Coats, 1976). In line with Coats' study, a significant decrease in oviposition was recorded after the 12th day (Coats, 1976). The emergence of houseflies in the presence of parasitoid species was higher in *M. zaraptor* than in *S. cameroni,* according to the parasitization rate. The highest percentage of housefly emergence for *M. zaraptor* was recorded in the last days of the experiment, corresponding to the lower parasitization rate. In *S. cameroni*, the emergence of houseflies was almost constant over the whole period as far as the parasitization trend is concerned. In both species, the presence of female parasitoids had reduced the emergence of houseflies compared to the control. Pupal parasitoids may be effective in suppressing the fly population in biological control program because of their ability to reduce the population of houseflies. The true potential of parasitoids as biological control agents can be assessed after field trials (Malik et al., 2007). The effectiveness of these parasitoids in the field differs from their performance under laboratory conditions because of environmental factors that influence parasitoid abundance, host location survival and distribution (Skovgård and Nachman, 2004). Parameters such as sensitivity to insecticides, use of low quality commercial colonies, microhabitat preferences, host availability and lack of optimal timing and methods of release (Petersen and Meyer, 1985; Machtinger et al., 2015) must be taken into consideration. Furthermore, fly immigration from neighboring livestock areas can rapidly increase fly populations (Machtinger et al., 2015). A monitoring program should be established to assess fluctuations in fly populations, to aid decisions on when to implement additional pest management strategies and to evaluate the effectiveness of the pest management program.

Monitoring records can be maintained and used to anticipate increases in fly populations in subsequent years (Machtinger et al., 2015).

5.7 Conclusion

The results of our investigation indicate that female parasitoids exhibit a decline in their parasitization capacity with each passing day. This implies that aged parasitoids produce fewer offspring over time. However, this decline did not result in a proportional increase in fly emergence compared to the control group, which is a significant finding. A reduction in flies was observed, but with significant variations between days. These results emphasize the need to consider not only the age of female parasitoids, but also the period of observation, as such dynamics may influence the overall effectiveness of fly population management. In addition to the age-related dynamics of female parasitoids, it was found that the sex ratio favors females relative to males. Despite a decline in female parasitization ability over time, the predominance of females may still be significant for managing the emerging fly population. Further research should investigate the complex relationship between sex ratio, age of female parasitoids, and their effectiveness, taking into account other individuals. Investigating intra- and interspecific competition dynamics, as well as the potential influence of males on these factors, would be beneficial. Investigating how parasitoids' interactions with other individuals, of the same or different species, influence their efficacy in regulating the housefly population may provide a more comprehensive picture. This investigation could provide insights into optimizing biological control strategies by considering not only the intrinsic characteristics of parasitoids, but also their interactions within a more global ecological context.

Author Contributions: Conceptualization, S.D, E.C.; Methodology, S.D, L.P, T.Y; Formal Analysis, S.D, E.C; Writing – Original Draft Preparation, S.D.; Writing – Review & Editing, All authors.; Supervision, L.M; Project Administration, L. M. Funding Acquisition, L.M.

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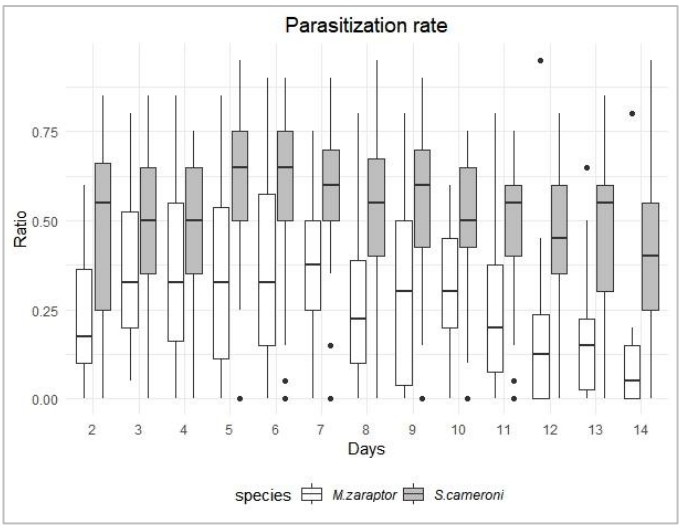

Figure 28. Trend of parasitization for *M. zaraptor* (white) and *S. cameroni* (grey)during a two-weeks experiment with the daily number of emerged parasitoids for each species. In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate 1.5× the interquartile range, and the dots are outliers.

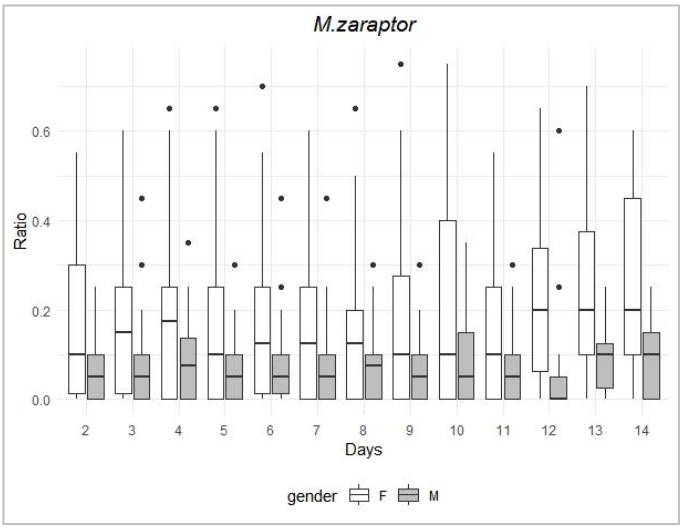

Figure 29. Ratio of F1 generation emergences for *M. zaraptor* during a two-weeks experiment with number of emerged parasitoids for female (white) and for male (grey). In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate 1.5× the interquartile range, and the dots are outliers.

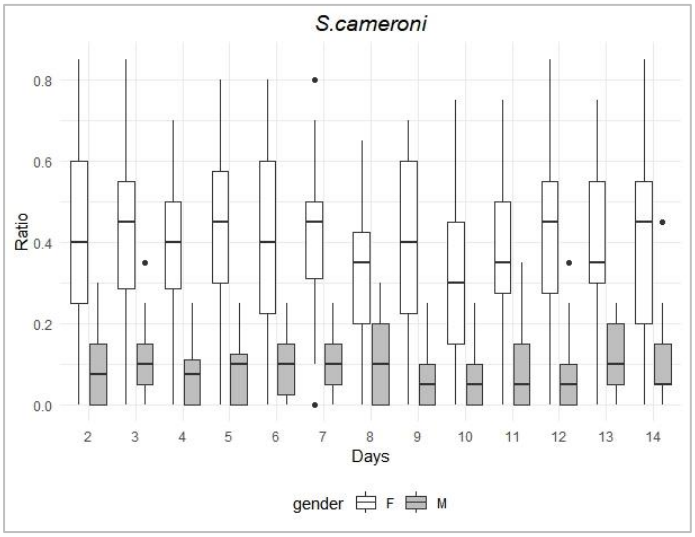

Figure 30. Ratio of F1 generation emergences for *S.cameroni* during a two-weeks experiment showing the number of emerged parasitoids belong to female (white) and male(grey). In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate 1.5× the interquartile range, and the dots are outliers.

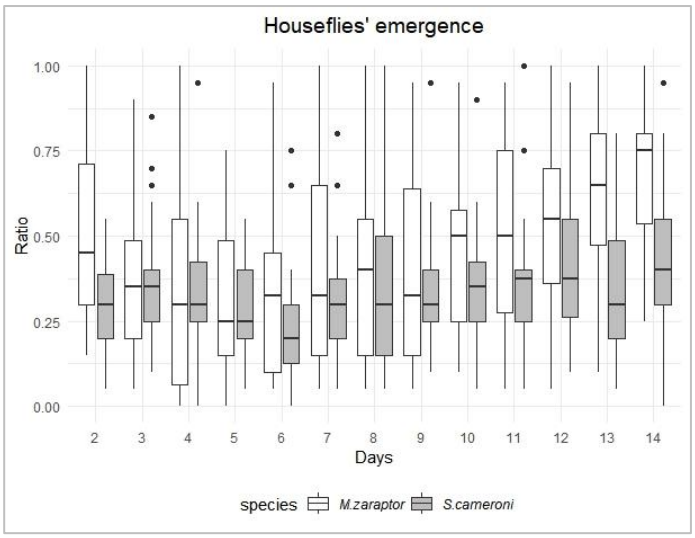

Figure 31. Trend of houseflies emergences in presence of both parasitoids species during a two-weeks experiment showing the number of emerged houseflies belong to *M.zaraptor* (white) and *S.cameroni*(grey). In the boxplots the boxes indicate the first and third quartile, the thick line in between shows the median, whiskers indicate 1.5× the interquartile range, and the dots are outliers.

Table 19 Parasitized pupae by *M. zaraptor*, expressed as the median of the proportion of parasitized pupae and the interquartile range in squares. The GLMM's estimate and p-value in bold, in case of P<0.05, are reported to compare the parasitization ratio between day 2 and other days.

Days	*M.zaraptor*	Estimate	P
3	9 [4.75-12]	0.4	**0.01**
4	10.5 [6-12]	0.41	**0.01**
5	7 [5-12]	0.36	**0.03**
6	9 [5.75-12.25]	0.42	**0.01**
7	10 [5.75-12.25]	0.45	**0.006**
8	6 [3.75-8.75]	0.1	0.57
9	8.5 [2-12]	0.22	0.21
10	6 [4.75-10]	0.2	0.25
11	5 [2.75-9]	-0.14	0.48
12	2.5 [0-4.25]	-0.59	**0.01**
13	1.5 [0-4]	-0.58	**0.01**
14	0 [0-2]	-1.25	**<0.0001**

Table 20 Parasitized pupae by *S.cameroni*, expressed as the median of the proportion of parasitized pupae and the interquartile range in squares. The GLMM's estimate and p-value in bold, in case of P<0.05, are reported to compare the parasitization ratio between day 2 and other days.

Days	*S.cameroni*	Estimate	P
3	10 [7-13]	-0.04	0.69
4	10 [7.25-13]	0.01	0.87
5	13.5 [10.25-15]	0.24	**0.03**
6	13[10.25-15]	0.15	0.2
7	12 [10-14]	0.11	0.34
8	11.5 [8.25-13.75]	0.12	0.28
9	12[9.25-14.5]	0.16	0.16
10	10.5 [9-13]	0.04	0.7
11	11 [8-12]	-0.004	0.96
12	9.5 [7.25-12]	-0.04	0.74
13	11 [6-12]	-0.05	0.64
14	8 [4.25-11]	-0.23	0.08

Table 21 Parasitization rate by M.*zaraptor* compared between day 2 and other days. GLMM's estimate and p- value in bold in case of P<0.05, are reported to compare rate of parasitization within days between the two parasitoid species.

	Parasitization rate	
Fixed effects	Estimate	P
Day 3	0.12	0.26
Day 4	0.15	0.17
Day 5	0.25	**0.02**
Day 6	0.17	0.12
Day 7	0.18	0.09
Day 8	0.09	0.41
Day 9	0.14	0.2
Day 10	0.09	0.43
Day 11	-0.04	0.7
Day 12	-0.2	0.12
Day 13	-0.17	0.18
Day 14	-0.47	**0.0009**
S.cameroni	0.6	**<0.0001**

Table 22 Median (+ interquartile range in parentheses) of the number of female and male parasitoid emerged in each day in a two-weeks period of parasitization by *M. zaraptor*. GLMM' s estimate and p- values are reported in bold in the case of P<0.05, as results of the comparison of the sex ratio between days in *M. zaraptor.*

Specie	Day	Females	Males	Estimate	P
M.zaraptor	2	2 [1-3.75]	1 [1-2]	0.77	**0.002**
	3	5 [2-7]	2 [1-2]	0.98	**<0.0001**
	4	5 [2.25-9]	2 [0.25-3]	0.78	**0.0002**
	5	3 [1-6]	2[0-2.75]	0.71	**0.001**
	6	4.5 [2-7.75]	2 [0.25-3]	0.89	**<0.0001**
	7	5.5 [2-9]	1 [0-2.75]	1.28	**<0.0001**
	8	3 [1-5]	1 [0-2]	0.84	**0.0004**
	9	4 [0.75-7]	2 [0-3]	0.76	**0.0011**
	10	4 [2-6]	2 [1-2]	0.65	**0.004**
	11	3 [0-4]	1 [0-2]	0.67	**0.01**
	12	2 [0-3]	1 [0-1]	0.7	**0.03**
	13	1 [0-4]	0 [0-1.25]	1.16	**0.006**
	14	0 [0-1]	0 [0-1.5]	-0.09	0.83

Table 23 Median (+ interquartile range in parentheses) of the number of female and male parasitoid emerged in each day in a two-weeks period of parasitization by *S. cameroni*. GLMM' s estimate and p- values are reported as results of the comparison of the sex ratio between days in *S.cameroni* in squares. Estimate was the estimate of the fitted model and P, p-value < 0.05 as the result of comparison in case of significant statistical difference.

Specie	Day	Females	Males	Estimate	P
S.cameroni	2	8 [5.5-12]	2 [0-3.5]	1.38	**<0.0001**
	3	8 [6-10]	1 [0.5-2]	1.61	**<0.0001**
	4	9 [6.5-12]	1 [0-2]	1.98	**<0.0001**
	5	10 [8.25-13]	2 [1-3]	1.51	**<0.0001**
	6	9 [6.5-13]	2 [1-3]	1.36	**<0.0001**
	7	10 [6.25-11.75]	2 [1-3.75]	1.47	**<0.0001**
	8	8.5 [6.25-11.75]	2 [1-3]	1.4	**<0.0001**
	9	9 [7-10.75]	2 [1-3]	1.28	**<0.0001**
	10	9 [6.25-11]	2 [1-2]	1.62	**<0.0001**
	11	8.5 [6-9.75]	2 [1-3]	1.24	**<0.0001**
	12	7 [4-9.25]	2 [1-4]	1.13	**<0.0001**
	13	7.5 [5-10]	2 [0.25-3]	1.41	**<0.0001**
	14	6.5 [4-9.25]	1.5 [0-3]	1.41	**<0.0001**

Table 24 Houseflies emergence in the control (absence of parasitoids) compared to presence of *M.zaraptor* and *S.cameroni*. The GLMM estimate and p-value in bold in case of P<0.05, are reported to compare the emergence of houseflies between day 2 and other days for all groups.

	Houseflies emergence	
Indipendent variables	Estimate	P
M.zaraptor	-0.78	**<0.0001**
S.cameroni	-0.97	**<0.0001**
day 3	-0.06	0.4
day 4	-0.06	0.36
day 5	-0.12	0.09
day 6	-0.12	0.09
day 7	-0.03	0.66
day 8	-0.003	0.95
day 9	-0.07	0.29
day 10	0.02	0.7
day 11	-0.006	0.93
day 12	0.14	**0.04**
day 13	0.12	0.08
day 14	0.16	**0.02**

Table 25 Median (+ interquartile range in parentheses) of houseflies daily emerged from pupae parasitized by *S. cameroni* and *M. zaraptor* in a two-weeks period of parasitization. GLMM' s estimate and p- values in bold in case of P<0.05, are reported as results of the comparison of the number of houseflies emerged between the two parasitoid species within the same day of parasitization.

Days	N emerg. houseflies with *M.zaraptor*	N emerg. houseflies with *S.cameroni*	Estimate	P
2	9 [6.5-15]	6[4-7]	4.39	**0.0001**
3	7 [4-11]	7 [5-8]	0.21	0.84
4	6 [1.5-10]	6 [5-8]	0.153	0.88
5	5 [3-8.75]	6 [3-8]	0.57	0.6
6	6 [2-9]	4.5 [3-6.5]	0.87	0.42
7	6 [3-13]	6 [4-7]	1.16	0.29
8	8 [3-11]	6 [3-10]	1.3	0.23
9	5 [3-12.25]	5.5 [5-7.5]	1.23	0.27
10	10 [6-11]	6.5[5-8]	1.67	0.15
11	10.5 [6-15]	7 [5-8]	2.74	**0.02**
12	12 [7.75-14]	7 [5-10]	3.149	**0.008**
13	12 [9.25-14.5]	6 [4-10]	5.78	**<.0001**
14	13 [3.75-16]	8 [6-11]	2.48	**0.05**

Table 26 Number of oviposition (binar events) and mortality rate (cumulative sum of binar events as 1) by *M. zaraptor* comparing with *S.cameroni* .*The* GLMM's estimate and p-value in bold, in case of P<0.05, are reported to compare the parameters between day 2 and other days.

	Mortality rate		**N oviposition**	
Fixed effects	Estimate	P	Estimate	P
S.cameroni	-0.17	0.99	-1.17	0.32
day 3	-0.17	0.99	14.27	0.98
day 4	-0.17	0.99	-1.42	0.21
day 5	-0.17	0.99	-1.09	0.35
day 6	-0.17	0.99	-0.65	0.6
day 7	-0.17	0.99	-0.65	0.6
day 8	-0.17	0.99	-1.68	0.13
day 9	0.0004	1	-1.17	0.32
day 10	1.03	0.24	-1.3	0.27
day 11	1.67	**0.05**	-2.01	0.07
day 12	1.67	**0.05**	-2.46	**0.02**
day 13	2.093	**0.01**	-2.44	**0.03**
day 14	2.37	**0.003**	-3.17	**0.0048**
Day 3:*S.cameroni*	0.17	0.99	-13.82	0.98
Day 4:*S.cameroni*	0.17	0.99	1.86	0.21
Day 5:*S.cameroni*	0.03	0.99	1.5	0.35
Day 6:*S.cameroni*	0.17	0.99	0.65	0.66
Day 7:*S.cameroni*	0.32	0.99	0.61	0.68
Day 8:*S.cameroni*	0.32	0.99	2.82	0.08
Day 9:*S.cameroni*	0.17	0.99	2.31	0.16
Day 10:*S.cameroni*	0.16	0.99	1.7	0.26
Day 11:*S.cameroni*	0.15	0.99	2.01	0.15
Day 12:*S.cameroni*	0.16	0.99	2.83	**0.05**
Day 13:*S.cameroni*	0.51	0.99	3.58	**0.02**
Day 14:*S.cameroni*	0.16	0.99	3.05	**0.03**

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6. Conclusions

Houseflies are considered pests because they can carry out many pathogens and cause nuisance to both humans and livestock. Therefore, there is a growing need to develop appropriate control strategies. In Italy and other parts of the world, insecticide control is a widely used method. However, this type of management can become ineffective in a short time as flies can develop resistance to insecticides within a few generations. To overcome the resistance issue, we conducted a behavioral study to test how flies of a resistant California strain responded to different concentrations of imidacloprid, an insecticide widely used in the USA. The aim was to determine the impact of behavioral resistance on responses (proboscis extension) when flies were exposed to imidacloprid through tarsal or proboscis contact. The study showed that resistant flies could discriminate insecticide concentrations within 2 seconds with their proboscis, but not with their tarsi. This finding is significant in the field of resistance because it provides an understanding of how flies behave in the presence of insecticides. Rotating the use of different types of insecticides has been proposed as a potential solution to reduce resistance. This study is the first to investigate the resistance of California flies in terms of their behavior and the systems involved in discriminating the concentration of imidacloprid.

There is a lack of data on the abundance of houseflies and their natural enemies, the pupal parasitoids, in dairy farms in the Parmigiano Reggiano production area in Italy. The aim of my study was to estimate the level of infestation by houseflies and pupal parasitoids, in dairy farms in this area. Two methods of housefly management were compared: organic, which used biological control with pupal parasitoids, and conventional, which used insecticides. The levels of infestation were found to be comparable between farms with different management. Therefore, it is important to consider not only the type of control but also the cleaning practices and organization of the farms. Additionally, flies can travel for long distances and can arrive from neighbors, which is another crucial aspect to consider in housefly management programs.

Considering the abundance of pupal parasitoids, *Muscidifurax* sp. was found mainly in farms where it was released for biological control, whereas *Spalangia* sp., was found in all farms, with a higher prevalence in conventional ones, where it was not released as a biological control agent. The presence of these parasitoids has not reduced the emergence of flies in both typology of farms, suggesting that there is a need to increase the parasitoids populations so that they can effectively reduce fly infestations to acceptable levels.

There was a lack of knowledge regarding the characterization and identification of pupal parasitoid species. We performed a molecular and taxonomic characterization of the pupal parasitoid species present in dairy farms, as well as those marketed by private companies. Our study found that the population of *Muscidifurax zaraptor* from private companies was the same as that of conventional farms where these populations had never been released.

However, the most abundant population in all farms differed from those in commercial production. *Muscidifurax raptorellus*, which was present in both commercial bags provided by companies in the USA and Italy, was not found in the farms. However, *M. raptor* was found in one organic farm where this species had never been released. *Spalangia sp*. was found predominantly in one conventional farm, with the same population network as *Spalangia cameroni* in the US commercial bag. The presence of *Spalangia nigroaenea* in this conventional farm represents the first record of this species in Italy. However, prior to this study, the wild populations of these parasitoids in conventional farms in Italy were unknown.

When comparing the parasitization behavior of different haplotypes and species, it is noteworthy that some behaviors, such as acceptance latency, varied between different haplotypes of the same species, while others, such as drumming activity, remained the same for the same genus. Within a short period of time, the number of parasitized pupae was similar between species and populations, but differences were found when comparing the species over a two-week period. An experiment was conducted to compare the efficiency of *Muscidifurax zaraptor* and *Spalangia cameroni* over a period of time. The results showed significant differences between the two species. The age of the *M. zaraptor* females affected the parasitism rate compared to *S. cameroni*, which remained almost constant over the two weeks. Under controlled conditions, the presence of parasitoids led to a drastic reduction in the emergence of house flies compared to the control. This work confirms the potential reduction of the fly population using pupal parasitoids. This information can aid in the implementation of an integrated pest management plan for house flies in Italy, considering the population of parasitoid species already present in these areas and their varying efficacy over time. Understanding parasitoids behavior and its temporal influence can provide valuable information for biocontrol programs, providing positive feedback and avoiding competition between species. It is crucial to have a comprehensive understanding of the different situations so that timely action can be taken to reduce the disturbances caused by flies in affected areas.

Overall, this work provides findings that fill gaps in the knowledge of insecticide resistance developed by house flies and in the characterization of the species and populations of pupal parasitoids from farms in the Parmigiano Reggiano production area, using taxonomic, molecular, and behavioral approaches. All together, these results are useful for the implementation of sustainable management of house flies.

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