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A holistic approach for the investigation of bee health

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Abstract

Beekeeping is defined by the Italian legislation (law n. 313, December 24th, 2004) as the zootechnical conduction of honey bees (*Apis mellifera*). The importance of this activity is not limited to delivering hive products but also to environment protection and agriculture through the act of pollination. For this reason, it is not surprising that honey bees issues have drawn so much attention by the media.

Despite current evidence is in strong disagreement with an emergency for the conservation of the species, great concern is related to profitability of beekeeping operations and thus for the positive externalities coming from them. A growing involvement of veterinary science in addressing bee health topics will therefore be fundamental to preserve and protect the entire sector.

With these premises in mind, the experiments in this thesis focused on two different and interdependent levels related to bee health: the biochemical level and the parasitological level. At the biochemical level the impact of plant protection products on bee physiology and survival was studied, elucidating synergistic interactions between poor nutrition and pesticide exposure in *A. mellifera* and between an insecticide and a fungicide in *Osmia bicornis*. These trials are particularly relevant for an evidence-based discussion of the registration process of new agrochemicals. Moreover, an innovative fingerprinting approach on honey bee haemolymph was applied to detect population imbalances in the hive. The sampling process and the subsequent analysis with an algorithm could be implemented in monitoring projects and, with specific training, also in the public veterinary service.

The control of *Varroa* infestations was studied both at the biochemical and parasitological level. A panel of biomarkers in honey bee haemolymph was applied to compare different mite control protocols. This resulted in relevant indications for beekeeping operations pursuing the least impact on nutritional status of the colonies. To guide the decision making of beekeepers, a new formic acid evaporator was tested in comparison with a more established one.

Considering its widespread distribution in the country, efforts were directed also towards *N. ceranae* an emergent pathogen with an impact on beekeeping still under debate. In

particular, the pivotal aspect of diagnosis was studied, proposing a new qPCR method to overcome some limits of the existing ones.

In conclusion, this works fills some of the knowledge gaps of the beekeeping sector. However, many of them still need to be addressed and the upcoming menaces of climate change and dispersal of pathogens via globalization should be targeted by research efforts in the near future. Therefore, a multifaceted vision of bee health is of capital importance, aware of the complementarity of reductionist and holistic approaches.

Chapter 1: Prelude

Introduction

Beekeeping is defined by the Italian legislation (law n. 313, December 24th, 2004) as the zootechnical conduction of honey bees (*Apis mellifera*). However, the general importance of this activity is highlighted in the law text by citing its importance in environment protection and agriculture through the act of pollination. Gallai et al. (2009) showed that at least 80% of the 264 plants cultivated in the European Union relies on insect pollination with an estimated value of 153 billion €. Even considering that honey bees are not "one size fits all" pollinators (National research council, 2007) they are undoubtedly the most important for monocultures (Delaplane & Mayer, 2000).

For this reason, it is not surprising that honey bees decline has drawn so much attention by the media. But is it really the case? This is a fairly complicated issue that deserves an analysis free of oversimplifications. Considering the data of the FAOSTAT database (http://www.fao.org/faostat) the number of beehives all over the world raised from under 50 million in 1961 to over 90 million in 2017. This is enough evidence to rule out the "beepocalypse" argument. However, this growing trend is not generalized in all continents. The serious decline in managed honey bees, in the same time frame, seen in Europe (from 21 million colonies to 19 million colonies) and North America (from under 6 million colonies to about 3.5 million colonies) is concealed globally by the growth of other continents. Specifically, South America (from about 1.5 million colonies to over 5 million colonies), Africa (from under 9 million colonies to about 18 million colonies), Oceania (from about 0.5 million colonies to 1.2 million colonies) and, most notably, Asia (from 10 million colonies to over 40 million colonies). The long-term declines observed in Europe and North America are most likely related to political and socioeconomic drivers (K. M. Smith et al., 2013). When U.S.S.R collapsed, soviet countries of eastern Europe experienced a cut in financing and this led to a 50% reduction in honey bee stocks (Aizen & Harder, 2009). On the other hand, western countries suffered the competition of cheaper alternatives like imported honey and beet sugar (Potts et al., 2010). Similar difficulties can be found in North America, with competition from imported honey and cuts in government support as main drivers of the decline (vanEngelsdorp & Meixner, 2010).

However, the phenomenon of colony losses must be put into context in order to have an insight in the current situation of beekeeping industry. Each year, some colonies die and

must be replaced in order to keep stable the stock. To do so beekeepers usually “split” healthy colonies. Therefore, measuring colony losses with a yearly survey could lead to a strong bias since beekeeping operations suffering losses could simply compensate and mask the event by weaken remaining colonies. This bias is overcome by considering only colonies failing to overwinter since these losses take place in a period where restock by splitting is impossible, at least in temperate climates. The extent of the colony losses phenomenon is quite variable, and its comprehension undermined by the abovementioned beekeeping practises and by the lack of standardization in the surveys throughout the world. The recent work of Gray et al. (2019) summarizes the results of the COLOSS survey obtained in winter 2017/2018 from 36 countries (33 in Europe). The overall winter loss rate reported is 16.4% (95% CI 16.1–16.6%). The losses in Italy are almost double the European average, 29.4 % (95% CI 27.2-31.7%). Higher losses are also reported by (Kulhanek et al., 2017) regarding 2015-2016 in the USA with a percentage of 26.9% (95% CI 26.4–27.4%). On the basis of these different values, the relevant question is: “To what extent losses are considered acceptable?”. Different answers can be given. In the USA, beekeepers-defined acceptable losses ranged from 13.2 to 19.1 (Kulhanek et al., 2017), while in a recent epidemiological study in Europe, a 10% threshold was chosen (Chauzat et al., 2016). Considering that managed colonies are virtually immortal through the practice of requeening, it is obvious that the acceptable losses are an economic parameter rather than a biological one.

The sustainability of losses is cause of concern not only for beekeepers but also for farmers relying on pollination and therefore for global population gaining food from this sector. It has been calculated that the need for pollination is growing much more rapidly (>300%) than the global domesticated honey bee population (Aizen & Harder, 2009). This hypothesis is confirmed by (Breeze et al., 2014) that highlighted that honeybee stocks are inadequate to supply 90% of demands in 22 over 41 European countries studied. Cabbri & Baracani (2018, unpublished data) used a similar approach to study the situation in Italy, obtaining a slightly different result than (Breeze et al., 2014). The latter calculated a pollination service capacity of 81% while Cabbri & Baracani a pollination service capacity of 55%. This discrepancy is related to the number of beehives/hectar needed for each culture; Cabbri & Baracani obtained the data froma a restricted geographical area, while Breeze et al. from an area as large as the USA. In conclusion, current evidence is in strong disagreement with an emergency for the conservation of the species. Rather, great

concern is related to the profitability of beekeeping operations and for the positive externalities coming from them.

The aim of this work is to investigate, both at the biochemical and parasitological level, some of the issues of beekeeping sector. In order to introduce the reader to this intricate scenario, current knowledge about them is summarized in the following paragraphs.

Varroa

Varroa is an obligatory ectoparasitic mite, living in honey bee colonies and reproducing in sealed brood. Initially recognized as *Varroa jacobsoni*, it was reported outside its natural range (Southeast Asia) in 1949, and it is now widespread all over the world except Australia and some minor islands. Considering its high pathogenicity and its distribution range it is considered as one of the main threats of apiculture (Rosenkranz, Aumeier, & Ziegelmann, 2010). The shift from its natural host (Apis cerana) to A. mellifera, took probably place in Asia during the first decades of the twentieth century (Matheson, 1995). This process was facilitated by the presence of the European honey bee in Asia, imported for its higher productivity compared to the native one. The current taxonomy of *Varroa* was established only in 2000 when Anderson & Trueman (2000) demonstrated that among the 18 haplotypes of *V. jacobsoni*, only 2 of them (Japanese and Korean) were able to reproduce on *A. mellifera*, justifying the creation of a new species, *Varroa destructor*. The first official appearance of *V. destructor* in Italy dates back to 1981, near the Slovenian border (Barbattini, 1981) and it rapidly spread all over the peninsula.

Life cycle

The life cycle of *V. destructor* can be divided in two distinct phases. The phase on adult bees, previously named “phoretic phase” until Ramsey et al. (2019) demonstrated that mites can actively feed during this stage. The other phase takes place inside brood cells, invaded when the larva is at the fifth stage (L5) (Rosenkranz et al., 2010). The pivotal identification of this stage is mediated by pheromones produced by the larva itself (Aumeier, Rosenkranz, & Francke, 2002). Since these pheromones benefits an individual of another species harming at the same time the emitter, they can be classified as kairomones. Seventy hours after capping, the mite lays a first haploid egg and afterwards a diploid egg every 30 hours; from the first unfertilized egg will hatch a male, from the

subsequent fertilized ones only females (Ifantidis, 1983). The immature forms of the mite feed on the pupae exploiting the wound made by the foundress (Donzé & Guerin, 1994).
When the male is mature, it fertilizes the progressively mature sister, mating multiple times until their spermatheca is full (Donzé & Guerin, 1994). The number of fertilized females is variable and it has been estimated to be, on average, 1.3-1.45 in female brood and 2-2.2 in drone brood (Martin, 1994).
The reason of the increased fertility in male brood is related to the longer pupation time compared to worker brood. Given this important reproductive advantage it is not surprising that a strong preference of foundresses for male brood has been elucidated: infestation rates of male brood is 8 to 10 times higher than the infestation on female brood (Boot, Driessen, Calis, & Beetsma, 1995; Calderone & Kuenen, 2001; Fuchs, 1990).
Interestingly, queen larva are rarely parasitized, probably because of the higher content of ottanoic acid in royal jelly, a compound with a strong repellent action towards the mites (Nazzi et al., 2009).

Population dynamics

The population dynamics of this parasite is strictly related to the host. It has been elucidated a role of climate and food supply, acting on the honey bee colony and, indirectly, on *Varroa* (Currie e Tahmasbi, 2008). In temperate climates, it is possible to approximate a doubling of the mite population each month, if brood is present (Calis, Fries, & Ryrie, 1999). The collapse of the colony can take place at any moment of the year, however the highest toll of *Varroa* infestation is paid in late summer/autumn. At this time of the year the mite can compromise the transition from short-lived summer bees to long-lived winter bees (Amdam et al., 2004), jeopardizing the surviving of the entire colony. However, the mite population inside the hive is influenced also by interaction with other hives via robbing or drift (Goodwin, Taylor, Mcbrydie, & Cox, 2006). Recently (Frey & Rosenkranz, 2014) measured the impact of density of neighbouring colonies on *Varroa* infestation, concluding that the infestation at the end of the year was significantly higher in apiaries with high density of neighbouring colonies. The most probable route of reinfestation is trough robbing of dying colonies by strong healthy colonies (Rosenkranz et al., 2010).

Pathogenicity

The pathogenicity of *Varroa* can be studied at the individual level and at the colony level. At the individual level, the negative effect of the mite is mainly related to the depletion of fat body during the pre-imaginal stages (Ramsey et al., 2019). This action leads to a lowered weight adult bee, associated to precocious foraging and reduced longevity (Amdam et al., 2004). More subtle effects include a reduced cognitive ability of workers (Kralj & Fuchs, 2006) and reduced flight capacity and semen production in drones (Duay, De Jong, & Engels, 2002). Unfortunately, these direct damages are coupled with those related to the virus conveyed by the mite. During feeding the mite can transmit different viruses to the host: Sacbrood virus (SBV), Kashmir virus (KBV), Israeli acute paralysis virus (IAPV), Acute paralysis virus (ABPV) and, most notably, deformed wing virus (DWV) (Boecking e Genersch, 2008). Although these viruses are normally present in the hive, the mite facilitates the infection by injecting them directly into the hemocoel of honey bee. Moreover, the transition from covert to over infection is facilitated by the immunosuppression induced by the mite on parasitized pupae (Yang & Cox-Foster, 2007). Near collapsing colonies are characterized by symptoms as scattered brood, high number of bees with crippled wings, supersedure of queens, absconding (Shimanuki, Calderone, & Knox, 1994). These symptoms are frequently summarized in the "parasitic mite syndrome". The collapse threshold is related to the mite to honey bees ratio and 30% has been proposed (Fries et al., 2003; Rosenkranz et al., 2010).

Control

Without proper intervention, colonies collapse in 3 to 4 years in temperate climates (Korpela, Aarhus, Fries, & Hansen, 1996); therefore beekeepers need to treat beehives in order to keep them healthy and productive. Usually two acaricidal treatments per year are enough to achieve this goal but further treatments could be needed according to different epidemiological conditions, as also recently stated by a note of the Italian Ministry of Health (n. 8845/2019). The autumn/winter treatment should exploit the absence of brood (where the climate is sufficiently cold to induce brood interruption) since this condition makes the mites much more vulnerable, as they are all on adult bees. This treatment has the aim of reducing as much as possible the mite number in the colony in order to reach the summer treatment without damage. In our latitudes the summer treatment should be administered within the first weeks of August to anticipate, and thus allowing, the rearing of the long-lived winter phenotype.

To date, synthetic and organic drugs are available for beekeepers. The first have the longest history and the active ingredients amitraz, fluvalinate and flumethrin are still on the market in Italy. Organic drugs are represented by organic acids (lactic, formic, oxalic) and thymol. The use of oxalic acid based treatments can be extended to summer if the absence of brood is obtained. In the work in chapter 4 (Cabbri et al., 2018), we compared two different techniques to achieve this, using colony level traits and biomarkers in haemolymph. Another organic option for summer treatment is formic acid aqueous solutions provided in appropriate evaporators. In chapter 5 we tested a new evaporator (Aspro-Novar-Form®) comparing its efficacy and tolerability to those of the long known Nassenheider professional evaporator.

Nosema

Nosemosis, is caused in honey bees by two species of microsporidian fungus: *Nosema apis* and *Nosema ceranae* (Higes et al., 2008). *N. apis* was described in *A. mellifera* by Zander in 1909 while *N. ceranae* was first detected in *A. cerana* (Fries et al., 1996) and some years later in *A. mellifera* (Higes, Martín, & Meana, 2006). Recently, a new microsporidian belonging to *Nosema* genus, *N. neumannii* was discovered in honey bees in Uganda but its presence could not be associated with clinical manifestations (Chemurot, De Smet, Brunain, De Rycke, & de Graaf, 2017).

Microsporidia are a phylum of intracellular obligate parasites. To date 1500 species are known, belonging to 187 different genera and able to infect all the range of eukaryotes, from protists to man (Vávra e Lukeš, 2013). The most peculiar structure of microsporidia is the polar tube, used to pierce the host cell and inject the genetic material to initiate reproduction (Keeling & Fast, 2002).

The parasitic life heavily conditioned these organisms, reducing their genome and proteome to essentiality and leaving behind everything they can obtain from the host (Corradi & Slamovits, 2011; Peyretaillade et al., 2011).

Life cycle

The entire cycle starts with the ingestion of mature spores of the parasite by the adult honey bee; this can happen by trophallaxis, auto-grooming or robbing of honey (Chen, Evans, Smith, & Pettis, 2008; Higes, Martín-Hernández, & Meana, 2010). In the ventriculus, spores germinate and inject genetic material (sporoplasm) in the epithelial

cells of the host through polar tube (Higes, Martín-Hernández, & Meana, 2010; Texier, Vidau, Viguès, El Alaoui, & Delbac, 2010). Factors influencing germination are still largely unknown but in vitro studies suggested that hydration, pH, osmotic conditions and UV light may induce germination (Keeling & Fast, 2002). After entering the cell, an asexual reproduction takes place (merogony), to date the exact number of merogonic cycles for *N. apis* and *N. ceranae* is unknown (Higes, Martín-Hernández, & Meana, 2010). The subsequent sporogonic phase generate sporonts that originate sporoblasts by binary replication (Gisder et al., 2010). Sporoblasts mature into spores that are released in the intestinal lumen trough cell disruption (Gisder et al., 2010). For *N. ceranae* the entire cycle takes about 36 hours to complete (Higes, García-Palencia, Martín-Hernández, & Meana, 2007) and the infective dose has been estimated to be 94.3 spore/bee for *N. apis* (Fries, 1988) and 1.28 spores/bee for *N. ceranae* (McGowan et al., 2016).

Epidemiology

To date, the exact timing of the host shift of *N. ceranae* is unknown, however there is some evidence dating its arrive in Europe back in 2003 (Klee et al., 2007) while another work based on historic specimens, found the parasite in a sample from Italy, collected in 1993 (Ferroglio et al., 2013).

In 2007 was detected in samples from USA, Brazil, China, Vietnam and some European countries (Klee et al., 2007; Paxton, Klee, Korpela, & Fries, 2007). Recently its presence was verified in Canada (Williams, Sampson, Shutler, & Rogers, 2008), Australia (Giersch, Berg, Galea, & Hornitzky, 2009) South America (Invernizzi et al., 2009) and Saudi Arabia (Ansari, Al-Ghamdi, Nuru, Khan, & Alattal, 2017).

Dispersion of this pathogen is facilitated by its wide host range; it has been detected in bumblebees

(Plischuk et al., 2009) *Apis florea* (Suwannapong, Yemor, Boonpakdee, & Benbow, 2011), *Apis dorsata* (Chaimanee, Chen, Pettis, Scott Cornman, & Chantawannakul, 2011) and solitary bees (Ravoet et al., 2014). Despite the perception of a displacement of *N. apis*, this microsporidia is still present in honeybee colonies and prevalent in Sweden and United Kingdom (Forsgren & Fries, 2013; Gisder et al., 2010). The reason behind this difference could be related to the higher susceptibility of *N. ceranae* spores to low temperatures (they are inactivated under 4 °C as shown by Gisder et al., 2010) and its

increased biotic potential at higher temperatures (around 37 °C, as shown by Martín-Hernández et al., 2009).

Another major difference between the two microsporidia is related to seasonality. *N. apis* exhibit a strong seasonal pattern with a peak in spring and a lower one in autumn (Bailey, 1955). *N. ceranae* on the other hand, can be found throughout the year (Martín-Hernández et al., 2012) but with different intensities. The peak is reached during winter in Spain (Higes et al., 2008), during spring in Serbia (Stevanovic et al., 2013) and Germany (Gisder, Schüler, Horchler, Groth, & Genersch, 2017), during summer in Canada (Copley, Chen, Giovenazzo, Houle, & Jabaji, 2012).

Pathogenicity

The effects of *N. ceranae* infection on honey bees are still under investigation. The majority of trials showed a negative effect on lifespan of honey bees (Alaux et al., 2010; Marina Basualdo, Barragán, & Antúnez, 2014; Dussaubat et al., 2012; Goblirsch, Huang, & Spivak, 2013; Huang, Solter, Aronstein, & Huang, 2015; Martín-Hernández et al., 2011; Mayack & Naug, 2009) with only few experiments failing to show this effect (Garrido et al., 2016; Milbrath, Xie, & Huang, 2013; Retschnig, Neumann, & Williams, 2014).

The reduction in life expectancy could be related to the high energetic stress determined by the parasite in the host (Mayack e Naug, 2009). Epithelial cells of the ventriculus are affected by the infection with degeneration leading to rupture of the cell wall (García-Palencia et al., 2010). The parasite is able to manipulate host cells preventing apoptosis and thus favouring its reproduction (Higes, Meana, Bartolomé, Botías, & Martín-Hernández, 2013). *N. ceranae* has been found in other organs like hypopharyngeal and salivary glands, malpighian tubules and fat bodies although reproduction in this sites has not been demonstrated (Chen et al., 2009; Gisder et al., 2010).

At the colony level, great concern is caused by the effect of the parasite on the transition of house bees to forager bees, anticipating this physiological process, and greatly reducing the longevity of the bee (Goblirsch et al., 2013). Moreover, the homeostasis of the colony could be impaired by alterations of the ethyl-oleate hormone, needed for regulation of the number of foragers (Dussaubat et al., 2010).

All the colony members are susceptible to the infection of *N. ceranae* (Chen et al., 2009).

Transmission inside the hive is mediated by cleaning of the combs (Higes et al., 2010) and trophallaxis (Copley e Jabaji, 2012; Smith, 2012). As with other diseases, transmission between hives is probably due to drift and robbing (Fries e Camazine, 2001).

Diagnosis

While clinical diagnosis of N. apis is quite straightforward given the typical symptoms (diarrhoea, crawling bees in front of the hive with enlarged abdomens, unable to fly); diagnosis of N. ceranae is much more cumbersome since the symptoms are subtle and aspecific: lower longevity (Higes et al., 2006; Alaux et al., 2010), sudden depopulation and low honey production (Higes et al., 2008), wintering problems (Chen e Huang, 2010). Spores of *N. ceranae* can be observed microscopically, they are 3.3-5.5 µm long and 2.3-3.0 µm wide, slightly smaller than those of *N.apis* but with a dimensional overlap between the two species making impossible a certain discrimination. Transmission electron microscopy (TEM) could be used instead utilising the number of coils of the polar tube as a discriminant (Chen et al., 2009). However, the most widely used techniques for diagnosis of nosemosis are those based on PCR. Different protocols have been proposed: PCR-RFLP (Higes et al., 2006; Klee et al., 2007), a uniplex PCR (Chen et al., 2008); a multiplex PCR for simultaneous amplification of both species (Higes et al., 2007) and real time PCR protocols (Cox-Foster et al., 2007; Chen et al., 2009). Moreover Forsgren e Fries, (2010) and Bourgeois et al., (2010) tuned protocols for quantitative PCR. Most of the abovementioned protocols are based on amplification of the 16S rRNA (Higes et al., 2006). Considering the high variability of this gene in N. ceranae, concern was raised about the reliability of the protocols (Sagastume, Del Águila, Martín-Hernández, Higes, & Henriques-Gil, 2011; Tay, O'Mahony, & Paxton, 2005). In this thesis, a new method was created for N. ceranae diagnosis based on the highly conserved, single copy Hsp70 gene (Cabbri et al., 2018). In order to increase sensitivity of the process, sampling of foragers should be preferred; alternatively older bees on outer combs can be used (Higes, 2010).

Therapy

Fumagillin is an antibiotic effective on both species of *Nosema* (Higes, 2010; Williams et al., 2011). However (Huang et al., 2013) showed that when the concentration in the hive drops, the proliferation of N. ceranae is higher than in untreatead hives. Moreover, this

antibiotic is effective only on vegetative forms, promoting the presence of the infection (Higes, 2010; Fries, 2010). To date in EU no drugs are registered with this active principle for beehives.

For this reason, dedicated effort took place to find alternative control protocols. Maistrello et al., (2008) showed a good efficacy of resveratrol and thymol in a cage setup. Botías et al., (2013) on the other hand, found no efficacy of Nosestat®, Phenyl salycilate and Vitafeed Gold® against N. ceranae.

API-Herb, a mixture of medicinal plants, showed a good capacity of reducing spore loads in caged bees (Nanetti et al., 2014). Recently, the same author investigated the effect of oxalic acid in caged bees and free-flying colonies founding a remarkable efficacy in reducing spore loads and preventing collapses (Nanetti, Rodriguez-García, Meana, Martín-Hernández, & Higes, 2015). A new promising field in N. ceranae control is related to the use of beneficial microbes as highlighted by (Arredondo et al., 2018; Baffoni et al., 2016).

Plant protection products

Plant protection products (PPPs) are widely used all over the world to control pests in agriculture and have been blamed as great contributors to honey bee colony mortalities since the late 19th century (Berenbaum, 2016). The scientific literature produced a great number of publications dealing with complex aspects, including combined exposure (Mullin et al., 2010), interaction and synergisms (Johnson, Dahlgren, Siegfried, & Ellis, 2013; Sánchez-Bayo et al., 2016) and sublethal effects (Sánchez-Bayo et al., 2016). These studies added evidence to the hypothesis of a crucial role of these products on bee health, but it is still debatable the impact of this factor compared to others, like pathogens, habitat fragmentation and climate change.

Neonicotinoids are undoubtedly the most debated class of pesticides, with about 200 papers in the scientific literature dealing with their relationship with bees. These insecticides, derived from nicotine, are agonist for the Ach nicotinic receptors with a strong affinity with those of insects rather than vertebrates (Tomizawa & Casida, 2005). The toxicity is very high, for example the oral LD50 in honey bees is 5 ng/bee for imidacloprid, 1/10000 compared to the LD50 of DDT. The value of the market of these products is about 2.6 billion dollars with imidacloprid alone covering about 41 % of this share, making it the second most widely used plant protection product in the world

(Jeschke, Nauen, Schindler, & Elbert, 2011). While lethal effects at field-realistic doses are probably negligible (Cresswell, 2011), there is a growing body of evidence regarding sub-lethal effects. These effects are those elicited by an exposure to a dose lower than LD50. Sub-lethal effects on reproduction are reported for imidacloprid, this molecule can delay honey bee development from preimaginal state to adult (Decourtye et al., 2005). Similar observations were made on *Osmia lignaria* (Abbott, Nadeau, Higo, & Winston, 2008).

Behavioural effects like reduction in learning capacity were reported for chronic exposure to imidacloprid (48 μg/kg; chronic oral administration) (Axel Decourtye, Lacassie, & Pham-Delégue, 2003) and thiamethoxam (0.1 ng/bee; contact) (Aliouane et al., 2009). Memory could also be affected by these pesticides as demonstrated by El Hassani et al. (2008) who observed long-term impairments after oral uptake of 0.1 μg/bee of acetamiprid. Orientation is probably the most cognitive intensive task for honey bees, therefore neurotoxic compounds could alter this ability. For example Decourtye & Devillers (2010) demonstrated a disruption in the process of associative learning between a visual mark and a reward: only 38% of the bees found the food source after oral ingestion of thiamethoxam at 3 ng/bee compared to 61% in the control group. However, field studies failed to report similar effects for clothianidin (Cutler & Scott-Dupree, 2007) imidacloprid (Faucon et al., 2005; Nguyen et al., 2009; Schmuck, Schning, Stork, & Schramel, 2001). Recently, a flupyradifurone-based insecticide was registered. Flupyradifurone is a butanolide compound, with a structure similar to neonicotinoids with which shares same target site (they are acetilcolinic agonist) and the systemic nature. In a recent work, an increased susceptibility of foragers and a synergistic effect with propiconazole was elucidated (Tosi & Nieh, 2019). Despite the alarming evidence of negative effects of neonicotinoids, the definition of field-realistic doses is still under debate. The recent work of Carreck & Ratnieks (2014) concluded that they have probably been overestimated. To complicate an already intricate scenario, it has been demonstrated that these molecules could attract insects and be addictive (Kessler et al., 2015).

While the danger related to the use of insecticides is self-explanatory, the negative effects of fungicides on honey bees are less obvious. Their LD50 is quite high but concerns are raised by their effects when combined with pesticides in both honey bees and wild bees (Johnson et al., 2013; Sgolastra et al., 2017). In chapter 3 we studied the effect of field realistic doses of clothianidin (neonicotinoid insecticide) and propiconazole (fungicide)

on *Osmia bicornis* (Sgolastra et al., 2018). In addition, negative interactions in the field can also be related to stressors other than PPPs. In the experiment reported in chapter 2 a synergistic interaction between poor nutrition and pesticide exposure was elucidated, with an important impact on bee survival.

Biomarkers

Biomarkers are routinely used in human medicine and represent a rapidly growing research field in veterinary medicine. The term biomarker is a shortening of the term biological marker, which has been cited for the first time in literature in the late 1960s (Figueroa & Rawls, 1969). In 1999 The NIH defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" later refined in the BEST (Biomarkers, EndpointS and other Tools) glossary as "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions". This latter definition is much broader as it includes environmental or toxicological exposures. Currently, the number of studies dealing with biomarker discovery in honey bees is limited. Specifically, biomarkers proposed so far can be classified in three different classes: biomarkers of exposure to xenobiotics, biomarkers of exposure to metals and biomarkers of nutritional status. The seminal paper of the first category is that of Badiou & Belzunces (2008) who observed an important increase in Acetylcholinesterase(AChE) activity in response to deltamethrin exposure. The same effect is obtained during imidacloprid or clothianidin intoxication (Boily, Sarrasin, DeBlois, Aras, & Chagnon, 2013). Chloroacetate esterase (CaE), Glutathione S-transferase (GST), Catalase (CAT) and Alkaline phosphatase (ALP) activity is modified in bees exposed to thiamethoxam (Badiou-Bénéteau et al., 2012). In africanized bees, fipronil at a sublethal dose (0.01 ng/bee) modulated the activity of CaE but didn't affect the activity of AChE or GST. Wegener et al. (2016) found that concentration of 10-hydroxy-2-decenoic acid (10HDA) in worker bee heads not only was influenced by imidacloprid but was also predictive of overwintering strength. The exposure to the fungicide Chlorothalonil was found to increase the level of Glucose oxidase (GOx) (O'Neal, Reeves, Fell, Brewster, & Anderson, 2019). The protein Vitellogenin was upregulated after exposure to Clothianidin (Christen, Vogel, Hettich, & Fent, 2019). A

potential biomarker of exposure to metals was studied by Purać et al. (2019) who demonstrated a dose-dependent relationship between Cd, Cu and Pb concentrations present in food and metallothionein (AmMT) expression. Regarding nutritional status, haemolymph protein concentration is a good candidate as it relates with the quality of food proteins (De Jong, 2009). Moreover, being vitellogenin the main protein of the haemolymph in adult bees (Amdam, Norberg, Hagen, & Omholt, 2003), its concentration is also a valuable tool to evaluate nutritional status due to its relation with quality (Cremonez, Jong, & Bitondi, 1998) and quantity (M. Basualdo et al., 2013; Bitondi & Simões, 1996) of proteins in the diet.

In chapter 5 we used a novel approach to biomarker discovery in haemolymph, based on SDS-PAGE electrophoresis and the application of machine learning algorithms.

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Chapter 2: Neonicotinoid pesticides and nutritional stress synergistically reduce survival in honey bees

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Abstract

The honey bee is a major pollinator whose health is of global concern. Declines in honey bee health are related to multiple factors, including resource quality and pesticide contamination. Intensive agricultural areas with crop monocultures potentially reduce the quality and quantity of available nutrients and expose bee foragers to pesticides. However, there is no evidence for synergistic effects between pesticides and nutritional stressors in animals. The neonicotinoids clothianidin (CLO) and thiamethoxam (TMX) are common systemic pesticides that are used worldwide and found in nectar and pollen. We therefore tested if nutritional stress (limited access to nectar, and access to nectar with low sugar concentrations) and sublethal, field-realistic acute exposures to two neonicotinoids (CLO and TMX at 1/5 and 1/25 of LD_{50}) could alter bee survival, food consumption, and hemolymph sugar levels. Bee survival was synergistically reduced by the combination of poor nutrition and pesticide exposure (-50%). Nutritional and pesticide stressors reduced also food consumption (-48%) and hemolymph levels of glucose (-60%) and trehalose (-27%). Our results provide the first demonstration that field-realistic nutritional stress and pesticide exposure can synergistically interact and cause significant harm to animal survival. These findings have implications for current pesticide risk-assessment procedures and pollinator protection.

Keywords

bee health, carbohydrates, clothianidin, thiamethoxam, hemolymph, food consumption

Introduction

Pollinators provide essential ecosystem services, contributing to wild plant biodiversity [1] and sustaining agricultural productivity [2]. The honey bee is a major pollinator species, and its poor health is related to multiple factors [3,4], including resource quality [5] and pesticide contamination [6]. Concern is therefore growing about honey bee nutrition and the potential for synergistic effects between pesticide exposure and nutrition [7,8].

Intensive agriculture with crop monocultures modifies natural land use, reduces natural habitats and plant diversity [9], and decreases the quality and quantity of nutrients in nectar and pollen [7,10]. Honey bees pollinate multiple crops and can therefore be vulnerable to such reduced food quality. Nutritional stress plays a crucial role in bee losses and poor colony health [7,11,12]. In fact, nutritional deficits were identified as a major cause of colony losses in the USA between 2007 and 2015 (21-58%) [13].

Agriculture also exposes foragers to pesticides [14]. Attention has focused on the neonicotinoid pesticides [15] because of their adverse impacts on pollinator health [16]. Neonicotinoids are globally used systemic insecticides [17] that can be found in the nectar and pollen collected by foragers [18] and are highly toxic to bees [17]. Bees can be exposed to pesticides that drift from treated fields when they forage on flowering strips, buffer zones, and cover or catch crops [19,20]. Furthermore, neonicotinoids are highly persistent [18] and are found in environmental reservoirs such as water and soil [21–23]. Consequently, plants could take up neonicotinoids years after the actual treatment, resulting in prolonged contamination [14].

Clothianidin (CLO) and thiamethoxam (TMX) are commonly used neonicotinoids, and CLO is also a degradation product of TMX [17]. These neurotoxic insecticides are agonists of nicotinic acetylcholine receptors (nAChRs) [17] and impair bees in multiple ways [16,24]. Neonicotinoids have additive and synergistic effects on honey bees in combination with health stressors such as nosemosis and *Varroa* infestation (see review by [25]). Moreover, the combination of poor nutrition and pesticide exposure may be especially problematic given that some genes can be up-regulated by pesticide or pollen stresses [26]. There is no evidence for negative synergistic effects between pesticides and nutritional stressors in any animal studies [32]. However, good nutrition can help: bees were typically more resistant to pesticides when fed pollen diets [26,27]. Food quality can influence the effect of toxins on the health of other arthropods, such as

Daphnia [28–30] and *Diaptornus* [31]. A few studies have demonstrated synergies between starvation and contamination from heavy metals, PAHs, or PCBs on aquatic animals (fish, amphipods, and mollusks) (see review by [32]). However, these are not pesticides. We therefore decided to study the interactive effects of field-realistic neonicotinoids and nutritional stressors on a major pollinator species.

We focused on honey bees because they are an important pollinator and are an indicator of how insect pollinators can respond to environmental stressors [33]. Bee foragers are particularly important because they are the only colony members that spend a significant proportion of their time flying [34] and therefore have significant energy needs. Unlike other insects, in which flight is initially powered by glycogen and subsequently by lipids, honey bee flight is entirely powered by sugars in the honey stomach after the depletion of glycogen reserves [35]. Sugar is therefore essential for foraging because flight has high energetic demands [36,37]: forager metabolic activity increases 50-100 times during flight [36]. A bee could need up to 12 mg of sugar to sustain itself per each 1 h of flight [38]. To deal with such high energy demands, sugars are quickly absorbed into bee's hemolymph [39].

Honey bees store only small amounts of glycogen in their flight muscles [40], and thus have high hemolymph sugar levels relative to other insects [41]. Hemolymph sugar content is therefore a good indicator of bee nutritional and physiological status. Trehalose, a disaccharide composed of 2 D-glucose molecules, is the most abundant sugar in honey bee hemolymph [41,42] and can be rapidly metabolized into D-glucose to release energy [42]. D-glucose is another major component of bee hemolymph [43], and is used to power motor activities directly [44].

We therefore tested the combined effects of sublethal, field-realistic acute exposures (see Methods) to two neonicotinoids (CLO and TMX at 1/5 and 1/25 of their LD_{50}) and nutritional stress (limited sugar quantity and quality) on forager survival, food consumption, and hemolymph sugar levels. Hemolymph sugar levels were assessed 2 h after treatment to test for potential rapid alterations caused by pesticide administration. Survival and sugar consumption were assessed over a longer period (4 days). We studied foragers because they spend a majority of their time foraging, an energy intensive task [36] that can also expose bees to neonicotinoid-contaminated nectar.

Methods

This study was conducted in the summer of 2015 in Bologna, Italy. We used five queen-right honey bee (*Apis mellifera ligustica*) colonies located in the experimental apiary of the Council for Agricultural Research and Economics, Agriculture and Environment Research Centre (CREA-AA). The colonies were healthy, produced honey, and showed no sign of disease throughout the season. They were managed according to an organic production protocol [45], and we used standard inspection techniques [46] to confirm that our colonies did not have detectable disease or parasite infestations. Colonies were inspected at least once per week.

We exposed bees to a nutritional stress (*limited* access to nectar or *ad libitum* access to nectar with low sugar concentrations) and a neonicotinoid treatment. These treatments were administered individually and in combination to test for synergistic interactions [47]. After exposure, we measured the effects of the nutritional and neonicotinoid stressors on survival (up to four days after treatment), food consumption (up to four days after treatment), and glucose and trehalose hemolymph levels (2 h after treatment). We repeated the experiment four times (twice per each pesticide), using a total of 2,840 foragers from five different colonies. We report mean ± 1 standard error (SE), and we indicate with ^{DS} the statistical tests that passed the Dunn-Sidak correction for multiple pairwise comparisons. Further details are reported in the Electronic Supplementary Material (ESM).

Sugar diet treatments

We define nutritional stress as limited access to nectar or access to nectar with low sugar concentrations. We tested sugar diets with different quantities (amounts) and qualities (concentrations) of sucrose. We provided the bees either *ad libitum* or *limited* (10 µL) quantities of sugar solution. The quality of the sugar diet was either *rich* (50% w/w sucrose solution), *intermediate* (32.5%) or *poor* (15%).

Our nutritional stresses are field realistic. Foragers can be exposed to the sugar concentrations that we tested when foraging for nectar or consuming non-ripened honey stored in the nest. Bees collect nectar containing 5-80% (w/v) sugar concentration [48,49], but sugar concentrations can be as low as 2% [48]. Nectar is converted to honey in the hive via ripening, a process that increases sugar concentrations [49]. However, this process starts in the hive only [49]. Counterintuitively, foragers even dilute the sugar

concentration in nectar by about 1% during nectar collection [50]. Thus, foragers can consume nectar containing less than 5% sugar while foraging and flying outside the nest.

Inside the nest, nectar is ripened gradually over a period taking up to 5 [50] or even 21 days [49]. When nectar is rapidly collected in large quantities, bees do not immediately ripen it, instead they deposit the nectar, largely unconcentrated, into storage cells [50]. Ripening is therefore influenced by multiple factors: weather, honey flow conditions, collection rates, colony strength, amount and concentration of nectar, extent of available storage cells, temperature, humidity, and ventilation conditions [50]. Bees can thus be exposed to largely unconcentrated nectar for several days when consuming carbohydrates stored in the hive.

Individual carbohydrate intake can also be limited by non-foraging periods. In fact, lack of sufficient food stores is a common cause of winter colony losses [11] (i.e. involved in 58% of the colony lost in USA in 2014-2015 [13]). In our study, we therefore tested this limited carbohydrate scenario in two ways: feeding bees with limited amount of sucrose solution or, in a separate treatment, feeding bees *no nutrients* (0% sucrose).

Neonicotinoid treatments

We followed the most recent international guidelines for pesticide tests on bees [51]. We tested sublethal acute oral exposure to field-realistic doses of two neonicotinoid pesticides: CLO and TMX. Our doses were field-realistic because bees can consume higher doses of CLO and TMX while collecting contaminated nectar in the field for a short period (1 h) (see details below). Treatments consisted of a control dose (pesticide-free), or a neonicotinoid dose (dose) that was either 1/25 (lower dose, TMX = 0.2 ng/bee, CLO = 0.16 ng/bee,) or 1/5 (higher dose, TMX = 1 ng/bee, CLO = 0.8 ng/bee) of their respective LD_{50} (TMX = 5 ng/bee, CLO = 4 ng/bee) [52,53]. The *no nutrients* diet was pesticide-free. The higher doses used per each neonicotinoid reflect field-realistic scenarios with elevated neonicotinoid contamination. Calculations based on EFSA [54] confirm that our sublethal doses were lower than the worst-case scenario in which bees foraged for 1 h on nectar that was contaminated with CLO or TMX after a seed treatment (maximum field-realistic doses: CLO = 1 ng/bee/1 h, TMX = 0.66 ng/bee/1 h) or a transplant-drip application (maximum field-realistic dose of TMX = 1.80 ng/bee/1 h).

For CLO, the European Food Safety Authority (EFSA) [54] calculated that foragers can consume up to 1 ng/bee in 1 h of nectar foraging. This calculation was based

on the field-realistic concentration of CLO in nectar (9 ppb, found in oilseed rape nectar after seed treatment application [54]) and sugar in oilseed rape nectar (10% w/w [49,55]). A previous study similarly estimated that a forager can acutely consume up to 1.36 ng of CLO in a foraging trip when collecting nectar on oilseed rape fields grown from seeds treated with CLO [47]. In fact, CLO can occur at even higher field-realistic concentrations in nectar (e.g. 10 ppb [18,56]) and pollen (e.g. 41 ppb [57]) than those used in our study.

Similarly, for TMX, EFSA [54] calculated that foragers can consume up to 0.66 ng/bee in 1 h of foraging for nectar (10% sugar w/w, oilseed rape) with 5 ppb of TMX (concentration found in nectar after seed treatment application [54]). However, foragers can consume up to 1.80 ng/bee in 1 h of foraging for nectar with 15 ppb of TMX (concentration found in nectar after transplant-drip application [56]). TMX also is found at higher concentrations in nectar (e.g. 17 ppb [57]; 19 ppb [56]; 20 ppb [58]) and pollen (e.g. 127 ppb [57]) than those used in our study. Further details on our calculations are provided in the ESM.

Foragers have a lower sucrose requirement when incubated in cages, leading to decreased sucrose consumption in cages as compared to the field. Thus, to test field-relevant CLO and TMX doses approaching a realistic worst-case scenario, we fed foragers with pesticide solutions that were more concentrated (CLO lower: 16 ppb; CLO higher: 80 ppb; TMX lower: 20 ppb; TMX higher: 100 ppb) than those typically found in field nectar. However, we focused on the field-realistic acute doses of CLO and TMX actually ingested by our bees, as recommended by the most recent international guidelines for testing acute oral pesticide exposure [51].

Results

Combined nutritional and neonicotinoid stressors synergistically reduced survival

Survival was monitored up to four days after exposure to the neonicotinoids. Sublethal and field realistic doses of neonicotinoids did not significantly reduce survival when foragers were fed *ad libitum rich* diets (Kaplan-Meier, $p > 0.13$, table 1, figure 1A, F). However, neonicotinoids significantly reduced the survival of bees fed the *ad libitum* diets with qualities that were *intermediate* (CLO, figure 1B) or *poor* (CLO and TMX, Kaplan-Meier, $p < 0.01$, figure 1C, H). Bees fed *higher* pesticide doses had significantly lower survival as compared to *control* bees (CLO: within *poor* and *intermediate* quality

diets groups; TMX: within the *poor* quality diet group) and *lower* dose (CLO: within the *poor* quality diet group) ($p < 0.0170$, Kaplan-Meier [DS]).

CLO and TMX also reduced the survival of bees fed *limited* quantity diets with either *rich* (figure 1D, I) or *poor* (figure 1E, L) sugar qualities (Kaplan-Meier, $p < 0.0001$, table 1). Specifically, *higher* doses of both neonicotinoids significantly reduced survival as compared to *control* and *lower* doses, at all diet qualities ($p < 0.0170$, Kaplan-Meier [DS]). Increased death of bees fed neonicotinoids and *poor* quality diets occurred 2-3 h after treatment (up to 0%, 6% and 19% mortality respectively 1 h, 2 h and 3 h after treatment, ESM table S1).

There was a significant synergistic reduction of survival elicited by all combinations of nutritional stresses (*ad libitum intermediate*, *ad libitum poor*, *limited high*, *limited poor*) and the *higher* pesticide dose (binomial proportion test, Holm correction, figure 2, ESM table S2). *Ad libitum poor* diets synergistically reduced survival between 2-24 h (CLO and TMX, SES_{range} = 5-33%, figure 2C, H), and *ad libitum intermediate* diets synergistically reduced survival between 3-24 h (CLO, SES_{range} = 9-21%, figure 2B). There was no significant synergistic effect on the survival of bees exposed to the *ad libitum intermediate* diet and TMX. *Limited poor* diets synergistically reduced survival between 2-10 h (CLO, SES_{range} = 8-36%, figure 2E) and 3-8 h (TMX, SES_{range} = 11-48%, figure 2L), and *limited rich* diets synergistically reduced survival between 4-5 h (CLO, SES_{range} = 39-50%, figure 2D) and 3-6 h (TMX, SES_{range} = 10-24%, figure 2I).

Receiving no nutrients (i.e. starvation) was better than receiving some nutrients with pesticides. Within the *limited* quantity diet trial, we tested an additional diet containing *no nutrients* (10 µL pure water). Bees fed the *no nutrients* diet had significantly higher survival than bees fed the *limited* quantity diet of *poor* quality (10 µL of 15% sucrose solution) containing the *higher* pesticide dose of either CLO and TMX (table 1, figure 1E, L). The survival of bees fed the *no nutrients* diet was significantly lower than bees fed *limited poor* diets containing the *control* and *lower* dose (TMX: at 15-50%; CLO: at 50%, figure 1).

Combined nutritional and neonicotinoid stressors reduced sugar consumption

We assessed the sucrose consumption of bees fed the *ad libitum* diet only because bees that received a *limited* quantity diet only had access to a fixed amount of food (10

µL). We calculated the actual mass of pure sucrose consumed per bee per day. There was no significant effect of CLO on sugar consumption of foragers fed *rich* and *intermediate* quality diets (GLMs, $p > 1.40$, table 2, figure 3A). However, there was a significant effect of CLO on consumption of bees fed *poor* quality diet (GLMs, $p < 0.0001$, figure 3A). Specifically, *control* bees consumed significantly more sucrose than *lower* (-31%) and *higher* (-48%) dose bees, and *lower* bees consumed more than bees treated with *higher* doses (-25%, Contrast test ^{DS}). There was no significant effect of TMX on sucrose consumption at any diet quality (GLM, $p > 0.3$, table 2, figure 3B).

Sublethal doses of neonicotinoids reduced glucose and trehalose hemolymph levels

Glucose and trehalose hemolymph levels were only assessed on bees fed the *ad libitum* quantity diet, because insufficient hemolymph was extractable from bees that were only fed the *limited* quantity diet (10 µL). The hemolymph was extracted 2 h after the neonicotinoid exposure. There was a significant effect of CLO on glucose ($p = 0.0092$) and trehalose ($p = 0.0021$) hemolymph levels when foragers were fed a diet of *rich* quality (50% sucrose) (GLM, table 3, figure 4A). Specifically, the hemolymph of *control* bees contained higher levels of glucose than bees fed the *higher* (+26%) and *lower* (+27%) CLO doses. *Control* bee hemolymph also contained higher levels of trehalose than the hemolymph of bees fed the CLO *higher* dose (+26%, Contrast test ^{DS}).

Likewise, there was a significant effect of TMX on glucose ($p = 0.0122$) hemolymph levels when foragers were fed diets of *rich* quality (GLM, table 3, figure 4B). Specifically, *control* bee hemolymph contained higher levels of glucose than that of bees exposed to *lower* (+55%) and *higher* (+60%) TMX doses (Contrast test ^{DS}).

Effects of nutritional deficits on pesticide-free bees

Nutritional deficits decreased the survival of pesticide-free bees

As expected, the survival of pesticide-free bees fed the *limited* quantity diet was significantly lower than the survival of pesticide-free bees fed the *ad libitum* diet (Kaplan-Meier, $\chi^2 = 762.32$, d.f. = 1, $p < 0.0001$).

There was a significant effect of diet quality on the survival of pesticide-free foragers fed *ad libitum* (Kaplan-Meier, $p < 0.0001$, ESM figure S5A, ESM table S3). Specifically, foragers fed lower quality diets had a significantly shorter survival (Kaplan-

Meier[DS], d.f. = 1, $p < 0.0001$; *poor* vs *intermediate*: $\chi^2 = 35.62$; *poor* vs *rich*: $\chi^2 = 100.16$; *intermediate* vs *rich*: $\chi^2 = 41.43$; ESM figure S5A).

There was a significant effect of diet quality on the survival of pesticide-free foragers fed *limited* quantity diets (Kaplan-Meier, $p < 0.0001$, ESM figure S5B, ESM table S3). Specifically, bees fed lower quality diets had significantly reduced survival (Kaplan-Meier, d.f. =1; *poor* vs *rich*: $\chi^2 = 5.45$, $p = 0.0196$; *no nutrients* vs *rich*: $\chi^2 = 37.30$, $p < 0.0001$; *no nutrients* vs *poor*: $\chi^2 = 9.02$, $p = 0.0027$; ESM figure S5B).

Lower quality diets reduced glucose and trehalose levels in the hemolymph

In pesticide-free foragers, there was a significant effect of diet quality on glucose (GLM, $\chi_{7,2} = 22.42$, $p < 0.0001$) and trehalose (GLM, $\chi_{7,2} = 37.30$, $p < 0.0001$) levels (ESM figure S5E, F). As expected, forager hemolymph of bees fed *rich* diets contained significantly higher levels of both glucose and trehalose than bees fed *intermediate* (+49% and +23%, respectively) and *poor* (+68% and +48%) diets (contrast tests [DS]).

Diet quality influenced sucrose consumption

There was a significant effect of diet quality on sucrose consumption of pesticide-free foragers (GLMs, $\chi_{7,2} = 171.09$, $p < 0.0001$, ESM figure S5C). Foragers consumed significantly less sucrose when they were fed lower quality diets (*rich* vs. *poor*: -72%; *rich* vs *intermediate: -33%; intermediate* vs. *poor*: -58%, contrast tests [DS], ESM figure S5C). There was no significant effect of diet quality on the volume of the sucrose solutions consumed daily by the foragers (GLMs, $\chi_{7,2} = 1.43$, $p = 0.488$, ESM figure S5D).

Discussion

One of the most common routes of honey bee pesticide exposure is via foragers collecting nectar and pollen. We demonstrate, for the first time, that nutritional stresses can act synergistically with a sublethal, field-realistic pesticide exposure, and reduce honey bee survival. We also show that the exposure to nutritional and pesticide stressors impairs bee hemolymph energy levels and food consumption. Although prior research demonstrated that a good pollen diet can increase bee resistance to pesticides [26,27], and that food quality influences the effect of toxins on arthropods health [28–31], this is the

first study to demonstrate the negative synergistic effects of sugar caloric restriction and pesticides in animals.

Bees that did not undergo nutritional stress, were not significantly impaired by TMX or CLO. Forager survival was not significantly altered by any field-realistic doses of these neonicotinoids when they were fed optimal quality and quantity sugar diets (table 1, figure 1A, F). This result also confirms that our doses were sublethal. However, bees fed a poor nutritional diet experienced detrimental synergistic effects, up to a 50% mortality increase as compared to the expected non-synergistic (additive) effects. Each neonicotinoid synergistically reduced survival of bees fed diets of low quality (32.5% and 15% sugar concentration) or quantity (*limited* 10 µL of sugar solution) (table 1, figure 1, 2). This adverse synergistic effect of neonicotinoids and poor nutrition appeared rapidly after treatment (2 h, ESM table S1) and lasted up to one day (figure 2). Interestingly, starvation was less severe than pesticide exposure: bees survived longer when fed a pesticide-free diet containing no nutrients (pure water), as compared to bees that consumed a sugar diet of poor nutritional value, but containing a sublethal dose of pesticide (table 1, figure 1E, L).

The combination of nutritional and neonicotinoid stressors also reduced food consumption (figure 3). In all of our consumption experiments, bees only fed from pure sucrose solutions. Neonicotinoids were administered separately, prior to measuring consumption. Consumption was therefore not influenced by the presence of neonicotinoids in the sucrose solutions [59]. When foragers were fed the richest quality diets, their consumption was not significantly altered by any prior neonicotinoid exposure. However, all acute doses of CLO significantly reduced subsequent food consumption when bees were exposed to the poorest quality diet, suggesting that neonicotinoids alter foragers' energy metabolism or feeding behaviour.

What accounts for this change in feeding? TMX reduced forager motor functioning (acute exposure, 1.34 ng/bee; 2-day chronic exposure, $\text{Range}_{\text{TMX daily doses}}$ = 1.42-3.48 ng/bee/day), and food consumption (one day of chronic exposure) [60]. The reduced motor functioning of neonicotinoid-treated bees may lead to decreased energy consumption and food intake [60]. Similarly, Kessler et al. [59] showed that chronic exposure to CLO (0.1-1 µM, 25-250 ppb) and TMX (0.1-1 µM, 29-292 ppb) reduced honey bee food consumption.

Neonicotinoid consumption also reduced sugar levels in the hemolymph of bees, measured 2 h after pesticide exposure (figure 4). CLO exposure significantly decreased both trehalose and glucose titers. TMX significantly reduced glucose levels, although TMX did not alter sucrose consumption at any diet quality. TMX may have altered sugar metabolism. These alterations were only significant when bees were fed *ad libitum* diets of the richest quality. Bees fed *ad libitum* diets of poorer qualities had very low hemolymph sugar levels (2 h after treatment) across all pesticide treatments. A likely explanation is that the poorer quality diets could not fulfill bee nutritional requirements.

The food consumption and hemolymph sugar level alterations caused by neonicotinoids can disrupt forager energy metabolism, which is important for honey bee colony health [61]. Specifically, the neonicotinoid, imidacloprid, inhibits mitochondria respiration and ATP synthesis [62], and increases brain oxidative metabolism [63]. Similarly, another pesticide (a triazole fungicide, myclobutanil) disrupts energy production through a reduced mitochondrial regeneration and ATP production [64]. These energetic changes may have broader behavioural effects, interfering with thermoregulation [65], and locomotion [60], and flight [66]. Flight is one of the most energy intensive tasks [36], is fueled by sugar oxidation [37], requires flight muscle thermoregulation [67], and is impaired by acute and chronic sublethal TMX exposures [66].

Although CLO and TMX elicited similar results, CLO exerted consistently stronger effects, which also appeared earlier after exposure, as compared to TMX. This may have occurred because TMX targets different nAChR subtypes with a lower affinity than CLO [17]. In fact, CLO (LD_{50} = 4 ng/bee [52]) is more toxic than TMX (LD_{50} = 5 ng/bee [53]). Because approximately 36% of TMX degrades to its main metabolic by-product, CLO [17,68], the toxicity of TMX may be enhanced, to a degree, by its degradation to CLO. In cockroaches, the impairing effect of TMX on locomotion is correlated with its degradation to CLO [69].

As expected, richer sugar diets significantly increased survival (ESM figure S5A, B) and hemolymph energy levels (ESM figure S5E, F) in pesticide-free bees. Foragers consumed roughly the same maximum amounts of sucrose solution by volume because they consumed similar volumes of food across diet treatments (64 ± 1 µL/bee/day, mean of all pesticide-free diets, ESM figure S5D). Bees are evidently unable to compensate for a diet with low sugar concentration by simply consuming a higher volume of sugar

solution. In fact, although the mean sugar levels in the hemolymph of our bees were within the typical concentrations of glucose (2-20 µg/µL) and trehalose (2-40 µg/µL) [41,70–73], pesticide-free bees fed lower quality diets had also lower hemolymph energy levels (ESM figure S5E, F).

Prior insect studies showed that nutritional deprivation impairs the immune functions of the mealworm beetle (*Tenebrio molitor* L.) [74], and decreases the longevity of the housefly (*Musca domestica* L.) [75]. Sugar scarcity affects the survival [76] and behaviour [77] of organisms with complex sociality, such as ants. Our results show that nutrient deprivation reduces the lifespan of honey bees, and also compromises their resistance and resilience (i.e. ability to recover from the acute sublethal exposure) to pesticides. These data highlight the fundamental importance of high quality carbohydrate food for bees.

The behavioural and physiological impairments showed in our study likely compromise bee health, contributing to a broader variety of sublethal side-effects (see reviews by [16,24]). Nutrition and pesticide stressors could trigger synergistic effects on other bee species. As compared to honey bees, bumblebees consume more food, while storing lower quantity of it. They are therefore more dependent from available nectar sources than honey bees, while being similarly exposed to pesticides. In addition, bumblebee food consumption can be widely altered by chronic exposures to neonicotinoids, such as CLO (0.1-1 µM, and 10 µg/L), TMX (1, 4, 39, 98 µg/kg), and imidacloprid (0.001-1 µM, and 0.8-125 µg/L) [59,78–80].

Current risk-assessment (RA) procedures used for testing chemicals do not fully take into account our current understanding of bee toxicology and health [30,32,81–83]. Our results raise further concerns by suggesting that the sugar diet regime typically used for RA toxicity tests may strongly influence pesticide toxicity. For example, the standard RA guideline for LD_{50} toxicity tests require feeding bees with 50% (w/v) sucrose solutions *ad libitum* [51]. The results of these toxicity tests, obtained feeding bees with an optimal nutritional diet, may underestimate the toxic effect that chemicals elicit on bees in the field, where foragers can be exposed to a combined nutritional stress (i.e. low sugar nectar) [7,10,18,24]. Thus, the consequences of low sugar nectar and neonicotinoid (TMX and CLO) exposure should be considered in assessing risks on insect pollinators. We suggest that RA procedures should test pesticide effects at various nutritional quality

levels. More broadly, the combined exposure to xenobiotic and nutritional stressors is highly relevant ecological scenario which effects on animals deserve more attention.

Competing interests

We have no competing interests.

Authors' contributions

ST conceived the experiments. ST, JN and FS designed the experiments. ST and RC collected the data. ST and JN analysed the data. PM provided materials, reagents. ST, JN, FS, RC, and PM wrote and reviewed the manuscript.

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Electronic supplementary material (ESM)

All details concerning honey bee preparation, sugar diets, neonicotinoid treatments, survival, sugar consumption, glucose and trehalose hemolymph levels, and statistical methods are reported in the ESM. Further details on the survival results are reported in the ESM tables S1, S2, and S3.

Data accessibility

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.kc680 [84].

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TABLES

Table 1. Survival of bees exposed to sublethal field-realistic neonicotinoid doses and fed sugar diets of different quantity and quality. We report the Lethal Time at which 25%, 50%, and 75% (LT_{25}, LT_{50}, and LT_{75}) of bees died for each treatment, as well as their short term (1, 2 and 3 h after treatment) mortality as percentages. We tested the *no nutrients* diet (0% sucrose concentration) to include a scenario in which bees had no nutrients available. We state Not Applicable (NA^1) when the respective LT was not reached because of bee mortality, or (NA^2) to indicate that the comparisons between the *no nutrients* and the *ad libitum* diets were not tested, given the extreme survival difference between treatments. Different letters next to the lethal times indicate significant differences of each respective treatment (Kaplan-Meier^{DS} test).

Diet quantity	Neonicotinoid	Diet quality (%)	N	DF	χ^2	*P*-value	LT_{25} - LT_{50} - LT_{75} (h)			
							Control dose	Lower dose	Higher dose	No nutrients
Ad libitum	TMX	Rich	270	2	1.3	0.5164	96-NA^1-NA^1	96-NA^1-NA^1	96-NA^1-NA^1	
		Intermediate	270	2	0.1	0.9904	48-72-96	48-72-NA^1	48-72-96	NA^2
		Poor	272	2	19.0	0.0003	24-48-72 a	24-48-72 a	4-48-72 b	
	CLO	Rich	270	2	4.2	0.1250	96-NA^1-NA^1	72-NA^1-NA^1	72-96-NA^1	
		Intermediate	270	2	14.4	0.0025	48-72-NA^1 a	48-48-NA^1 ab	24-48-NA^1 b	NA^2
		Poor	270	2	42.4	<0.0001	8-48-96 a	8-36-48 a	3-8-48 b	
Limited	TMX	Rich	405	2	31.4	<0.0001	4-5-7 a	4-5-6 a	4-4-5 b	4-4-5 b
		Poor	407	2	68.3	<0.0001	4-5-6 a	4-5-7 a	2-3-5 b	4-4-5 c
	CLO	Rich	405	2	58.2	<0.0001	5-5-6 a	4-5-6 a	4-4-5 b	4-4-5 b
		Poor	406	2	46.2	<0.0001	4-5-6 a	4-5-6 a	2-3-5 b	4-4-5 a

Table 2. Main effects of sublethal field-realistic neonicotinoid dose on average daily sucrose consumption of foragers fed different diet qualities. The asterisk indicates a significant effect of dose (GLMs).

Neonicotinoid	Diet quality	N	DF numerator	DF denominator	L-R χ^2	*P*-value
CLO	Rich	18	5	2	0.24	0.8875
	Intermediate	18	5	2	3.95	0.1391
	Poor	16	5	2	63.52	<0.0001*
TMX	Rich	18	5	2	1.92	0.3820
	Intermediate	18	5	2	2.01	0.3667
	Poor	17	5	2	1.47	0.4805

Table 3. Main effects of sublethal field-realistic dose of two neonicotinoids (CLO and TMX) on glucose and trehalose levels in forager hemolymph. Results are shown for each diet quality. Asterisks indicate significant effects of dose (GLMs).

Neonicotinoid	Carbohydrate	Diet quality	N	DF numerator	DF denominator	L-R χ^2	*P*-value
TMX	Glucose	Rich	36	5	2	8.82	0.0122*
		Intermediate	36	5	2	0.22	0.8945
		Poor	36	5	2	0.49	0.7822
	Trehalose	Rich	36	5	2	1.16	0.5598
		Intermediate	36	5	2	0.40	0.8194
		Poor	36	5	2	2.53	0.2827
CLO	Glucose	Rich	36	5	2	9.38	0.0092*
		Intermediate	36	5	2	0.89	0.6392
		Poor	36	5	2	2.74	0.2535
	Trehalose	Rich	36	6	2	12.35	0.0021*
		Intermediate	36	6	2	2.33	0.3124
		Poor	36	6	2	5.92	0.0517

FIGURES

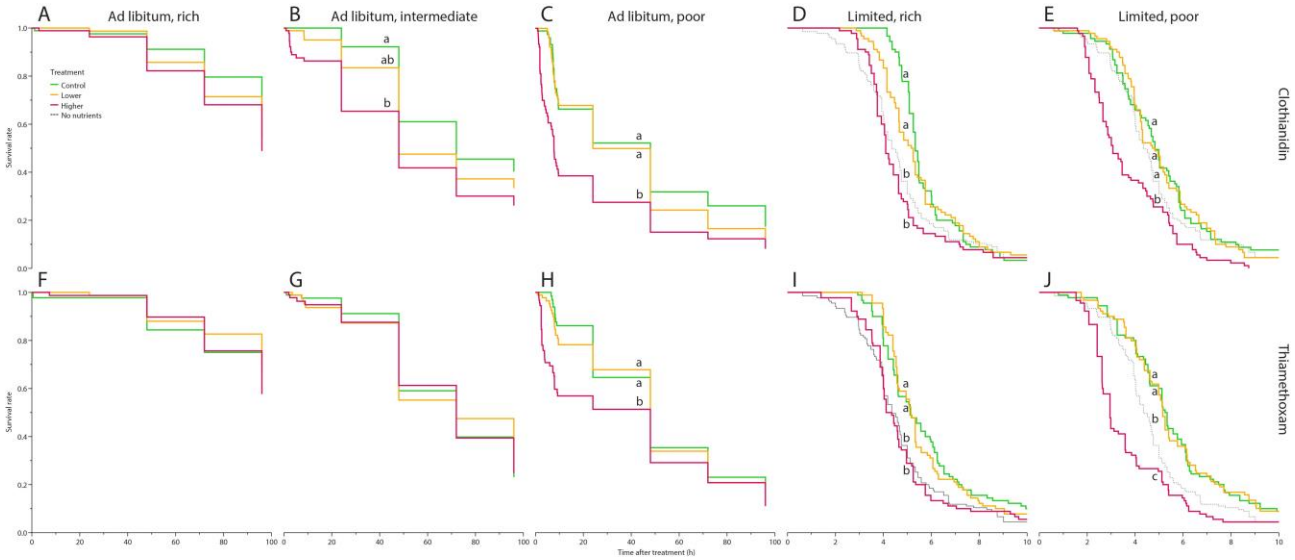

Figure 1. Survival of bees exposed to combined nutritional and pesticide stressors. We exposed bees to a control (green lines), lower (orange) or higher (red) field-realistic sublethal dose of CLO (A, B, C, D, E) or TMX (F, G, H, I, L). We fed bees *ad libitum* quantity diets of *rich* (A, F), *intermediate* (B, G) or *poor* (C, H) quality, and *limited* quantity diets of *rich* (D, I) or *poor* (E, L) quality. Because of the low survival rate and to facilitate graphical display, we show the survival of bees fed *limited* quantity diets until 10 h after treatment only. We also fed bees a *no nutrients* diet (0% sucrose concentration, dotted dark gray lines), and their survival was compared to bees fed *limited* quality diets. Different letters indicate significant differences (Kaplan-Meier^{DS} test). Main effects and sample sizes are shown in table 1.

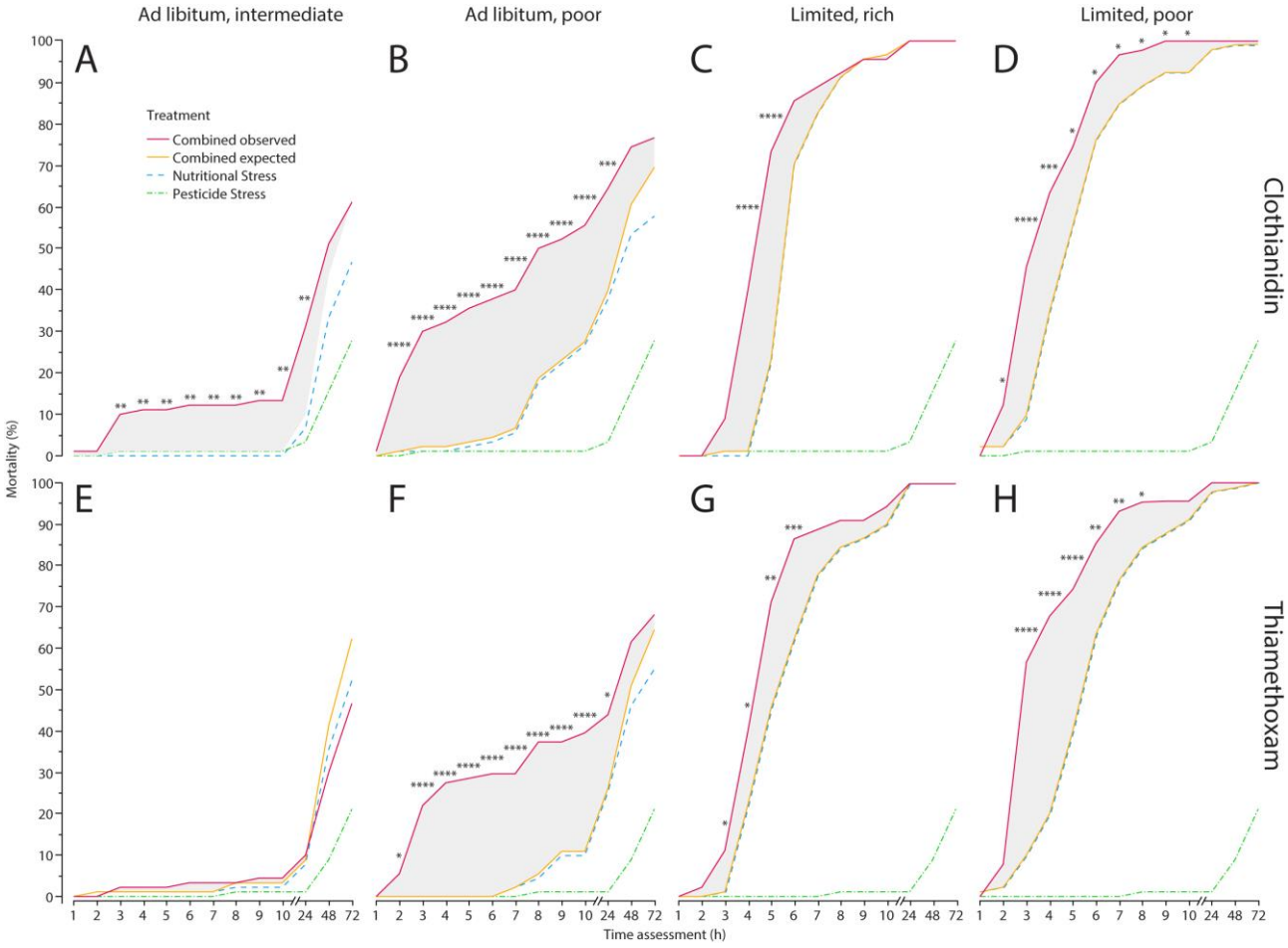

Figure 2. Synergistic effects of nutritional and neonicotinoid stressors on bee survival across time. We tested the individual and combined effects of each nutritional stress (treatment A, blue, dashed lines) and the higher neonicotinoid sublethal field-realistic dose of either CLO (A, B, C, D) or TMX (E, F, G, H) (treatment B, green, dashed and dotted lines), and compared their expected (orange, full lines) and observed (red, full lines) combined effects (treatment AB). The size of the synergistic effects is highlighted by the grey shaded area between expected and observed mortality. Asterisks indicate significant synergistic effects (i.e. significant difference between mortality of expected and observed combined treatment) at specific time assessments (binomal proportion tests, Holm corrected, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Synergistic Effect Sizes for each time assessment are shown in ESM table S2.

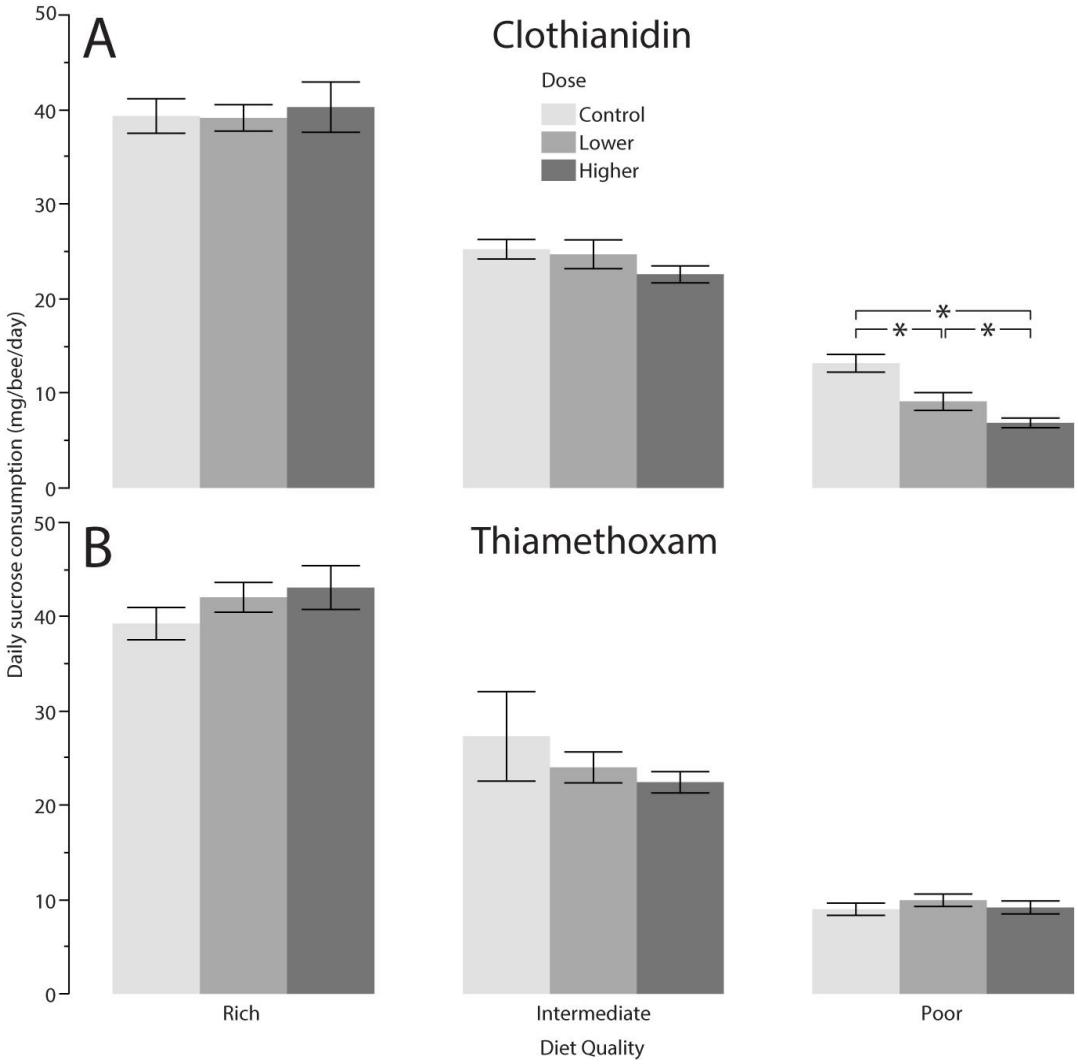

Figure 3. Daily sucrose consumption of bees exposed to combined nutritional and pesticide stressors. We exposed bees to three sublethal doses of either CLO (A) or TMX (B), and then we fed them *ad libitum* quantity diets of three different qualities for four days. Darker shading reflects higher doses of pesticide. Asterisks indicate significant differences (GLM, Least-Square Means contrast^{DS} tests). Main effects and sample sizes are shown in table 2. Error bars show standard errors.

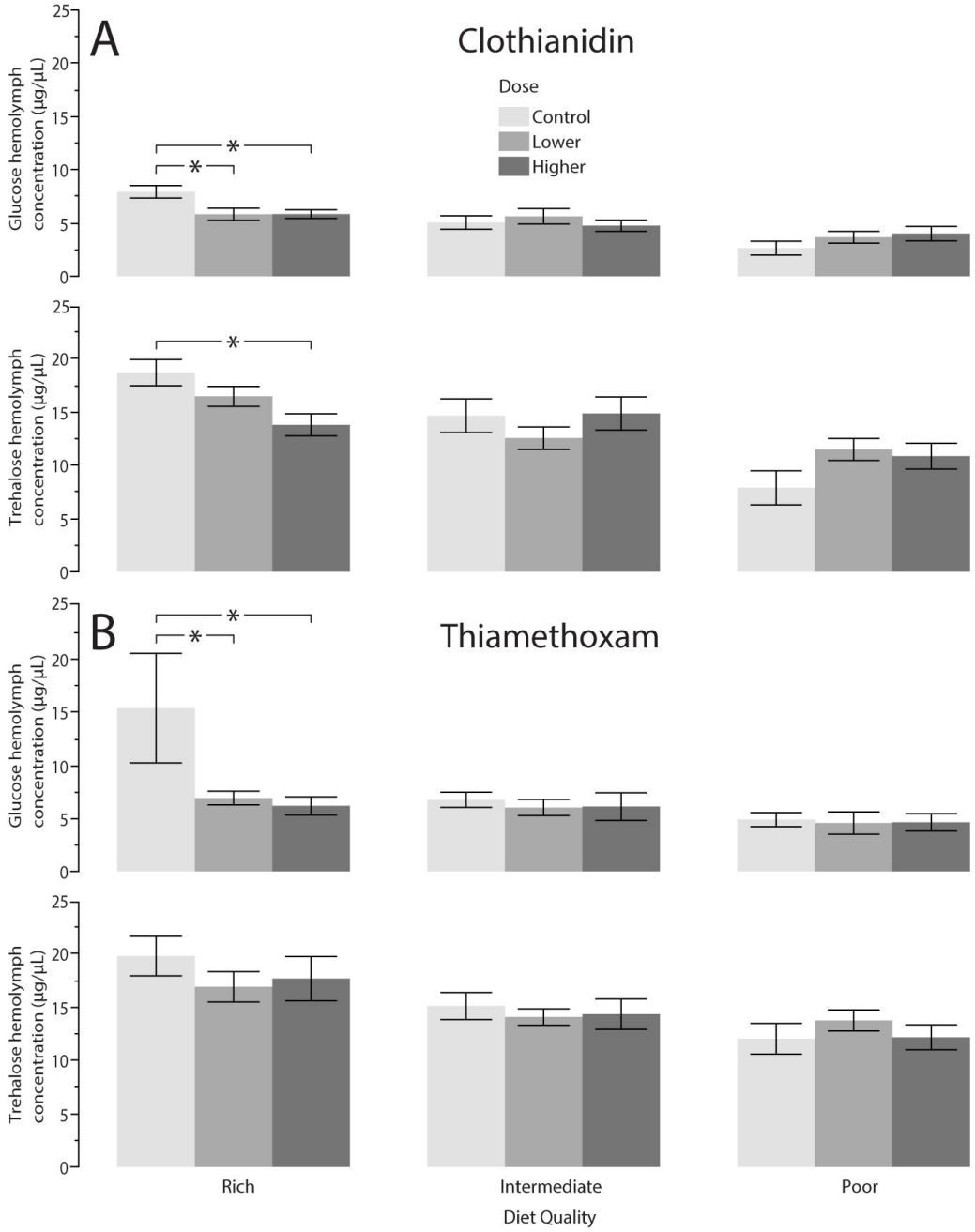

Figure 4. Glucose and trehalose concentrations in the hemolymph of bees exposed to a combination of nutritional and pesticide stressors. We exposed bees to three sublethal doses of either CLO (A) or TMX (B), and then we fed them *ad libitum* quantity diets of three qualities. The hemolymph was sampled 2 h after the pesticide treatment. Darker shading reflects higher doses of pesticide. Asterisks indicate significant differences (GLM, Least-Square Means contrast^{DS} tests). Main effects and sample sizes are shown in table 3. Error bars show standard errors.

Chapter 3: Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee

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Abstract

Pollinators in agroecosystems are often exposed to pesticide mixtures. Even at low concentrations, the effects of these mixtures on bee populations are difficult to predict due to potential synergistic interactions. We orally exposed newly-emerged females of the solitary bee *Osmia bicornis* to environmentally-realistic levels of clothianidin (neonicotinoid insecticide) and propiconazole (fungicide), singly and in combination. The amount of feeding solution consumed was highest in bees exposed to the neonicotinoid, and lowest in bees exposed to the pesticide mixture. Ovary maturation and longevity of bees of the neonicotinoid and the fungicide treatments did not differ from those of control bees. In contrast, bees exposed to the pesticide mixture showed slow ovary maturation and decreased longevity. We found a synergistic interaction between the neonicotinoid and the fungicide on survival probability. We also found an interaction between treatment and emergence time (an indicator of physiological condition) on longevity. Longevity was negatively correlated to physiological condition only in the fungicide and the mixture treatments. Delayed ovary maturation and premature death imply a shortened nesting period (highly correlated to fecundity in *Osmia*). Our findings provide a mechanism to explain the observed dynamics of solitary bee populations exposed to multiple chemical residues in agricultural environments.

Key words: neonicotinoids, insecticide, ergosterol-biosynthesis-inhibiting fungicide, synergism, ecotoxicology, *Osmia bicornis*

Introduction

The last decades have seen significant declines in wild bee diversity at local and regional scales [1-3], together with abnormal honey bee colony losses in various parts of the world [4,5]. Although these declines are undoubtedly caused by a combination of factors, pesticides in general, and neonicotinoid insecticides in particular, have often been signalled as one of the main drivers of the population declines experienced by both wild and managed species. For this reason, the use of neonicotinoids has been recently restricted in the European Union [6]. Nonetheless, neonicotinoids are still used on a wide range of crops and account for more than 30% of the global insecticide market [7]. Neonicotinoids are highly toxic to insects [8-10]. However, studies testing lethal and sublethal effects of neonicotinoids on bees often yield inconsistent results [11-14]. There are several important challenges when assessing the potential hazards of pesticides on bees. First, in as much as possible, bees should be subjected to realistic exposure conditions, likely to be experienced in field situations. In relation to this, some studies have been criticized based on allegedly overestimated exposure in terms of concentration and duration (e.g., studies testing acute exposure to high doses rather than chronic exposure to low doses) [15]. Second, in agricultural environments bees are often exposed to combinations of chemicals [16]. This is important because certain pesticide mixtures have been shown to produce synergistic effects [17-19]. Yet, with some exceptions [e.g., 17-20], ecotoxicological studies usually test single compounds. Third, sensitivity to pesticides may be highly influenced by the physiological condition of the bee. A recent review [21] shows that response to pesticide exposure in honey bees is highly variable at the individual level and dependent on several endogenous factors such as genetic background, body size and age. Fourth, the effects of pesticides may be species-dependent. Most bee ecotoxicological studies have been conducted on a single species, the western honey bee, *Apis mellifera* [16,22]. However, there is increasing evidence that solitary bees (*Osmia bicornis*) are more sensitive to certain pesticide treatments than honey bees and bumblebees [12,13,18,23].

In this study, we tested the effects of environmentally-realistic oral exposure to clothianidin (a neonicotinoid insecticide) and propiconazole (an ergosterol-biosynthesis-inhibiting (EBI) fungicide), singly and in combination, in the solitary bee *O. bicornis.* In agricultural environments, bees are likely to be exposed simultaneously to both

compounds because these two groups of agrochemicals are commonly applied to various crops [24,25].

A key question in ecotoxicological studies is whether the test doses applied in the laboratory can be considered to be field realistic. However, estimating field realistic pesticide doses is not easy. The amount of nectar collected in a foraging bout by a nesting *Osmia* female can be estimated from the literature [26], and concentrations of pesticides in nectar can be measured (e.g., [27,28]). However, it is difficult to establish how much of the nectar collected is actually ingested by the foraging female *versus* regurgitated onto the larval food provision. Nonetheless, we know that upon emergence out of the natal nest, and prior to engaging in nesting activities, *Osmia* females collect nectar exclusively for their own consumption [29]. Therefore, we provided newly-emerged *Osmia* females in the laboratory with *ad libitum* feeding solution to simulate this "first nectar meal". To account for the physiological condition of the bees, we measured body size and emergence time. Adult body size in *Osmia* is strongly correlated to the amount of food ingested during the larval period [30]. Large bees have higher lipid content [31], and are more likely to survive the winter [32]. As for emergence time, *Osmia* females lose ~7.5% of their body weight during the process of emerging out of the cocoon [31]. Previous studies have shown that the probability to start a nest and reproduce decreases with emergence time [33], indicating that females that take longer to emerge are less vigorous than females that emerge promptly.

Upon feeding at the flowers, newly-emerged *Osmia* females undergo a short period (2-4 days) during which they complete ovary maturation prior to initiating nesting activities [33,34]. During this period ovary size and vitellogenin concentration in the hemolymph increase in parallel for up to six days [35]. On average, individual *Osmia* females live for about 20 days, and their fecundity is low (10-20 eggs) and highly correlated to the duration of the nesting period [33,34]. Therefore, any effects on ovary maturation during this pre-nesting phase may significantly delay the onset of nesting activities, with important consequences on reproductive success. Consequently, we measured vitellogenin levels, ovary maturation and longevity in females exposed to the neonicotinoid insecticide and the EBI fungicide, singly and in combination. Based on previous studies showing synergistic mortality effects between clothianidin and

propiconazole [18], we hypothesize lower vitellogenin levels, slower ovary maturation and shorter life span in newly-emerged *O. bicornis* females taking their first meal on the neonicotinoid-fungicide mixture. We also hypothesize that these effects will be stronger on bees in poor physiological condition (smaller bees and/or bees taking longer to emerge).

Material and Methods

(a) Bee population and treatments

Osmia bicornis cocoons were obtained from a population nesting in a pesticide free area in Kazimierz Landscape Park, Poland. In January 2016, wintering adults within their cocoons were shipped to the CREA-AA in Bologna, Italy, where they were transferred to a 3 °C cabinet. In early April 2016, cocoons were taken to the laboratory of Agricultural Entomology at the University of Bologna. In mid-April 2016, cocoons presumed to contain females (generally larger than those containing males) were incubated at 21±2 °C and 55±10% RH under natural light. Emergence was checked daily. Since most males emerge a few days before females, any emerging males were discarded. We recorded the days each female took to emerge out of the cocoon following incubation (henceforth emergence time). Upon emergence, females were transferred to a Plexiglas laboratory cage (50 x 50 x 50 cm) to allow them to deposit the meconium. Females emerging on any given day were equally distributed among four treatments: control (feeding solution with 1%-acetone, CON), propiconazole (PRO), clothianidin (CLO) and mixture (propiconazole + clothianidin, MIX). Throughout the study bees were maintained at 21-23 °C, 40-50 % RH under natural light.

(b) Test solution preparation

We used clothianidin active ingredient (purity 99%) from Dr Ehrenstorfer Gmbh. A stock solution was prepared by dissolving technical grade clothianidin (99% pure) in acetone at a nominal concentration of 1000 mg/L (actual concentration: 1090 mg/L), which was then diluted to 1 mg/L (actual concentration: 0.983 mg/L). The stock solution was then diluted in a 38% w:v (33% w:w) sugar + distilled water solution to achieve the desired concentration of 10 µg/L (corresponding to 8.6 µg/Kg). This concentration is within the range of clothianidin residues found in nectar collected from flowers of oilseed rape

grown from clothianidin-coated seeds (6.7-16 µg/L [12]; 5-16 µg/Kg [24]; 2.3-10.1 µg/Kg [36]; <0.7-13.2 µg/Kg [37]);

We tested a propiconazole concentration of 62.5 mg/L. This concentration corresponds to the field application rate of the commercial formulation Protil ® EC (250 g/L of a.i.) in orchards (25 mL/hL or 0.25 L/ha). To obtain this concentration we prepared a stock solution with a propiconazole concentration of 25 g/L by dissolving Protil ® EC in distilled water. The stock solution was then diluted with 38% w:v (33% w:w) sugar solution to achieve the desired concentration.

The final concentration of acetone in the feeding solution was adjusted to 1% (v:v) with pure acetone in all treatments.

(c) Exposure phase

Previous studies have shown that upon emergence out of the cocoon, *Osmia* females take about one day to come out of their natal nest [38]. Therefore, 24 hours after emergence, meconium-free females were individually housed in small plastic cylinders (width: 3.5 cm; high: 5.5 cm) with a transparent plastic lid through which a feeder made with a 1-mL syringe was inserted. Each feeder contained ~150 µL of feeding solution (33% sucrose concentration w:w) with or without pesticides. A flower petal (*Euryops*, Asteraceae) was attached to the tip of the syringe to ensure the bees located the feeder quickly (see [18,39] for details). To simulate a first nectar meal, bees were maintained in these cylinders for 4 hours. Preliminary trials showed that extending this exposure phase up to 8 h did not result in increased solution consumption. To measure the amount of solution ingested by each bee, syringes were weighed before and after the exposure phase. Three cages without bees served as controls to account for potential evaporation. Only bees that fed were included in the statistical analyses. In natural conditions, newly-emerged bees have to fly to reach flowers on which to sip nectar. In our laboratory set-up bees only had to walk a very short distance to have access to a feeding solution source. Therefore, if anything, our method can be assumed to underestimate the amount of nectar and chemical residue ingested by a newly-emerged bee in her first nectar meal. Sample size were 35-50 bees per treatment.

(d) Experiment 1

After the exposure phase, each bee was individually transferred to a plastic ice cream cup (width: 5.5-8 cm; high: 7 cm) with a transparent lid through which a 2.5 mL syringe filled with sucrose solution (33% sugar concentration, w:w) was inserted. Again, a flower petal was attached to the tip of the feeder to ensure the bees located the feeder quickly. Bees were allowed to feed *ad libitum* and the sucrose solution in the feeder was renewed every 3 days. Solution consumption was visually assessed every day. Mortality was monitored daily until all bees died. Upon death, the head width of each bee was measured under a stereomicroscope at 32 X. Head size is strongly correlated to body weight in *Osmia* [30]. Sample sizes were ~30 bees per treatment.

(e) Experiment 2

We followed the same procedure as the experiment 1 with two modifications. First, because pollen consumption enhances ovary maturation in *Osmia* [40], bees of this experiment were provided with a source of pollen throughout the post-exposure phase. In each ice cream cup we provided ~55 mg of pollen in a 1.5 ml Eppendorf tube cap. Pollen was obtained from nests of an *O. bicornis* population nesting in a pear/apple orchard near Bologna. Several provision masses (pollen mixed with nectar) from various nests were mixed to obtain a common homogeneous pollen source from which 55 mg portions were taken. Samples of this pollen source were subjected to palynological and chemical multiresidue analyses (see details in the electronic supplementary material). Chemical analyses revealed that the provisions contained several pesticide residues, including insecticides, fungicides and herbicides at very low concentrations (electronic supplementary material, Table S1). Although unplanned, the presence of these residues resulted in a more realistic exposure, congruent with the co-occurrence of multiple compounds in pollen-nectar matrices in agricultural environments [41,42]. Importantly, no obvious negative effects were observed in the nesting *O. bicornis* population from which the provisions were taken or its progeny.

Second, in this experiment the post exposure phase was interrupted after 3 days to measure vitellogenin levels in the haemolymph and ovary maturation. Details on vitellogenin and ovary maturation measurements are available in the electronic supplementary material.

All statistical analyses are described in the electronic supplementary material.

Results

(a) Exposure phase feeding

The amount of feeding solution ingested during the 4-hour exposure phase differed among treatments (Table 1). Bees of the CLO treatment fed significantly more than bees of the other treatments, and feeding levels were lowest in the MIX treatment (Fig. 1). Solution ingestion during this phase also depended on body size (larger bees ingested more syrup), but not on emergence time (Table 1). However, the interaction between treatment and emergence time was significant. As emergence time increased, feeding increased in CLO bees, whereas it decreased in PRO and MIX bees, and did not change in CON bees (electronic supplementary material, figure S1).

Experiment 1

Differences among treatments in feeding rate (µL of syrup per day) during the post-exposure phase approached significance (Table 1), again with bees of the MIX treatment tending to feed less (Fig. 2). Both body size and emergence time affected post-exposure feeding (Table 1). Feeding rates were higher in larger bees and lower in bees that took longer to emerge.

Cumulative survival curves differed significantly among treatments ($df = 3$, $\chi^2 = 12.99$, $P = 0.005$) (Fig. 3). Throughout the first days following exposure, mortality in the MIX treatment was much greater than mortality in the other treatments, yielding a significant synergistic interaction between clothianidin and propiconazole on day 4 (day 4: $p = 0.045$; day 8: $p = 0.075$; day 17: $p = 0.44$). That is, the CLO–PRO combination was significantly more toxic than the sum of the toxicity of the two compounds separately. Consequently, longevity differed significantly across treatments (Table 1), and was shortest in the MIX treatment (Fig. 2). Body size had no effect on longevity, but bees that took longer to emerge tended to have shorter longevity (Table 1). In addition, there was a significant interaction between treatment and emergence time. As emergence time increased, longevity decreased in PRO and MIX bees, but did not change in CON and CLO bees (Table 1, electronic supplementary material, Fig. S2).

Experiment 2

Nectar feeding rate during the three-day post-exposure phase significantly differed among treatments (Table 1). As in experiment 1, it was highest in the CON treatment and lowest in the MIX treatment (Fig. 4). In contrast to experiment 1, body size and emergence time

did not affect post-exposure feeding (Table 1), but it is important to note that the post-exposure phase lasted only three days in this experiment. We repeatedly observed *O. bicornis* females feeding on the pollen provided. However, the amount of pollen consumed could not be measured because bees spread the pollen all over the hoarding cage.

Three-day cumulative survival curves differed among treatments ($df = 3$, $\chi^2 = 45.72$, $P < 0.001$). Survival was again lowest in the MIX treatment (Fig. 5), and there was a significant synergistic interaction between clothianidin and propiconazole on all three assessment time points (day 1: $p < 0.001$; day 2: $p < 0.001$; day 3: $p = 0.002$). Oocyte length and vitellogenin concentration were measured in all the bees that survived the 3-day post-exposure period (n=55). We found significant differences among treatments in basal oocyte mean length (Table 1), with bees of the MIX treatment having shorter oocytes than bees of the other treatments (Fig. 4). Ovary size was positively related to head size, but was not related to emergence time (Table 1). We found no differences among treatments in vitellogenin concentration (Table 1). Larger bees had higher vitellogenin concentrations, but emergence time did not affect vitellogenin levels (Table 1). No interactions between treatment and head size or emergence time were apparent in this experiment (Table 1).

Discussion

Wild and managed bees are exposed to pesticide mixtures in agricultural and urban areas [41,43-45]. Neonicotinoids and EBI fungicides, in particular, are routinely used on many crops [24,25], and have often been found together in the nectar and pollen of both cultivated and wild flowers [37,41], in honey bee-collected pollen and on bee body surfaces [41,46,47]. In a previous study [18] we showed synergistic mortality effects in honey bees, bumblebees and solitary bees (*Osmia bicornis)* acutely exposed to sublethal doses of CLO (0.63 ng/bee) and PRO (7 µg/bee) in a fixed amount of syrup (10 µL). The amount of CLO ingested by bees in that study was within the range of CLO potentially ingested in a foraging bout. However, the tested concentration (63 µg/L of CLO) was higher than concentrations likely to be found in nectar (<0.7-16 µg/L) [12,24,36,37,48]. On the other hand, considering the honey stomach capacity of honey bees (~ 30 µL) and bumblebees (80 µL) [49,50] it is conceivable that a bee could ingest more than 10 µL of

nectar in a single foraging bout. At any rate, given the difficulty to estimate what proportion of the nectar collected by a nesting female bee is ingested versus regurgitated in the nest, in this study we worked with pre-nesting females, which consume all the nectar they collect. Our study provides first-time-evidence that oral exposure to field-relevant concentrations of an insecticide and a fungicide mixture affect feeding behavior, ovary maturation and longevity in a solitary bee.

Results of syrup consumption during the exposure phase show that *O. bicornis* females not only did not avoid but even preferred neonicotinoid-laced syrup. This behavior has also been observed in bumblebees and honey bees [51,52]. Interestingly, syrup consumption during this phase was lowest in bees of the MIX treatment, indicating that the attractiveness of clothianidin was lost when propiconazole was added. Post-exposure feeding rate (ml of syrup consumed per day) was also lowest in the MIX treatment in both experiments (although differences among treatments narrowly failed significance in Experiment 1), suggesting that the clothianidin-propiconazole combination alters the feeding behavior of *O. bicornis*.

Vitellogenin is a fat-body-synthesized glycolipophosphoprotein that constitutes a significant part of the yolk protein of insect eggs [53]. In *Osmia*, vitellogenin concentration in the hemolymph increases with ovary maturation, reaching maximum levels 3-6 days after adult emergence and gradually declining thereafter [35]. Studies on honey bee and bumblebee queens have reported a strong up-regulation of vitellogenin genes [54] but slower ovary maturation following experimental neonicotinoid exposure [55,52]. Because pollen feeding enhances ovary development in bumblebees [56], Baron *et al.* [52] hypothesized a reduction in pollen consumption in bees exposed to neonicotinoids. *Osmia* females also require pollen to mature their oocytes [40]. Our bees clearly fed on the pollen supplied in experiment 2, but we could not establish whether pollen consumption differed among treatments because bees spread the pollen over the hoarding cages. At any rate, we did not find differences in vitellogenin concentration or ovary maturation between clothianidin-exposed and control bees. On the other hand, we found that ovary maturation was slowest in bees of the MIX treatment, even if this reduction was not accompanied by increased levels of vitellogenin concentration.

In experiment 1, longevity of propiconazole- and clothianidin-exposed bees (mean: 17 and 19 days, respectively) did not differ from that of control bees (mean: 17.5 days). These life spans are similar to those recorded in field and greenhouse populations (17.5 - 24 days [33,34,57]; though mean longevity can be extended up to 30.5 days under bad weather conditions; [34]). Bees of the CLO treatment consumed larger amounts of feeding solution, thus ingesting greater amounts of sugar, which could have buffered any negative effect of clothianidin [58]. By contrast, exposure to the MIX treatment resulted in significantly reduced longevity. Life span of bees of the MIX treatment in experiment 1 was 10 days, that is 0.5-0.6 times shorter than that of control bees (17.5 days) and bees exposed to single compounds (17 and 19 days, respectively). The negative effect of the pesticide mixture was further evidenced by the comparison of the survival curves of the various treatments, revealing a synergistic interaction between clothianidin and propiconazole on survival probability in both experiments. Three days after exposure, mortality in the MIX treatment of experiment 2 was 78%, more than twice higher than expected under additive (non-synergistic) effects (36%). Bees of experiment 2 were fed pollen during the post-exposure phase whereas bees of the experiment 1 were not, and the pollen supplied was contaminated with pesticide residues (electronic supplementary material, Table S1). This pollen was obtained from *O. bicornis* provisions from a population nesting in a pear/apple orchard that was sprayed during bloom with boscalid. This fungicide was the main chemical residue found in the pollen, but four other chemicals that were not sprayed in the orchard were also found. Pollen analysis of the provisions revealed that *O. bicornis* females foraged mostly on wild plants (*Quercus robur* (39%), *Ranunculus* spp. (27%), *Cercis* spp. (25%), apple/pear (2 %)). Thus, our study provides further evidence of pesticide exposure affecting not only bees foraging on sprayed crops, but also those foraging on the accompanying flora [13,59,60].

Differences between experiments 1 and 2 in survival probability at day 3 were very small for the CON (87% *vs* 87%) and PRO (82% *vs* 88%) treatments. By contrast, these differences were very pronounced for the CLO (93% *vs* 73 %) and the MIX treatments (48% and 22%), suggesting that, even at the low concentrations recorded, the presence of additional pesticides in the pollen supplied in experiment 2 interacted with the clothianidin ingested during the exposure phase.

We used body size and timing of emergence as proxies of physiological condition. Not surprisingly, large bees consumed more feeding solution during the exposure phase and during the post-exposure phase of experiment 1. No such relationship was found in experiment 2, but the post-exposure phase of this experiment lasted only three days. Larger bees also had higher levels of vitellogenin in the hemolymph and, in agreement with previous studies [33], produced larger oocytes. However, large bees did not live longer than small bees. Studies on *Osmia* populations nesting in field and greenhouse conditions have also failed to find a relationship between female body size and longevity (or nesting period) [33,34,61-63].

Emergence time affected post-exposure feeding solution consumption rate and longevity in experiment 1, both of which were lower in females with long emergence periods. These results are congruent with the reduced ability of bees that take long to emerge to start nesting activities [33]. As with body size, such a relationship was not apparent in experiment 2, possibly due to the short post-exposure phase of this experiment. Despite their lower feeding solution consumption, we did not find lower vitellogenin levels or slower ovary maturation in bees with long emergence times.

Physiological condition may influence sensitivity to pesticides [21]. Our results show that the negative effects of emergence time on longevity occurred only in the MIX and PRO treatments. The suboptimal physiological condition of bees with long pre-emergence periods could have reduced their detoxification capacity making them more vulnerable to these two treatments. To our knowledge, this is the first time an effect of physiological condition on sensitivity to pesticides is shown for a solitary bee. Ecotoxicological studies are often carried out under conditions that are assumed to be optimal for the test organisms (e.g., healthy individuals kept at adequate temperatures with *ad libitum* feeding). In the field, however, bees may be exposed to various stress factors, such as parasites, diseases, and limiting food resources, which could magnify the negative effects of pesticides. In their review, Holmstrup *et al.* [64] argue that synergistic interactions between toxic compounds and natural stressors are frequent and should be considered in risk assessment schemes.

Our study shows that a single meal with a cocktail of pesticides at sublethal doses and realistic concentrations during the pre-nesting period affects feeding behavior, ovary maturation and longevity in a solitary bee. Importantly, none of these effects were

observed when bees were exposed to either compound singly. The pre-nesting period is a critical stage in the life cycle of solitary bees for two reasons. First, females in poor physiological condition are less likely to start nesting activities and reproduce [33]. Our results show that nesting success of these weakened females may be further compromised by exposure to pesticide mixtures at realistic field concentrations. Second, fecundity of females that do successfully nest is highly correlated to the duration of the nesting period [33,34], which is constrained by ovary maturation at one end [33,35] and by death at the other end. Our insecticide-fungicide mixture had negative effects on both ovary maturation and longevity, thus affecting the duration of the nesting period at both ends. Under field conditions, *Osmia* females live ~ 20 days on average [34]. Of this time, ~ 5 days are spent maturing the ovaries [35], prior to the initiation of nesting activities (pre-nesting period) [33,34]. During the rest of their life time (nesting period), females build and provision nest cells and lay eggs at a rate of ~ 0.7 per day [34]. If we assume that mean longevities recorded in our study are representative of longevities under field conditions, females of our MIX treatment would have laid a mean of 3.5 eggs compared to 8.4 in control bees. We conclude that our findings have direct repercussions on the reproductive success of solitary bees, and provide a potential mechanism to explain observed negative dynamics of *Osmia* populations in agricultural environments [12,13,65]. Our study has also important implications for pesticide regulation. Current risk assessment schemes rely on tests of single compounds [27,28]. Our results underscore the need to consider pesticide combinations likely to occur in agricultural environments.

Data accessibility: Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.895pn6p [66].

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FIGURES

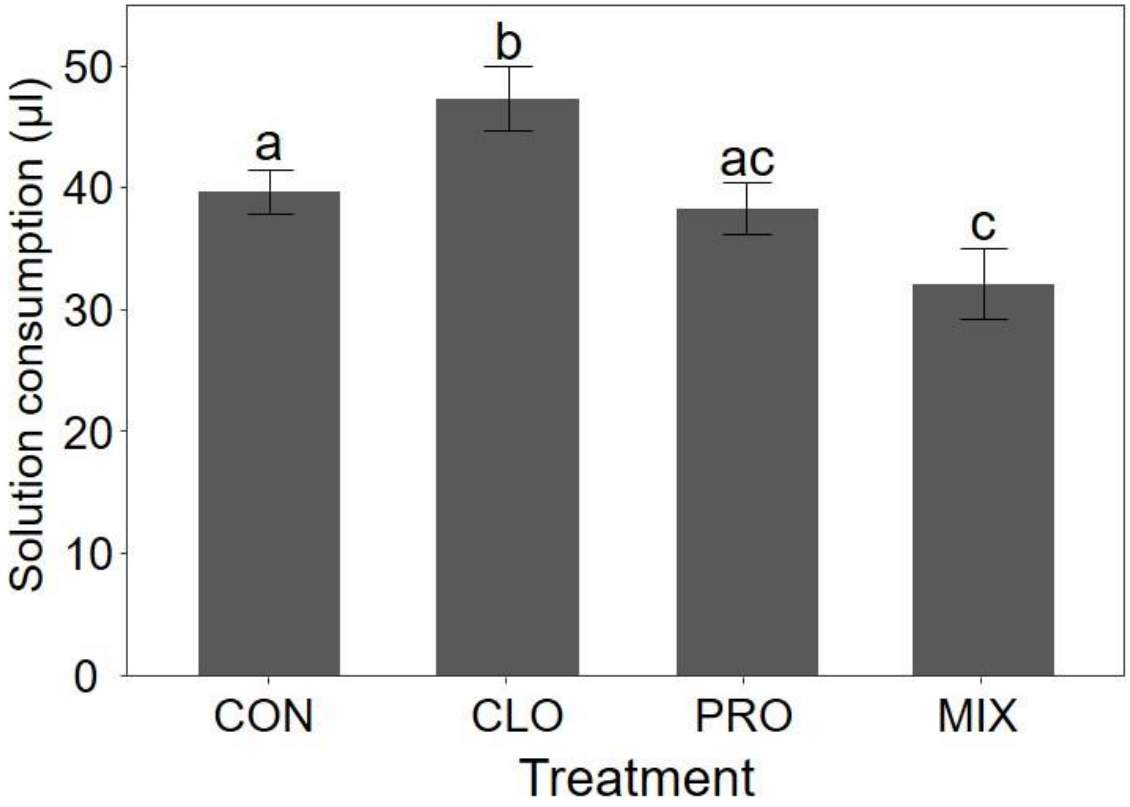

Figure 1. Mean + SE test solution ingested during the 4-hour exposure phase in *O. bicornis* females orally exposed to four treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX: clothianidin + propiconazole mixture). Different letters denote significant differences (Fisher LSD Post-hoc, $P<0.05$).

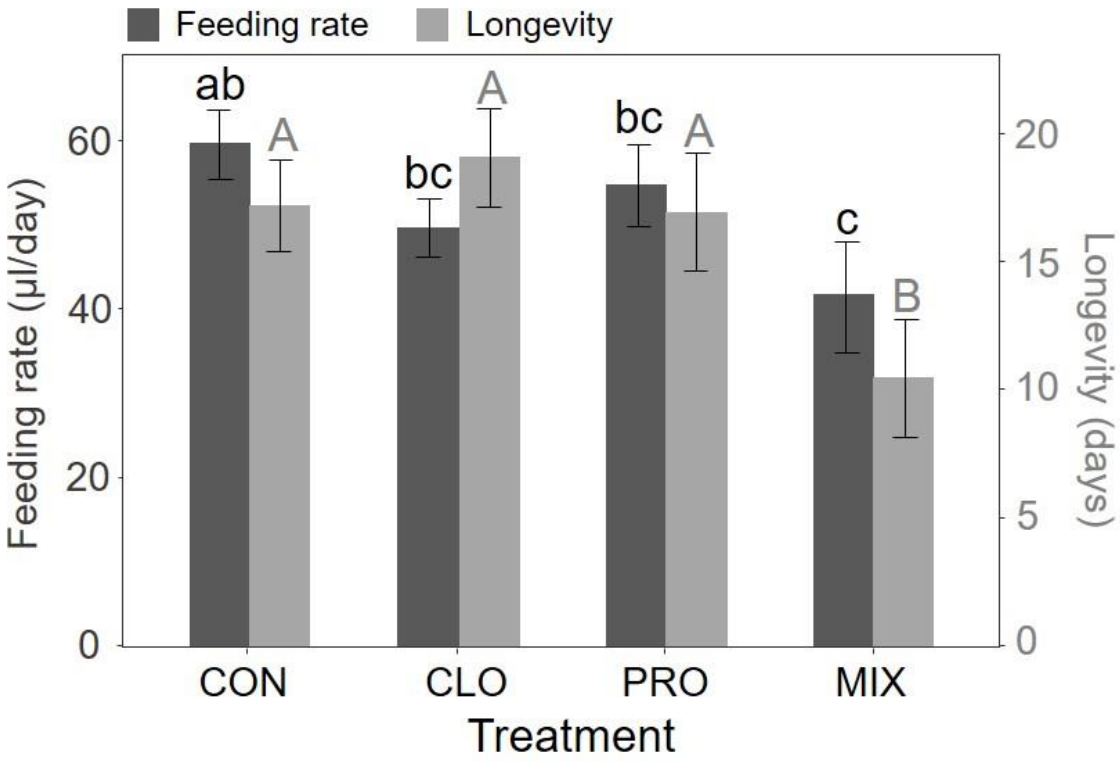

Figure 2. Experiment 1- Mean + SE post-exposure feeding rate (µl of feeding solution ingested per day) and longevity in *O. bicornis* females orally exposed to four treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX: clothianidin + propiconazole mixture). Different letters denote significant differences (Fisher LSD Post-hoc, $P<0.05$).

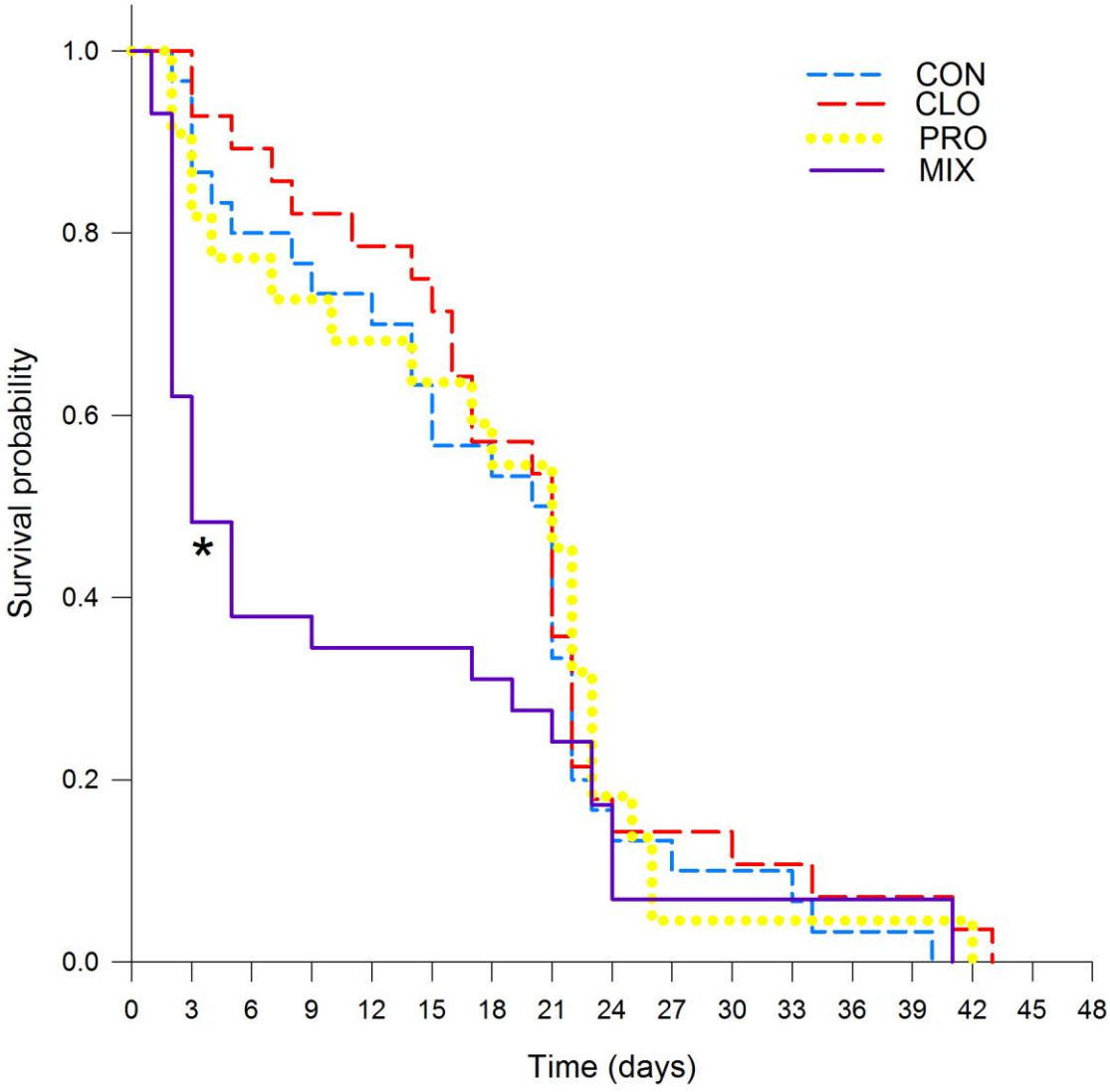

Figure 3. Experiment 1 - Cumulative survival probability of *O. bicornis* females orally exposed to four treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX: clothianidin + propiconazole mixture). Synergistic interactions between CLO and PRO treatments ($P < 0.05$; one-tailed binomial proportion test; assessment times: 4, 8, and 17 days) are marked with an asterisk.

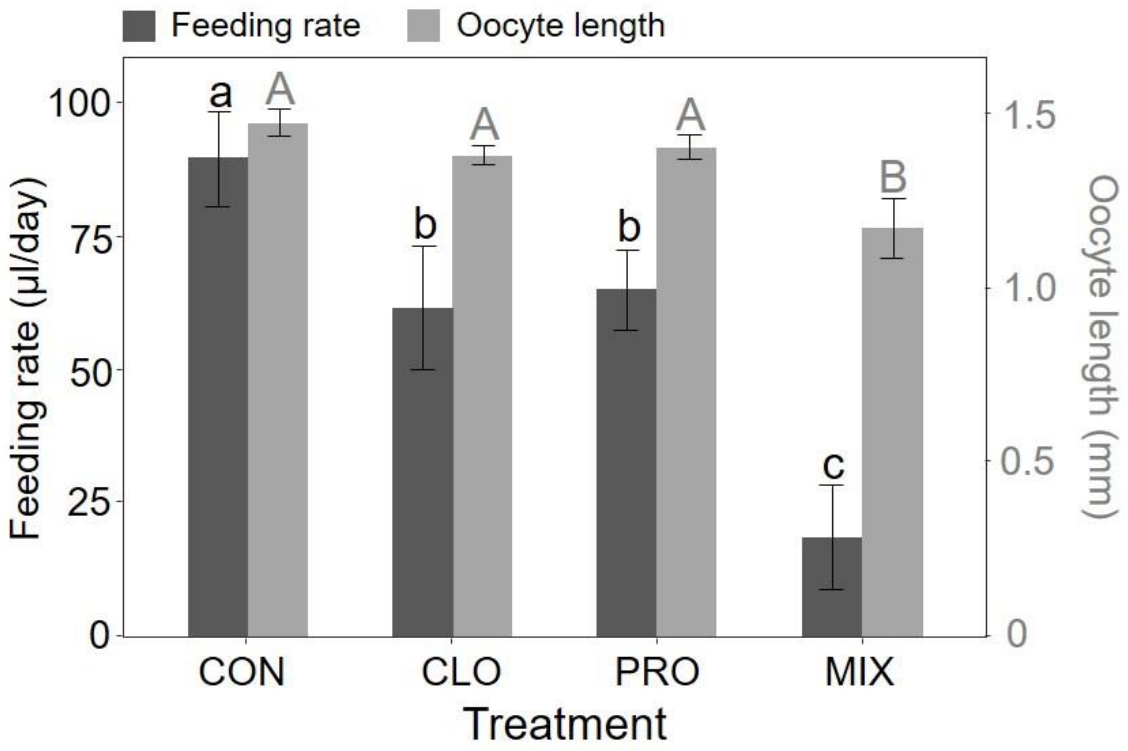

Figure 4. Experiment 2 - Mean + SE post-exposure feeding rate and basal oocyte length in *O. bicornis* females orally exposed to four different treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX: mixture). Different letters denote significant differences (Fisher LSD Post-hoc, $P<0.05$).

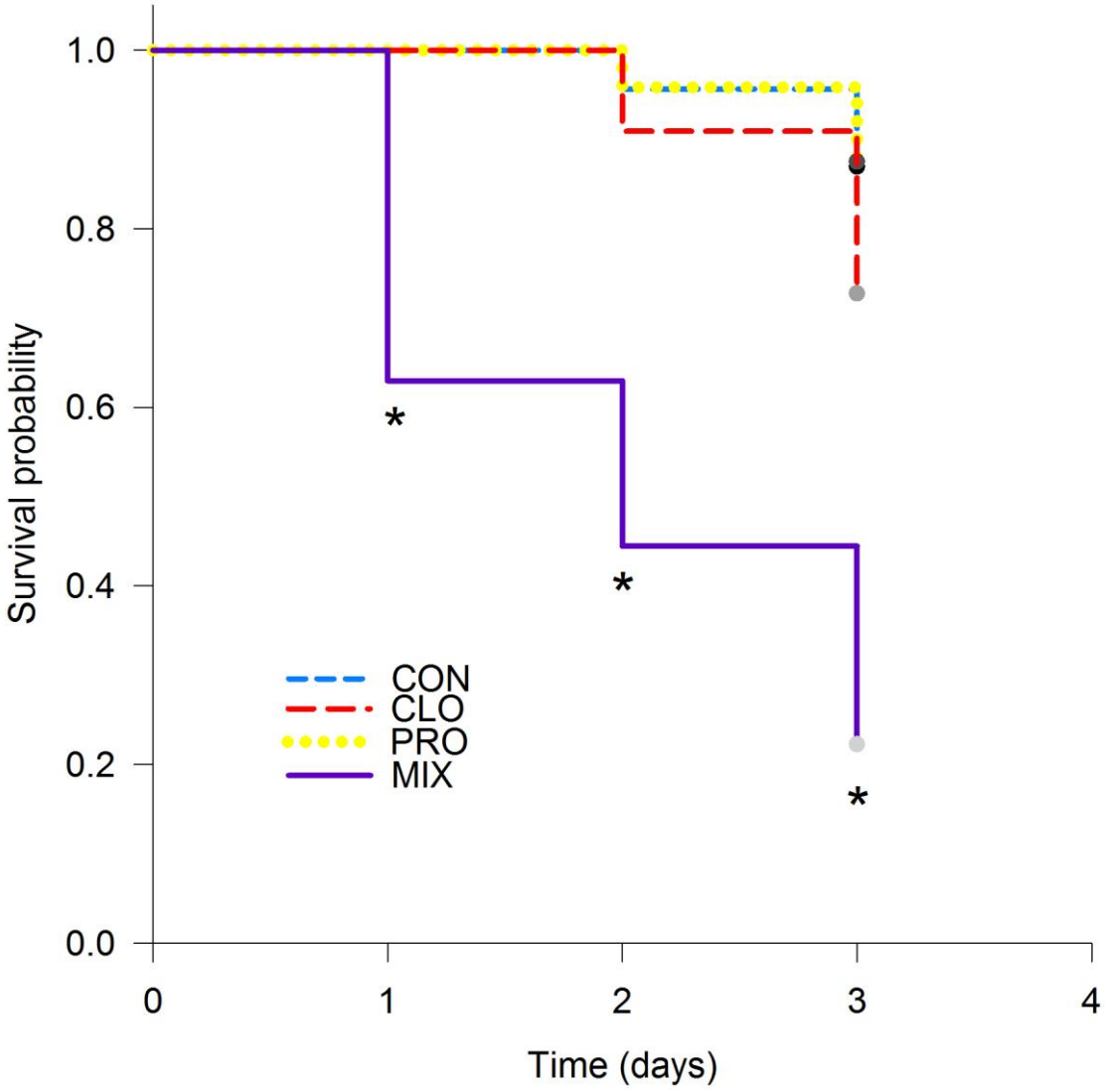

Figure 5. Experiment 2 - Cumulative survival probability of *O. bicornis* females orally exposed to four treatments (CON: control; CLO: clothianidin; PRO: propiconazole; MIX: clothianidin + propiconazole mixture). Synergistic interactions between CLO and PRO treatments ($P < 0.05$; one-tailed binomial proportion test; assessment times: 1, 2, 3 days) are marked with an asterisk.

TABLES

Table 1. Best selected (ΔAICc < 2) general linear models explaining the effects of treatment (Tr), emergence time (ET), head size (HS) and the interactions between treatment and emergence time and treatment and head size on each response variable. Significant predictors ($p < 0.05$) in bold, marginally significant predictors ($p=0.05 – 0.1$) in italics. Positive and negative signs in brackets denote the direction of the relationship.

	Response variable		*Model components*	AIC_c	ΔAIC_c	w_i	R^2 (%)
Experiment 1	Exposure feeding	1	**Tr** + ET (+) + **HS (+)** + **Tr:ET**	1376.7	0.00	0.592	22
		2	**Tr** + **HS (+)**	1378.4	1.73	0.249	17
	Post-exposure feeding rate	1	*Tr* + **ET (-)** + **HS (+)**	707.1	0.00	0.463	21
		2	**ET (-)** + **HS (+)**	707.5	0.44	0.371	14
	Longevity (sqrt-transformed)	1	**Tr** + ET (+) + **Tr:ET**	380.3	0.00	0.358	26
		2	**Tr** + **ET (-)** + HS (+) + *Tr:ET*	381.3	0.99	0.218	27
		3	**Tr** + **ET (-)** + HS (+)	381.9	1.62	0.159	21
		4	**Tr** + **ET** (-)	382.2	1.89	0.139	19
Experiment 2	Post-exposure feeding rate	1	**Tr**	647.5	0.00	0.562	22
	Oocyte length	1	**Tr + HS (+)**	-51.0	0.00	0.667	37
		2	**Tr** + ET (+) + **HS (+)**	-49.3	1.78	0.273	38
	Vitellogenin concentration (sqrt-transformed)	1	**HS (+)**	123.1	0.00	0.467	27
		2	ET (-) + **HS (+)**	123.1	0.03	0.460	31

Chapter 4: Biomarkers of nutritional status in honey bee haemolymph: effects of different biotechnical approaches for Varroa destructor treatment and wintering phase

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Short title

Nutritional biomarkers in honey bee haemolymph

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Abstract

Oxalic acid achieves its maximum efficacy during the active season only when coupled with brood manipulation techniques like brood interruption and brood removal. This study aimed to assess the impact of these manipulations on the colony nutritional status and the subsequent wintering phase, focusing on selected haemolymph biomarkers: total proteins (TP) , zinc (Zn), vitellogenin (VG), and apolipophorin (APO). Twenty-five days after the manipulations (T1) colonies that underwent brood interruption (BI) stored more TP and VG than colonies in the brood removal groups (BR), with a lower APO percentage, suggesting a lower metabolic effort in summer. In winter, honey bee colonies of all groups reached similar concentrations of the abovementioned parameters, but colonies in the BI group showed a higher population. TP, VG and APO are shown to be promising biomarkers of nutritional status of the colony. Basing on the results obtained we suggest brood interruption coupled with oxalic acid as the preferred organic method for the control of *Varroa destructor* in summer.

Keywords

haemolymph proteins/apolipophorin/vitellogenin/nutritional status/*V. destructor* control

Introduction

Biomarkers are a common tool to evaluate health, diagnose disease, monitor therapies and predict outcomes both in human and veterinary medicine (Myers et al., 2017). Few biomarkers have been proposed in apidology and none of them made the way to clinical practice. The activity of some enzymes has been described in literature as biomarker of xenobiotic exposure: AChE activity increases during imidacloprid or clothianidin intoxication (Boily et al., 2013), while CaEs, GST, CAT and ALP activity is modified in bees exposed to thiamethoxam (Badiou-Bénéteau et al., 2012). Regarding nutritional status, haemolymph protein concentration is a good candidate as it relates with the quality of food proteins (De Jong, 2009). Moreover, being vitellogenin the main protein of the haemolymph in adult bees (Amdam et al., 2003), its concentration is also a valuable tool to evaluate nutritional status due to its relation with quality (Cremonez et al., 1998) and quantity (Basualdo et al., 2013; Bitondi and Simões, 1996) of proteins in the diet.

The nutritional status is of capital importance in the wintering phase to allow colony survival across several months, relying on carbohydrates only. In fact, in temperate climates, honey bees exhibit two different phenotypes: short lived bees, reared from late spring to summer and long lived bees, derived from the generation produced in late summer and autumn (Fluri et al., 1982; Maurizio, 1950). The lifespan of the first type is about 25-35 days, while the lifespan of the latter can exceed 6 months (Free and Spencer-Booth, 1959). The longer lifespan of winter bees is related to a higher haemolymph protein concentration, mainly due to the accumulation of vitellogenin (Amdam et al., 2004; Fluri et al., 1982, 1977). The long-lived phenotype can be also obtained, regardless of the season, by manipulating the colony into interrupting brood rearing, for example by caging the queen and thus preventing oviposition (Amdam et al., 2004; Fluri et al., 1982, 1977; Maurizio, 1950). The absence of brood is a prerequisite to achieve maximum efficacy in summer of oxalic acid, of one of the most widely adopted organic acaricides; in fact, this compound is active only on phoretic mites (Nanetti et al. 2003).

Gregorc et al. (2017) recently studied the efficacy of the two most popular solutions proposed by beekeepers: brood interruption by queen caging and brood removal. Less is known about the impact of these two techniques on the nutritional status of the colony, in the short term and, more importantly, in the wintering phase.

Considering the expected effects of brood manipulations on honeybee physiology, the aim of this paper is to study these modifications in the context of the treatment against *V. destructor*, using a panel of haemolymph biomarkers: total proteins, vitellogenin and apolipophorin and Zn.

Materials and Methods

All the colonies were initially housed at the CREA-AA apiary in Bologna, Italy. All the analyses were performed at the Department of Veterinary Medical Sciences, Alma Mater Studiorum - University of Bologna. All reagents were purchased from Thermo Fisher Scientific unless otherwise specified.

Study design, sample collection and colony level traits

Ten homogeneous *Apis mellifera ligustica* derived colonies, naturally infested with *V. destructor* and with similar anamnesis were enrolled in the present study; all of them consisted of nine frames covered with bees and seven frames with brood of variable age, honey and pollen stores were sufficient and normal for the season. Five colonies were randomly assigned to group BI that underwent 25 days of brood interruption by queen caging, while the remaining five colonies were assigned to group BR that underwent brood removal. Since in our climatic conditions colonies left untreated for mites usually collapse before winter (Rosenkranz et al., 2010), no control group was used as the death of these colonies would have prevented a complete and useful collection of data. Moreover, colonies with uncontrolled mite infestation can result in increased risk of mite exchange with treated colonies.

All the brood frames of each BR colony were separated from the original colony and used to establish a single nucleus; a small number of bees (ca. 700 workers per comb, approximately 3 portions of frame) was left on these combs for brood tending. Care was taken during nucleus formation to guarantee that a sufficient amount of honey was present on the brood frames in order to satisfy colony needs. Therefore, from BR group derived two sub-groups: original colony (BR-oc), consisting of adult bees, the queen and frames of stores; and nucleus (BR-n), consisting of a small number of adults and the frames with brood and stores. The BR-oc colonies were immediately transferred to another apiary in order to prevent robbery and mite exchange between them (just treated) and the untreated colonies of the other two groups. The second apiary was distant about

three kilometres from the first one and was selected for similarity of micro-climatic conditions and trophic sources.

Each treatment was administered to the colonies in absence of brood by trickling a solution of sucrose and API-Bioxal (Chemicals Laif) according to the manufacturer instructions. The BR-oc colonies were treated after 5 days instead of at day 0 to allow them to build new combs and recover the internal dynamics. After 5 days no receptive brood was present so full treatment efficacy could be achieved. The BR-n and BI hives were treated after 25 days when all the brood was hatched. To ensure the absence of receptive brood in BR-n colonies, natural queen cells were removed on day 7 and replaced with artificial ones at day 13. Samplings were performed at three critical time-points: pre-manipulation (T0, end of July), post-manipulation (T1, 25th day, August) and winter (T2, 148th day, December). At T0 and T2, colony-level traits (areas covered with bees, brood, sealed brood and pollen) were estimated. A modified Liebefelder standard method (Delaplane et al., 2013) was used: during the inspection, every face of the frame was divided into six portions and the number of portions covered with each matrix recorded. The number of mites killed by the treatments was used to estimate the *V. destructor* population size, as suggested by (Branco et al., 2006). The mites were counted carefully on sticky boards, placed under mesh floor equipped hives, from the beginning of the trial until the 8th day after the treatment.

The evaluation of mite fall started at day 0 for the colonies of all groups and continued until day 33 for the colonies of BI and BR-n groups (25 days of natural mite fall, until colonies were broodless, and 8 days of treatment effect) and until day 13 for the colonies of BR-oc group (5 days of natural mite fall + 8 days of treatment effect). The estimation of the *V. destructor* population of the colonies of BR group resulted from the sum of the respective BR-n and BR-oc colonies.

The graphic timeline of the field trial is reported in Figure 1.

Haemolymph collection

For each colony, 30 worker bees were collected between the last brood frame and the stores as suggested by van der Steen, et al., (2016). At T1 when no brood was present in BR-n and BI colonies, bees were collected between a store frame and the adjacent frame where brood is expected to be in presence of a fertile queen. Worker honeybees were narcotized with gaseous CO_2, held between forceps by the thorax while a glass disposable

graduated microcapillary (125 mm length, accuracy ± 0.30%, reproducibility ± 0.6%, Blaubrand®) was inserted between fourth and fifth tergite. Two microliters of transparent uncontaminated haemolymph were collected from each bee. Haemolymph samples collected from 30 specimens were pooled together, added with glutathione to a final concentration of 0.1% p/v (L-glutathione reduced, Sigma) centrifuged at 3000 *g* for 15 min in order to separate the cells and stored at -80°C for subsequent analysis.

Total protein and Zn analysis

Total protein (TP) concentration was measured by the Bradford method (Coomassie protein assay) following manufacturer instruction.

The quantification of Zn was conducted by graphite furnace atomic absorption spectroscopy (GFAAS) (Varian Spectra AA 20 Plus) using palladium, magnesium and EDTA matrix modification with high temperature pyrolysis (Stevens et al. 2017). The haemolymph samples were centrifuged into small tubes at 3000 *g* for 15 min to avoid possible solid material and 2.5 µl were dissolved in 1000 µl 0.1% Pd/Mg/EDTA matrix modifier for the estimation of Zn concentration.

SDS-PAGE

For each sample, three µg of proteins were loaded and analysed with the protocol that assured the best protein separation in our experimental conditions (4–12% gradient gels, in MOPS buffer; NuPAGE, Invitrogen). The gels were stained with Coomassie G250 compatible with mass spectrometry analysis, digitalized by ChemiDoc™MP (BioRad) and the pherograms obtained using ImageLab 5.2.1 software (BioRad). The software determines the volume of each protein band through the analysis of the pixel values in the digital image, meaning as volume the sum of all the pixel intensities within the band boundaries. The band volumes were subsequently compared to the entire volume of the lane and the relative abundance of each protein band was reported as percentage, representing therefore the respective relative abundance as a percentage of the total proteins. The concentration of vitellogenin and apolipophorin expressed as mg/mL was obtained by multiplying the total protein concentration by the corresponding relative abundance in percentage.

Protein identification by mass spectrometry

The bands which molecular weight corresponded to vitellogenin (180 kDa) and apolipophorins (250 kDa) were cut form the gels, digested in-gel with trypsin and finally analysed by a Nano LC-CHIP-MS system (ESI-Q-TOF 6520; Agilent Technologies) as previously described (Bellei et al. 2013). Raw mass spectrometry data were processed to obtain the Mascot generic files for database searching. Since the honeybee haemolymph protein database is not well annotated, a broader taxonomy was selected for identification to be based on sequence homology. Protein-identification peak lists were generated using the Mascot search engine (http://mascot.cigs.unimo.it/mascot) against the UniProt database (UniProt.org) specifying the following parameters: all entries, parent ion tolerance ±40 ppm, MS/MS error tolerance ±0.12 Da, alkylated cysteine as fixed modification and oxidized methionine as variable modification, and two potential missed trypsin cleavages. Proteins with a score >80 or identified with at least two or more significant sequences were selected. The significant threshold in Mascot searches was set to obtain a false discovery rate <5% (5% probability of false match for each protein with a score above 80).

Statistical analysis

Statistical analysis was performed with R software (3.2.5). Data are reported with mean (M) and standard deviation (SD). Normal distribution was tested by Shapiro-Wilk normality test. Correlation between laboratory (TP, Zn, VG, APO) and colony-level traits (*Bees*, *Total brood*, *Sealed brood*, *Pollen*, *V. destructor population size*) was performed by a Pearson's correlation test. Two-way ANOVA for repeated measures was performed to evaluate the effect of the interaction of the factors group and time on the laboratory parameters. One-way ANOVA with Tukey's post-hoc test for pairwise comparison was performed to evaluate differences among groups (BI, BR-oc, BR-n) within time-points (T0, T1, T2) for colony-level traits and laboratory parameter or among time-points within groups for laboratory parameters. Paired *t*-test was performed to evaluate differences between time-points (T0 and T2) within groups for colony-level traits and to evaluate differences between the groups BI and BR at T0. Values of $P<0.05$ were considered statistically significant.

Results

Colony level traits

Data on *Bees*, *Total brood*, *Sealed brood* and *Pollen* are reported in Table I. At T0 there were no statistically significant differences between BI and BR. Also *V. destructor* population size did not show significant differences between BI (M = 2691, SD = 2070) and BR (M = 2823, SD = 1202). At T2 only the parameter *Bees* differed significantly between groups (F [2,12] = 7.35, P=0.008); portions of the frame covered with bees was significantly higher in the colonies of BI group (M = 45.9, SD = 14.08) than those from BR-oc group (M = 20.8, SD = 4.42).

Regarding differences between T0 and T2, all the parameters showed a significant decrease in the wintering phase (T2).

Total protein and Zn concentrations

Total protein and Zn concentrations in honeybee haemolymph are reported in Figure 2 and Table II. TP ranged from 12.4 at T0 to 33.7 mg/mL at T2, while Zn from 2.7 at T0 to 4.8 µg/mL at T2.

A two way analysis of variance showed that the effect of the interaction between time and group on TP was significant (F[2,11] = 4.302, P = . 041670).

As regards significant differences among groups, at T0 and T2 there were no significant differences. At T1, TP concentration differed significantly between groups (F [2,12] = 33.38, P= 1.25e-05); protein concentration was significantly higher in the colonies of BI group (M = 31.72, SD = 4.55) than in those from BR-oc group (M = 14.44, SD = 3.09) and BR-n group (M = 19.84, SD = 2.22).

An analysis of variance showed also that the effect of time on TP was significant for group BI (F[2,11] = 8.71, P = .0054), BR-n (F[1,7]= 26.06 P = .000108) and BR-oc, (F[2,11] = 20.67 P = . 000188). Protein concentration was significantly higher at T1 (M = 31.72, SD = 4.55) and T2 (M = 33.70, SD = 11.03) than at T0 (M = 14.90, SD = 1.72) for BI colonies and significantly higher at T2 (M = 36.25, SD = 7.75) than T1(M = 19.84, SD = 2.22) and T0 (M = 13.60, SD = 2.02) for BR-n. Same as BR-n can be said about BR-oc with T2 (M = 33.40, SD = 8.21) higher than T0 (M = 13.60, SD = 2.019901) and T1 (M = 14.44, SD = 3.086746).

Regarding Zinc, there were no significant differences between groups or between time points.

Protein separation by SDS-PAGE

The optimized protocol resulted in the separation of different protein bands in samples of worker honeybee haemolymph. All samples presented a similar pattern characterized by the presence of five most abundant protein bands (Fig. 3). The two most consistent bands were identified by mass spectrometry: the band at an apparent molecular weight >200 kDa was unambiguously identified as apolipophorin (APO), while the band at 180 kDa was identified as vitellogenin. Other important protein bands were present at 72, 70 and 67 kDa in all the analysed samples. Less abundant bands were present in particular at molecular weights less than 60 kDa.

Percentage and concentration of VG and APO

Data on VG and APO are reported in Figures 4 and 5, respectively and in Table II. VG concentration in haemolymph ranged from 2.8 to 17 mg/mL accounting for 43-46 % of haemolymph TP at the wintering phase (T2). On the contrary, APO concentration showed minor variations ranging from 3.6 to 5.8 mg/mL and at T2 this protein represented only 11-13%. When evaluating the differences among BI, BR-n and BR-oc within each time-point, at T0 and T2, there were no significant differences.

A two way analysis of variance showed that the effect of the interaction between time and group was significant on VG percentage ($F[2,11] = 14.55$, $P = .000813$) and APO percentage ($F[2,11] = 19.61$, $P = .000236$) but not on VG concentration and APO concentration.

At T1, after the manipulations, there were statistically significant differences among groups regarding VG percentage ($F [2,12] = 13.54$, $P = .000837$) and VG concentration ($F [2, 12] = 30.94$, $P = 1.84e-05$). VG concentration in colonies of the group BR-oc ($M = 26.98$, $SD = 5.84$) was significantly lower than that of groups BR-n ($M = 35.08$, $SD = 1.79$) and BI ($M = 39.48$, $SD = 2.68$). On the other hand, VG concentration differed among all the groups with the highest value in BI group ($M = 12.55$, $SD = 2.18$) followed by BR-n group ($M = 6.99$, $SD = 1.08$) and BR-oc ($M = 4.03$, $SD = 1.78$). A similar pattern could be observed regarding APO percentage ($F [2,12]$, $P = 6.51e-05$), with highest value in BI group ($M = 5.83$, $SD = 0.53$) followed by BR-n group ($M = 4.60$, $SD = 0.45$) and BR-oc ($M = 3.50$, $SD = 0.60$).

APO percentage showed also significant differences among groups (F [2,12] = 17.87, P = .000252). In this case, the value for the BI group (M = 18.50, SD = 1.27) was significantly lower than those from BR-n (M = 23.32, SD = 2.30) and BR-oc (M = 24.46, SD = 1.22).

Regarding variations among time-points, there were significant differences in VG percentage for BI group (F [2,11] = 22.1, P = .00014), BR-n (F [2,11] = 36.41, P = 2.57e-05) and BR-oc (F [2,11] = 20.46, P = .000196). In BI group the percentage was significantly higher at T1 (M = 39.48, SD = 2.68) and T2 (M = 43.68, SD = 3.97) than at T0 (M = 23.63, SD = 7.03). In BR-n group all the time points differed significantly from each other with, in ascending order, T0 (M = 23.60, SD = 5.33), T1 (M = 35.08, SD = 1.79) and T2 (M = 46.43, SD = 3.87). A totally different pattern can be observed in BR-oc group with only the percentage at T2 (M = 44.12, SD = 4.62) significantly higher than those at T0 (M = 23.60, SD = 5.33) and T1 (M = 26.98, SD = 5.84).

There were significant differences in VG concentration for BI group (F [2,11] = 9.85, P = .00353), BR-n (F [2,11] = 36.41, P = 2.57e-05) and BR-oc (F [2,11] = 20.46, P = .000196). In BI group the percentage was significantly higher at T1 (M = 12.55, SD = 2.18) and T2 (M = 15.03, SD = 6.14) than at T0 (M = 3.58, SD = 1.31). In BR-n group only T2 (M = 36.25, SD = 7.75) differed significantly from T0 (M = 13.60, SD = 2.02) and T1 (M = 19.84, SD = 2.22) while in BR-oc group only the percentage at T2 (M = 15.03, SD = 5.62) significantly higher than those at T0 (M = 3.23, SD = 1.04) and T1 (M = 4.03, SD = 1.78).

APO percentage showed also significant differences among time points in BI group (F [2,11] = 25.72, P = 7.12e-05), BR-n (F [2,11] = 30.61, P = 5.46e-05) and BR-oc (F [2,11] = 48.87, P = 3.37e-06). In BI group the percentages at T1 (M = 18.50, SD = 1.27) and T2 (M = 13.12, SD = 2.09) were significantly lower than that at T0 (M = 28.25, SD = 5.36).

In BR-n group only the percentage at T2 (M = 11.30, SD = 1.89) was significantly lower than those at T0 (M = 28.88, SD = 4.97) and T1 (M = 23.320, SD = 2.30).

The same pattern can be observed for BR-oc group where only the percentage at T2 (M = 11.08, SD = 1.57) was significantly lower than those at T0 (M = 28.88, SD = 4.97) and T1 (M = 24.460, SD = 1.22).

Correlations were studied using all the values in the dataset, independently from treatment groups or time of sampling. There were significant positive correlations between TP and VG concentration (r=0.991, $P<0.01$), TP and VG percentage (r =0.89,

P<0.01), TP and Zn concentration (*r* =0.533 *P*<0.05), Zn and VG concentration (*r* =0.55, *P*<0.01), Zn and VG percentage (*r* =0.526, *P*<0.05). There were significant negative correlations between APO percentage and TP (*r* =-0.839, p<0.01), Zn (*r* =-0.498, *P*<0.05), VG concentration (*r* =-0.864, *P*<0.01) and VG percentage (*r* =-0.929, *P*<0.01). Regarding the correlation between field and laboratory data, *Total brood* was negatively correlated with TP (*r*=-0.75, *P*<0.01), VG concentration (*r* =-0.77, *P*<0.01) and VG percentage (*r* =-0.72, *P*<0.01) and positively correlated with APO percentage (*r* =0.867, *P*<0.01). Similarly, *Sealed brood* was negatively correlated with TP (*r* =-0.77, *P*<0.01), VG concentration (*r* =-0.79, *P*<0.01) and VG percentage (*r* =-0.73, *P*<0.01) and positively with APO percentage (*r* =0.874, *P*<0.01).

Discussion

The aim of the present paper was to assess the impact of the manipulations needed for the summer *V. destructor* treatment (T1) and the subsequent wintering phase (T2) on the colony nutritional status, using an innovative panel of biomarkers.

At T1 the adopted manipulations determined a different impact on colony nutritional status: hives from the BI group showed significantly higher TP concentration, VG concentration and percentage than the other groups. The consistency among the above-mentioned biomarkers is not surprising since vitellogenin is the most abundant protein in honey bee haemolymph (Amdam et al., 2003). In insects, this protein plays an important role in lipid transport, immune function, longevity and production of royal jelly (Blacklock and Ryan 1994; Amdam et al. 2004a; Havukainen et al. 2013). Therefore, the accumulation of vitellogenin in haemolymph of workers subjected to gradual reduction of brood caring is predictable. In fact, colonies of BI and BR-n groups, where oviposition was interrupted, showed significantly higher concentration and percentage of vitellogenin, than colonies of BR-oc group where oviposition continued throughout summer.

Notably, only colonies of BI group reached in summer TP and VG concentrations comparable to those found in winter. This confirms previous findings regarding the possibility of obtaining winter bees in summer by controlling brood rearing (Amdam et al., 2004; Fluri et al., 1982, 1977; Maurizio, 1950). This phenomenon probably resembles what physiologically happens in temperate zones when winter is approaching: during autumn, the unfavourable environmental conditions drive the colony to cease brood

rearing and store protein in their fat bodies (Fluri et al., 1982). A further evidence of the relationship between a specific nutritional status and the presence of brood is provided by the correlations found in the present study between brood amount (total and sealed) and TP, VG concentration and VG%.

Brood interruption is often considered with concern by beekeepers, due to the loss of 25 days of queen oviposition. However, considering that Amdam et al (2005) found a positive correlation between vitellogenin concentration and worker longevity and that longevity is a key factor for colonies (DeGrandi-Hoffman and Curry 2004), it is possible that the loss of oviposition is at least partially compensated by the increase in honey bee longevity.

BI differed significantly from the others groups also regarding APO %: the percentage at T1 was significantly lower than values found in the other groups, and from the percentage found at T0, approaching the winter value determined at T2. Apolipophorins are the major lipoproteins in insects and their presence in haemolymph is closely related to lipid mobilisation (Robbs et al. 1985). According to Arrese and Soulages (2010), the mobilisation of lipids in insects can be related to several causes, including embryogenesis, immune response, and starvation. In the present study, the cause of the higher lipid mobilisation observed in BR-n and BR-oc is probably related to nutritional stress. In fact, subgroup BR-n underwent depletion of most of adult bees with their endogenous reserves, whose function is to produce royal jelly to feed the young larvae (Haydak 1970), and of part of the pollen reserves, contained mainly in the frames left in the original colony. At the same time, the subgroup BR-oc is deprived of part of honey stores contained in the removed brood frames and of drawn combs, therefore it is forced to build new combs, an energetically expensive task. Schulz, Huang & Robinson (1998) showed how carbohydrate starvation causes a precocious transition from house bees to foragers. Moreover, Huang and Robinson (1996) demonstrated that wax deprivation, obtained by removing drawn combs, produced the same effect. Interestingly this physiological transition has been correlated to a decrease in the vitellogenin endogenous reserves and with a precocious senescence (Amdam et al. 2003). It is possible that the manipulation elicited an effect on the nutritional status both directly and indirectly via an alteration of the division of labor in the colony. In addition, the recovery of the nutritional stress is probably impaired by the late summer condition of the zone where the experiment took place, characterised by dry climate with scarce flowering.

In honey bee colonies, there is a strict relation between nutritional status and immunity (Münch et al. 2013). Basualdo et al. (2014) showed that the use of "beebread" (pollen supplemented with enzymes and bacteria of the salivary bee glands, fermented and stored in honey cells) promotes a significant increase of haemolymph total proteins and a higher survival in bees subjected to artificial infection by *Nosema ceranae* compared to honeybee fed with a protein substitute. This supports the hypothesis that bee resilience to pathogens is related to the protein content in haemolymph. In this context the infestation by *V. destructor* could play an important role. In particular, a negative effect of this parasite on the ability of bees in accumulating vitellogenin has been reported (Amdam et al. 2004b). Differently, in the present paper, *V. destructor* infestation did not correlate with vitellogenin. The discrepancy could be ascribed to the fact that Amdam et al. (2004b) evaluated the effect of the parasite on single bees individually, while in our study the evaluation was performed on the entire colony. Moreover, the relatively low level of infestation in the colonies, that was also not different among groups, could have been not sufficient to influence the concentration of vitellogenin in haemolymph.

Vitellogenin is the main haemolymph zinc binding molecule and a relationship between this protein, haemolymph zinc concentration and cellular immunity has been suggested (Amdam et al., 2004). Our study adds more evidence of the positive relation between vitellogenin and circulating zinc.

Strict relation between zinc and immune function in insects has been demonstrated also in *Manduca sexta* (Willott and Tran, 2002). The levels found in our study are similar to those reported by Amdam et al. (2004) in foragers and lower than the levels found in hive bees and winter bees.

At winter time (T2), no significant differences in total proteins, vitellogenin and apolipophorinVG and VG concentration among the three groups were measured, suggesting that bees of all the colonies were able to store similar protein reserves. The data obtained in the present study are in accord with those reported for winter bees by other authors (van der Steen et al. 2016). Interestingly, hive population was the only parameter showing significant differences among groups at T2 . While BI and BR-n groups showed a population in line with the expectations for *Apis mellifera ligustica* colonies, colonies of the BR-oc group overwintered with a mean population of only approximately 5000 adult bees, roughly corresponding to three Dadant-Blatt frames covered with bees. Such small cluster is very similar to that studied by Harbo (1983)

(approximately 4400 bees) which is reported to be less capable in thermoregulation and less efficient in the use of reserves; indeed the single bee consumption of honey is inversely correlated with the number of individuals in the cluster itself. Despite the low population, all the colonies of BR-oc group survived until next spring, thanks to the relatively warm winter of 2015.

In conclusion, the innovative panel of biomarkers presented in this study, including also for the first time apolipophorin, proved to be useful in assessing the impact of brood manipulation on the colonies. Our data suggest that the duplication of the colonies by brood removal could determine a greater risk of colony losses facing the winter, due to the impact on endogenous stores in summer and on hive population in winter. Supplemental feeding might be an option to mitigate the negative effects of brood removal even if scientific consensus on pollen substitutes is lacking. The better nutritional status obtained with brood interruption in summer should be furher investigated in order to confirm the supposed benefits on bee immunity and resilience to pathogens.

For the above-mentioned reasons, we suggest the brood interruption protocol as an organic, effective and less stressful method to control *V. destructor* in summer.

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Authors contribution

RC, EF, AN, GA, RG, GI conceived the experiment; RC performed the experiment; RC, EF, EM and GA performed the analyses; RC, EF, GA, RG, GI wrote the paper and participated in the revision of it. All authors read and approved the final manuscript.

Conflict of interest

None of the authors of this paper has any financial or personal relationship that could inappropriately influence or bias the content of the paper.

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TABLES

Table I. *Bees*, *Total brood*, *Sealed brood* and *Pollen* evaluated at T0 and T2 following a modified Liebefeld method (Delaplane et al., 2013). Data are reported in portions of frame and as mean ± SD (*n*=5).

Bees **(portions of frame)**	**Group\Time**	**T0**	**T2**
	BI	100.8±26.4 A a	45.9±12.6 A b
	BR-n	112.6±34.8 A a	33.6±9.1 AB b
	BR-oc	112.6±34.8 A a †	20.8±4.0 B b
Total Brood **(portions of frame)**	**Group\Time**	**T0**	**T2**
	BI	49.5±7.6 A a	4.9±4.4 A b
	BR-n	60.7±10.9 A a	3.8±4.2 A b
	BR-oc	60.7±10.9 A a †	0.4±0.8 A b
Sealed Brood **(portions of frame)**	**Group\Time**	**T0**	**T2**
	BI	34.8±5.1 A a	4.1±3.8 A b
	BR-n	39.5±6.9 A a	3.0±3.3 A b
	BR-oc	39.5±6.9 A a †	0.4±0.8 A b
Pollen **(portions of frame)**	**Group\Time**	**T0**	**T2**
	BI	5.7±4.6 A a	0.6±0.5 A b
	BR-n	7.0±3.2 A a	0.2±0.2 A b
	BR-oc	7.0±3.2 A a †	0.1±0.2 A b

Different lower case letters within rows indicate significant differences ($P<0.05$) among time-points within groups. Different capital letters in the column indicate significant differences ($P<0.05$) among groups within time-points. BI, brood interruption; BR, brood removal; BR-n, nucleus; BR-oc, original colony. † since BR-n derived from BR-oc after the sampling at T0, the values of the analytes at T0 are the same and refer to the BR colonies before the removal of the brood frames.

Table II. Total proteins (TP), Zn, vitellogenin (VG) and apolipophorin (APO) determined in honeybee haemolymph at different time-points. Data are reported as mean ± SD (n=5).

TP mg/mL	**Group\Time**	**T0**	**T1**	**T2**
	BI	13.6±2.9 A a	31.7±4.1 A b	33.7±9.9 A b
	BR-n	12.4±2.8 A a	19.8±2.0 B a	30.7±12.6 A b
	BR-oc	12.4±2.8 A a †	14.4±2.8 B a	33.4±7.3 A b
Zn µg/mL	**Group\Time**	**T0**	**T1**	**T2**
	BI	2.7±0.7 A a	2.9±0.7 A a	4.8±1.8 A a
	BR-n	2.9±1.1 A a	2.9±1.9 A a	4.4±1.2 A a
	BR-oc	2.9±1.1 A a †	1.6±0.7 A a	4.2±0.5 A a
VG mg/mL	**Group\Time**	**T0**	**T1**	**T2**
	BI	3.1±1.4 A a	12.6±1.9 A b	15.0±5.5 A b
	BR-n	2.8±1.2 A a	7.0±1.0 B a	17.0±4.3 A b
	BR-oc	2.8±1.2 A a †	4.0±1.6 C a	15.0±5.0 A b
APO mg/mL	**Group\Time**	**T0**	**T1**	**T2**
	BI	3.9±0.7 A a	5.8±0.5 A b	4.3±0.8 A a
	BR-n	3.7±0.9 A a	4.6±0.4 B a	4.0±0.8 A a
	BR-oc	3.7±0.9 A a †	3.5±0.5 C a	3.6±0.2 A a
VG %	**Group\Time**	**T0**	**T1**	**T2**
	BI	21.5±6.9 A a	39.5±2.4 A b	43.7±3.5 A b
	BR-n	21.5±5.9 A a	35.1±1.6 A ab	46.4±3.3 A b
	BR-oc	21.5±5.9 A a †	27.0±5.2 B a	44.1±4.1 A b
APO %	**Group\Time**	**T0**	**T1**	**T2**
	BI	29.7±5.1 A a	18.5±1.1 A b	13.1±1.9 A b
	BR-n	29.7±4.2 A a	23.3±2.1 B a	11.3±1.6 A b
	BR-oc	29.7±4.2 A a †	24.5±1.1 B a	11.1±1.4 A b

Different lower case letters within rows indicate significant differences ($P<0.05$) among time-points within groups. Different capital letters in the column indicate significant differences ($P<0.05$) among groups within time-points. BI brood interruption; BR-n, brood removal nucleus; BR-oc, brood removal original colony. † since BR-n derived from BR-oc after the sampling at T0, the values of the analytes at T0 are the same and refer to the BR colonies before the removal of the brood frames.

FIGURES

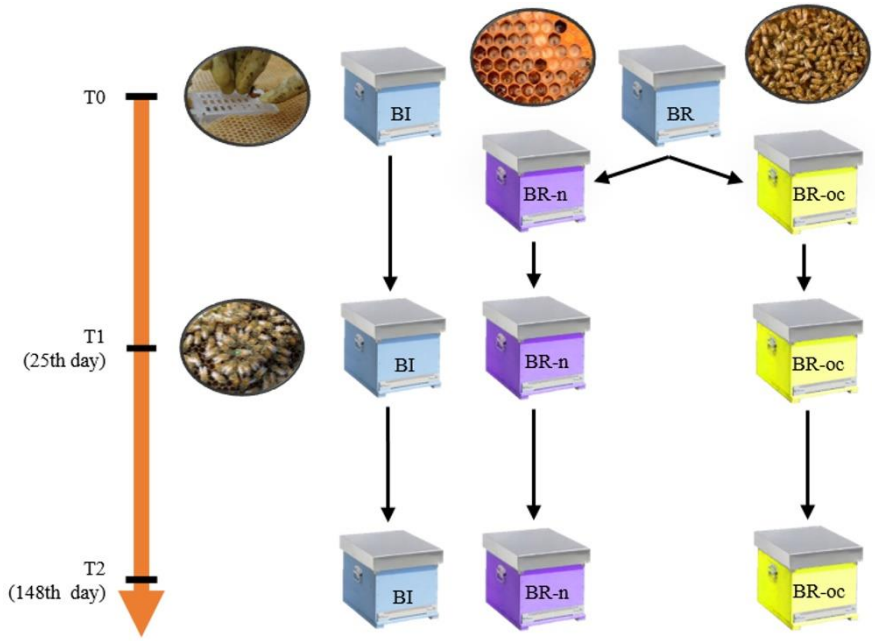

Fig. 1 Graphical timeline of the field trial. At T0 (July) colony-level traits inspection and haemolymph collection, queen caging on BI, brood removal on BR establishing BR-n and BR-oc, chemical treatment on BR-oc were performed. At T1 (August) haemolymph collection, queen releasing, chemical treatment on BI and BR-n were performed. At T2 (December) colony-level traits inspection and haemolymph collection were performed. BI, brood interruption; BR, brood removal; BR-n, nucleus; BR-oc, original colony.

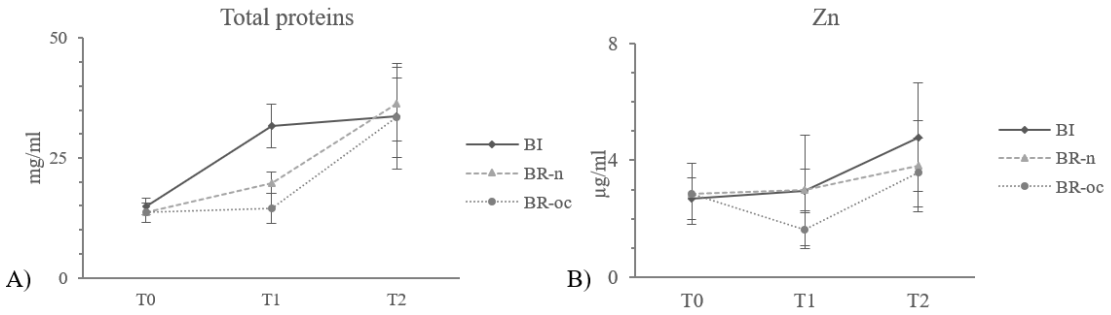

Fig. 2 Variations of total protein and Zn concentrations in honeybee haemolymph at different time-points. Data are expressed in (a) mg/mL (TP) and (b) µg/mL (Zn) and reported as mean ± SD (n=5). BI, brood interruption; BR-n, nucleus; BR-oc, original colony.

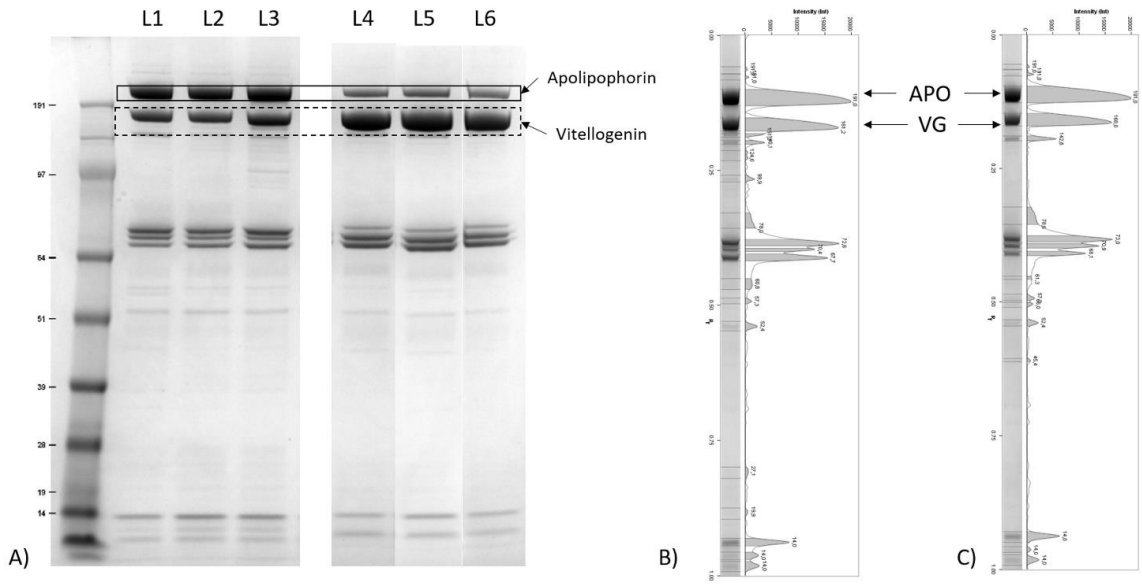

Fig. 3 Representative SDS-PAGE gel and pherograms of honeybee haemolymph. (a) SDS-PAGE: lanes 1, 2 haemolymph samples from BR group at T0; lanes 3, haemolymph sample from BI group at T0; lanes 4,5,6 haemolymph samples from BR-n, BR-oc and BI, respectively, at T2. (b) pherogram of lane 3 (BI, T0); (c) pherogram of lane 6 (BI, T2). BI, brood interruption; BR, brood removal; BR-n, nucleus; BR-oc, original colony.

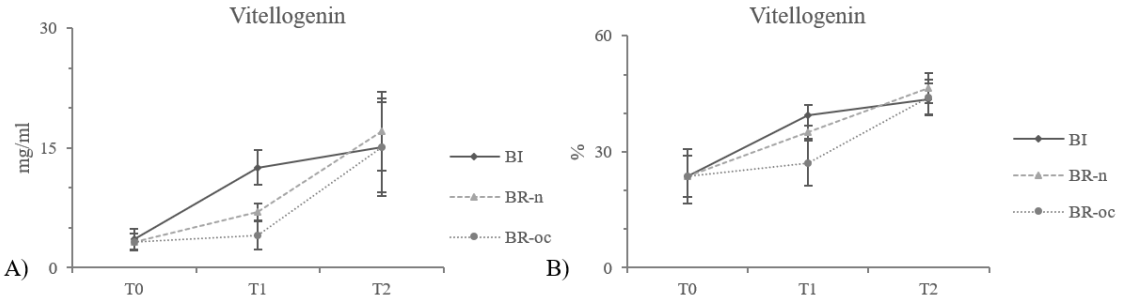

Fig. 4 Variations of vitellogenin in honeybee haemolymph at different time-points. Data are expressed in (a) mg/mL and (b) percentage and are reported as mean ± SD (n=5). BI, brood interruption; BR-n, nucleus; BR-oc, original colony.

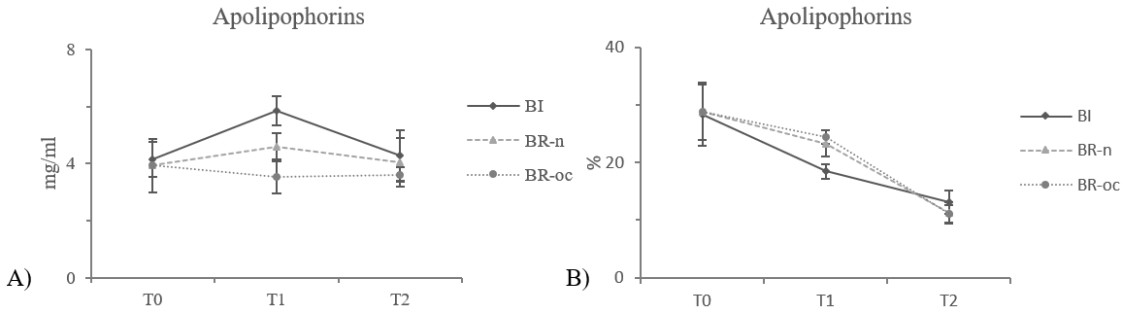

Fig. 5 Variations of apolipophorin in honeybee haemolymph groups at different time-points. Data are expressed in (a) mg/mL and (b) percentage and are reported as mean ±SD (n=5). BI brood interruption; BR-n, brood removal nucleus; BR-oc, brood removal original colony.

Chapter 5: A machine learning approach to study demographic alterations in the hive by SDS–PAGE fingerprinting

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Short title

Study of demographic alterations of the hive

Abstract

Honey bees are social insects, living in colonies often referred as superorganism in order to highlight the higher level of organization achieved. The adult members of the colony are mainly represented by female workers, found in the colony into three different phenotypes: nurse bees, foragers and diutinus workers. The accelerated maturation of hive bees into foragers is exerted by many negative stimuli and recent work proposed this mechanism as a common pathway of colony collapse stressing the need to shift from the study of single stressors to the study of the effect exerted by these stressors to the colony homeostasis.

The aim of this research is therefore to study colony population unbalance by discriminating overaged nurses from proper aged nurses and precocious foragers from proper aged foragers through the study of the electrophoretic pattern of haemolymph proteins and a machine learning algorithm.

The KNN model fitted on the foragers dataset showed a remarkably good predictive accuracy in discriminating precocious foragers from proper aged ones, making it an interesting candidate to monitor population imbalances in the hive and possibly to achieve an early detection of the collapse syndrome.

Introduction

The effect of the recently reported decline in insects (Hallmann et al. 2017) and particularly in wild pollinators (Biesmeijer et al. 2006) is alarming: agriculture and wild plants dependence on honey bees and beekeeping is spiking. However, the same drivers of the abovementioned decline, affect *Apis mellifera* managed colonies as well (Potts et al. 2010). Given the multifactorial nature of the problem (Goulson et al. 2015), approaches to objectively analyse the wellbeing of the colonies are urgently needed.

Honey bees are social insects, living in colonies often referred as superorganism in order to highlight the higher level of organization achieved. As stated by Wilson and Sober (Wilson and Sober 1989), a superorganism is “a collection of single creatures that together possess the functional organization implicit in the formal definition of organism”. The strength of this level of organization, are the emergent properties arising from interactions between individual members of the colony like thermoregulation, comb construction or foraging behaviour (Delaplane 2017).

The adult members of the colony are mainly represented by female workers, found in the colony into three different phenotypes: nurse bees, foragers and diutinus workers (G. V. Amdam and Page 2005). While the latter are reared in peculiar conditions, naturally occurring in autumn (in temperate climates) or artificially inducted trough the active season (Cabbri et al. 2018; G. V. Amdam and Page 2005; Omholt and Amdam 2004; Maurizio 1950); the belonging to the first two phenotypes is age related (so called "age polyethism").

Workers act as nurse bees within 3-4 days of emergence (Naiem, Hrassnigg, and Crailsheim 1999), they have hypertrophic hypopharyngeal glands and fat bodies (for royal jelly production and brood caring) and a high number of circulating hemocytes (G. V. Amdam 2011).

Nurse bees eventually become foragers (Seeley 1982), responsible for the nutrient supply to the colony. Contrary to nurse bees, foragers are characterized by atrophy of hypopharyngeal glands and fat body (G. V. Amdam 2011) and by a lower cellular immunity due to hemocyte apoptosis (G. V Amdam et al. 2005). The nurse-forager transition is socially regulated trough a feedback loop between vitellogenin and juvenile hormone (G. V. Amdam and Omholt 2003; Guidugli et al. 2005).

The timing of this transition is not fixed and can be anticipated or postponed following the hive needs (Z. Y. Huang and Robinson 1992; Z.-Y. Huang and Robinson 1996).

However, the flexibility of the system is not infinite, and some detrimental effects can occur, for example lowest flight performances have been reported for precocious foragers compared to normal aged foragers (Vance et al. 2009). The accelerated maturation of hive bees into foragers is exerted by many stimuli: denutrition, wax deprivation, lack of pollen and forager loss (Fergusson and Winston 1988; Janmaat and Winston 2000; Schulz, Huang, and Robinson 1998; Toth and Robinson 2005). Interestingly, the action of pathogens can also trigger this behaviour: both *Nosema* spp. and *Varroa destructor* have been proven to cause precocious foraging (Goblirsch, Huang, and Spivak 2013; Higes et al. 2008). Moreover, a recent work (Doublet et al. 2017) identified a "common host response" to different pathogens (including, together with *Nosema* spp. and *Varroa*, also IAPV, BQCV and DWV) which comprises a decrease in vitellogenin expression, as above stated a key protein in foraging regulation.

This evidence may suggest the presence of a common pathway of colony collapse, as recently proposed by (Perry et al. 2015b) and therefore the usefulness of a paradigm shift: from the study of single stressors to the study of the effect exerted by these stressors to the colony homeostasis. To date, only (Alaux et al. 2018) proposed a method to study demographic alterations in the colony, based on the estimation of biological age trough

gene expression of vitellogenin and adipokinetic hormone receptor. However, no studies have been conducted to deal with the problem using a proteomic approach.

The aim of this research is therefore to study colony population unbalance by discriminating over aged nurses from proper aged nurses and precocious foragers from proper aged foragers through the study of the electrophoretic pattern of haemolymph proteins and a machine learning algorithm. Machine learning is a supervised learning approach where an algorithm is trained on a dataset consisting of predictors and dependent variables in order to formulate prediction rules, those rules are then exploited to predict the dependent variables knowing only the predictors.

The experimental model to study this issue is represented by Single cohort colonies (SCCs). SCCs are colonies consisting of a variable number of same aged workers, obtained by controlled eclosion in incubator, and a fertile queen. In these colonies, after 8 to 10 days from eclosion some workers start foraging (precocious foragers) and some others initiate brood caring (proper aged nurses); after 21 days from eclosion foragers of proper age start foraging and brood caring relies on overaged nurses.

Materials and Methods

SCCs

Two trials with single cohort colonies (SCCs) were conducted: one in June-July 2017 and one September-October 2017. The reason behind the different timing in the two replicates is to collect data from different physiological moments of the colonies and thus be able to better generalize the results. Both experiments took place in a dedicated apiary at the Department of Veterinary Medical Sciences (DIMEVET) of the University of Bologna, Italy.

To obtain workers of the same age to populate the SCCs, the following protocol was employed: at day -21, four mated sister queens were caged on four different combs, drawn from organic-certified residue free wax, and placed inside four fully developed and healthy colonies; after two days (day -19) the queens were removed from the cages, in order to have a maximum difference of 48 hours among the brood laid; three days before the expected eclosion (day -18), the combs were moved to an incubator set to 32 celsius degrees. At day 1, newly eclosed workers were gently brushed from the combs, mixed to eliminate the mother colony factor, and used to prepare the SCCs. Two SCCs were made with 250 grams of bees (equivalent to approximately 2500 individuals), one queen and two combs: one empty and one with plenty of honey and pollen (both drawn from the same wax mentioned earlier). The queens were freshly mated and sisters. The SCCs were kept closed in a protected and shaded environment for 3 days post-eclosion, to allow complete maturation of the workers before being moved to the outdoor apiary.

With this setup, four categories of bees were sampled: proper aged foragers (n=35), precocious foragers (n=28), proper aged nurses (n=35), over aged nurses (n=36).

In order to increase specificity of the sampling, only bees displaying the behaviour of feeding larvae were sampled as nurses; only bees leaving the hive (caught with a home-made apparatus hanging in front of the flight entrance) outside the central hours of the day when usually orientation flights take place, were sampled as foragers.

Haemolymph collection and SDS-PAGE electrophoresis

Two microliters of haemolymph were drawn for each bee with a graduated glass microcapillary and stored at -80 Celsius degrees. For each of the 134 samples analysed, 3μg of proteins were loaded and separated with 4–12% gradient gels, in MOPS buffer (NuPAGE, Invitrogen). The gels were stained with Coomassie G250 and digitalised by

ChemiDoc™MP(BioRad). Protein identification by mass spectrometry was carried out according to (Cabbri et al. 2018).

Data preparation

Gel images were imported in Fiji, a software based on ImageJ 1.52i (Schindelin et al. 2012), coupled with the Bioformats 6.0.0 plugins in order to read the proprietary .scn files. The lanes of each gel were manually delimited drawing a segmented line through the centre (from the loading well to the end of the gel), adding it as a ROI (Region of Interest) and specifying the width. Afterwards, the electropherograms were plotted using the Multi-plot command and exported to an Excel spreadsheet. In this file, columns represent the samples, rows represent distances from the loading well. The intersection of the two contains the intensities of the pixels in that area of the gel. The data of each gel were collected in a comprehensive database and analysed with the statistical software R 3.6.0 (R Development Core Team, 2008), and the RStudio IDE (RStudio team, 2015).

The distance variable was binned in order to reduce the complexity and thus the computation time of the dataset. The initial dataset included about 800 distance values, corresponding to a resolution of 80 µm. 100 intervals were created inside the above-mentioned range, and the corresponding intensities were averaged. The new distance variable was created using the median value of every interval. With this manipulation, resolution dropped to about 650 µm.

To compensate for the differences in migration patterns of the various gels, the GCalignR (Ottensmann et al. 2018) library was used. The areas of the pherograms below 5000 microns and exceeding 45000 microns of migration distance were excluded from the alignment in order to avoid high noise zones. The aligned data matrix was used for the subsequent analysis.

Statistical analysis

Two different datasets were prepared, one containing nurses data and one with foragers data. Each dataset was randomly split in a train set (75% of the cases) and a test set (25% of the cases).

Recursive feature elimination (RFE) with random forests function was used to select relevant variables in the foragers and nurses datasets, separately. A conservative approach based on the empirical "one in ten rule" was used to choose the maximum number of features to retain; Considering a number of cases in the train set of approximately 50, the maximum number was set to 5.

The external validation needed to avoid selection bias was achieved through 10 fold cross-validation (Ambroise and McLachlan 2002). Considering the important deviation of data from normality, non-parametric algorithms were chosen. Three different models based on three different algorithms were fit using the train dataset: Support Vector Machines with Linear Kernel (SVM), k-Nearest Neighbors (KNN) and Random Forest (RF).

The performances of the models were evaluated by repeating ten times a ten-fold cross-validation, parameters considered were (AUC, Sensitivity, Specificity); differences were computed, then a t-test was used to evaluate the null hypothesis that there is no difference between models.

The generalization error was then assessed on the test set by building a confusion matrix and computing again AUC, Accuracy (with 95% CI), Sensitivity and Specificity for each model. The null Accuracy was compared with the obtained Accuracy and a p-value was computed to know if the classifier is significantly better than a random classifier.

Results

A representative gel and the relative pherogram are reported in Fig.1. The identified proteins are reported in Fig. 8 and Tab. 7.

Nurses

The results of the feature selection process on the nurses dataset showed a negligible improvement in accuracy with a number of variables exceeding three (Fig. 2) in the 1:5 range chosen to limit overfitting. For this reason, the first three variables were chosen to build the models. Those with the highest ranking were: X23040, X24336, X38014 (Fig. 4).

Performances obtained through resampling on the train dataset are summarized in Tab 1. The differences in mean AUC are not statistically significant, while the mean sensitivity achieved through the KNN model is significantly higher than the sensitivity of the SVM model. Regarding mean specificity, the value of the KNN model is significantly lower than that of the other models. The best overall performance is obtained with the RF model.

Performances calculated on the test set for the RF model are summarized in Tab. 3 and in Fig. 8. The AUC value in Tab. 3 refers to the area under the ROC curve (Fig. 6).

Foragers

The results of the feature selection process on the foragers dataset showed a negligible improvement in accuracy with a number of variables exceeding three (Fig. 3), so the first three variables were chosen to build the models. Those with the highest ranking were: X23690, X23040, X22392 (Fig. 5). Performances obtained through resampling on the train dataset are summarized in Tab. 3. The differences in mean AUC are not statistically significant, while the mean sensitivity achieved through the RF model is significantly

lower than the sensitivity of the other models. Regarding mean specificity, no significant differences were found between models. The best overall performances are obtained with SVM and KNN, the latter is preferred as is less computationally intensive. Performances calculated on the test set for the KNN model are summarized in Tab. 4 and in Fig. 9. The AUC value refers to the area under the ROC curve (Fig. 7).

Discussion

The timing of nurse to forager maturation is of capital importance in determining longevity of honey bees (Robinson et al. 1989). A wide variety of stress factors exert an influence on this timing and thus greatly impacts on colony population dynamics, possibly leading to colony collapse (Perry et al. 2015a).

For this reason, a machine learning method was developed with the aim of discriminating bees whose age matches role held in the colony from bees whose age does not. In this study the predictors are represented by the intensities found at specific migration distances, while the dependent variable is a categorical binary outcome: precocious forager – correct aged forager or over aged nurse – correct aged nurse. A proteomic fingerprinting approach to gel analysis was chosen to explore the overall contribution of the main haemolymph proteins, without the bias related to an "*a priori"* selection.

The objective of the analysis on the nurses dataset is trying to discriminate over aged nurses from normal aged ones. Although this shift in nurses age is probably not directly related to collapse, over aged nurses are not functionally equivalent to young ones; the degeneration of mandibular glands leads to phenotypic differences in the reared workers which exhibit higher ovary development (Wegener, Lorenz, and Bienefeld 2009). Higher ovary development in workers was linked to a suboptimal foraging behaviour, leading to an overall decrease in performance of the colony (Mattila, Reeve, and Smith 2012).

The RFE algorithm indicated the variables X23040, X24336, X38014 as the most relevant to discriminate the two categories. The maximum accuracy was achieved with all the 60 variables, but in the range 1:5 chosen to avoid overfitting the model, no significant improvement of accuracy was achieved using a number of predictors exceeding three. Despite the good performances exhibited in the train dataset (Tab. 3) a conspicuous generalization error affected the chosen RF model as shown by the poor predictive performance on the test dataset. The accuracy obtained is not significantly different from the null accuracy and so this model has no predictive power nor practical use.

Better results were obtained with the foragers dataset. The RFE algorithm indicated the variables X23690, X23040, X22392 as the most relevant to discriminate the two categories. In this case also, the maximum accuracy was achieved with all the 60 variables, but in the range 1:5 chosen to avoid overfitting the model, no significant improvement of accuracy is achieved using a number of predictors exceeding three. The excellent performances of the KNN model in the train dataset (Tab. 4) were confirmed on the test dataset (Tab. 6). As seen in the fourfold plot (Fig. 9) the model was able to discriminate with 100 % accuracy (7/7) the precocious foragers in the test set. The features chosen for the foragers model correspond to three contiguous zones of the gel, comprised in the range 22064 – 24015 μm of migration distance, containing a band of ~75 kDa (Fig. 1). This area overlaps with the area of a protein not annotated for *A. mellifera* in the UniProt database (UniProt.org) but showing an elevated degree (91.8%) of homology with apolipophorin of *A. cerana*. Apolipophorins are the major lipoproteins in insects and their presence in haemolymph is closely related to lipid mobilisation (Robbs et al. 1985). An apolipophorin showing this molecular weight has been described as apolipophorin II (ApoLp-II) in *Manduca sexta* (Kawooya et al. 1986). Apolipophorin I (ApoLp-I) and Apolipophorin II ApoLp-II) are produced from a posttranslational

cleavage of a precursor protein, apolipophorin II/I (apoLp-II/I) during lipoprotein synthesis (Ryan and van der Horst 2000). To date, no studies deal with the specific function of ApoLp-II in *A. mellifera*. However, Wen et al., (2017) recently demonstrated an upregulation of Ap-apoLp-II/I gene in response to bacterial challenge and a novel role for Ap-apoLp-II/I in regulating prophenoloxidase activation system in *Antheraea pernyi*.

Despite the need for further studies to elucidate the role of ApoLp-II in honey bees, the great discriminatory capacity shown in this work suggests that the amount of this protein foragers haemolymph is more age related than task related.

Conclusions

This work deals with the often-neglected problem of demographic alterations in honey bee colonies. The KNN model fitted on the foragers dataset showed a remarkably good predictive accuracy making it an interesting candidate to monitor population imbalances in the hive and possibly to achieve an early detection of the collapse syndrome. Given the experimental setup, an experiment on fully developed colonies artificially deprived of foragers is needed to validate this tool. Moreover, a validation of a protocol with a pool of bees instead of single insects would be useful to reach an affordable solution for beekeepers and veterinarians operating in the field.

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Figures

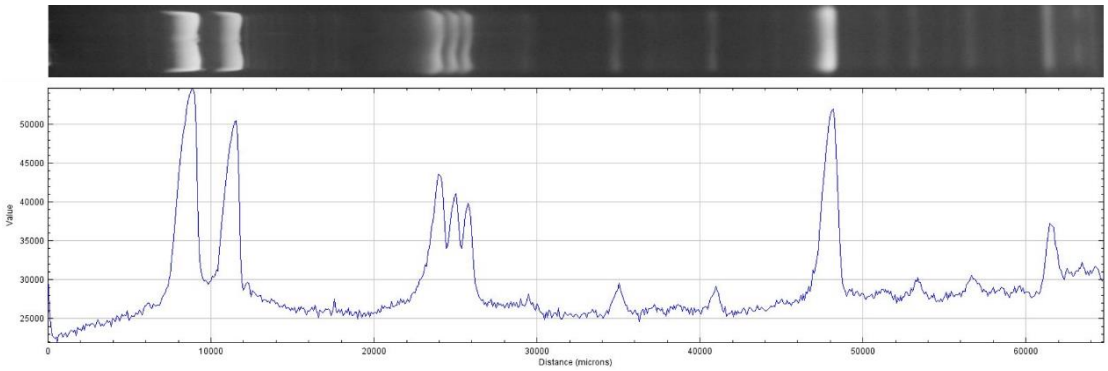

Figure 1. Electropherogram of a sample as acquired through Fiji software.

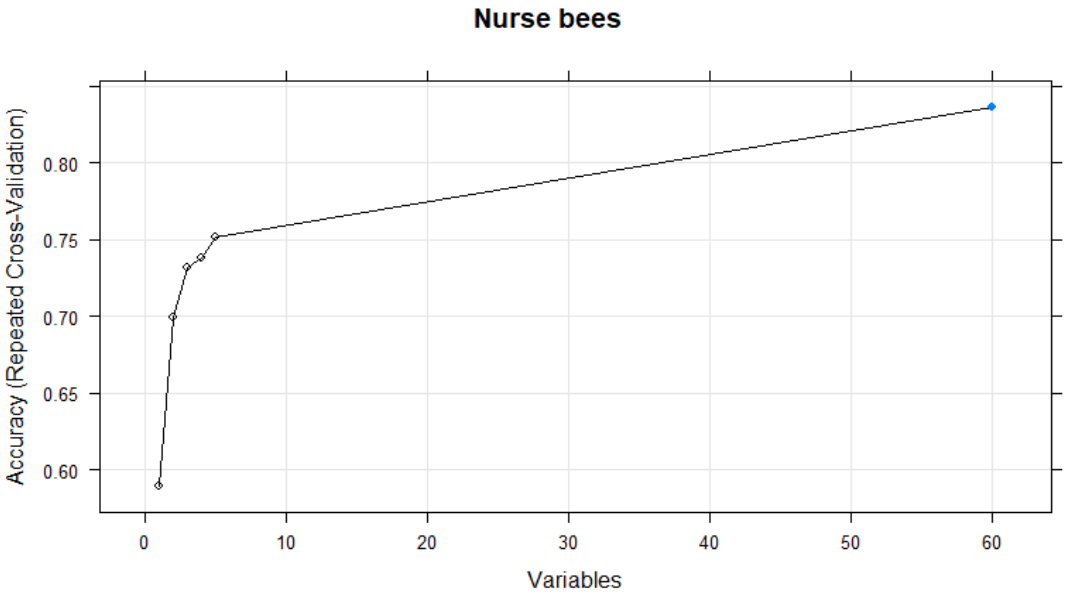

Figure 2. Nurses dataset. Relation between mean Accuracy of the model obtained with the train dataset (assessed through Repeated Cross-Validation) and number of variables used.

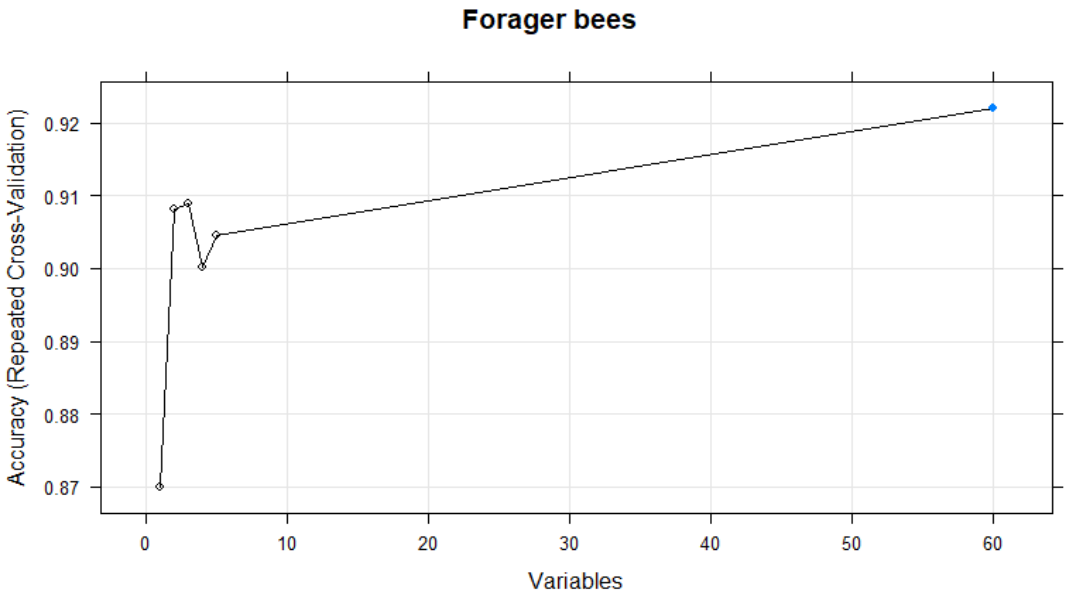

Figure 3. Foragers dataset. Relation between mean Accuracy of the model obtained with the train dataset (assessed through Repeated Cross-Validation) and number of variables used.

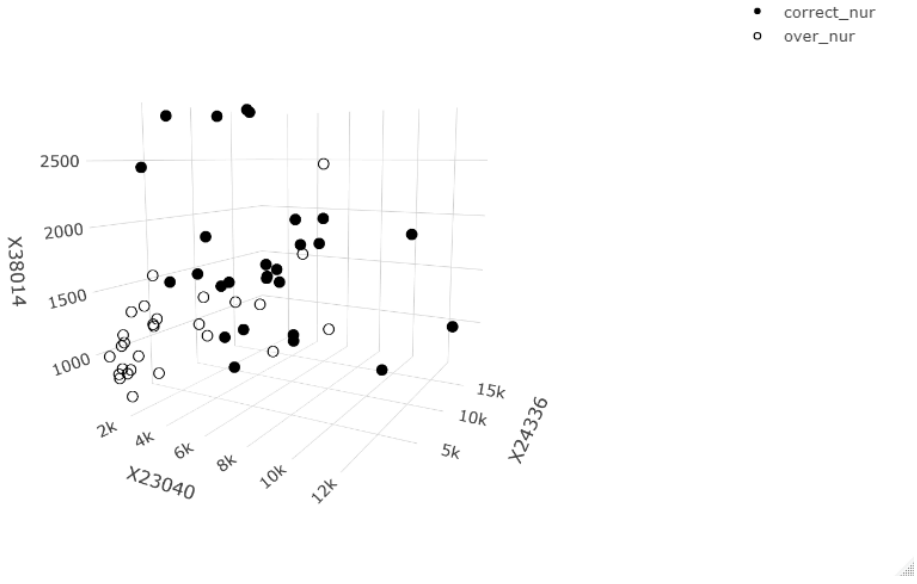

Figure 4. Nurses dataset. 3D plot showing the relation between the intensity values of three most informative variables and the category of the bees in the train dataset.

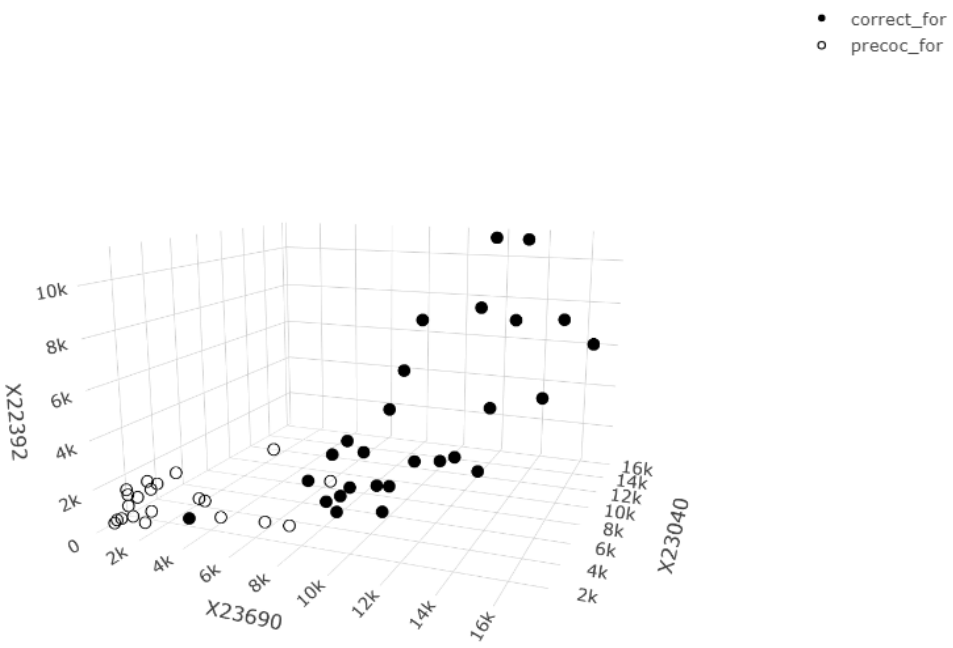

Figure 5. Foragers dataset. 3D plot showing the relation between the intensity values of the three most informative variables and the category of the bees in the train dataset.

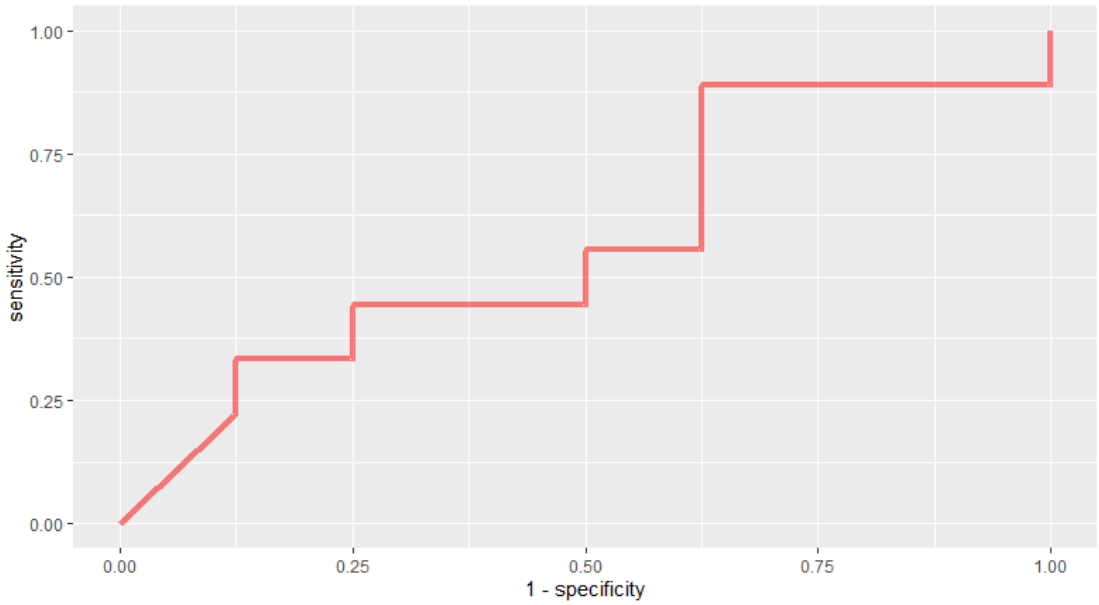

Figure 6. Nurses dataset. ROC curve computed with the prediction of the RF model on the test set.

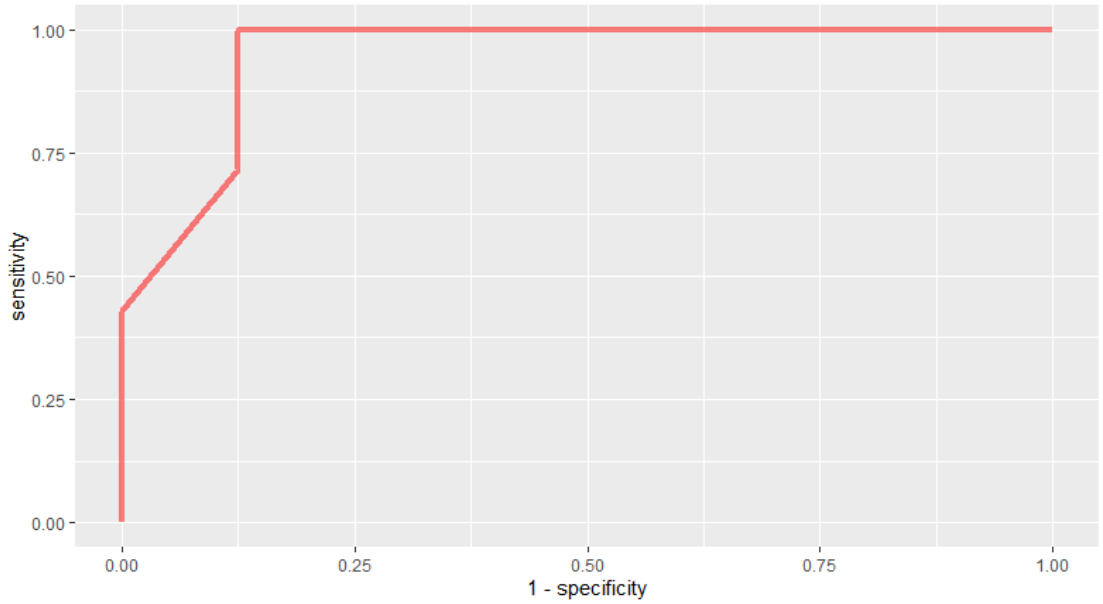

Figure 7. Foragers dataset. ROC curve computed with the prediction of the KNN model on the test set.

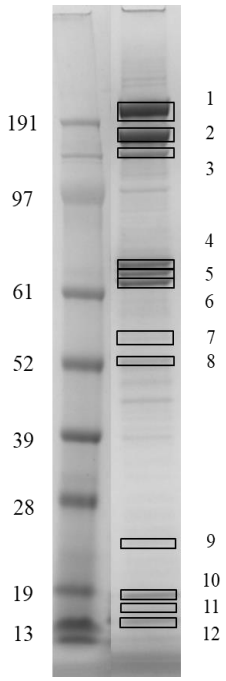

Figure 8. Representative SDS-PAGE (on the right) with molecular weight marker (on the left). Each band is indicated with a progressive number, for the identifications see Table 7.

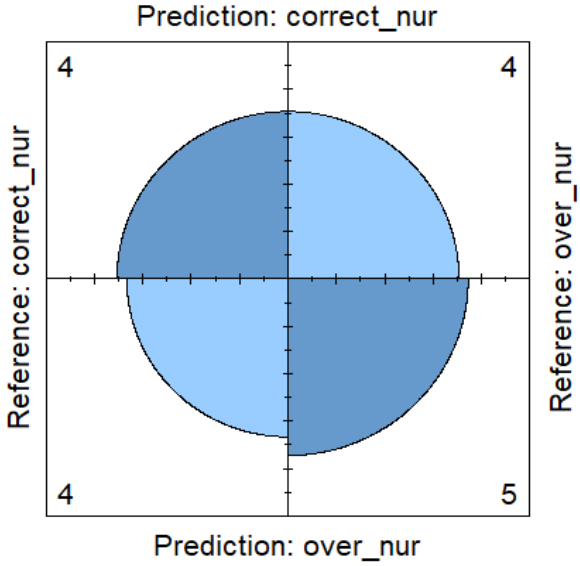

Figure 8. Nurses dataset. Fourfold plot showing the results of the confusion matrix produced with the prediction of the RF model on the test set.

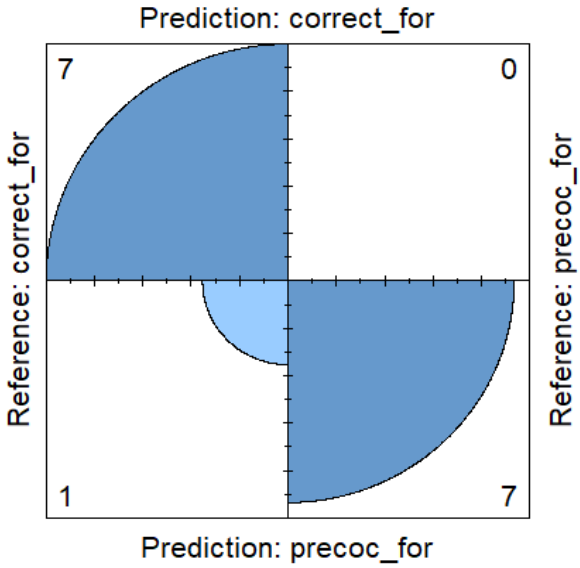

Figure 9. Foragers dataset. Fourfold plot showing the results of the confusion matrix produced with the prediction of the KNN model on the test set.

Tables

Model	*AUC Mean*	*Sens Mean*	*Spec Mean*
SVM	0.87 (a)	0.76 (a)	0.83 (a)
KNN	0.83 (a)	0.9 (b)	0.67 (b)
RF	0.82 (a)	0.82 (ab)	0.75 (ab)

Table 1. Nurses dataset. Performances of the models assessed through resampling on the train dataset. Different lowercase letter in columns means statistically significant ($p < 0.05$) difference.

	AUC Mean	*Sens Mean*	*Spec Mean*
SVM	0.98 (a)	0.96 (a)	0.86 (a)
KNN	0.98 (a)	0.96 (a)	0.87 (a)
RF	0.96 (a)	0.89 (b)	0.90 (a)

Table 2. Foragers dataset. Performances of the models assessed through resampling on the train dataset. Different lowercase letter in columns means statistically significant ($p < 0.05$) difference.

RF	
Accuracy	0.53
AccuracyLower	0.28
AccuracyUpper	0.77
AccuracyNull	0.53
AccuracyPValue	0.6
Sensitivity	0.5
Specificity	0.56
AUC	0.57

Table 3. Nurses dataset. Performances of the selected model on the test set.

KNN	
Accuracy	0.93
AccuracyLower	0.68
AccuracyUpper	1
AccuracyNull	0.53
AccuracyPValue	0.00113
Sensitivity	0.88
Specificity	1
AUC	0.95

Table 4. Foragers dataset. Performances of the selected model on the test set.

Supplementary material

Variables	*Accuracy*	*SD*
1	0.59	0.18
2	0.7	0.17
3	0.73	0.16
4	0.74	0.16
5	0.75	0.17
60	0.84	0.16

Table 5. Nurses dataset. Mean accuracy and standard deviation (SD) of the model obtained with the train dataset (assessed through Repeated Cross-Validation) and number of variables used.

Variables	*Accuracy*	*SD*
1	0.87	0.13
2	0.91	0.14
3	0.91	0.13
4	0.9	0.14
5	0.9	0.13
60	0.92	0.11

Table 6. Foragers dataset. Mean accuracy and standard deviation (SD) of the model obtained with the train dataset (assessed through Repeated Cross-Validation) and number of variables used.

Band	Accession	Score	Mass	Matches	Pep(sig)	Sequences	Seq(sig)	emPAI	Description	Protein homologous	% identity	Species
1	A0A088AS56	10642	369557	998	579	182	116	3.07	Uncharacterized protein	Apolipophorins	91.8	Apis cerana
2	A0A088ADL8	3244	202025	478	232	88	54	2.38	Vitellogenin			
2	Q868N5	2951	202117	478	233	87	54	2.32	Vitellogenin			
2	G5D3E6	825	25466	99	61	15	12	7.16	Vitellogenin (Fragment)			
3	A0A088ADL8	106	202025	34	7	13	3	0.05	Vitellogenin			
4	A0A088AS56	3848	369557	410	224	48	35	0.59	Uncharacterized protein	Apolipophorins	91.8	Apis cerana
4	A0A088AQB0	1001	76627	98	59	19	13	0.96	Uncharacterized protein	Leucine-rich repeat-containing protein 15	98.4	Apis cerana
4	A0A088AFH7	340	80005	55	24	19	9	0.56	Transferrin			
5	A0A088AFH7	1790	80005	222	103	34	25	2.92	Transferrin			
6	A5YVK7	1429	81522	282	101	37	19	2.14	Hexamerin 70a			
6	A0A088AFH7	436	80005	45	21	17	7	0.49	Transferrin			
7	Q86MV4	2644	80443	265	149	32	20	2.45	Prophenoloxidase			
7	A0A088AMK2	194	50442	34	14	13	8	0.66	Uncharacterized protein	Chitinase-like protein Idgf4	98.4	Apis cerana
7	A0A087ZN44	103	46895	23	6	5	1	0.15	Carboxypeptidase			
7	A0A088A5I8	40	67150	10	3	6	2	0.10	Pyruvate kinase			
8	A0A088AMK2	2708	50442	236	149	26	16	4.19	Uncharacterized protein	Chitinase-like protein Idgf4	98.4	Apis cerana
8	MRJP1_APIME	511	49311	54	33	16	14	1.48	Major royal jelly protein 1			
8	C7AHQ2	430	55084	49	20	14	8	0.59	Gram-negative bacteria-binding protein 1-2			
8	A0A088AJR6	136	53028	26	11	8	4	0.35	Uncharacterized protein	Plasma glutamate carboxypeptidase	81.8	Apis cerana
8	A0A087ZQA2	130	47588	29	5	10	2	0.14	Dipeptidase			
9	B0LUE8	1723	21335	62	44	8	5	3.32	Apolipophorin-III-like protein			
9	B0LUE8	1514	21335	47	41	8	6	2.73	Apolipophorin-III-like protein			
10	Q1W640	1000	15216	149	57	11	6	8.19	OBP 14			
10	A0A087ZTA5	133	16454	15	7	5	3	0.75	Uncharacterized protein	Niemann-Pick disease type C2	94.6	Apis cerana
11	Q86MV4	112	80443	22	7	6	4	0.22	Prophenoloxidase			
11	Q1W641	106	15494	19	10	8	5	1.69	OBP13			
12	AMCI_APIME	1060	6539	57	43	3	3	7.83	Chymotrypsin inhibitor			

Table 7. Identification table.

Chapter 6: A Novel TaqMan® assay for Nosema ceranae quantification in honey bee, based on the protein coding gene Hsp70

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Abstract

Nosema ceranae is now a widespread honey bee pathogen with high incidence in apiculture. Rapid and reliable detection and quantification methods are a matter of concern for research community, nowadays mainly relying on the use of biomolecular techniques such as PCR, RT-PCR or HRMA. The aim of this technical paper is to provide a new qPCR assay, based on the highly-conserved protein coding gene *Hsp70*, to detect and quantify the microsporidian *Nosema ceranae* affecting the western honey bee *Apis mellifera.* The validation steps to assess efficiency, sensitivity, specificity and robustness of the assay are described also.

Keywords: *Apis mellifera*; *Hsp70* gene; *Nosema ceranae*; Nosemosis; qPCR; TaqMan® assay

Introduction

Nosemosis caused by *Nosema apis* (Zander, 1909) has been the only known microsporidiosis of honey bees (Klee et al. 2007; Paxton et al. 2007) until the first evidence of infection by *Nosema ceranae* (Fries et al. 1996) in *Apis ceranae*. Being detected in *Apis mellifera* (Higes et al. 2006), *N. ceranae* is now another widespread causative agent of nosemosis in the western honey bee (Ansari et al. 2017; Chen et al. 2009; Giersch et al. 2009; Higes et al. 2009b; Jack et al. 2016). The consequences of nosemosis are of increasing concern among both beekeepers and scientists, although the actual impact of this exotic parasite on *A. mellifera* is still debated (Botías et al. 2013). Besides, the microsporidium *N. ceranae* was detected in other Hymenoptera like stingless bee (Meliponini) and the social wasp *Polybia scutellaris* (Vespidae) (Porrini et al. 2017) and it may cause emerging infectious diseases (EIDs) in *Bombus* spp. (Brown 2017; Graystock et al. 2014).

In *A. mellifera,* higher pathogenicity was reported at individual level, leading to increased mortality compared to *N. apis* infections (Higes et al. 2007).

In the colony, correlation was found between *N. ceranae* infections and colony collapses (Higes et al. 2008; Martin-Hernandez et al. 2007). However a metagenomic survey of CCD-affected colonies failed to attribute to this microsporidium the role of primary causative agent (Cox-Foster et al. 2007).

The discrimination between the two *Nosema* parasites affecting the honey bees by light microscopy is hardly achievable and results may be highly biased. Thus, the use of biomolecular techniques has been deeply investigated: several PCR methods have been developed so far, most of them based on the amplification of the microsporidium's multi-copy gene *16s rRNA* (Chen et al. 2008; Forsgren and Fries 2010; Higes et al. 2006; Klee et al. 2007; Martin-Hernandez et al. 2007).

In principle, multi-copy genes are preferred for molecular diagnostics since they allow to design high sensitivity tests, with obvious advantage when the organism is present in a low number of copies. However, in our case, reliability of quantification may be deeply affected by the fact that the *Nosema* genome contains a highly variable number of *16s rRNA* gene copies (Sagastume et al. 2011).

Moreover, the design of PCR primers on a variable gene may obviously affect their capability to anneal to the template influencing the assay sensitivity. This makes the *16s*

rRNA gene a suboptimal candidate for both strain genotyping and designing of PCR diagnostic assays.

An end-point PCR assay has been designed on the gene coding for the DNA-dependent *RNA polymerase II largest subunit* (*RPB1*) (Gisder and Genersch 2013), which provides an alternate molecular tool for nosemosis detection.

However, to assess the effect of the parasite both at individual and colony level and to better understand the effect of multiple infections, quantification is pivotal (Fries et al. 2013). In quantitative diagnostics, probe-based assays are to be preferred over dye-based assays due to better specificity (Newby et al. 2003) and amplicon size ranging from 50-150bp should be used to maximize the reaction efficiency.

The aim of this investigation is to provide a reliable qPCR assay based on a highly-conserved region of the *N. ceranae* genome, namely single copy heat-shock protein coding gene *Hsp70* (Gomez-Moracho et al. 2014; Wang et al. 2017).

Material and Methods

qPCR assay design

An in silico assay design was performed by checking the sequences of 23 clones (Table 1) obtained from homogenates of *A. mellifera* workers, naturally infected with *N. ceranae*, from three colonies: 440 Hungary, 1251 Hawaii and 1324 Hawaii (Gomez-Moracho et al. 2014).

Polymorphisms in the annealing zones were investigated with BioEdit software v7.0.4 (Hall 1999) while potential primers and probe interactions were ruled-out by using AutoDimer software (Vallone and Butler 2004) using Temperature = 56 °C and Total score required = 7 as testing condition.

The primers pair and the TaqMan® MGB Probe were designed with Primer Express® Software v2.0 (Applied Biosystems) using AN: XM_002995382.1 as reference sequence (Table 2). The primers used in the study are located on scaffold NW_003314033.1 of the NcBRL01 genome.

qPCR optimization

Regardless the use of TaqMan® chemistry, SYBR® has been chosen in order to spot the presence of non-specific amplification through melting curve analysis.

All the reactions were carried out using an AB7300 thermocycler (Applied Biosystems) under the following cycling conditions: initial activation step 95 °C for 10 min, PCR cycling (40 cycles of 95 °C for 15 sec., 56 °C for 60 sec.) with a final dissociation stage for melting curve analysis.

The reaction was performed using 10 µl of SYBR® Green PCR Master Mix (Applied Biosystems), 2 µl of template (IDT Ultramer®, $1,5 * 10^4$ copies of DNA) and a matrix of all possible combinations between forward and reverse primers at the concentrations of 1000, 500, 250, 125 and 62,5 nM. Nuclease free water was added to adjust the final volume to 20 µl.

Each primer concentration pair had a corresponding well with no template as a control (NTC) to assess false positives.

qPCR final setup

All the reactions in the study were performed according to the following conditions: 4 µl of 5x HOT FIREPol® Probe qPCR Mix Plus (ROX) (Solis Biodyne), 2.5 µl of template, 250 nM of forward primer, reverse primer and probe, and a volume of nuclease free water to adjust to 20 µl.

The thermal cycle followed mix manufacturers' specifications with initial activation step 95 °C for 15 min, PCR cycling (40 cycles of 95 °C for 15 sec., 56 °C for 60 sec.).

Absolute quantification and Inhibition test

Synthesized oligonucleotide (IDT Ultramer®), having the sequence of the target fragment has been chosen as standard for absolute quantification.

Serial logarithmic dilutions were performed in order to obtain a reasonable range of copies to build the six-point standard curve ($1.88 * 10^6$ – $1.88 * 10^1$ copies).

All the solutions were diluted in IDTE buffer and kept at – 80 °C in single use aliquots, in order to avoid multiple thawing.

Possible bias in sample quantification due to the presence of inhibitors in the honey bee extracts has been investigated through the simultaneous amplification of buffer-eluted standards (as used in qPCR reactions) and standards eluted in DNA extracts from a single honey bee abdomen (previously tested and resulted negative to *N. ceranae* infection).

Each of the six dilutions were tested in triplicates with the qPCR method proposed in this paper, respectively in IDTE buffer and in three different negative matrixes.

The arithmetic means of the three replicates for each dilution were calculated and then the results of each matrix were compared with One-way ANOVA.

Performance of the assay

Efficiency and R^2

These assay parameters were evaluated by running 10 separate plates arranged with the 6 concentrations of the quantification standards ($1.88 * 10^6$ – $1.88 * 10^1$ copies) in triplicate, to evaluate also within run repeatability.

The percentage efficiency for every run examined was calculated with the formula:

E [%] = (10^(-1/Slope)-1)*100

Linear Dynamic Range (LDR), Limit of Detection (LOD), Limit of Quantification (LOQ)

The regression line to estimate these parameters has been calculated with the results of a plate arranged with quantification standards ($1.88 * 10^6$ – $1.88 * 10^2$ copies) in triplicate plus standards with 24, 12 and 6 copies in 20 replicates each.

Specificity

The specificity of the reaction has been investigated with 3 different methods:

1) In silico, searching for nearly exact match on primer set with NCBI-BLAST (Basic Local Alignment Search Tool) (Altschul et al. 1990); the amplicon sequence and both primers were tested with BLASTN algorithm using the "somewhat similar sequence" parameter.

2) Melting profile analysis was performed with AB Prism 7300 SDS software v1.4 and consisted in a gradual increase in temperature (1°C/15 s to 95°C) after the end of the amplification cycles, during which changes in fluorescence were monitored.

3) Experimental evaluation was performed versus the other relevant microsporidia of honey bees, *N. apis*; an end-point PCR assay (Martin-Hernandez et al. 2007) was used to confirm the spores as *N. apis*; two DNA extracts from different stocks of Percoll-purified, microscopy counted (8500 and 12000 spores per microliter respectively), spores have been evaluated in triplicate with SYBR chemistry. The reaction was performed using 10 µl of SYBR® Green PCR Master Mix (Applied Biosystems), 2 µl of template, 250 nM of forward primer and reverse primer and nuclease free water to adjust the volume to 20 µl.

Repeatability between runs (operator effect)

Quantification standards and PCR mastermix were prepared from three different operators. The operator involved in PCR mastermix preparation always used a quantification standard set prepared by a different operator.

Testing the Hsp70 gene-based method on realistic samples

The reliability of the method was also tested on real honey bee samples (N=100). Ten colonies were randomly selected in summer from apiaries located in the area of Bologna, Italy. Forager worker bees were sampled from each hive entrance and ten random individuals were chosen from each sample for further analysis.

The honey bees were processed individually. Each abdomen (= gastrum) was dissected and homogenized with Tissue Lyser II (Qiagen, Hilden, Germany) for 3 minutes at 30 Hz. DNA was extracted by ZR Tissue & Insect DNA Microprep© (Zymo Research, USA) following the manufacturer's instructions and the extracts were analysed in duplicate with Real Time PCR as previously described.

Results

qPCR assay design

Sequence alignment evidenced a highly-conserved region of 65bp (ranging from 498bp to 562pb of the reference sequence) that was used as target for the assay.

Among the oligos proposed by the Primer Express® Software v2.0, the ones falling on polymorphic sites were discarded whereas the ones in the conserved region were tested for potential interactions.

The resulting following oligo pairs when testing oligos for potential interactions (hairpin, homodimer, and heterodimer structures) satisfied the testing conditions.

qPCR optimization

The lowest Cq was obtained from the couple Forward 1000 nM / Reverse 500 nM followed by the couple Forward 1000 nM / Reverse 1000 nM and the couple Forward 250 nM / Reverse 250 nM. The NTC amplification failed for all of the primer pairs combination.

The couple chosen for the assay is F250/R250 because, despite the slightly higher Cq

(24.3771 vs 24.2632 of the F1000/R500 couple), it showed a considerably higher melting temperature of the amplicon (77.9 vs 77.4 of the F1000/R500 couple).

Quantification standards and Inhibition test

The risk in using synthetic oligos as standards lies in the possible presence of inhibitors in the samples extract, capable of distorting quantifications.

To verify this possibility, a comparison between standards eluted in IDTE buffer and eluted in negative samples has been arranged.

The statistical test (One-way ANOVA: $F_{3,20} = 0.007601$, $p = 0.999$) indicates no significant differences between the matrixes (Figure 1).

Although it is not possible to exclude the presence of inhibitors in every sample without testing them, our data suggest that there are no endogenous inhibitors for the assay in the honey bee abdomen extracts.

Performance of the assay

Efficiency and R^2

The mean value obtained for all the plates with the relative confidence interval is E [%] = 97.5 ± 2.1 %, very close to the ideal value of 100 %.

The mean R^2 value was 0.998 ± 0.002.

LDR, LOD, LOQ

The LDR covers 6 $\log_{10}$ with a maximum CI of 0.5.

The standard deviation was ≤ 0.250, which is compatible with the possibility to discriminate between a 2-fold dilution in more than 95% of cases for 100% efficient reactions.

The least concentrated standard with a SD ≤0.250 was selected as LOQ (188 copies).

The LOD, intended as the last dilution showing 100% response, was close to 6 copies (Figure 2).

Specificity

1) In silico BLAST search returned the following results:

- The whole amplicon sequence returned a BLAST Max score of 118 with 100% query coverage (E-value 8e-24). No other genes showed 100% coverage except

for *N. ceranae* Hsp70. Second best match had a max score of 64.4 and 96% coverage (E-value 1e-07).

- The Forward primer best matches against *N. ceranae Hsp70* gene with 100% query coverage (E-value 5e-04); following matches were with organisms not likely to be present in honey bee extract and returned query coverages lower than 76% (E-value lower than 1.9).
- The Reverse primer best matches with *N. ceranae Hsp70* gene with 100% query coverage (E-value 4.8e-02); following matches were with organisms not likely to be present in honey bee extract and (E-value lower than 0.75); the only ubiquitous bacteria (thus possibly present in bee extract) scored was *Bacillus subtilis* (AN: CP015004.1): query coverage 76%; E-value 46.

2) Melting profile analysis confirmed the presence of only one amplicon, melting at 77.7 ± 0.6°C.

3) Experimental evaluation of two different DNA extracts from *N. apis* spores were amplified in triplicates and revealed no amplification.

Repeatability between runs (operator effect)

Ct data from 10 runs for each of the three operators involved were analysed. Each of the six dilution points were independently tested and returned non-significant differences for LS-means value (Ct[10^6], P=0.40; Ct[10^5], P=0.35; Ct[10^4], P=0.44; Ct[10^3], P=0.5; Ct[10^2], P=0.17; Ct[10^1], P=0.53).

The complete Ct data set was tested, returning non-significant overall differenced for LS-means value as well (Ct[means], P=0.94). No evidence was attained that the assay results may be significantly affected by the operators.

Testing the Hsp70 gene-based method on realistic samples

Amplification of the *Hsp70* gene was achieved in 95 out of 100 honey bee samples (Table 3), with a number of *N. ceranae* copies covering all the orders of magnitude in the range log 10^0 to log 10^6 with respectively 4, 40, 31, 7, 2, 8 and 3 samples.

More precisely, the number of *N. ceranae* copies found with qPCR analysis in the positive foragers ranged from 7 to 1,555,570 and averaged 93,318.7 (± 29,903.0 s.e.) per individual honey bee with a standard deviation of 291,458.7.

Discussion

The novel qPCR assay exploits the functionality of the TaqMan® probe. It relies on the exonuclease activity of the polymerase, which is best at a lower temperature than the probe's annealing temperature. This also reduces enzyme processivity, hence the need of small amplicons to maintain high reaction efficiency. The amplicon size of 65bp chosen in our case fits the recommended range of 50-150 bp. Moreover, a shorter amplicon size implies lower sensitivity to sample degradation. It is also convenient to keep the probe short to makes it more likely that a single mismatch with the template prevents annealing and, consequently, a fluorescent signal. However, such a small probe (12bp) needs the use of the MGB moiety attached to the quencher molecule to raise the melting temperature (Kutyavin et al. 2000).

The assay proved to be specific and sensitive, the LOD is very low and the LOQ is far below the identified ID_{100} for the parasite (10^4 spores) (Forsgren and Fries 2010), which allows to discriminate between infected and diseased bees. A reliable quantification of the parasite load is an essential tool to increase knowledge towards this elusive enemy. A recent study (Graystock et al., 2014) showed considerable pathogen spill over between honey bees and bumblebees. It is not surprising that closely related species are susceptible to the same parasite, but only a quantitative approach can disclose the degree of susceptibility (and consequently of damage) of each involved species. Another field that could take advantage of this quantitative molecular approach is the study of genetic tolerance towards *N. ceranae* (Huang et al. 2014) where high accuracy in spore quantification is required.

The analysis of samples collected from colonies in the field confirmed that the method allows to detect *N. ceranae* infections far below the ID_{50} (85 spores) (Forsgren and Fries 2010) that are likely to correspond to subclinical conditions, and corroborated both LOD and LOQ that were calculated in the previous stages of setup.

This novel *Hsp70* gene-based qPCR detection and quantification assay relies on a sequence that is highly conserved (Gomez-Moracho et al. 2014; Wang et al. 2017) and not reported as subjected to intraspecific genetic variability. It provides reliable and sensitive assay to overt and subclinical *N. ceranae* infections in honey bees. Cross reaction with *Nosema apis* was not detected but, at the moment, this statement cannot be generalised for other *Nosema* species that may come into contact with honey bees. Indeed, sequences for the *Hsp70* gene of *Nosema neumanni*, a new species recently

identified only in Ugandan honey bees (Chemurot et al. 2017), and of other closely related species like *Nosema bombi* are not yet available. Further investigation is needed in this respect.

FIGURES

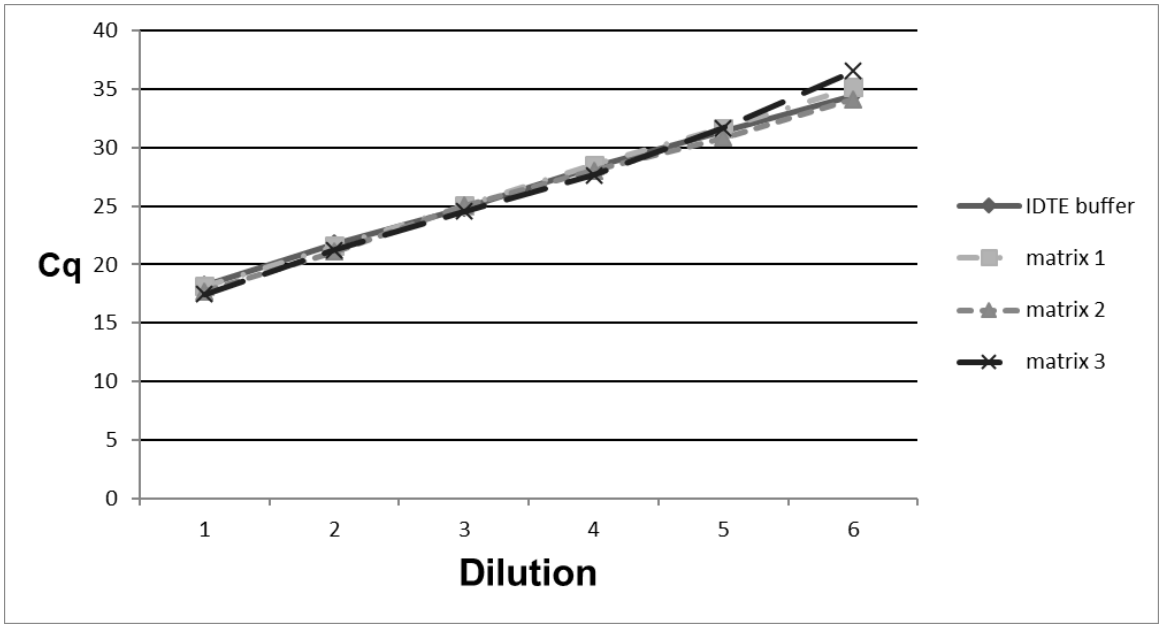

Figure 1: Comparison of six logarithmic dilutions of the standards in IDTE Buffer and in spiked honey bee extracts (matrixes).

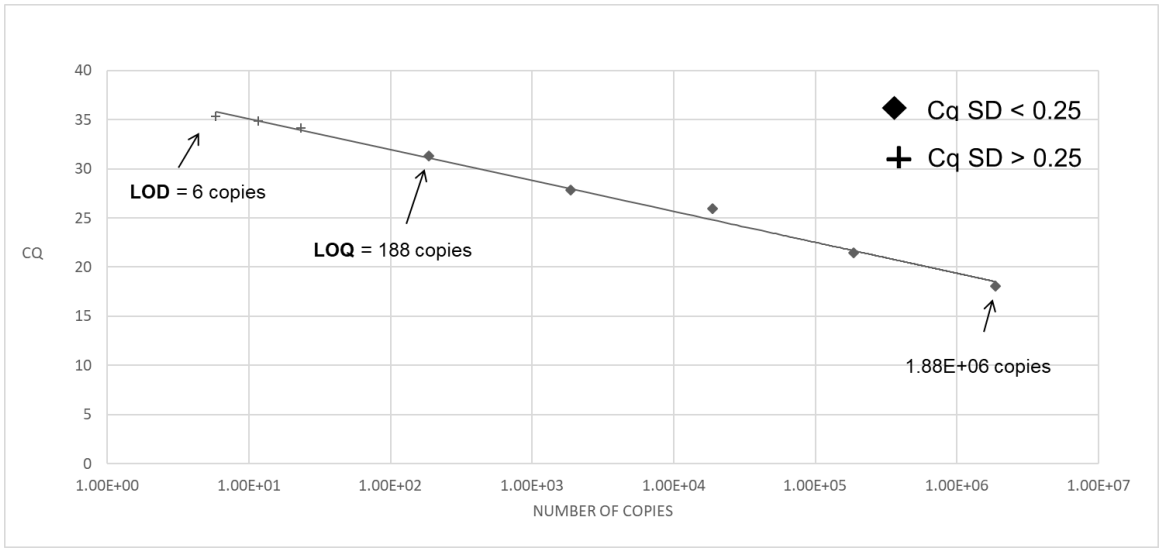

Figure 2: Regression line obtained from serial dilution of Standard.

TABLES

Table 1: 23 clones, obtained from homogenates of *Apis mellifera* workers, naturally infected with *Nosema ceranae*, from three colonies: 440 Hungary, 1251 Hawaii and 1324 Hawaii, used for in silico design.

Origin	Colony	Clone	Accession
Hungary	440	1	KF002438.1
		2	KF002439.1
		3	KF002440.1
		4	KF002441.1
		5	KF002442.1
		7	KF002443.1
		8	KF002444.1
		9	KF002445.1
		10	KF002446.1
Hawaii	1251	3	KF002447.1
		4	KF002448.1
		5	KF002449.1
		6	KF002450.1
		7	KF002451.1
		8	KF002452.1
		9	KF002453.1
		10	KF002454.1
Hawaii	1324	1	KF002455.1
		3	KF002456.1
		4	KF002457.1
		7	KF002458.1
		9	KF002459.1
		11	KF002460.1

Table 2: Primers and probe for *Nosema ceranae Hsp70* qPCR

Primers	**Sequence (5'-3')**
Forward	GGGATTACAAGTGCTTAGAGTGATT
Reverse	TGTCAAGCCCATAAGCAAGTG
Probe	TGAGCCTACTGCGGC

Table 3: *Nosema ceranae* copies detected in 100 honey bee samples.

Sample	*N.ceranae* copies	Sample	*N.ceranae* copies	Sample	*N.ceranae* copies
1	140	35	45	69	339
2	1110	36	2324	70	26
3	235	37	0	71	361524
4	190	38	698	72	1555570
5	7	39	462760	73	43
6	63	40	168	74	225
7	104	41	103	75	2480
8	299	42	75	76	14
9	152	43	15	77	0
10	1422	44	1165320	78	15
11	48	45	215	79	14
12	43	46	3869	80	70
13	781	47	134	81	187
14	150	48	669031	82	82130
15	16	49	73	83	15
16	0	50	960183	84	35
17	54	51	38	85	758
18	29	52	49	86	22
19	26351	53	216	87	48
20	151	54	34	88	53
21	85	55	727151	89	2873
22	34	56	144	90	39
23	8	57	510	91	113
24	137	58	650882	92	34
25	24	59	9	93	369
26	9	60	0	94	685
27	839	61	72	95	0
28	1444970	62	1059	96	83
29	28	63	536	97	53
30	251	64	164	98	251
31	10	65	32	99	97
32	410143	66	34	100	373
33	322829	67	23		
34	18	68	40		

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Chapter 7: Formic acid based treatment with two different evaporators: efficacy and tolerability comparison

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Abstract

Formic acid based treatments are active also against Varroas enclosed inside sealed brood. For its organic nature and the low risk of long-lasting residues in bee products, are very popular in Northern Europe, but could be problematic in Southern regions. In this study, the efficacy and the tolerability of aqueous solution of 60% formic acid using two different evaporator (Aspro-Novar-Form® - CMA Pitarresi, Italy, and Nassenheider professional® (Joachim Weiland Werkzeugbau GmbH & Co., Germany, named AN and –NH respectively) were compared. Total efficacy was on average high (> 80%), but with high variability among the different colonies, without significant differences between the two evaporators. No significant differences in tolerability between the two approaches were observed, however, the number of bees after treatment in AN group, is on average higher than in NH group. Further field comparisons should be conducted to explore the differences in tolerability arose in this study.

Keywords: formic acid; *Apis mellifera*, *Varroa destructor*; evaporators; efficacy; tolerability.

Introduction

Beekeeping is facing several menaces and *Varroa destructor* is one of the most dangerous (Nazzi et al., 2016). Its peculiar life cycle consists of two different phases: one on adult bees, where they actively feed on fat bodies of hosts (Ramsey et al., 2019) and a reproductive one inside sealed brood; in the latter, the parasite is relatively protected from the action of most of the treatments except from formic acid based ones (Rosenkranz et al., 2010). This reason, combined with its organic nature and the low risk of long-lasting residues in bee products made the latter very popular in Europe since the eighties (Wachendörfer et al., 1984; Mutinelli et al., 1994). However, due to the great volatility of this organic acid, particularly with high temperatures, its use in hot Mediterranean countries has always been troublesome. In fact, sudden evaporation of high amounts of formic acid can lead to adverse effects in the colony, like queen loss and brood mortality (Bolli et al., 1993). Thus, the choice of the evaporator is crucial in achieving the best efficacy combined with the highest tolerability.

Recently, a new evaporator became popular among Italian beekeepers: Aspro-Novar-Form® (CMA Pitarresi, Italy) is a simply made evaporator, consisting of three vials (200 ml of capacity each), fitted inside a Dadant-Blatt super frame. The peculiarity of this evaporator is that it can be positioned between brood frames, inside the brood chamber, where temperature and humidity are strictly regulated by honey bees. This could potentially lead to a more gradual evaporation of the active principle and therefore to a better tolerability without losing acaricidal efficacy.

To test this hypothesis, this new evaporator was compared with the long known Nassenheider professional® (Joachim Weiland Werkzeugbau GmbH & Co., Germany) as summer treatment in Bologna, Italy.

Material and Methods

Field trial

The trial took place in an apiary near Bologna, Northern Italy (44°24'18.2'' N, 11°35'56.1''E, 74 meters above sea level), between august 13th and October 14th 2017. Twenty colonies of *Apis mellifera ligustica* housed in ten frames Dadant-Blatt hives were used. Inclusion criteria were: at least six combs covered of bees and no symptoms of disease. Hives were randomly allocated to the two experimental groups: one treated with Aspro-Novar-Form® evaporator (subsequently called AN) and one treated with Nassenheider-professional® evaporator (subsequently called NH).

Each of the three bottles of the AN evaporator was filled with 200 ml (total volume 600 ml) of Apifor60® (Chemicals Laif, Italy), while the bottle of the NH evaporator was filled with 290 ml, as suggested by the manufacturers. Apifor60® is an aqueous solution of formic acid (60 % w/w).

The AN evaporator was placed in the middle of the colony, between two brood frames, while the NH was placed on top of the frames of the brood chamber. In order to have enough space for the NH evaporator, a super without frames was placed on the hive, as previously described by Pietropaoli and Formato (2017).

For the NH evaporator we chose the larger U-wick size provided with the evaporator, the one suggested for Dadant-Blatt hives by the producer. On the contrary, the AN evaporator was slightly modified according to the experience of UNAAPI (National Union of Italian Beekeepers) technicians who observed an erratic evaporation probably due to the inconstant origin of the wick provided by the manufacturer (personal communication). In fact, the wick provided with the evaporator is made of natural felt, probably more inconstant in composition compared to a synthetic one. For this reason, the original wick was replaced with one made of polyester (Myola srl, Italy) having a similar density (450

g/m^2) and thickness (3 mm). The wick was cut in stripes 75 mm long and 30 mm wide, put into the bottle and left out of the cap for about 15 mm.

The trial protocol is summarized in Table 1. On day 0, total brood and adult population was assessed in each hive with a slightly modified Liebefelder method (Delaplane et al. 2013): during the inspection, every face of the frame was divided into six portions and the number of portions covered with each matrix recorded. During the assessment the presence of the queen was verified, and other relevant traits of the colonies annotated. One hive per group was removed from the study in this phase due to queen issues. The same day, the treatment was administered to the colonies. After 14 days, the evaporators were removed and individually weighted in order to assess the mass of drug evaporated (drug_mass). Moreover, another assessment of brood and adult population was carried out.

To evaluate acaricidal efficacy, the number of fallen mites was counted throughout the trial on sticky sheets, placed under the mesh floor of the hives and changed according to the protocol in Table 1. The mite fallen from day 0 to day 26 were ascribed to the formic acid treatment: 14 days corresponding to the time with evaporators in place plus 12 days to consider the mite killed inside the sealed brood by the treatment.

The follow-up treatment chosen was a combination of 25 days queen caging and an oxalic acid based treatment. This treatment was carried out by trickling a sucrose solution with API-Bioxal® (Chemicals Laif, Italy), according to the manufacturer instructions. This treatment was chosen due to its high efficacy, over 90 % (Moro and Mutinelli, 2019) needed to comply with the EMA guidelines (EMA, 2010).

Total efficacy (TEf) and Cumulative efficacy (CEf) were calculated with the following formulas:

TEf = (Mites trt) / (Mites trt + Mites fu) * 100

CEf = (Mites trt day x) / (Mites trt + Mites fu) * 100

where “Mites trt” corresponds to the mites fallen because of the formic acid treatment, “Mites fu” corresponds to the mites fallen because of the follow-up treatment and “Mites trt day x” represents the mites fallen until day x (3, 7, 11 days).

To monitor the temperature throughout the treatment period, three HT160 probes (HT, Italy) were programmed to record the temperature every 10 minutes for 14 days. One probe was dislocated in a hive of the AN group, one in a hive of the NH group and another one was placed in an empty hive, near the others on trial, in order to assess the environmental temperature. The probes in the treated hives were positioned as close as possible to the evaporators.

Statistical analysis

Statistical analysis was performed with R (version 3.5.2), coupled with readxl, ggplot2, psych libraries.

The difference in total efficacy between groups was compared with Two- sample t-test, while differences in cumulative efficacy were studied with Welch’s Two-sample t-test. Regarding tolerability, adult bee population post-treatment (Bees_1) and brood amount post-treatment (Brood_1) were compared with ANCOVA fitting adult bee population pre-treatment (Bees_0) and brood amount pre-treatment (Brood_0) as covariates.

The amount of drug evaporated (drug_mass) was compared between the two groups with Wilcoxon’s test.

Temperature time series was decomposed in “trend”, “seasonal” and “random” factors with an additive model, chosen over a multiplicative model after visual checking the plots of the time series.

Parametric methods were applied only where the assumptions of normality (checked with Shapiro-Wilk's test) and homoscedasticity (checked with Bartlett's test) were met.

Differences between groups (AN and NH) were considered significant for p-values < 0.05.

Results

Total efficacy of Apifor60 dispensed through the two evaporators tested in this study was on average elevated (> 80%) however, the variability of this parameter among the different colonies was high in both groups. In fact, in three colonies of AN group and in two of NH group the efficacy was under 75 %, and one per group under 60 %.

The mean total efficacy in the NH group (M = 82.0 % ± SD = 13.7 %) and the mean total efficacy in the AN group (M = 81.5 % ± SD = 13.3 %) were not significantly different [$t(14) = -0.061$, $p = 0.95$] (Fig. 1).

The mean cumulative efficacy, did not differ between groups at days 3, 7 and 11 [$t(14) = 1.05$, $p = 0.31$]; [$t(14) = 0.37$, $p = 0.72$]; [$t(14) = - 0.01$, $p = 0.99$] (Fig. 2).

Regarding tolerability, the adult bee population post-treatment (Bees_1) was not significantly affected by the interaction between group (Grp) and adult bee population pre-treatment (Bees_0), [$F(3, 12) = 0.09$, $p = 0.77$], nor adult bee population pre-treatment (Bees_0), [$F(3, 12) = 0.15$, $p = 0.71$] or by group alone [$F(3, 12) = 4.25$, $p = 0.06$] (Fig. 3). However, the difference found between groups was conspicuous: in fact Bees_1 in AN group (M = 64.8 ± SD = 4.1), is on average 8 sixths of comb higher than in NH group (M = 56.5 ± SD = 9.0). This large effect size (Cohen's d = 1.17), corresponds to an adequate power of the experiment to detect differences with 10 hives per group, as planned for this study ($\alpha = 0.05$, $1\text{-}\beta = 0.80$). Unfortunately, one hive per group was

removed from the study at its first stages due to queen problems decreasing the statistical power.

The amount of brood post-treatment (Brood_1) was not significantly affected by the interaction between group (Grp) and the amount of brood pre-treatment (Brood_0), [$F(3, 12) = 0.05$, $p = 0.83$], nor by group alone [$F(3, 12) = 0.01$, $p = 0.91$] or amount of brood pre-treatment (Brood_0), [$F(3, 12) = 3.21$, $p = 0.10$] (Fig. 4).

The "trend" components of the temperature time series for the three different groups are plotted in Fig. 6.

The amount of drug evaporated (drug_mass) in the NH group (Mdn = 324.8 g, IQR = 5.8 g), compared with the amount evaporated in the AN group (Mdn = 275.5 g, IQR = 8.5 g) was significantly higher ($W = 0$, p-value = 0.0007853) (Fig. 5).

Discussion

Despite the recent increase in the number of acaricidal formulations on the European market, most of the active ingredients are long known, since the majority of them have been discovered in the 90's. In this scenario of paucity, it is of capital importance to have specific protocols in order to make the use of each registered active principle possible in all regions of Europe. Notwithstanding the popularity in central and northern Europe, formic acid based treatment struggled to gain popularity in the Mediterranean regions. In this work we tried a new evaporator for this molecule to assess the suitability of its use for the southern countries, comparing it with a more established one.

Our results showed that Total efficacy with both evaporators was on average high (> 80%), although under the minimum efficacy advised by the EMA guidelines for organic acaricides (> 90 %), and no significant differences in Total efficacy between the two evaporators was observed. Nevertheless, the variability in Total efficacy among the

different colonies is high in both groups.

The Total efficacy for NH evaporator reported in this study is higher than the one found by Pietropaoli and Formato (2017), who reported a minimum mean efficacy of 57.0 % and a maximum mean efficacy of 72.7 % in different apiaries. It is worth mentioning that in our work the treatment was carried out in the same period of the year and in locations with similar “mean annual temperature” and “max temperature of warmest month” of the cited work, according to Worldclim.org (Hijmans et al., 2005). So, probably the difference in Total efficacy is related to the length of the treatment (11 days for Pietropaoli and Formato, 2017 and 14 days in this study) though both in the range of 10 to 14 days suggested by the manufacturer of the evaporator. This hypothesis is confirmed by the cumulative efficacy at day 11 of the present study ((M = 68.6 % ± SD = 18.2 %) that falls in the range reported by the Pietropaoli and Formato (2017). The length of the treatment could be important in determining the efficacy of formic acid, that is not always considered sufficient to prevent wintering issues as highlighted by Beyer et al. (2018) who demonstrated, with a four-year epidemiological study in Luxembourg, the absence of an effect of 60 % formic acid treatments in reducing colony winter losses. This could be even more true in Mediterranean regions, where a longer season implies more reproductive cycles and greater summer varroa infestations.

Cumulative efficacy was also evaluated, taking into account the importance of the mite fall dynamics in addition to total efficacy at the end of the treatment (Fig. 2). The efficacy 3 days after the evaporators were placed is, on average, substantially higher in the AN group (M = 31.7 %) than in the NH group (M = 19.8 %); however, the variability is very high in both groups and the difference is not significant. In the following days the difference is negligible, as also confirmed by the statistical analysis. Despite suboptimal, it is interesting to note that about 68 % of efficacy is reached in 7 days and about 80 % of

it is reached in two weeks. This could leads to two considerations: firstly, the limited *Varroa* fall in the 12 days following the treatment, could be an indication of a poor effectiveness on the mites contained in the brood; secondarily, the fast action on varroa mite could be, in certain cases, an advantage compared to other commonly used summer treatments. For example, Apivar (AI: amitraz), in order to reach a comparable efficacy of 76.5 %, needed 8 days more, according to Al Naggar et al., (2015), while using oxalic acid coupled with queen caging a very high miticide effect (> 90 %) is reached only performing the treatment at the end of the caging period, when no more brood is present in the hive (Gregorc et al., 2017).

Regarding the tolerability parameters, the observations on the interaction between group and starting condition lead to the conclusion that any detectable effect of the group is not related to the starting conditions and vice versa.

The effect of group and starting condition was also studied separately: no statistically significant effect of the pre-treatment state on the post-treatment was found, highlighting homogeneous starting conditions and a good randomization.

No statistically significant effect of the group (evaporator used) was found on adult bee population (Bees_1) nor on amount of brood (Brood_1), does implying no differences in tolerability between the two approaches. However, the number of bees after treatment (Bees_1) in AN group, is on average 8 sixths of comb higher than in NH group, corresponding to about 2000 more bees. This difference may be related to the position of the evaporator in the hive; in fact, despite the higher average temperature experienced by the AN evaporator throughout the study, this parameter is less dependent on the external conditions than in the NH group, as shown in the comparison of time series. Probably the highly controlled conditions inside the brood chamber inhibit sudden evaporation of the active principle thus preventing the related acute toxicity on adults. The acute effects of

formic acid based treatments are described in several studies; in particular, 60-65 % formic acid is reported to cause reduction of worker population and worker brood area (Ostermann et al., 2004), reduced drone survival and production (de Guzman et al., 1999).

The hypothesis of higher tolerability on adult bees could be also related to the amount of drug evaporated (Drug_mass): the median for the NH group was about 50 grams higher than that of AN group. This difference is particularly relevant because highlights the capacity of the Aspro-Novar-Form® of delivering a comparable efficacy with a substantially lower amount of drug, thus increasing its therapeutic index.

Conclusion

In conclusion, the efficacy showed by both evaporators is quite low, especially compared with control strategies that couple a drug treatment with a manipulation of the colony like queen caging and oxalic acid based treatment. For this reason, the use of formic acid could not be recommended as the only summer treatment in the Mediterranean regions where high infestations and elevated variability is expected in the colonies. However, the rapid action on mite population achieved by both evaporators makes these treatments interesting as a feasible way to deal with uncontrolled infestations in spring where a long lasting approach hampers the honey harvest. Moreover, the Aspro-Novar-Form® evaporator could have some advantages over the competitor, such as higher tolerability on adult bees, so further field comparisons should be conducted to explore the differences in tolerability arose in this study.

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Disclosure statement

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Tables & Figures

Day	Date	Activity
-3	13/08/17	CA + SC
0	16/08/17	TRT start Apifor60® + SC
3	19/08/17	SC
7	23/08/17	SC
11	27/08/17	SC
14	30/08/17	TRT end Apifor60®, CA,
21	06/09/17	SC
26	11/09/17	QC + SC
33	18/09/17	SC
40	25/09/17	SC
47	02/10/17	SC
51	06/10/17	SC + TRT Api-Bioxal®
54	09/10/17	SC
59	14/10/17	SC

Table 1. Study protocol. CA: colony assessment, SC: sheets change, TRT: Treatment, QC: queen caging.

variables	mean	sd	median	min	max	range	se	IQR
Bees_0	73.1	7.9	74.8	62	84.5	22.5	2.8	11.2
Brood_0	35.8	6.7	32.5	30	47	17	2.4	10.6
Bees_1	64.8	4.1	64.8	59	72	13	1.4	3.6
Brood_1	16	8.1	19.2	3.5	25.5	22	2.9	13.1
Drug_mass	275.1	18.2	275.5	242	308	66	6.4	8.5
Efficacy	81.5	13.3	87.1	58	97.6	39.6	4.7	18.6
Efficacy_day_3	31.7	26.7	24.6	3.5	72	68.5	9.4	39.1
Efficacy_day_7	62.1	22.2	65.2	24.8	95.2	70.4	7.8	28.7
Efficacy_day_11	68.5	21.3	74.2	33.3	96	62.7	7.5	32.9

Table 2. Descriptive statistics for group AN.

variables	mean	sd	median	min	max	range	se	IQR
Bees_0	66.8	13	65.8	49.5	92	42.5	4.6	14.5
Brood_0	33.4	7.6	32	24	46	22	2.7	7.1
Bees_1	56.5	9	59.2	40	66.5	26.5	3.2	9
Brood_1	17.9	10.9	22.5	2	30	28	3.9	16.1
Drug_mass	319.9	8.5	324.8	304.8	324.8	20	3	5.8
Efficacy	82	13.7	86.2	51.6	92.8	41.2	4.8	9.5
Efficacy_day_3	19.8	17.8	15.1	5.8	59.2	53.4	6.3	14.4
Efficacy_day_7	57.9	23.6	61.2	24.3	86.8	62.5	8.4	25.5
Efficacy_day_11	68.6	18.2	70	37.5	89.7	52.2	6.4	20.5

Table 3. Descriptive statistics for group NH.

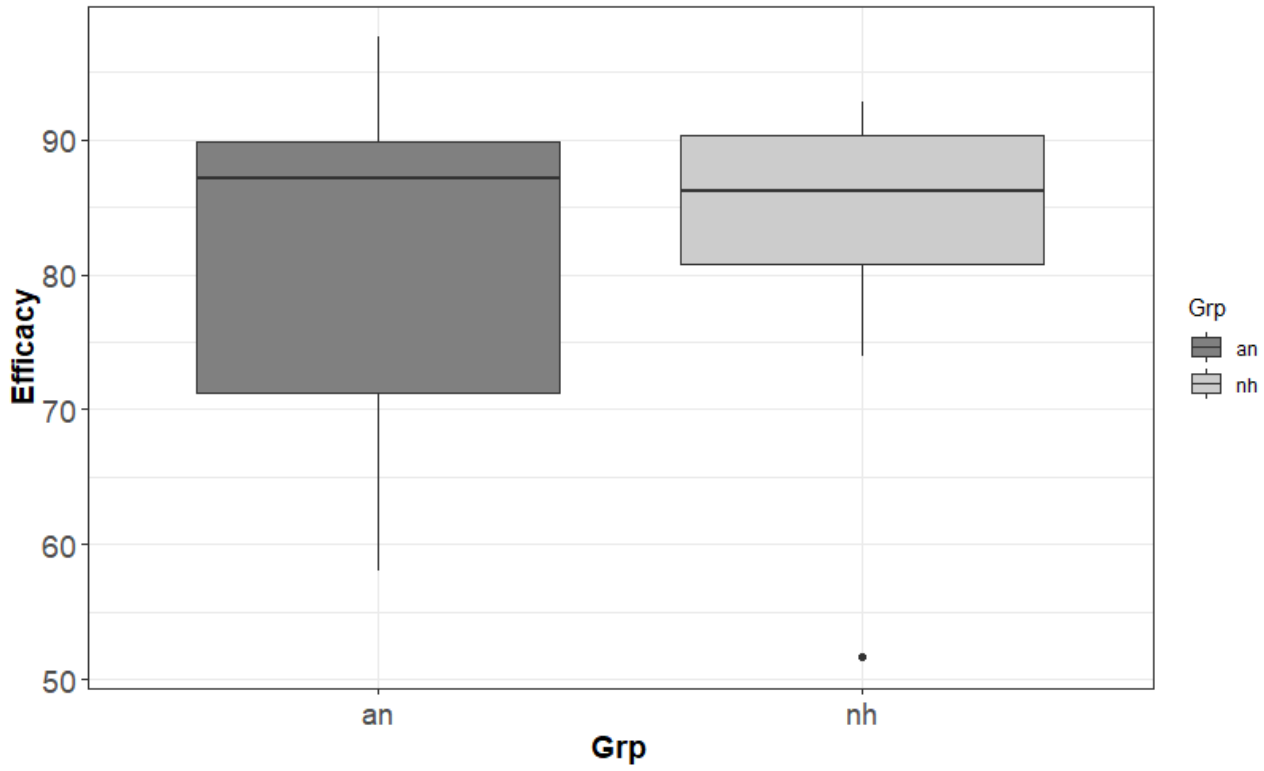

Figure 1. Box plots representing Total efficacy in the groups AN and NH. Horizontal bold line = median, box = IQR, lower whisker = Q1 – 1.5* IQR, upper whisker Q3 + 1.5 *IQR, dot = outlier

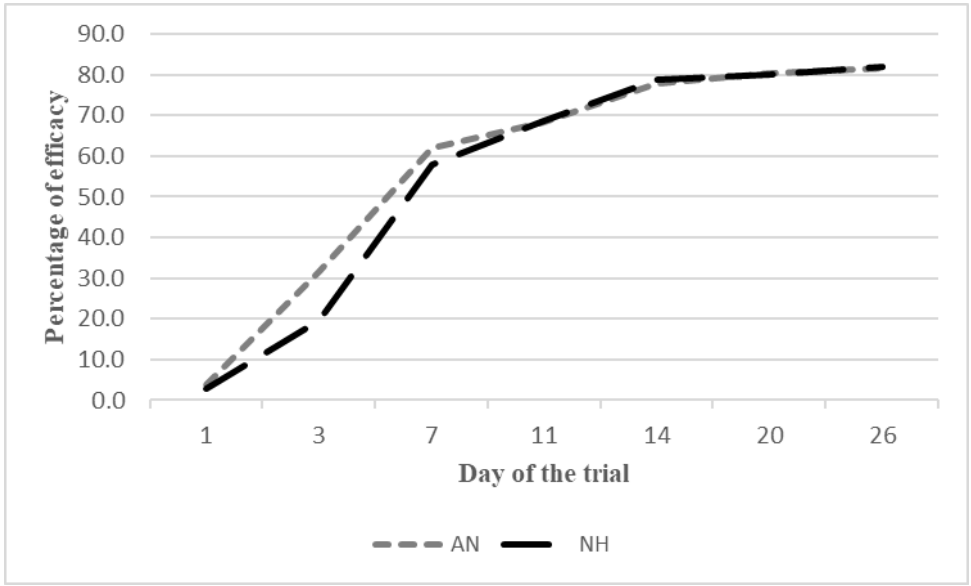

Figure 2. Cumulative efficacy in the groups, during the trial.

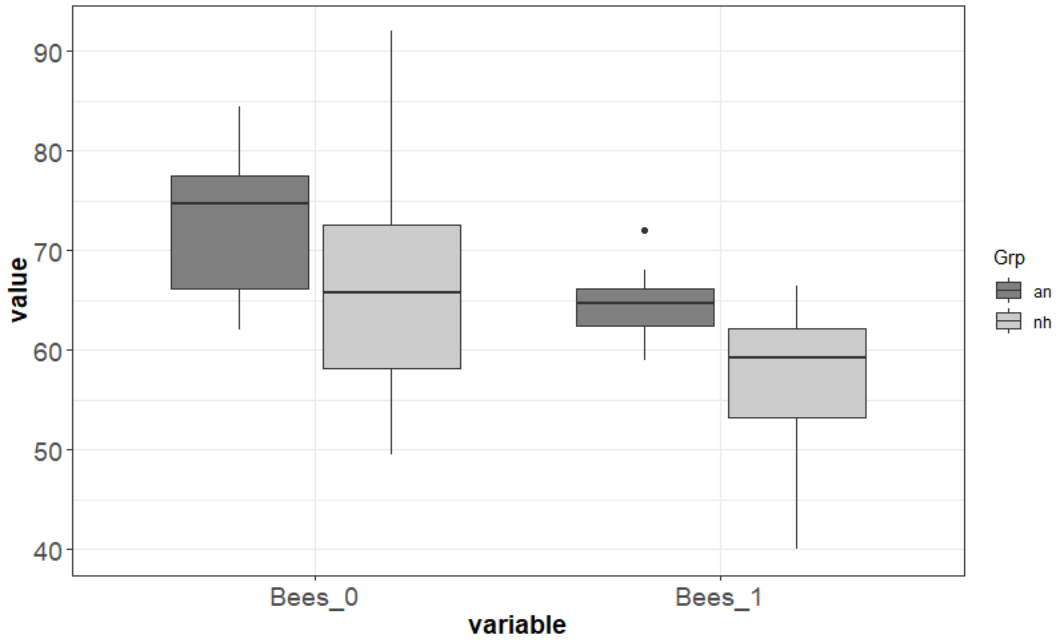

Figure 3. Boxplots representing the adult population pre-treatment (Bees_0) and post-treatment (Bees_1) in the groups; the unit of measurement is 1/6 of a frame surface. Horizontal bold line = median, box = IQR, lower whisker = Q1 – 1.5* IQR, upper whisker Q3 + 1.5 *IQR, dot = outlier

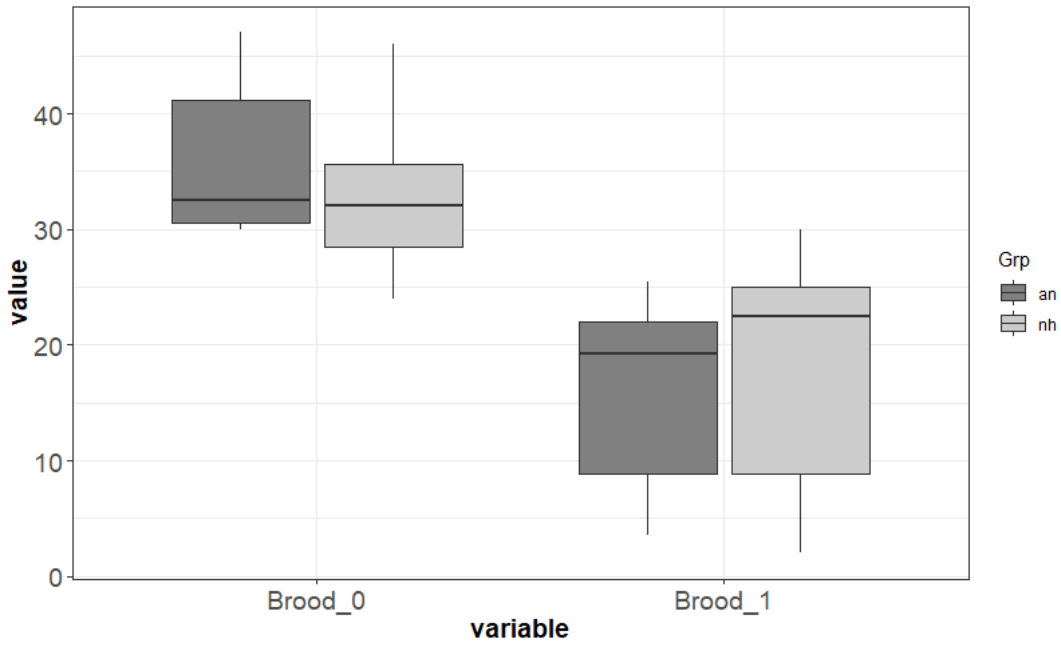

Figure 4. Boxplots representing amount of brood pre-treatment (Brood_0) and post-treatment (Brood_1) in the groups; the unit of measurement is 1/6 of a frame surface. Horizontal bold line = median, box = IQR, lower whisker = Q1 – 1.5* IQR, upper whisker Q3 + 1.5 *IQR, dot = outlier

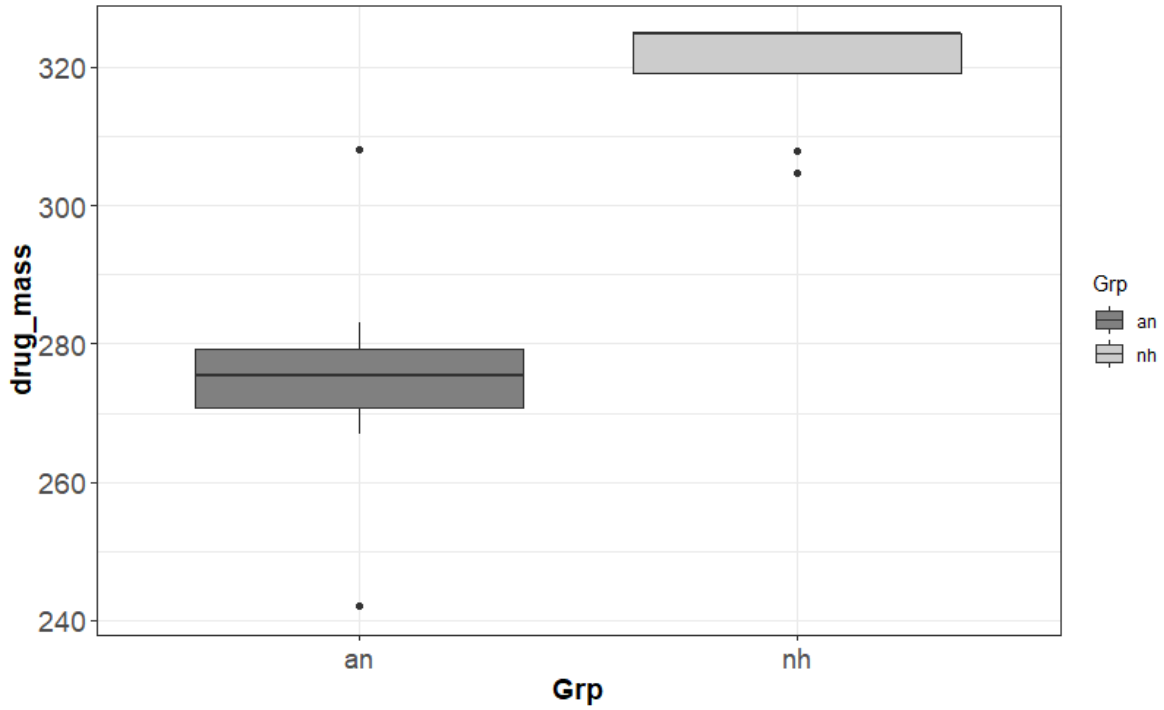

Figure 5. Boxplots representing mass of drug evaporated in the groups; the unit of measurement is grams. Horizontal bold line = median, box = IQR, lower whisker = Q1 – 1.5* IQR, upper whisker Q3 + 1.5 *IQR, dot = outlier.

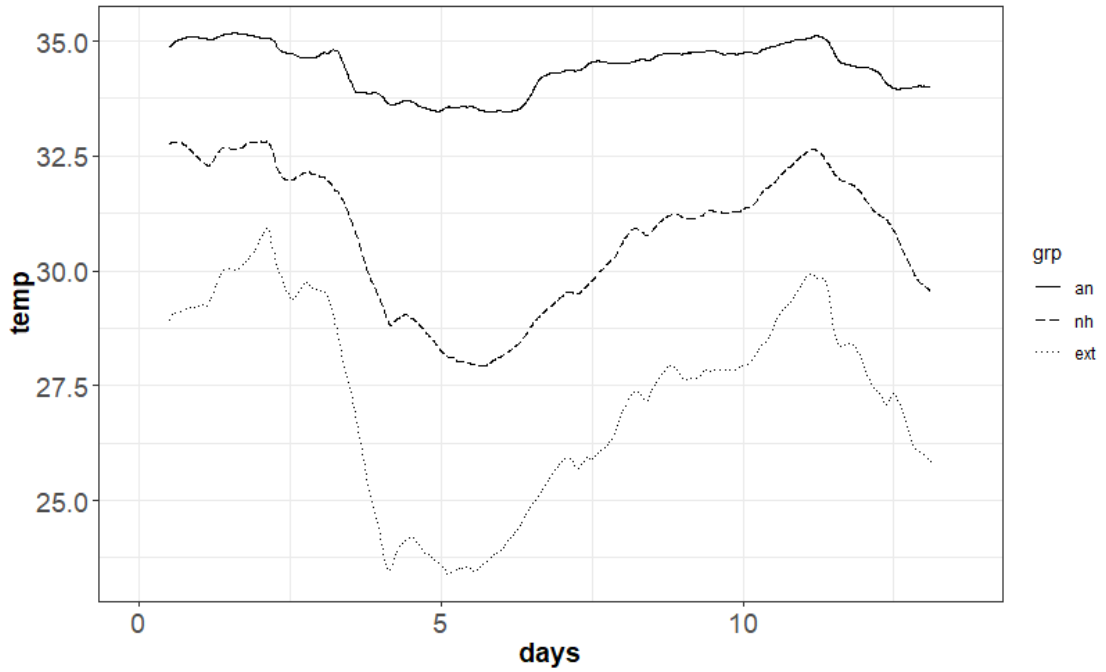

Figure 6. “Trend” component of the temperature time series by groups; “ext” corresponds to the control hive series. Temperature in Celsius degrees on the y axis, day of treatment on the x axis.

Chapter 8: Conclusions

In this work, the complexity of honey bee health assessment has been investigated with a holistic approach. The philosophical antecedents of holism can be traced back to Aristotle, who suggested that "the whole is more than the sum of its parts." This vision could be especially useful to study complex insect societies. In fact, while mathematically the whole colony is only equal to the sum of its parts, neither more nor less, this is not biologically true. Complex behaviours like thermoregulation, comb construction or foraging are best explained by emergent properties of the colony, arising from interactions between individual members.

With these premises in mind, the experiments in this thesis focused on two different and interdependent levels related to bee health: the biochemical level and the parasitological level. At the biochemical level the impact of plant protection products on bee physiology and survival was studied, elucidating synergistic interactions between poor nutrition and pesticide exposure in *A. mellifera* and between an insecticide and a fungicide in *Osmia bicornis*. These trials are particularly relevant for an evidence-based discussion of the registration process of new agrochemicals. Moreover, an innovative fingerprinting approach on honey bee haemolymph was applied to detect population imbalances in the hive. The sampling process and the subsequent analysis with an algorithm could be implemented in monitoring projects and, with specific training, also in the public veterinary service.

The control of *Varroa* infestations was studied both at the biochemical and parasitological level. A panel of biomarkers in honey bee haemolymph was applied to compare different mite control protocols. This resulted in relevant indications for beekeeping operations pursuing the least impact on nutritional status of the colonies. To guide the decision making of beekeepers, a new formic acid evaporator was tested in comparison with a more established one.

Considering its widespread distribution in the country, efforts were directed also towards *N. ceranae* an emergent pathogen with an impact on beekeeping still under debate. In particular, the pivotal aspect of diagnosis was studied, proposing a new qPCR method to overcome some limits of the existing ones.

In conclusion, this works fills some of the knowledge gaps of the beekeeping sector. However, many of them still need to be addressed and the upcoming menaces of climate change and dispersal of pathogens via globalization should be targeted by research efforts

in the near future. Therefore, a multifaceted vision of bee health is of capital importance, aware of the complementarity of reductionist and holistic approaches.