Altered Physiology in Worker Honey Bees (Hymenoptera: Apidae) Infested with the Mite *Varroa destructor* (Acari: Varroidae): A Factor in Colony Loss During Overwintering?

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ABSTRACT The ectoparasitic mite *Varroa destructor* (Anderson & Trueman) is the most destructive pest of the honey bee, *Apis mellifera* L., in Europe and the United States. In temperate zones, the main losses of colonies from the mites occur during colony overwintering. To obtain a deeper knowledge of this phenomenon, we studied the mites' impact on the vitellogenin titer, the total protein stores in the hemolymph, the hemocyte characteristics, and the ecdysteroid titer of adult honey bees. These physiological characteristics are indicators of long-time survival and endocrine function, and we show that they change if bees have been infested by mites during the pupal stage. Compared with noninfested workers, adult bees infested as pupae do not fully develop physiological features typical of long-lived wintering bees. Management procedures designed to kill *V. destructor* in late autumn may thus fail to prevent losses of colonies because many of the adult bees are no longer able to survive until spring. Beekeepers in temperate climates should therefore combine late autumn management strategies with treatment protocols that keep the mite population at low levels before and during the period when the winter bees emerge.

KEY WORDS winter bees, longevity, storage protein

THE MITE *Varroa destructor* (Anderson and Trueman 2000) poses the largest worldwide threat to the honey bee, *Apis mellifera* (L.) (Bailey and Ball 1991). The problem is most acute in temperate regions with bees of European origin, where the majority of colonies die within a few years if the population growth of the mite is not controlled (De Jong 1990). The large populations of *V. destructor* that build up in European colonies are associated with a variety of secondary diseases (Bailey and Ball 1991), and the severe effects of the mites in temperate climates have been linked to virus infections (Allen and Ball 1996, Martin 2001).

Bees infested by *V. destructor* as pupae show reduction in body weight, hemolymph volume, hemolymph protein content, and abdominal carbohydrate levels at emergence (De Jong et al. 1982, Weinberg and Madel 1985, Bowen-Walker and Gunn 2001). Other physiological characteristics are degenerated fat bodies and underdeveloped hypopharyngeal glands (Schneider and Drescher 1987, Beetsma et al. 1988, Drescher and Schneider 1988, Schatton-Gadelmayer and Engels 1988). The workers also may start foraging earlier in life (Janmaat and Winston 2000), and their longevity is reduced (De Jong and De Jong 1983, Schneider and Drescher 1987, Kovak and Crailsheim 1988).

In temperate climates, long-lived workers develop in autumn as the production of brood ceases before wintering (Maurizio 1950). However, a substantial population of *V. destructor* may be present over the period when these so called "winter bees" are reared. Although management guidelines normally recommend mid or late summer treatments against *Varroa* (including practical procedures such as drone removal), chemicals and organic acids are often applied only once a year because the alternative is more laborious (Tew 2001). This treatment is normally preformed in late autumn after the emergence of the winter bee population (Korpela et al. 1992). Infestation during the pupal stage has a negative impact on the ability of workers to survive until spring (Kovac and Crailsheim 1988), and this could explain losses of nontreated honey bee colonies during winter in Europe and the United States (Sammataro and Needham 1996, Bowen-Walker and Gunn 1998, Hunt 1998). It also suggests that treatment strategies that exclusively kill phoretic mites (mites feeding on adult bees) in late autumn have limited value.

Long-lived winter bees develop unique physiological features over a period of 3 to 4 weeks after emergence (Maurizio 1950, Fluri et al. 1982). These characteristics have been associated with worker longevity

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(Maurizio 1950; Fluri et al. 1977, 1982; Amdam and Omholt 2002; Amdam et al. 2003). If mite infestation during the pupal stage causes bees to live shorter lives, we would expect it to induce changes in features normally distinguishing winter bees from other workers. To test this hypothesis, we studied temporal changes in indicators of long-time survival and endocrine function, i.e., vitellogenin titer (Fluri et al. 1982, Amdam and Omholt 2002, Amdam et al. 2003), total protein stores in hemolymph (Maurizio 1950), hemocyte (immunocyte) characteristics (Rutz et al. 1974, Wille and Vecchi 1974, Fluri et al. 1977) and ecdysteroids (Hartfelder et al. 1995, Colonello and Hartfelder 2003). These traits were monitored during the adult life of bees that were either infested or not infested by *V. destructor* as pupae.

Materials and Methods

Bees. We used a technique developed by Maurizio (1950) to produce simulated winter bees in queenright summer colonies without brood. Maurizio (1950) found that long-lived bees develop independent of season if the amount of brood rearing is controlled experimentally. Workers build up the physiological characteristics of true wintering bees within 4 weeks under such conditions (Maurizio 1950). The validity of this method was later confirmed by Fluri et al. (1982).

Frames of sealed brood were collected from four mite-infested colonies that showed no clinical signs of deformed wing virus and acute paralysis virus. This was done to increase the probability of obtaining infested, but viable newly emerged bees with normal wing morphology. The combs were kept in an incubator (33°C, 60% RH). At the moment of emergence, the bees as well as their cells were checked for mites. Adult mites and nymphal forms were recorded. Any adhering mites were then gently removed from the bees with a paintbrush. The workers were marked with a color code conveying the date of emergence and the number of mites recorded for each individual bee.

Bees from all donor colonies were pooled (N = 1,500) and divided into equal samples before introduction into two noninfested colonies (referred to as colonies 1 and 2 in the following). Colonies 1 and 2 contained eight frames of pollen and honey, and two empty frames. The queens were caged in flat plastic boxes lined with queen excluder and placed between the central combs. The colonies were provided with synthetic queen pheromones (Bee Boost, Phero Tech Inc., Delta, BC, Canada) to help maintain colony integrity. The queens were checked regularly during the course of the experiment. Workers were sampled at emergence, and after 2, 5, 8, 12, 15, 20, 25, and 30 d in July 2000.

A second set of marked workers (N = 700) was introduced into a third colony (colony 3) for more frequent sampling of bees in June 2002. The bees were sampled at emergence and thereafter daily until 7 d old. Colony 3 contained nine frames of pollen and honey and one frame with pollen and a small number of sealed brood. The queen was caged as described previously. All colonies were kept in the apiary of the Agricultural University of Norway (Ås, Norway).

Sample Collection. Between 10 and 30 workers from each cohort were anesthetized on ice before fixation to a piece of Styrofoam with four crossed needles. In this position, hemolymph was extracted with Dummond micropipettes (1 ± 0.001 μl) after puncturing the abdomen between the third and the fourth tergite by using a sterile needle. Care was taken to avoid contaminating the samples with tissue fragments and foregut content from the bees.

For colonies 1 and 2, 1 μl of hemolymph was dissolved in 50 μl of Tris buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidin, 0.7 μM pepstatin, 8 μM chymostatin, 10 μM leupeptin, and 0.8 μM aprotinin, Sigma, St. Louis, MO) for quantification of total protein, and 1 μl of hemolymph was dissolved in 10 μl of Tris buffer for quantification of vitellogenin. One microliter of hemolymph was also dissolved in 3 μl of staining solution (16.2 g of NaH_2PO_4, 4.2 g of NaOH, 1 g of methylene blue in 0.1 liter of formalin [40%] and 0.9 liters of distilled H_2O) for classification and count of hemocytes. For colony 3, individual samples of 4 or 5 μl were dissolved in 500 μl of methanol for the ecdysone assay. As a result, only an additional 1 μl of hemolymph was generally available. It was dissolved in 10 μl of Tris buffer for quantification of vitellogenin.

Quantification of Physiological Characteristics. We did not conduct a full analysis of the bees from colonies 1 and 3 due to the high number of individual samples. Specifically, results were not obtained from 8- to 15-d-old workers in colony 1, nor from 1- and 3-d-old workers in colony 3, with the exception of ecdyseroid titers. Samples from bees younger than 8 d and older than 15 d in colony one were selected for processing because we assumed that the data from young workers could be linked to previous findings (De Jong et al. 1982, Weinberg and Madel 1985, Bowen-Walker and Gunn 2001), whereas the results from old bees were most relevant for testing our hypothesis. The vitellogenin samples from 1- and 3-d-old bees in colony three were not assayed because we expect them to convey little information when the titers of 0-, 2-, and 4–7-d-old bees were known.

The vitellogenin titers were determined by the method of Lin et al. (1999) by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a β-galactosidase standard (Sigma). The concentration of solubilized protein in 1 μl of hemolymph was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA) at 595 nm by using a bovine serum albumin standard.

Samples of stained hemocytes were loaded in a cell counter (Bürker, 0.05 mm), and the number of hemocytes in 1 μl of hemolymph was determined by light microscopy. In addition, hemocytes were classified as normal cells (NC) or pycnotic cells (PC) apparently undergoing apoptosis (Rutz et al. 1974, Wille and Vecchi 1974) as described by Price and Ratcliffe

(1974). The proportion of normal hemocytes was calculated as NC/(NC + PC) (Fluri et al. 1977) to indicate the integrity of the cellular immune system (Strand and Pech1995).

Samples in methanol were stored at −20°C in glass vials with a Teflon-lined screw cap until analysis. The precipitate containing hemolymph proteins was removed by centrifugation (14,000 × *g*, 4°C, 10 min), and the supernatant was transferred to 1-ml glass vials. After solvent evaporation by vacuum centrifugation, the samples were subjected to a highly sensitive ecdysone radioimmunoassay for quantification of honey bee ecdysteroids (Feldlaufer and Hartfelder 1997, Pinto et al. 2002). Radioimmunoassay standard curves were prepared with 20-hydroxyecdysone (20E, Simes, Milan, Italy) as the nonradioactive ligand. The results are therefore expressed as 20E equivalents (picograms per microliter of hemolymph).

Data Analysis. During the study, there was a drop in the number of heavily infested workers relative to less infested bees in colonies 1–3. This affected the availability of workers for sampling. To obtain statistical power in the subsequent data analyses, the number of mites recorded for each bee was not included as variable.

Except for the ecdysteroid titers, Tukey's test for multiple comparisons (Montgomery 1997) was used to compare all pairs of means within colonies 1–3. The standard errors for the tests were determined as weighted means, by using sample sizes as weights. Tukey's test for multiple comparisons determines a critical value T_α for all pairwise comparisons that is corrected for multiple tests. If the observed differences between pairs of means are larger than T_α, we concluded that the pairs of means in question were significantly different. When the distance between a pair of means is given in the text rather than displayed by a figure, the distance is denoted *D*. The level of the overall test was set to 0.05, and for each data set, the corresponding $T_{0.05}$ is reported.

Ecdysteroid titers of unknown samples were calculated by log linear regression analysis of standard curve doses (log pg 20E) on logit binding values. Pairwise comparisons for ecdysteroid titers of *Varroa*-infested and control workers of corresponding age were performed by the Mann–Whitney *U* test.

Results

The mean vitellogenin titer increased after emergence in both infested and noninfested bees (Fig. 1). However, as workers aged, bees infested during the pupal stage developed vitellogenin levels significantly lower than the noninfested controls (Fig. 1). In colony 3, the difference was significant as of 2 d (Fig. 1A). This was due to a lower overall variance and thus a smaller critical value for comparing groups in this particular colony.

The total protein titer and the vitellogenin level was highly correlated at the level of the individual bee (r = 0.9, df = 463). This is in agreement with previous results showing that vitellogenin is the main storage

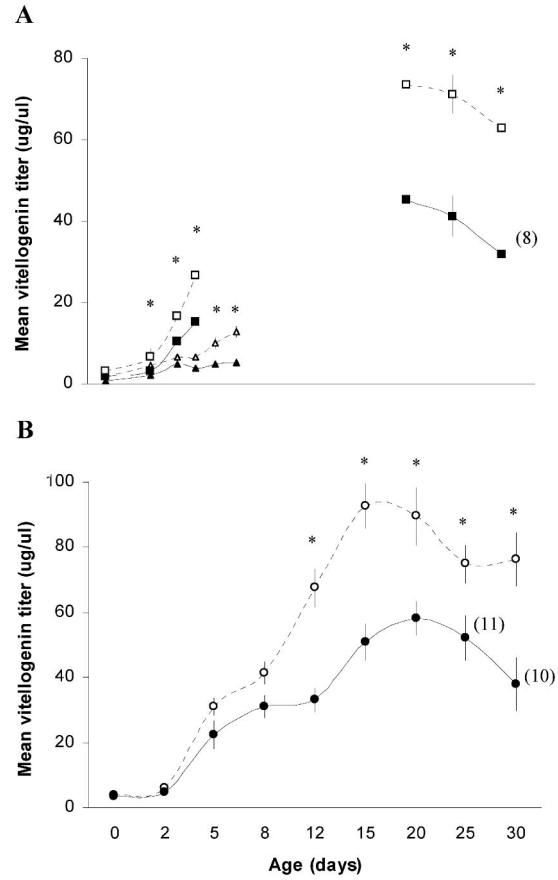

Fig. 1. Mean ± SE of vitellogenin titers (micrograms per microliter) for simulated winter bees, by using a β-galactosidase standard. Noninfested controls (white indicators) and workers infested by *V. destructor* during metamorphosis (black indicators) were kept in colonies 1–3, depicted by cubes, circles, and triangles, respectively. (A) Results obtained from colony 1 (N = 20 or indicated, $T_{0.05}$= 14.4) and colony 3 (N = 15, $T_{0.05}$ = 2.4). (B) Data from colony 2 (N = 20 or indicated, $T_{0.05}$ = 17.5). * indicates significant differences between groups and refers exclusively to colony three in the case of workers younger than 8 d.

protein in honey bee hemolymph (Fluri et al. 1977, 1982). Figure 1 therefore nicely reflects the differential pattern of total protein accumulation in the hemolymph of the bees. As for vitellogenin, total protein levels were significantly different in workers older than 8 d, and bees infested during the pupal stage had the lower mean titers ($D_{20\ d}$ = 21.6, $D_{25\ d}$ = 29.9, $D_{30\ d}$ = 38.1 with $T_{0.05}$ = 12.8, and $D_{12\ d}$ = 13.9, $D_{15\ d}$ = 19.0, $D_{20\ d}$ = 16.2, $D_{25\ d}$ = 24.2, $D_{30\ d}$ = 40.4 with $T_{0.05}$ = 13.2 for colony 1 and 2, respectively; N = 20). The relationship between total hemolymph protein and vitellogenin titer was apparent from the SDS-PAGE, and an example is given in Fig. 2. We also observed an increase in concentration of an ≈67-kDa protein (noticeable in Fig. 2), which we assumed to be a hexameric storage protein (Danty et al. 1998). However, the putative increase in hemolymph hexamerin seemed

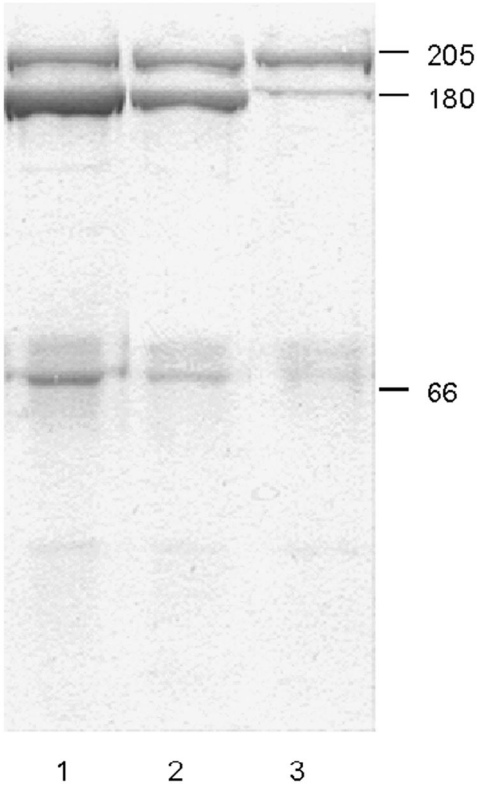

Fig. 2. Example of SDS-PAGE electrophoresis of hemolymph proteins from 12-d-old bees. An equal amount of hemolymph (1 μl) was loaded in each lane. The protein contents of the samples were lane 1, 48.0 μg; lane 2, 26.3 μg; and lane 3, 8.2 μg. Numbers to the right give molecular weights in kilodaltons. Vitellogenin is the unambiguous band at 180 kDa. Hexamerin may be apparent in lane 1 and 2 at ≈67 kDa.

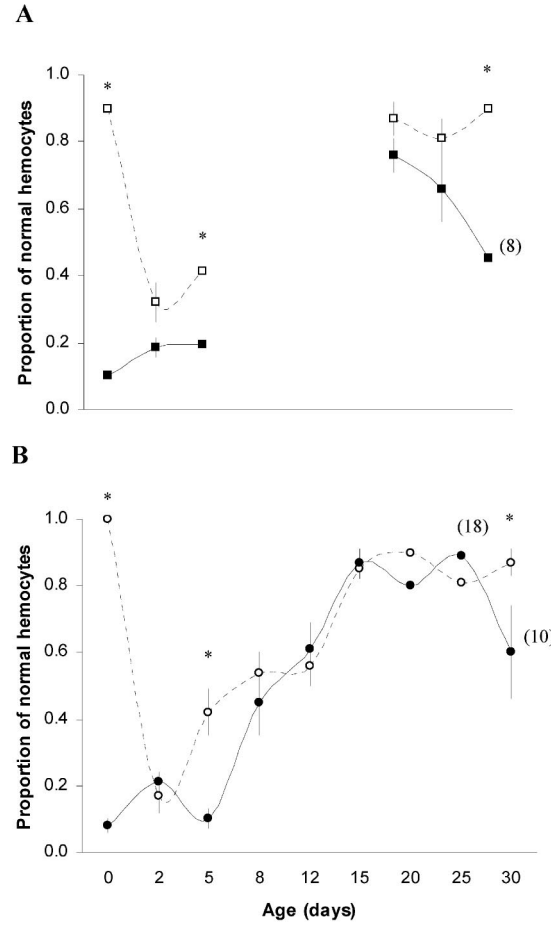

Fig. 3. Mean ± SE of the proportion of normal hemocytes (PNH) in simulated winter bees ($N = 20$ or indicated). Noninfested workers (white indicators) and workers infested by *V. destructor* during metamorphosis (black indicators) were kept in (A) colony 1 ($T_{0.05} = 0.21$) and (B) colony 2 ($T_{0.05} = 0.24$). * indicates significant differences between groups.

small compared with the accumulation of vitellogenin in the bees. From the SDS-PAGE, we approximated that noninfested workers showed a 30- to 40-fold increase in their vitellogenin level over the experimental period (also see Fig. 1), whereas the corresponding change in hemolymph hexamerin was 5- to 10-fold.

At emergence, the mean proportion of normal hemocytes was significantly lower in infested workers compared with the noninfested controls (Fig. 3). The difference was not significant 2 d after introduction, but apparent after 5 d (Fig. 3). Except for 30-d-old workers (Fig. 3), we found no significant differences in the succeeding cohorts of simulated winter bees. The 30-d-old infested workers had either high or very low proportions of normal hemocytes. This variation is partly conveyed by the large standard errors of the corresponding means (Fig. 3). A drop in the proportion of normal immunocytes is normally associated with the hive bee-to-forager transition (Rutz et al. 1974), which suggests that a proportion of the infested workers had initiated foraging. This is in agreement with observations of precocious foraging behavior in infested bees (Janmaat and Winston 2000).

During the first 48 h after emergence, infested bees seemed to have a higher ecdysteroid titer compared with the noninfested controls (Fig. 4): The mean was higher in the infested group at emergence, and the means were significantly different for 1-d-old bees (Mann–Whitney U test, $P = 0.05$). In the cohorts of 3-d-old workers, ecdysteroid levels were almost identical. This was probably because the titer continued to fall in the infested bees, whereas in the control group it began to go through the typical fluctuation pattern previously observed in queenless and queenright workers between days 3 and 5 (Hartfelder et al. 2002). As of day 4 after emergence, the infested bees had higher ecdysteroid levels than noninfested workers. The differences were statistically significant at days 4, 6, and 7 (Fig. 4). The age interval for sampling (0–7 d) was chosen because the major changes in the ecdysteroid titers of adult bees occur on a short temporal

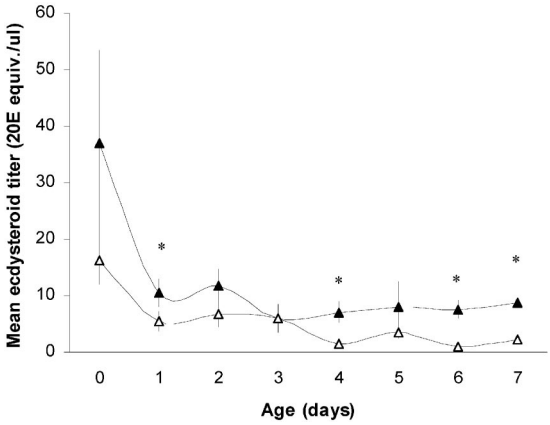

Fig. 4. Mean ± SE of ecdysteroid titer in 20E equivalents (picograms per microliter of hemolymph) for noninfested workers (white indicators) and workers infested by *V. destructor* during metamorphosis (black indicators) kept in colony three ($N = 10$). * indicates significant differences between groups.

scale during the first days of adult life (Hartfelder et al. 2002).

Discussion

Our results show that the majority of workers infested as pupae do not accumulate hemolymph proteins, including vitellogenin, to the same extent as in noninfested bees. The yolk protein vitellogenin is of special interest because it seems to have evolved alternative functions in honey bees (Engels et al. 1990, Amdam et al. 2003). The protein is metabolized as a storage protein and is used by nurse bees to produce brood food (Amdam et al. 2003). Cessation of brood rearing in late autumn is associated with an accumulation of vitellogenin in European workers (Fluri et al. 1977, 1982), and this may be a flexible colony level mechanism for producing bees with large enough protein stores to survive several months on honey only (Amdam and Omholt 2002, Amdam et al. 2003). Honey bee colonies normally overwinter with very limited pollen supplies (their only source of amino acids), and it is therefore likely that the protein stored in workers is crucial for survival as well as for brood production in early spring (Maurizio 1950, Fluri et al. 1982). A lower storage capacity in infested bees may thus indicate a physiological basis for the severe impact of *V. destructor* on honey bees in temperate zones.

The low mean proportion of normal hemocytes in newly emerged and 5-d-old infested bees suggests that the cellular immune system is also affected by the infestation. This is in accordance with previous findings (Wienands and Madel 1988). However, because we did not find significant differences in most of the subsequent age groups, it seems unlikely that infestations during the pupal stage have an irreversible impact on the cell-based immune system of the bees. In general, hemocytes are capable of rapidly reducing the number of circulating particles, including microorganisms, either by phagocytosis, nodule formation, or encapsulation (Gliñski and Jarosz 1995). Pycnotic hemocytes do not contribute to these defense mechanisms (Strand and Pech 1995, Bedick et al. 2001), and extensive pycnosis (or apoptosis) of hemocytes is apparently not part of the active immune response toward bacterial or viral infections in honey bees (Wille and Vecchi 1974). Our results may therefore suggest a down-regulated immune function in young workers infested as pupae. The phenomenon is normally observed in foragers (Rutz et al. 1974), where it may be part of a resource saving strategy (Amdam et al. 2003). However, the pycnotic hemocytes also may be the result of compounds secreted by the mite, or other forms of physiological stress caused by the infestation. Stress caused by handling (i.e., collection and marking) may thus partly explain the low mean proportion of hemocytes in the 2-d-old controls.

The elevated ecdysteroid titers in infested bees may affect general aspects of winter bee physiology in two ways; either by interfering with the long-term physiological program characterizing a winter bee or by direct effects, e.g., on general and specific protein synthesis. Although no significant effects on protein synthesis levels have been detected in fat body preparations of workers exposed to makisterone A in vitro (K.H., unpublished data), experimental evidence for ecdysteroid-induced suppression of general and specific protein synthesis has been demonstrated in two distinct aspects of honey bee development and reproduction. First, in the context of caste-specific differentiation of the honey bee ovary in the last larval instar, particularly at the onset of the prepupal stage (Hartfelder et al. 1995), and second, during sexual maturation of the mucus gland in adult honey bee drones (Colonello and Hartfelder 2003). The role played by ecdysteroids in the programming of a winter bee physiology thus clearly requires a more profound investigation.

Although our findings were obtained under simulated conditions, it is reasonable to assume that the observed effects apply to true winter bees (Maurizio 1950, Fluri et al. 1982). This suggests that workers infested by *V. destructor* as pupae fail to develop key physiological characteristics of long-lived winter bees, which in turn makes them less likely to survive until spring. If a substantial fraction of the wintering bee population is infested during the pupal stage, it is natural to ask how these doomed bees may affect the healthy winter bees and thereby the overall colony survival. There are several ways previously infested bees may have a negative impact on noninfested ones, even in the absence of *V. destructor:* 1) secondary infections affecting the levels of stored nutrients as well as the immune system may be transmitted between workers during wintering; 2) noninfested bees may deplete their protein stores by feeding already protein-depleted infested bees mimicking a nurse bee to forager relation (Crailsheim 1990), and 3) general deterioration of colony conditions due to severe reduction in colony size or disturbance of normal winter

cluster functioning. These considerations indicate a need for thorough investigations on how noninfested winter bees are affected by previously infested workers.

Overall, findings suggest that treatments with chemicals or organic acids intended exclusively to kill *V. destructor* mites in late autumn may fail to prevent losses of colonies because the physiology of the bees has already been impaired. Beekeepers in temperate climates should therefore combine late autumn management strategies with mid and late summer treatment protocols to keep the mite population at low levels before and during the period when the winter bees emerge.

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