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Varroa destructor exacerbates the negative effect of cold contributing to honey bee mortality

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ABSTRACT

Several concurrent stress factors can impact honey bee health and colony stability. Although a satisfactory knowledge of the effect of almost every single factor is now available, a mechanistic understanding of the many possible interactions between stressors is still largely lacking.

Here we studied, both at the individual and colony level, how honey bees are affected by concurrent exposure to cold and parasitic infection. We found that the parasitic mite *Varroa destructor*, further than increasing the natural mortality of bees, can induce an anorexia that reduces their capacity to thermoregulate and thus react to sub-optimal temperatures. This, in turn, could affect the collective response of the bee colony to cold temperatures aggravating the effect already observed at the individual level. These results highlight the important role that biotic factors can have by shaping the response to abiotic factors and the strategic need to consider the potential interactions between stressors at all levels of the biological organization to better understand their impact.

1. Introduction

Honey bees represent a vital resource both for natural and agricultural ecosystems due to their key role as pollinators of many plant species (Russo, 2016; Hung et al., 2018). In recent years, large colony losses have become a global issue (Le Conte et al., 2010; Neumann and Carreck, 2010) threatening the sustainability of our food production system (Aizen et al., 2009; Potts et al., 2010). It has been suggested that colony losses are caused by multiple stressors (Goulson et al., 2015), however, a mechanistic understanding of the possible interactions between different stressors is still largely lacking (Nazzi and Pennacchio, 2014). In particular, the potential combination of biotic and abiotic stressors, like parasites and adverse environmental conditions has been poorly investigated (Chen et al., 2012; Retschnig et al., 2017) but is becoming increasingly important given the growing impact of climate change on the already complex interactions within the ecosystems (Grimm et al., 2013). This lack of knowledge is not surprising in view of the complexity of the necessary multifactorial studies. On the other hand, given the detailed knowledge of their biology, the large suite of molecular tools available (Grozinger and Robinson, 2015) and thanks to their structure, which can be studied from individual to colony level, honey bees could be considered an ideal model organism for such studies.

In the northern hemisphere, where honey bee colony losses are mostly reported, they normally occur during the autumn–winter period (Amdam et al., 2004; Genersch et al., 2010; Jacques et al., 2017). Monitoring programs in the US highlighted higher mortality of bee colonies in northern states (Kulhanek et al., 2017) and in some cases, a correlation was found between winter temperature and colony losses (vanEngelsdorp et al., 2008). To our knowledge, in Europe, where extensive surveys of colony losses were carried out, a similar pattern has not been reported so far, although published data support the hypothesis that colony losses are somewhat higher in northern Europe as compared to southern European countries (Jacques et al., 2017).

It has been shown that colony losses are related to the progressive build-up of viral infections promoted by the increasing *Varroa destructor* infestation (Nazzi et al., 2012). Both mite infestation and deformed wing virus (DWV) prevalence and abundance gradually increase along the season, peaking at the end of Summer, when thousands of mites can be present in each colony and DWV prevalence reaches 100% (Nazzi et al., 2012). This, in turn, causes increased honey bee mortality, leading to the progressive weakening of the colony, which eventually collapses during

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autumn or the following winter (Genersch et al., 2010; Dainat et al., 2012; Nazzi et al., 2012). Concurrently, under temperate climatic conditions, a decrease in temperatures in the autumn–winter period is also observed.

Thermoregulation is essential for honey bees both at the individual and colony level. Regardless of the external fluctuations, nest temperature is constantly maintained at around 34.5 °C for the whole season (Heinrich, 1981; Seeley, 1985; Bujok et al., 2002; Human et al., 2006). Honey bee nest thermoregulation, under lower external temperatures, is made possible by the capacity of a cohort of bees (i.e. >50 bees; Simpson, 1961) to warm up their thorax after consuming an adequate supply of honey (Rothe and Nachtigall, 1989). In wintertime, when the temperature drops below 10 °C, bees form a cluster whose internal temperature is optimal (Döke et al., 2015). Also, individual bees are capable of thermoregulation; indeed, bees need optimal temperatures to perform flight and recruitment dance (Stabentheiner et al., 1995; Schmaranzer, 2000; Stabentheiner, 2001).

Mite infestation can influence the physiology of individual bees, the number of bees involved in colony thermoregulation as well as the supply of honey that is needed to perform such function. Therefore, we hypothesize that the capacity of bees to maintain an optimal temperature can be impaired by *Varroa* parasitization; in turn, this can influence the conditions of the bee colony further aggravating the already negative impact of the parasite.

To gain insight into how an abiotic factor, like the environmental temperature, can shape the influence of a biotic factor such as a common ectoparasite on honey bees, we investigated the possible interaction both at colony and individual level. At the colony level, we studied nest temperature as affected by *Varroa* infestation, under the decreasing environmental temperatures observed during the Autumn months. At the individual level, we investigated how the lower temperature observed in mite-infested colonies at that time can affect the survival of individual bees infested or not with the mite during the pupal stage. Given the essential role of a convenient sugar supply for thermoregulation, we also studied sugar consumption in uninfested and mite-infested bees maintained at low and normal temperatures and investigated how this could influence thermoregulation in individual bees.

This work aims to add to the growing body of research about the effects of interacting stress factors on honey bee health (Nazzi and Pennacchio, 2014; Goulson et al., 2015) but has got the ambition to provide useful clues to interpret similar interactions in other insects that are similarly affected by multiple stress factors (for a review see Wagner et al., 2021). We believe that these kinds of studies should be further intensified in view of the growing pressure that climate change puts on the existing host-parasite interaction (Morales-Castilla et al., 2021).

2. Materials and methods

2.1. Field experiment

2.1.1. Bee colonies used in the study and field observations

Two apiaries, made of 5 colonies each, housed into ten frames Dadant Blatt hives (385 × 452 × 310 mm), were set up 4 km apart from each other in northeastern Italy. In the untreated apiary, no acaricidal treatments were carried out during Summer (note that one colony collapsed in early October), so that mite infestation could naturally increase along the season. In the treated apiary, the mite population was kept under control with different acaricides; in particular, 2 strips of an amitraz-based product (Apitraz, Laboratorios Calier S.A.) were used in August, followed by three treatments with a thymol-based product in tablets (ApiLife Var, Chemical Laif S.p.A.) from September to early-October (Fig. S1). Our decision to maintain mite infested and uninfested colonies in two separate apiaries was dictated by the need to prevent the possible flow of mites from the first to the latter due to drift of mite infested bees and, moreover, the robbing of the infested colonies by the healthier and stronger ones (Greatti et al., 1992). Unfortunately, this may introduce a problem related to the confusion of treatment and location. One way to address this issue would be to increase the number of apiaries, and thus the number of honey bee colonies; however, this would come at the expense of precision because our research involved a number of periodic detailed observations on each experimental hive that would have been impossible on a bigger number of colonies. Therefore, we opted for the simpler solution that is to select two locations that are distant enough to prevent robbing but not too distant to introduce any relevant difference as far as the response variable is concerned. In this particular case, the response variable was the nest temperature of the colonies belonging to the two groups which, in principle, could be affected by the location if the two sampling sites were very far from each other. However, this was not the case since the distance between the two apiaries, located in a flat area, was only 4 Km and we can assume that the external temperature was similar if not identical.

The bee population in the experimental hives was estimated approximately once a month from August to December, by counting the number of full or partial "sixth of frames" covered by bees in each hive at sunset and calculating the overall bee population, based on the correlation which indicates that one fully covered sixth of comb corresponds to 253 adult bees (Marchetti, 1985; Nazzi et al., 2012).

To assess bee mortality, dead bees found in under basket cages placed in front of the colonies were counted every week from August to December, taking note of the number of individuals showing deformed wings (Nazzi et al., 2012). During the experiment, infestation levels were estimated by counting the number of mites fallen on a vaseline-coated bottom board (Nazzi et al., 2012). To assess total mite infestation in both apiaries, on the 9th of October 2 strips of a fluvalinate-based product (Apistan, Vita Europe Ltd) were used for two months. Counting dead mites on bottom boards can be considered as rough method to estimate mite infestation in a bee colony as compared to other available methods (Dietemann et al., 2013). However, in this case, this method was regarded as a sufficiently precise for the purpose of checking the difference between untreated colonies (that were supposedly heavily mite infested) and bee colonies treated with acaricides (that supposedly hosted only a limited number of mites). Moreover, this method allowed to reduce to the minimum the disturbance to the bee colony and the consequent possible effect on the response variable we were studying (i.e. internal temperature). The use of a fluvalinate-based product for testing total infestation, despite the acaricide resistance developed by Varroa over the years to this active principle (Milani, 1995) is justified by the reversion of resistance demonstrated in the area (Milani and Della Vedova, 2002). Furthermore, resistance is currently managed in the area suspending the use of fluvalinate based products for 3–4 years after use and combining fluvalinate with other acaricides.

In order to compare the two groups of hives, the total number of mites, the bee mortality, the deformed bees and the colony strength were analysed in the same period (i.e. from the 12th of October to the 3rd of December). Throughout the whole experiment, no symptoms of other impacting pathogens (e.g. *Nosema cerane*, *Ascosphaera apis*, and *Paenibacillus larvae*) were recorded during the weekly inspection of the colonies. Data collected from the field (i.e. infestation level, proportion of deformed wing bees, bee mortality and population) were analysed using a Mann-Whitney *U* test.

2.1.2. Temperature measurement inside the experimental hives

In order to monitor the temperature inside the hives, a temperature probe (Maxim integrated, US; ± 0.0625 °C) inserted in a queen caged held by a metal wire fasten at the top of the frame was positioned in the central part of each hive; the probe was set to provide the actual temperature every 600 s. Twice a week the probe position was monitored to make sure it was located in the center of the nest where most bees congregate and the brood is reared; this operation was made it simpler by the progressive removal of unoccupied frames from the hive, such that, at each time, there were bees on each of the combs and the cluster's center was normally positioned about mid-way between the lateral

combs. Monitoring took place from August to November. At the end of the experiment, data collected by the probes were downloaded and the average daily temperature inside the hives with variability coefficient (standard deviation/mean) was calculated. Following already published studies (Vandame and Belzunces, 1998; Schäfer et al., 2011; Tan et al., 2012; Tosi et al., 2016), the statistical significance of the differences in the internal temperature of the colonies of the two groups (acaricide-treated colonies vs. untreated colonies) was analysed using repeated Mann-Whitney U tests. In order to evaluate field weather conditions, average daily external temperature data were derived from the regional meteorological observatory (ARPA FVG – OSMER and GRN, https://www.meteo.fvg.it/).

2.2. Laboratory experiments

2.2.1. Artificial infestation of bee larvae and maintenance of adult bees in cages at different temperatures

Honey bee larvae were artificially infested and reared according to standard methods (Dietemann et al., 2013; Williams et al., 2015). In particular, honey bees and mites were collected from July to September from the same experimental apiary cited above (same apiary but different colonies). Mites and last instar bee larvae were collected from brood cells capped in the preceding 15 h and obtained as follows. In the evening of the day preceding the experiment, the capped brood cells of a comb were marked. The following morning the combs were transferred to the lab and unmarked cells, that had been capped overnight, were manually unsealed. The comb was then placed in a position similar to that in the hive, in an incubator at 34.5 °C and 75% R.H., where 5th instar larvae and mites spontaneously crawled out. Last instar bee larvae were transferred into gelatin capsules (Agar Scientific ltd., 6.5 mm diameter) with no mites (uninfested bees) or 1 mite (infested bees) and maintained at 34.5 °C, 75% R.H. for 12 days (Nazzi and Milani, 1994; Dietemann et al., 2013). Upon eclosion, newly emerged adult bees were separated from the infesting mite and transferred into four plastic cages (185 × 105 × 85 mm) with water and sugar candy (Apifonda®), supplied *ad libitum* through a small plastic container (Ø = 1.5 cm), refilled every 2 days and placed on the floor of the cages. To prevent the exsiccation of the candy, containers were wrapped with laboratory film (Parafilm®); a small cut was made on the top, to ensure bee feeding.

Two of the four cages, one with bees that were mite-infested at the pupal stage and the other with the same number of uninfested bees were maintained in a climatic chamber at 34.5 °C, 75% R.H.; the two other cages with mite-infested and uninfested bees were maintained in a climatic chamber at 32 °C, 75% R.H.; each cage hosted from 20 to 25 adult bees. Sugar candy consumption was recorded by weighing the containers with a precision balance (10^{-4} g), monitored from day 4, when the sugar intake of the bee is stabilized, to day 15 and normalized according to the different weights of infested and control bees (Annoscia et al., 2012).

For virus evaluation, seven days after the eclosion, at least eight bees per treatment were collected, killed with liquid nitrogen and stored at −80 °C until RNA extraction.

The experiment was repeated six times from July to September. In total, between 112 and 134 bees per group were used. The hazard ratio was calculated using the "coxme" function (R software version 3.6.2) with "cage" as a random factor. Sugar consumption under different treatments was normalized and analysed utilizing the two-way ANOVA test.

2.2.2. Honey bee thermoregulation

To test if mite-infested bees thermoregulate their body temperature as well as uninfested bees, in an additional experiment, honey bee larvae were artificially infested using the protocol described above or maintained uninfested as a control. Upon eclosion, newly emerged adult bees were separated from the infesting mite and transferred into plastic cages (185 × 105 × 85 mm). Both uninfested and mite-infested honey bees were maintained in a climatic chamber (34.5 °C, 75% R.H.). Bees with deformed wings were excluded from the experiment since their heat production capacity may be impaired by anatomical deficiencies. Starting from day 4, when the sugar intake of the bee is stabilized, we evaluated the body temperature of the single bee placed at room temperature (Tosi et al., 2016). A single honey bee was collected randomly from the mite-infested and uninfested groups. Then it was placed in a polystyrene box, transferred to room temperature (25 °C) and then photographed with an infrared thermographic camera (brand: FLIR; model: i5; thermal resolution = ± 0.1 °C) with emissivity settled at 0.97 (Stabentheiner et al., 2010). Pictures were taken through a hole in the polystyrene lid to reduce the possible interference of light radiation. Pictures were taken for four consecutive days with three biological replicates (i.e. three honey bees per day per group) per time point. Every biological replicate was calculated using the average of three technical replicates (i.e. three pictures). Images were analysed with FLIR Tools® software and temperature data were collected, considering the average value of the warmer part of the bee which always corresponded to the thorax. The area used to calculate the mean temperature was equal for each bee. The recorded temperatures were compared using the Mann-Whitney *U* test.

2.2.3. DWV relative quantification

Sampled bees collected in the survival experiment were defrosted in RNA later (Ambion®). The whole body of the bees was homogenized using mortar and pestle in liquid nitrogen, after gut dissection. Total RNA was extracted from each bee, according to the method suggested by the producer of the RNeasy Plus mini kit (Qiagen®, Germany). The amount of RNA in each sample was quantified using a NanoDrop® spectrophotometer (ThermoFisher™, US) and integrity was verified by means of agarose gel electrophoresis. cDNA was synthesized from 500 ng of RNA per sample, following the manufacturer's specifications (PROMEGA, Italy). Additional negative control samples containing no RT enzyme were included. 10 ng of cDNA from each sample were analysed by qRT-PCR with the primers reported in Table S1, using SYBR®green dye (Ambion®), according to the manufacturer specifications, on a BioRad CFX96 Touch™ Real-time PCR Detector. All samples were run in triplicate. An inter-plate calibrator (i.e. a control sample that was run in every analyzed plate) was used. The following thermal cycling profiles were adopted: one cycle at 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and one cycle at 68 °C for 7 min. Primer efficiency was calculated according to the formula E = 10^(-1/slope)-1)*100. Primers' efficiency was between 94% and 97%. Primer sequence can be found in Table S1. Relative viral load and gene expression data were analysed using the $2 - \Delta\Delta Ct$ method (Livak and Schmittgen, 2001) using actin and GAPDH as housekeeping genes. Relative expression data were transformed, normalized and analysed using the two-way ANOVA test. At least eight individual bees per experimental group were analysed.

2.2.4. IRS-1 expression in mite-infested and uninfested honey bees

To investigate at the molecular level the impaired sugar intake due to *Varroa* infestation, we studied the expression of the insulin receptor substrate 1 (IRS-1), a key gene in insulin/insulin-like signalling (IIS). To evaluate its expression level in mite-infested bees, we artificially infested or not honey bee larvae as described before. The two groups of bee larvae (uninfested and mite-infested) were maintained in a climatic chamber at 34.5 °C, 75% R.H., dark, for 12 days. Upon eclosion, mite-infested and uninfested newly emerged bees were reared separately in plastic cages at 34.5 °C, 75% R.H.. On day 7, bees were sampled to assess the expression of the selected gene.

To assess the effect of rearing temperature on IRS-1 expression, an additional experiment with the same protocol was performed with uninfested bees reared at 34.5 °C and 32 °C. The protocols used for RNA extraction, cDNA synthesis and qRT-PCR are the same as those described previously. Primer sequences can be found in Table S1. Relative

expression data were analysed using the Mann-Whitney *U* test. Three replicates per experiment were performed. Two to four bees per replicate were analysed.

3. Results

3.1. Bee mortality in mite-infested and uninfested colonies

To study the effect of the external temperature on honey bee colonies as affected by *V. destructor* infestation, we established two groups of hives one of which was treated with acaricides throughout the season, to maintain mite infestation at the lowest possible level, while the other was left untreated until October when an acaricide treatment was carried out in both groups of colonies to assess mite population in the hives (Fig. S1).

A high number of mites was found in the untreated group, whereas a significantly lower mite infestation was recorded in the colonies where an appropriate acaricide treatment was carried out (Mann-Whitney *U* test: n_1=4, n_2=5, U=0, *P*=0.007; Fig. 1a). In turn, higher mite infestation caused increasing viral load in bees from the colonies where the parasite population was higher, as revealed by the higher number of individuals with deformed wings following DWV infection (Mann-Whitney *U* test: n_1=4, n_2=5, U=0, *P*=0.007; Fig. 1b). As a result of the increasing mite infestation and the associated viral infection, higher bee mortality was observed in the group of colonies suffering from higher parasitic pressure (Mann-Whitney *U* test: n_1=4, n_2=5, U=0, *P*=0.007; Fig. 1c). This, in turn, accelerated seasonal depopulation in mite-infested colonies, such that in November a significantly lower number of bees was found in untreated colonies as compared to treated ones (Mann-Whitney *U* test: n_1=4, n_2=5, U=0, *P*=0.007; Fig. 1d).

3.2. Thermoregulation in mite-infested and uninfested colonies

The average daily temperature gradually decreased from August, when 26 °C was recorded, to November, when it reached 15 °C (Fig. 2). In the same period the temperature inside the central part of the nest, where brood was present, showed a concurrent, albeit less marked decrease, starting from 35.5 °C registered in August. However, in November, after the end of the brood rearing, the temperature of the central part of the nest, where most bees congregated, was around 30 °C, while in untreated colonies it dropped to 24 °C (Fig. 2; Table S2).

3.3. Survival of uninfested and mite-infested bees exposed to normal and sub-optimal temperatures

The field trial revealed that, during the Autumn months, the temperature within the nest can be lower than optimal in both treated and untreated colonies. In particular, in untreated colonies, where mite infestation was higher, a temperature lower by a few Celsius degrees was observed. Therefore, we investigated how a temperature lower by 2 Celsius degrees can affect the survival of adult bees that were mite-infested or not during the pupal stage.

To this aim, in a lab experiment, we artificially infested honey bees at the larval stage. Then, at eclosion, mites were removed from the adult

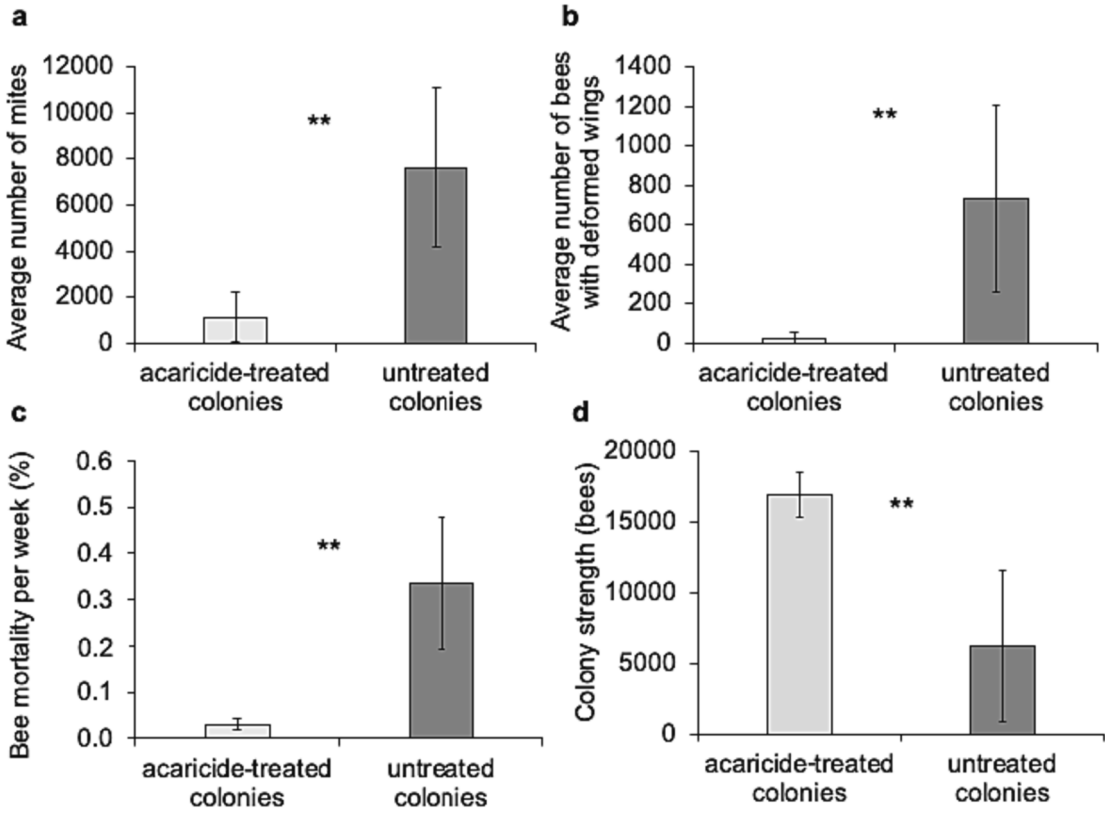

Fig. 1. Effects of mite infestation on honeybee colonies. All the studied parameters were recorded in the same period, from the 12th of October to the 3rd of December. (a) Average number of mites collected from untreated colonies (n=4) and acaricide-treated colonies (n=5). A significantly lower mite infestation was recorded in the colonies where an appropriate acaricide treatment was carried out (Mann-Whitney *U* test: n_1=4, n_2=5, U=0, *P*=0.007). (b) Average number of deformed wing bees collected in the under-basket cages in untreated (n=4) and acaricide treated (n=5) colonies. In untreated colonies, a higher number of individuals with deformed wings was found (Mann-Whitney *U* test: n_1=4, n_2=5, U=0, *P*=0.007). (c) Average percentage of honey bee mortality in untreated (n=4) and treated (n=5) colonies. Higher bee mortality was observed in the group of colonies suffering from higher parasitic pressure (Mann-Whitney *U* test: n_1=4, n_2=5, U=0, *P*=0.007). (d) Number of bees in untreated (n=4) and treated (n=5) colonies. In November a significantly lower number of bees was found in untreated colonies as compared to treated ones (Mann-Whitney *U* test: n_1=4, n_2=5, U=0, *P*=0.007).

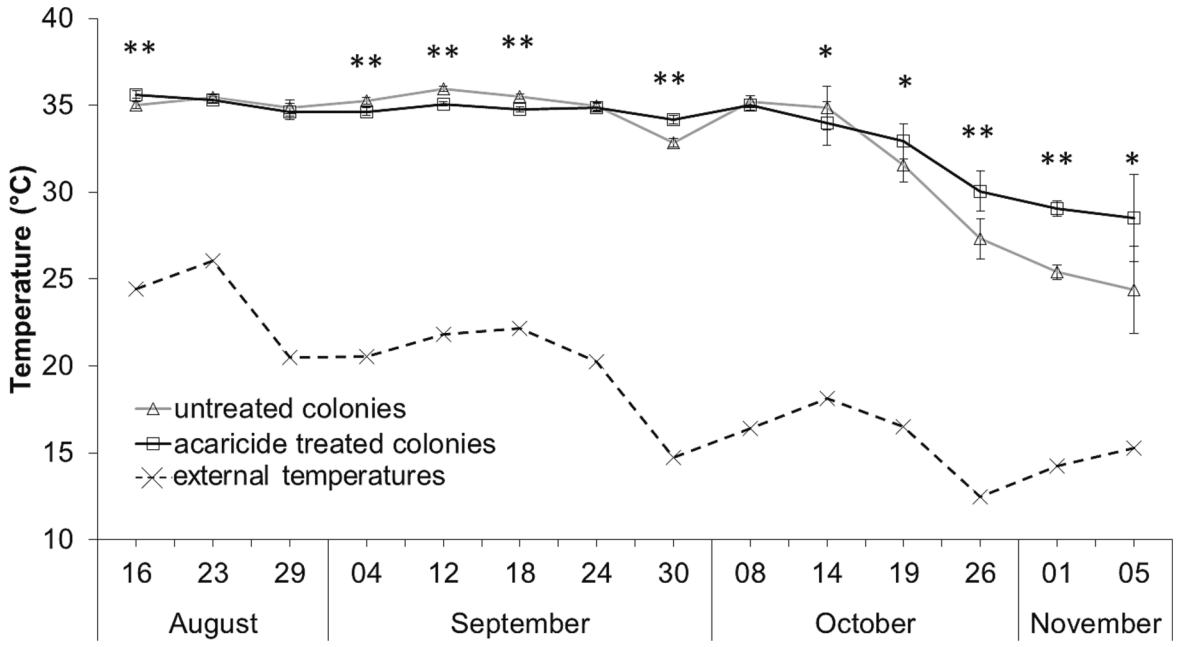

Fig. 2. Temperatures recorded in the center of untreated and acaricide-treated hives during the trial as compared to the external temperature (Table S3). The measurements were performed with temperature probes (Maxim integrated, US; ± 0.0625 °C). Untreated colonies show lower temperatures than acaricide treated ones.

bees and both mite-infested and control honey bees were exposed to different temperature regimes under laboratory conditions. As expected, mite infestation significantly reduced the survival of parasitized bees (Cox regression analysis: HR=1.843, $P<0.001$; Fig. 3) while a similar but smaller effect of a lower rearing temperature was observed (Cox regression analysis: HR=1.338, $P=0.028$; Fig. 3). Interestingly, mite-infested bees exposed to sub-optimal temperatures (i.e. 32 °C) had reduced longevity compared to control bees and bees exposed to either mite infestation or sub-optimal temperature. Since the interaction between the infestation and low temperature is not significant (Cox regression analysis: HR=0.911, $P=0.631$; Fig. 3) the observed reduced survival seems to be due to an additive effect of the two stressors.

As expected, varroa parasitisation increased viral load as compared to unparasitized bees (two-way ANOVA test: d.f.=1, F=16.873, $P<0.001$; Fig. S2) while sub-optimal temperature and the interaction between the two stressors did not affect viral dynamics (two-way ANOVA test: d.f.=1, F=2.799, $P=0.106$; Interaction; two-way ANOVA test: d.f.=1, F=1.381, $P=0.250$; Fig. S2).

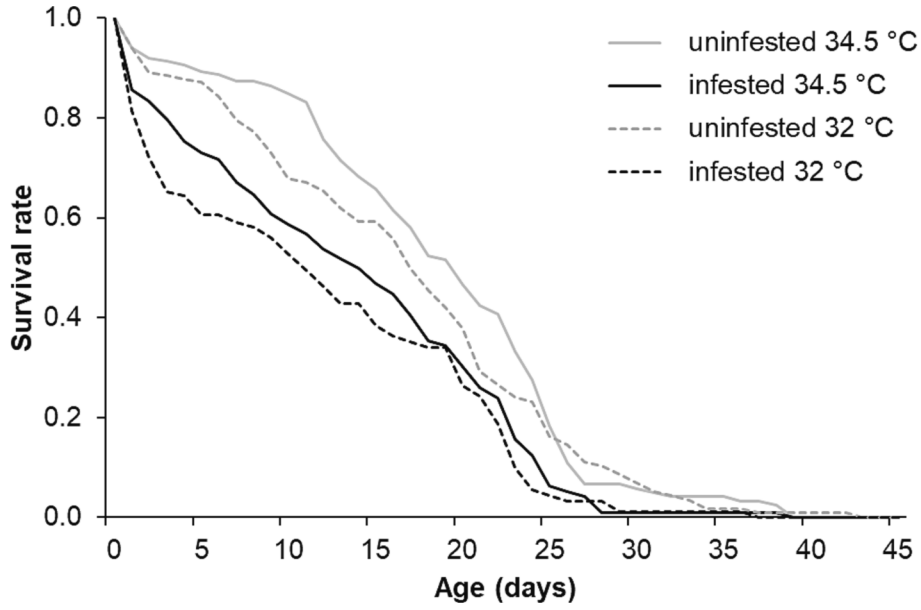

Fig. 3. Survival of caged honey bees infested or not with *V. destructor* during the pupal stage and exposed to two temperature regimes at the adult stage. *Varroa* and sub-optimal temperatures decreased the survival of bees (*Varroa*, Cox regression analysis: HR=1.843, $P<0.001$; Temperature, Cox regression analysis: HR=1.338, $P=0.028$) while the interaction between the two stressors did not influence lifespan (Interaction; Cox regression analysis: HR=0.911, $P=0.631$). The hazard ratio was calculated using the "coxme" function (R software version 3.6.2) with "cage" as a random factor.

3.4. Sugar consumption of uninfested and mite-infested individual bees exposed to normal and sub-optimal temperatures

Heat production in honey bees depends on the availability of a convenient supply of sugar to fuel this process; therefore, we investigated sugar consumption in the uninfested and mite-infested bees used in the preceding experiment.

As expected, we found that, at sub-optimal temperatures, both uninfested and mite-infested bees increased sugar consumption (two-way ANOVA test: d.f. den.=53, d.f. num.=1, F=7.412, P=0.009; Fig. 4); however, in the case of mite infestation, sugar consumption was significantly reduced (two-way ANOVA test: d.f. den.=53, d.f. num.=1, F=21.09, P<0.001; Fig. 4).

3.5. Thermoregulation of uninfested and mite-infested individual bees

Provided that a convenient sugar supply is available, honey bees are capable of producing heat in response to low external temperatures by contracting their thoracic flight muscles. Therefore, we investigated if mite-infested bees are as efficient as uninfested bees in this activity when exposed to a sub-optimal temperature.

We found that bees infested at the pupal stage responded less efficiently to a lower temperature than uninfested bees (Mann-Whitney U test: n_1=12, n_2=12, U=19, P=0.001; Fig. 5).

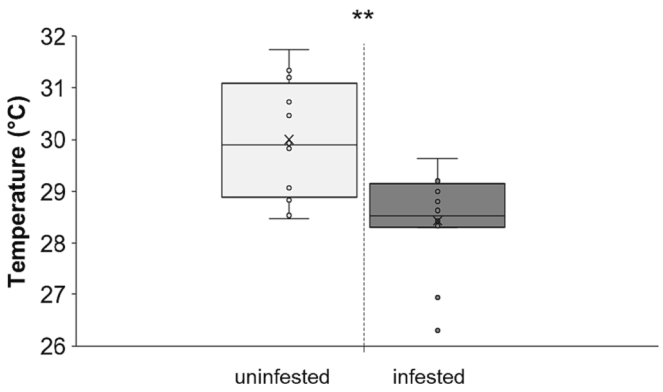

Fig. 5. The average body temperature of mite-infested and uninfested honey bees exposed to a sub-optimal temperature (i.e. 25 °C) was assessed through a thermographic camera (FLIR, model i5). Adult bees that were mite-infested at the pupal stage thermoregulate less efficiently than uninfested bees (Mann-Whitney U test: n_1=12, n_2=12, U=19, P=0.001).

3.6. Effect of Varroa parasitization on insulin receptor substrate 1 (IRS-1)

To gain insight into the reduced sugar intake of mite-infested bees we studied the expression of insulin receptor substrate 1 (IRS-1), a key protein in the insulin/insulin-like signalling (IIS) pathway as affected by mite infestation. IRS-1 appeared to be up-regulated in mite-infested bees at 34.5 °C (Mann-Whitney U test: n_1=12, n_2=12, U=38, P=0.025; Fig. 6a) while sugar intake decreased (Mann-Whitney U test: n_1=11, n_2=11, U=30, P=0.023), highlighting an influence of *Varroa* mite infestation on bee metabolism. Furthermore, to verify that the greater expression of IRS-1 in mite-infested bees was not influenced by the reduced dietary input previously observed in those bees, we studied IRS-1 expression in uninfested bees reared at the standard temperature of 34.5 °C and at 32 °C, a condition under which increased sugar consumption is observed (Mann-Whitney U test: n_1=12, n_2=12, U=28, P=0.005). In this case, the relative expression of IRS-1 was not different between bees maintained at normal and sub-optimal temperatures (Mann-Whitney U test: n_1=8, n_2=10, U=34, P=0.297; Fig. 6b).

4. Discussion

The progressive weakening of mite-infested honey bee colonies towards the end of Summer is a very common situation under temperate climates in the Northern Hemisphere (Amdam et al., 2004; Genersch et al., 2010; Jacques et al., 2017) and was confirmed here. In particular, we provided further evidence that this decline is related to the increased mortality of bees associated with the high DWV infection levels associated with the parasitic activity of the mite *V. destructor*, vectoring the virus and triggering its replication in infected bees (de Miranda and Genersch, 2010; Nazzi et al., 2012; Annoscia et al., 2019). Smaller colonies may be worse at warming their hive or the cavity hosting their nest

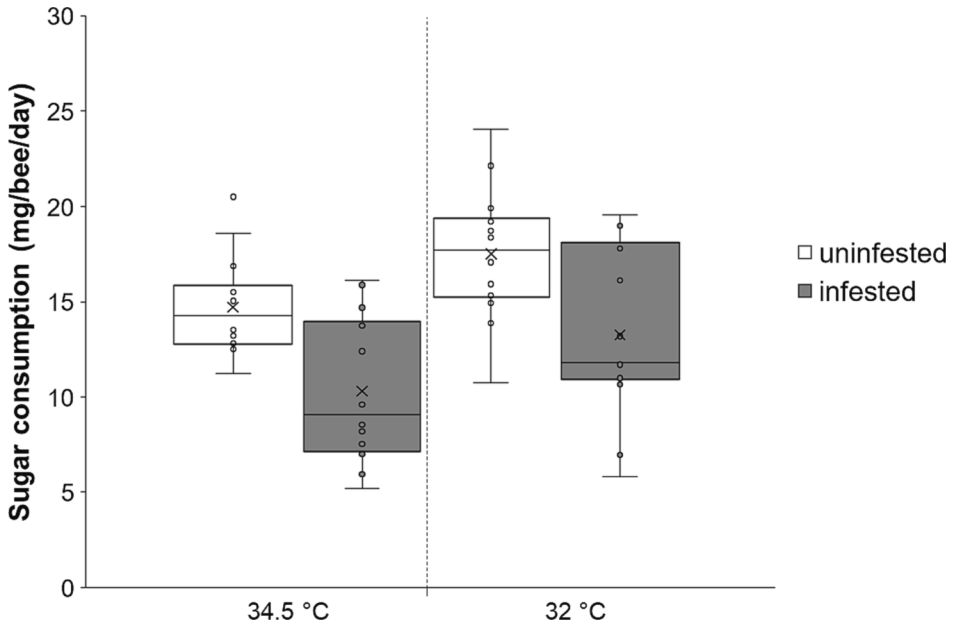

Fig. 4. Sugar consumption of caged mite-infested and uninfested bees exposed to two temperature regimes. Sub-optimal temperatures stimulate sugar intake (two-way ANOVA test: d.f. den.=53, d.f. num.=1, F=7.412, P=0.009) while *Varroa* infestation reduced feeding in adult honey bees (two-way ANOVA test: d.f. den.=53, d.f. num.=1, F=21.09, P<0.001).

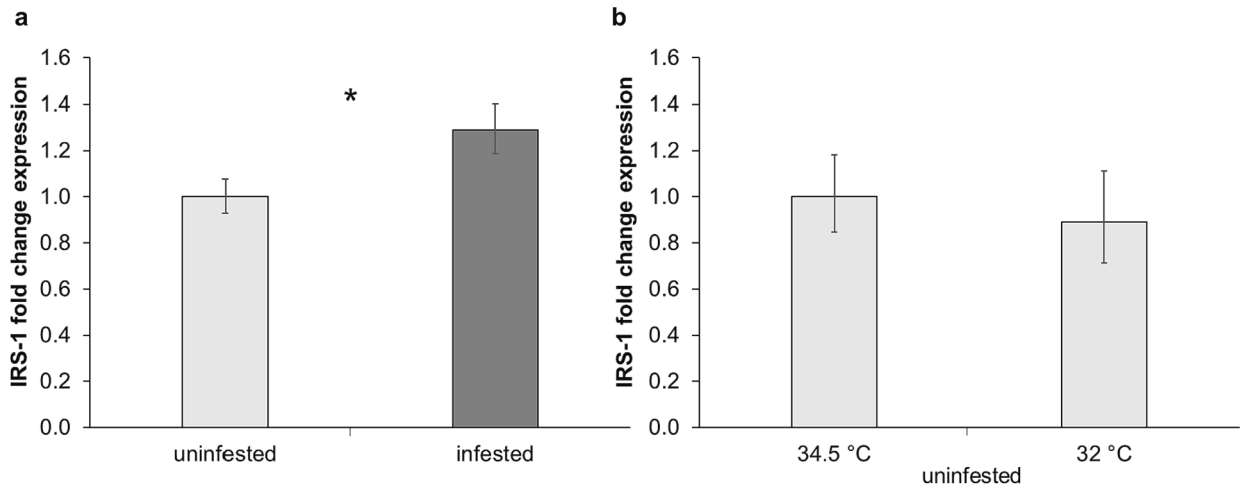

Fig. 6. Relative expression of IRS-1 in bees infested or not with *V. destructor* at 34.5 °C (Mann-Whitney *U* test: n_1=12, n_2=12, U=38, *P*=0.025) (a) and in uninfested bees reared at 34.5 °C and 32 °C (Mann-Whitney *U* test: n_1=8, n_2=10, U=34, *P*=0.297) (b). Actin and GAPDH were used as housekeeping genes.

because of the disadvantageous cavity volume/cluster size ratio. However, while we acknowledge the importance of cluster size for thermoregulation, we suggest that the lower temperature recorded in the nest of mite infested colonies in our study is not related to the progressive reduction of the number of bees observed in mite infested colonies, because we tried to record temperatures in the center of the cluster by checking the probes' position twice a week and, according to several authors (Southwick, 1985; Kleinhenz et al., 2003; Stabentheiner et al., 2010), even honeybee clusters smaller than those observed during our experiment would be able to maintain an optimal core cluster temperature (i.e. 35 °C). As a matter of fact, a significant and stable difference in colony temperature between the two experimental groups was observed as early as mid-October when colony size was not significantly different yet. Towards the end of the experiment, in November, temperatures cooler by up to four degrees were observed in the center of the nest of colonies infested by the mite.

The detected lower temperature in the nest's center can have multiple negative effects. In particular, our investigation into the effects of the concurrent exposition of bees to parasitic infection and sub-optimal temperatures showed that the negative effects of these two stressors add up to reduce the survival of bees. Differently from our results, Schäfer and colleagues (2011) did not report a significantly different temperature of mite-infested small bee colonies as compared to uninfested ones. However, these results are hardly comparable with those reported here which regard individual mite-infested bees in the lab and normal-sized bee colonies, at higher temperatures, under field conditions.

To understand the possible causes of the reduced capacity of mite-infested bees to maintain a convenient temperature in the nest's center, we recorded the temperature of both individual mite-infested and uninfested adult bees upon exposition to 25 °C and noted that parasitized bees have a reduced capacity to warm up their bodies to counteract a lower external temperature. It has been shown that the capacity of thermoregulation of single bees is influenced by the number of surrounding bees (Southwick, 1991); however, this is critical for temperatures below 15 °C and should not influence the effect noted here at 25 °C. The reduced thermoregulation of mite-infested bees has not been reported before, despite the thermoregulatory capacity of bees has been investigated in considerable detail (Southwick and Mugaas, 1971). According to the results of our experiment on the effects of mite infestation on sugar consumption in bees exposed to low external temperatures, we suggest that the impaired capacity of mite-infested bees to maintain an appropriate temperature may depend on the reduced sugar intake of mite-infested bees. This effect previously noted in other insects (Bernardo and Singer, 2017) points towards an interesting parasite-induced anorexia. The expression pattern of IRS-1, a key gene in the insulin/insulin-like signalling pathway, confirms a limited but significant effect of *Varroa* parasitization on the bee's metabolism at the molecular level. These results seem to confirm a recent study by Cournoyer and colleagues (2022) showing that in autumn, the sugar concentration in the honey bees' haemolymph is significantly lower in highly infested hives as compared to control hives.

The parasite-altered feeding behaviour is an important factor that can contribute to the balance of host-parasite interactions (Bernardo and Singer, 2017) and could generate benefits for the fitness of the host or the parasite, or both or neither (Poulin, 1995; Hurd, 2001; Moore, 2012). In general, anorexia seems to be a therapeutic behavioural adaptation to ameliorate host survival (for a review of parasite-altered feeding behaviour in insects see Bernardo and Singer (2017)), but in this case, the *Varroa*-induced anorexia seems to negatively interfere with the survival of bees at the colony level, exacerbating the effect of parasite infestation. Indeed, it appears that mite infestation beyond increasing bee mortality *per se* also reduces their capacity to warm up their thorax, further exposing both the single bees and the whole colony to the detrimental effect of lower temperatures. In sum, dangerous positive feedback loops may be generated, with potentially devastating effects on the survival of the colony, as suggested by our field results which however may also be related to further aspects we did not consider in our study. Therefore, the *Varroa*-induced anorexia reported here and the decreasing temperature observed during the cold season can enhance the negative effect of the increasing mite infestation, further reducing the survival of bees and thus impairing the very sustainability of the colony. These results are consistent with the improved winter survival after warmer autumn months (Switanek et al., 2017) and allow a more comprehensive understanding of the reported higher colony losses in northern regions (Amdam et al., 2004; Genersch et al., 2010; Jacques et al., 2017), where lower temperatures are observed during the cold season (vanEngelsdorp et al., 2008).

Previous studies indicated that the ultimate responsibility for the loss of bee colonies observed in the northern hemisphere is the deformed wing virus, which has a worldwide distribution (Wilfert et al., 2016; Grozinger and Flenniken, 2019). Here we would like to suggest an additional aspect that could enhance our understanding of winter colony losses. It is evident that these losses are primary caused by the impact of the *Varroa*-DWV association. However, it is worth noting that the lower temperatures observed in the northern hemisphere during the autumn months may further exacerbate this impact.

Finally and importantly, we demonstrated that a secondary effect of individual parasitization (i.e. parasite-induced anorexia) can affect an

upper level of biological organization (i.e. colony thermoregulation) exposing the bees to the effects of an abiotic factor (i.e. low temperature) that exacerbates the detrimental effects of the parasite.

Although some conclusions of this work are restricted to honey bees with their peculiar biology, we believe that the holistic and multilevel experimental approach adopted here can represent a useful template for similar studies on other insect species, aiming at elucidating the critical positive feedback loops triggered by the interaction between abiotic and biotic stress factors. We hope that similar studies will become more common given the alarming news regarding climate change and its potential impact on terrestrial ecosystems (Nolan et al., 2018).

CRediT authorship contribution statement

Davide Frizzera: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Virginia Zanni:** Investigation, Formal analysis. **Mauro D'Agaro:** Investigation. **Giulia Boaro:** Investigation. **Laura Andreuzza:** Investigation. **Simone Del Fabbro:** Investigation. **Desiderato Annoscia:** Conceptualization, Investigation, Formal analysis, Writing – original draft. **Francesco Nazzi:** Conceptualization, Investigation, Formal analysis, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2023.104571.

References

Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., Klein, A.M., 2009. How much does agriculture depend on pollinators? Lessons from long-term trends in crop production. Ann. Bot. 103 (9), 1579–1588.

Amdam, G., Hartfelder, K., Norberg, K., Hagen, A., Omholt, S.W., 2004. Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite *Varroa destructor* (Acari: Varroidae): a factor in colony loss during overwintering? J. Econ. Entomol. 97 (3), 741–747.

Annoscia, D., Del Piccolo, F., Nazzi, F., 2012. How does the mite *Varroa destructor* kill the honeybee *Apis mellifera*? Alteration of cuticular hydrocarbons and water loss in infested honeybees. J. Insect Physiol. 58 (12), 1548–1555.

Annoscia, D., Brown, S.P., di Prisco, G., de Paoli, E., del Fabbro, S., Frizzera, D., Zanni, V., Galbraith, D.A., Caprio, E., Grozinger, C.M., Pennacchio, F., Nazzi, F., 2019. Haemolymph removal by *Varroa* mite destabilizes the dynamical interaction between immune effectors and virus in bees, as predicted by Volterra's model. Proc. Royal Soc. b. 286, 20190331.

Bernardo, M.A., Singer, M.S., 2017. Parasite-altered feeding behavior in insects: integrating functional and mechanistic research frontiers. J. Exp. Biol. 220 (16), 2848–2857.

Bujok, B., Kleinhenz, M., Fuchs, S., Tautz, J., 2002. Hot spots in the bee hive. Sci. Nat. 89 (7), 299–301.

Chen, Y.W., Chung, W.P., Wang, C.H., Solter, L.F., Huang, W.F., 2012. *Nosema ceranae* infection intensity highly correlates with temperature. J. Invertebr. Pathol. 111 (3), 264–267.

Cournoyer, A., Plamondon, L., Bau-Gaudreault, L., Deschamps, A., Dubreuil, P., Benoit-Biancamano, M.O., 2022. Effects of *Varroa destructor* on hemolymph sugars and secondary infections in honeybees (*Apis mellifera*). Appl. Sci. 12, 11630.

Dainat, B., Evans, J.D., Chen, Y.P., Gauthier, L., Neumann, P., 2012. Dead or alive: Deformed wing virus and *Varroa destructor* reduce the life span of winter honeybees. Appl. Environ. Microbiol. 78 (4), 981–987.

de Miranda, J.R., Genersch, E., 2010. Deformed wing virus. J. Invertebr. Pathol. 103, S48–S61.

Dietemann, V., Nazzi, F., Martin, S.J., Anderson, D.L., Locke, B., Delaplane, K.S., Wauquiez, Q., Tannahill, C., Frey, E., Ziegelmann, B., Rosenkranz, P., Ellis, J.D., 2013. Standard methods for varroa research. J. Apic. Res. 52 (1), 1–54.

Döke, M.A., Frazier, M., Grozinger, C.M., 2015. Overwintering honey bees: biology and management. Curr. Opin. Insect Sci. 10, 185–193.

Genersch, E., von der Ohe, W., Kaatz, H., Schroeder, A., Otten, C., Büchler, R., Berg, S., Ritter, W., Mühlen, W., Gisder, S., Meixner, M., Liebig, G., Rosenkranz, P., 2010. The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies. Apidologie 41 (3), 332–352.

Goulson, D., Nicholls, E., Botías, C., Rotheray, E.L., 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347 (6229).

Greatti, M., Milani, N., Nazzi, F., 1992. Reinfestation of an acaricide-treated apiary by *Varroa jacobsoni* Oud. Exp. Appl. Acarol. 16, 279–286.

Grimm, N.B., Chapin, F.S., Bierwagen, B., Gonzalez, P., Groffman, P.M., Luo, Y., Melton, F., Nadelhoffer, K., Pairis, A., Raymond, P.A., Schimel, J., Williamson, C.E., 2013. The impacts of climate change on ecosystem structure and function. Front. Ecol. Environ. 11 (9), 474–482.

Grozinger, C.M., Robinson, G.E., 2015. The power and promise of applying genomics to honey bee health. Curr. Opin. Insect Sci. 10, 124–132.

Grozinger, C.M., Flenniken, M.L., 2019. Bee Viruses: Ecology, Pathogenicity, and Impacts. Annu. Rev. Entomol. 64 (1), 205–226.

Heinrich, B., 1981. The mechanism and energetics of honeybee swarm temperature regulation. J. Exp. Biol. 91, 25–55.

Human, H., Nicolson, S.W., Dietemann, V., 2006. Do honeybees, *Apis mellifera scutellata*, regulate humidity in their nest? Sci. Nat. 93, 397–401.

Hung, K.L.J., Kingston, J.M., Albrecht, M., Holway, D.A., Kohn, J.R., 2018. The worldwide importance of honey bees as pollinators in natural habitats. Proc. Royal Soc. B 285 (1870).

Hurd, H., 2001. Host fecundity reduction: a strategy for damage limitation? Trends Parasitol. 17 (8), 363–368.

Jacques, A., Laurent, M., Ribière-Chabert, M., Saussac, M., Bougeard, S., Budge, G.E., Hendrikx, P., Chauzat, M.P., 2017. A pan-European epidemiological study reveals honey bee colony survival depends on beekeeper education and disease control. PLoS One 12 (3), e0172591.

Kleinhenz, M., Bujok, B., Fuchs, S., Tautz, J., 2003. Hot bees in empty broodnest cells: heating from within. J. Exp. Biol. 206 (23), 4217–4231.

Kulhanek, K., Steinhauer, N., Rennich, K., Caron, D.M., Sagili, R.R., Pettis, J.S., Ellis, J.D., Wilson, M.E., Wilkes, J.T., Tarpy, D.R., Rose, R., Lee, K., Rangel, J., vanEngelsdorp, D., 2017. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. J. Apic. Res. 56 (4), 328–340.

Le Conte, Y., Ellis, M., Ritter, W., 2010. Varroa mites and honey bee health: can Varroa explain part of the colony losses? Apidologie 41 (3), 353–363.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods 25 (4), 402–408.

Marchetti, S., 1985. Il 'metodo dei sesti' per la valutazione numerica degli adulti in famiglie di *Apis mellifera*. Apicoltura 1, 41–46.

Milani, N., 1995. The resistance of *Varroa jacobsoni* Oud. to acaricides. Apidologie 30 (2–3), 229–234.

Milani, N., Della Vedova, G., 2002. Decline in the proportion of mites resistant to fluvalinate in a population of *Varroa destructor* not treated with pyrethroids. Apidologie 33 (4), 417–422.

Moore, J., 2012. Host Manipulation by Parasites. Oxford University Press.

Morales-Castilla, I., Pappalardo, P., Farrell, M.J., Aguirre, A.A., Huang, S., Gehman, A.L. M., Dallas, T., Gravel, D., Davies, T.J., 2021. Forecasting parasite sharing under climate change. Philos. Trans. R. Soc. Lond., B 376 (1837), 20200360.

Nazzi, F., Milani, N., 1994. A technique for reproduction of *Varroa jacobsoni* Oud under laboratory conditions. Apidologie 25 (6), 579–584.

Nazzi, F., Brown, S.P., Annoscia, D., del Piccolo, F., di Prisco, G., Varricchio, P., della Vedova, G., Cattonaro, F., Caprio, E., Pennacchio, F., 2012. Synergistic Parasite-Pathogen Interactions Mediated by Host Immunity Can Drive the Collapse of Honeybee Colonies. PLoS Pathog. 8 (6), e1002735.

Nazzi, F., Pennacchio, F., 2014. Disentangling multiple interactions in the hive ecosystem. Trends Parasitol. 30 (12), 556–561.

Neumann, P., Carreck, N.L., 2010. Honey bee colony losses. J. Apic. Res. 49 (1), 1–6.

Nolan, C., Overpeck, J.T., Allen, J.R.M., Anderson, P.M., Betancourt, J.L., Binney, H.A., Brewer, S., Bush, M.B., Chase, B.M., Cheddadi, R., Djamali, M., Dodson, J., Edwards, M.E., Gosling, W.D., Haberle, S., Hotchkiss, S.C., Huntley, B., Ivory, S.J., Kershaw, A.P., Jackson, S.T., 2018. Past and future global transformation of terrestrial ecosystems under climate change. Science 361 (6405), 920–923.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25 (6), 345–353.

Poulin, R., 1995. Adaptive" changes in the behaviour of parasitized animals: A critical review. Int. J. Parasitol. 25 (12), 1371–1383.

Retschnig, G., Williams, G., Schneeberger, A., Neumann, P., 2017. Cold Ambient Temperature Promotes Nosema spp. Intensity in Honey Bees (Apis mellifera). Insects 8 (1), 20.

Rothe, U., Nachtigall, W., 1989. Flight of the honey bee. J. Comp. Physiol. B 158 (6), 739–749.
Russo, L., 2016. Positive and Negative Impacts of Non-Native Bee Species around the World. Insects 7 (4), 69.
Schäfer, M.O., Ritter, W., Pettis, J.S., Neumann, P., 2011. Concurrent parasitism alters thermoregulation in honey bee (Hymenoptera: Apidae) winter clusters. Ann. Entomol. Soc. Am. 104 (3), 476–482.
Schmaranzer, S., 2000. Thermoregulation of water collecting honey bees (*Apis mellifera*). J. Insect Physiol. 46, 1187–1194.
Seeley, T.D., 1985. Honeybee Ecology. Princeton University Press.
Simpson, J., 1961. Nest climate regulation in honey bee colonies. Science 133 (3461), 1327–1333.
Southwick, E.E., Mugaas, J.N., 1971. A hypothetical homeotherm: The honeybee hive. CBP 40 (4), 935–944.
Southwick, E.E., 1985. Allometric relations, metabolism and heart conductance in clusters of honey bees at cool temperatures. J. Comp. Physiol. B 156, 143–149.
Southwick, E.E., 1991. Overwintering in honey bees: implications for apiculture. In: Lee, R.E., Denlinger, D.L. (Eds.), Insects at Low Temperature. Springer, Boston, MA.
Stabentheiner, A., Kovac, H., Hagmuller, K., 1995. Thermal behavior of round and wagtail dancing honeybees. J. Comp. Physiol. B 165, 433–444.
Stabentheiner, A., 2001. Thermoregulation of dancing bees: thoracic temperature of pollen and nectar foragers in relation to profitability of foraging and colony need. J. Insect Physiol. 47, 385–392.
Stabentheiner, A., Kovac, H., Brodschneider, R., 2010. Honeybee Colony Thermoregulation – Regulatory Mechanisms and Contribution of Individuals in Dependence on Age, Location and Thermal Stress. Plos ONE 5 (1), e8967.
Switanek, M., Crailsheim, K., Truhetz, H., Brodschneider, R., 2017. Modelling seasonal effects of temperature and precipitation on honey bee winter mortality in a temperate climate. Sci. Total Environ. 579, 1581–1587.
Tan, K., Yang, S., Wang, Z., Radloff, S.E., Oldroyd, B.P., 2012. Differences in foraging and broodnest temperature in the honey bees *Apis cerana* and *A. mellifera*. Apidologie 43, 618–623.
Tosi, S., Demares, F.j., Nicolson, S.W., Medrzycki, P., Pirk, C.W.W., Human, H., 2016. Effects of a neonicotinoid pesticide on thermoregulation of African honey bees (*Apis mellifera scutellata*). J. Insect Physiol. 93 (94), 56–63.
Vandame, R., Belzunces, L.P., 1998. Joint actions of deltamethrin and azole fungicides on honey bee thermoregulation. Neurosci. Lett. 251, 57–60.
vanEngelsdorp, D., Hayes, J., Underwood, R.M., Pettis, J., 2008. A Survey of Honey Bee Colony Losses in the U.S. PLoS One 3 (12), e4071. Fall 2007 to Spring 2008.
Wagner, D.L., Grames, E.M., Forister, M.L., Berenbaum, M.R., Stopak, D., 2021. Insect decline in the Anthropocene: Death by a thousand cuts. PNAS 118 (2) e2023989118.
Wilfert, L., Long, G., Leggett, H.C., Schmid-Hempel, P., Butlin, R., Martin, S.J.M., Boots, M., 2016. Deformed wing virus is a recent global epidemic in honeybees driven by Varroa mites. Science 351 (6273), 594–597.
Williams, G.R., Alaux, C., Costa, C., Csáki, C., Doublet, V., Eisenhardt, D., Fries, I., Kuhn, R., McMahon, D.P., Medrzycki, P., Murray, T.E., Natsopoulou, M.E., Neumann, P., Oliver, R., Paxton, R.J., Pernal, S.F., Shutler, D., Tanner, G., van der Steen, J.J.M., Brodschneider, R., 2015. Standard methods for maintaining adult *Apis mellifera* in cages under in vitro laboratory conditions. J. Apic. Res. 52 (1), 1–36.