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Dead or Alive: Deformed Wing Virus and *Varroa destructor* Reduce the Life Span of Winter Honeybees

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Elevated winter losses of managed honeybee colonies are a major concern, but the underlying mechanisms remain controversial. Among the suspects are the parasitic mite *Varroa destructor*, the microsporidian *Nosema ceranae*, and associated viruses. Here we hypothesize that pathogens reduce the life expectancy of winter bees, thereby constituting a proximate mechanism for colony losses. A monitoring of colonies was performed over 6 months in Switzerland from summer 2007 to winter 2007/2008. Individual dead workers were collected daily and quantitatively analyzed for deformed wing virus (DWV), acute bee paralysis virus (ABPV), *N. ceranae*, and expression levels of the *vitellogenin* gene as a biomarker for honeybee longevity. Workers from colonies that failed to survive winter had a reduced life span beginning in late fall, were more likely to be infected with DWV, and had higher DWV loads. Colony levels of infection with the parasitic mite *Varroa destructor* and individual infections with DWV were also associated with reduced honeybee life expectancy. In sharp contrast, the level of *N. ceranae* infection was not correlated with longevity. In addition, *vitellogenin* gene expression was significantly positively correlated with ABPV and *N. ceranae* loads. The findings strongly suggest that *V. destructor* and DWV (but neither *N. ceranae* nor ABPV) reduce the life span of winter bees, thereby constituting a parsimonious possible mechanism for honeybee colony losses.

Losses of managed honeybee, *Apis mellifera*, colonies have become a major threat to the apicultural industry and ultimately to food production (25). In temperate regions, such losses occur mainly during winter and pathogens are considered key factors (57). In particular, the ectoparasitic mite *Varroa destructor*, an invasive species from Asia, is considered the main candidate (61). It has been reported to have an impact on the bee immune system (69) and to serve as a vector for several harmful positive-strand RNA viruses (10), which is likely to enhance the deleterious action of mites on colonies. Deformed wing virus (DWV) and acute bee paralysis virus (ABPV) are transmitted by this mite after feeding on bee pupae or adults (16, 17, 62, 67) and have been suspected to cause colony losses in association with this vector (16, 17). Another potential candidate involved in these losses is the microsporidian *Nosema ceranae*, another invasive species from Asia (24), which is currently considered a main factor in colony losses in Spain (31, 32), although its impact on colony health remains controversial. While several candidates for honeybee colony collapse have been identified by now, the actual proximate mechanisms remain unclear.

Obviously, individual bees have to die before the entire colony collapses. Therefore, one parsimonious mechanism appears to be that the number of individual workers is gradually (and/or suddenly) decreasing below the social minimum to ensure colony functionality (22) and especially the effectiveness of thermoregulation during winter (40, 44, 63). Therefore, the intrinsic life span of individual winter bees in surviving and dying colonies may actually be a key issue for honeybee colony losses, and this trait has not yet been investigated. Indeed, the life expectancy of winter bees can extend for up to 10 months (49, 50), contrasting drastically with bees reared during the warm season, which live only 3 to 4 weeks on average. In general, honeybee workers perform successive temporal tasks, roughly taking care of the brood inside of the nest in the first part of their life (nurse bees) and then switching to foraging outside (68). This shift between nurse bees and foragers is driven by physiological modifications (6), which are governed by the egg yolk protein vitellogenin (1, 52, 54). Vitellogenin displays various functions in workers, drones, and queens (7, 11, 66). It is processed in the nurse hypopharyngeal glands to produce royal jelly polypeptides (3) and seems to play essential roles in bee immunity (5, 43) and in bee ageing (53, 55). It has been shown that the vitellogenin content of worker bees starts at a high level in autumn and decreases during winter (23) and that its synthesis is higher in nurse bees than in winter bees since the former have to produce jelly to feed the brood (52). Thus, vitellogenin is an important parameter acting on winter bee longevity. In addition to direct or indirect immunosuppressive effects (29, 69), *V. destructor* appears to reduce the vitellogenin levels in the hemolymph of infected bees (2), suggesting an impact on bee life expectancy (42). Although these effects can be directly related to the simple feeding activity of the mite, they can also result from the replication of viruses transmitted by the mite into the bee hemolymph (10, 59). Indeed, at least one virus, DWV, has been identified in fat body cells by *in situ* hybridization (21), suggesting an impact on vitellogenin synthesis and on genes involved in immunity. Here we empirically tested whether infections levels of *Nosema ceranae*, *V. destructor*, and associated viruses, as well as changes in *vitellogenin* gene expression, are negatively correlated with the life span of winter bees, thereby suggesting the proximate mechanism by which colonies die in winter.

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MATERIALS AND METHODS

Experimental setup. In summer 2007, 29 queenright honeybee colonies (predominantly *Apis mellifera carnica*) were divided into two groups, located at two different experimental apiaries at the Swiss Bee Research Centre in Liebefeld, Canton of Bern (Switzerland). The two experimental sites were within the same foraging range but spatially separated by ~500 m, one four-story building and vegetation to minimize potential interactions between the two groups, such as drifting and robbing. The local area is characterized by a typical central European climate and winter temperatures usually ranging from −2°C to +7°C. A set of these colonies ($n = 18$ colonies) received routine treatment against the mite *V. destructor* at regular intervals using organic acids (38, 39), while the others were left untreated ($n = 11$ colonies). These colonies had similar population levels (~14,000 workers) at the beginning of the experiment in August 2007. In September, 500 freshly emerged workers were collected from each of the 29 experimental colonies, given colony-specific marks on the thorax using commercial acrylic paint, and reintroduced into their respective mother colonies. Each hive entrance was then equipped with modified Münster bee traps (35). On a daily basis, all dead bees (including marked and unmarked ones) were collected from these traps and immediately frozen at −20°C. Bee colony sizes were estimated from August 2007 to April 2008 using the Liebefelder standard method (36). The level of infestation of each colony with *V. destructor* was estimated by counting the natural mite fall every week on bottom boards placed underneath the colony (12, 37) using adequate ant control (15).

Molecular analyses. From the sampled workers, 558 were randomly chosen for the quantification of DWV, ABPV, and *N. ceranae*, as well as gene expression of *vitellogenin*. Bees were individually homogenized in a 2-ml Eppendorf tube containing a 5-mm metal bead and 250 μl of 10 mM Tris–400 mM NaCl buffer (pH 7.5) using a TissueLyser apparatus (Qiagen). Total RNA was extracted from 50 μl of the homogenate using the RNA II Nucleospin kit (Macherey-Nagel), and cDNA was immediately synthesized using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen) using random hexamers as anchors. Quantification was performed using the SYBR-green kit (Eurogentec) according to published protocols (19, 27). The sequences of primers for measuring DWV, ABPV, *N. ceranae*, and vitellogenin were the same as those described elsewhere (14, 19). For all targets except DWV, in order to normalize the data according to the amount of RNA in the sample, analysis of the transcript for β-actin was performed in parallel for each sample (45). For relative quantification (ΔC_T relative value), the cDNA levels were normalized by subtracting the target threshold cycle numbers from that of actin. Conversely, DWV loads in samples were calculated from standard curves made of serial dilutions of known amounts of PCR amplicons (27) and presented as equivalent copies of DWV genome. All the quantitative PCRs were conducted using a thermal profile of 50°C (2 min) and then 95°C (10 min), followed by 40 cycles of 95°C (15 s) and 60°C (1 min). A melting curve was performed at the end of each run to verify the amplification of the target.

Statistics. Variation across colony groups in pathogen and *vitellogenin* transcript abundances was evaluated using two-tailed *t* tests or nonparametric Kruskal-Wallis (or Mann-Whitney) tests when the data did not fit the parametric assumptions. DWV values were log transformed since the data covered a wide range of values of several magnitudes. Correlations were studied by Spearman's rank correlation (r_s). For survival analysis, Kaplan-Meier survival analysis was performed, followed by a Mantel-Haenszel test to compare the live and dead groups. The Cox regression model was applied to identify factors related to the shortening of life expectancy. The Cox regression model ranks the effect of several variables on survival. Each factor was assessed to evaluate if it affects survival (56). *P* values below 0.05 were considered significant. To distinguish natural death from death due to handling, all bees which died during the first 8 days were excluded, giving a total of 404 workers. To compare pathogen prevalences, a chi-square test, with Yates' correction when the samples were small, was used. The statistical analyses were performed using the R, Systat 12 (Cranes), and JMP (SAS) software programs (58).

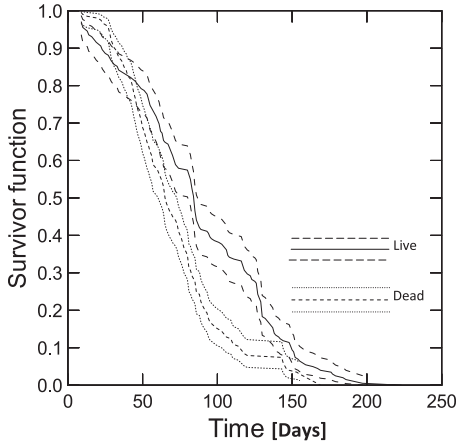


FIG 1 Life span of workers collected from colonies that died (Dead; dotted line) or survived (Live; continuous line) during winter. The time in days is indicated at the *x* axis, and the *y* axis shows the survivor function, representing the proportion of bees alive (1.0 = 100%). The lines bracketing "Live" and "Dead" show the 95% confidence interval.

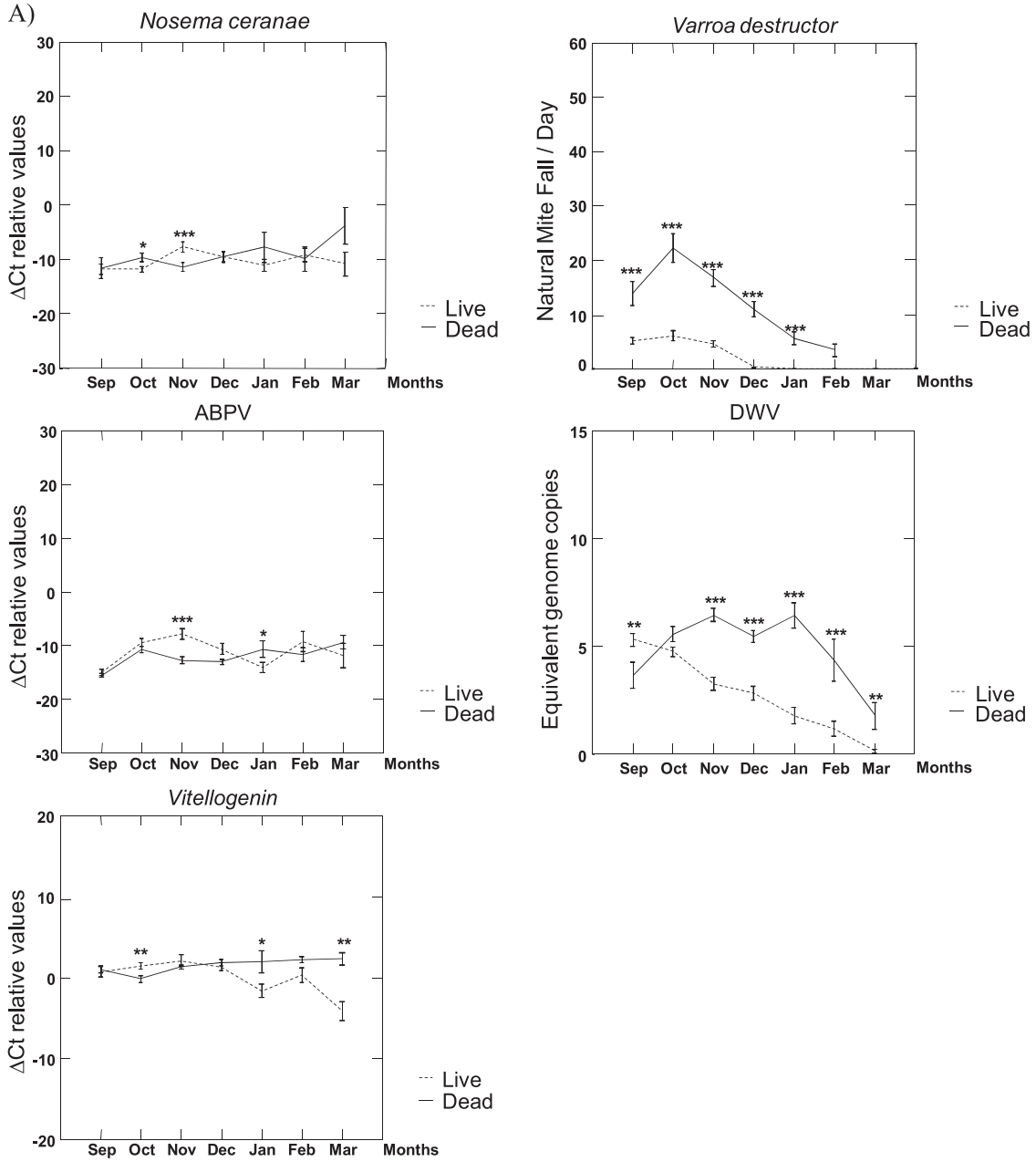
RESULTS

In the Canton of Bern, winter 2007/2008 was characterized by snow in November/December and average temperatures ranging between −1°C, 7°C, and 3°C (minimum, maximum, and median).

Once the experiment ended in April 2008, two groups "live" and "dead," were assigned. The live group ($n = 16$) consisted of all colonies that survived until April and coincided with the colonies treated for *V. destructor*. The dead group ($n = 13$) consisted of all colonies that collapsed during the experiment. This group was composed of all the untreated colonies and two colonies which were treated for the mite.

Workers from colonies that died in winter had a shortened life expectancy. The results show that bees collected from colonies that died during winter had a significantly shorter life span than those originating with surviving colonies (Fig. 1) (survival mean, 71.3 days and 88.4 days, respectively; $P < 0.001$; 95% confidence interval for dead colonies, 66.5394, 76.0448; for live colonies, 81.9190, 94.8038). No differences were observed between the two groups until 50 days following bee emergence (corresponding to mid-November), and about 80% of the labeled bees were still alive at this time. By the end of November, the data showed that ~55% of labeled bees were alive in colonies that did not survive winter, whereas ~70% were still present in the other group. After 100 days (December), 40% of the labeled bees were alive in the surviving colonies, as opposed to 15% in the colonies that would later perish.

***Varroa destructor* infestation and DWV prevalence were higher in the group of colonies that died during winter.** From September until January, the dead group of colonies had significantly higher levels of mite infestation than the group of colonies that survived ($P < 0.001$) (Fig. 2). In the latter, the mite levels recorded in the colonies increased to reach a peak in October and decreased thereafter. Likewise, from November until January, DWV prevalence in individual bees was significantly higher in



B)

Months	Sep	Oct	Nov	Dec	Jan	Feb	Mar
Live	47	32	41	51	38	28	9
Dead	12	33	76	63	14	10	6

FIG 2 (A) Quantitative analysis of honeybee pathogen loads for individual workers between surviving colonies (Live) and colonies that died during winter (Dead) from September 2007 to March 2008. Included is the corresponding level of *vitellogenin* mRNA expression levels. The level of significance are as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Error bars show standard errors (Mann-Whitney test). (B) Sample sizes of workers analyzed.

colonies which died during winter than in the other group (Table 1). No significant differences were observed in October and in February. Conversely, in November and in December, ABPV prevalence was higher in surviving colonies than in dead colonies. The prevalence of *N. ceranae* was equivalent in the two groups from October until February. In surviving colonies, a significant decrease in the percentage of DWV-positive bees was observed between December and January (χ^2, $P < 0.005$).

Bees collected from colonies that died during winter had higher DWV loads and expressed higher *vitellogenin* levels. As shown in Fig. 2, the levels of parasites, as well as the expression of the *vitellogenin* gene, varied between the two groups of colonies. Except

TABLE 1 Percentages of DWV-, *Nosema ceranae*-, and ABPV-positive individual workers collected in front of hives from October 2007 to February 2008[a]

Mo	Colony fate (*n*)	% DWV positive	*P* value	% ABPV positive	*P* value	% *N. ceranae* positive	*P* value
Oct.	Live (32)	89	0.792	28	0.847	28	0.722
	Dead (33)	88		30		24	
Nov.	Live (41)	88	**0.035**	46	**0.004**	39	0.418
	Dead (76)	99		21		32	
Dec.	Live (51)	73	**<0.001**	29	**0.049**	37	0.438
	Dead (63)	100		14		44	
Jan.	Live (38)	42	**<0.001**	16	0.949	37	0.693
	Dead (14)	93		21		43	
Feb.	Live (28)	41	0.080	43	0.366	46	0.596
	Dead (10)	80		20		30	

[a] Data are grouped according to the sampling month and the fate of the colony (surviving [live] or dead). Significant *P* values are in bold ($P < 0.05$, χ^2 tests with Yates corrections in cases of small sample sizes; *n*, sample size).

for October, where *vitellogenin* mRNA expression levels were significantly lower, a higher expression of *vitellogenin* mRNA was observed in January and in March in the group of colonies that collapsed (except for February; Mann-Whitney test, $P = 0.397$). In surviving colonies, DWV loads decreased linearly from September to March, while DWV levels increased in survivors to reach a maximum from November until January. Significantly higher levels of DWV were observed in colonies that did not survive winter than in the other group during the whole duration of the experiment (Mann-Whitney test, $P < 0.001$). Conversely, very few significant ABPV and *N. ceranae* differences were observed between the two groups, with higher levels of both parasites recorded only in November in surviving colonies (Mann-Whitney test, $P < 0.001$) (Fig. 2). The seasonal variation of pathogens and vitellogenin mRNA loads in workers is given elsewhere (see Fig. S1 in the supplemental material).

Vitellogenin gene expression is correlated with parasite loads. Correlations were detected when the data were analyzed without distinction between month or seasons or outcome of the colonies after winter. A significant positive association was observed between *vitellogenin* expression and the *N. ceranae* load ($r_s = 0.321$; $P < 0.001$) and between *vitellogenin* expression and the ABPV load ($r_s = 0.317$; $P < 0.001$), as shown in Fig. 3. However, no correlation was detected between *vitellogenin* expression and the DWV load ($r_s = -0.048$; $P = 0.551$).

Another pattern emerged when the data were analyzed on a monthly basis according to death or survival of the colonies during winter (Table 2). A negative nonsignificant trend between *vitellogenin* expression and the DWV load was observed, but only in December in the surviving colonies. Expression of the *vitellogenin* gene was also significant negatively correlated with *V. destructor* infestations. This relationship was significant only in January in surviving colonies, with a trend toward significance in the other months, starting in November in dead colonies. A positive correlation was observed in December, January, and February between ABPV and *vitellogenin* gene expression in surviving colonies and in November and December in colonies that died during winter. Positive correlations were also observed between *N. ceranae* and *vitellogenin* gene expression in December in the dead group and in January and February in the other group.

DWV has an impact on honeybee life expectancy. The Cox regression model was appropriate for testing the effect of multiple

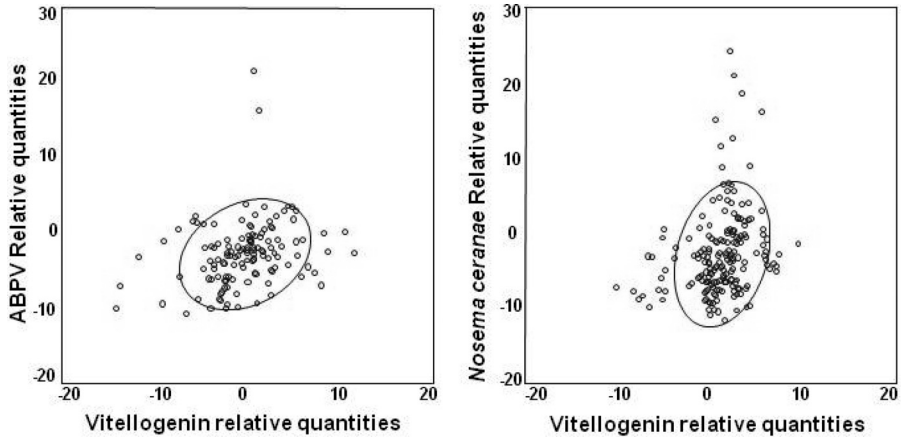


FIG 3 Correlations between ABPV and *N. ceranae* loads in workers and *vitellogenin* mRNA expression levels. The total workers collected during the experiments were used without distinction of season or month (Spearman $r_s = 0.317$ and $P < 0.001$ for ABPV; $r_s = 0.321$ and $P < 0.001$ for *N. ceranae*).

TABLE 2 Spearman rank correlations between *vitellogenin* mRNA expression and DWV, ABPV, and *N. ceranae* loads in individual workers[a]

Mo and colony fate	Parameter	Value for Vg and load correlation for: DWV	ABPV	*N. ceranae*	*V. destructor*
Oct.					
Live	r_s	−0.169	0.337	0.091	−0.051
	P value	0.443	***0.063***	0.624	0.849
Dead	r_s	−0.029	0.034	−0.080	0.133
	P value	0.890	0.850	0.657	0.573
Nov.					
Live	r_s	0.197	0.228	0.088	−0.137
	P value	0.316	0.174	0.604	0.494
Dead	r_s	−0.032	0.317	0.116	−0.202
	P value	0.786	**0.005**	0.324	0.204
Dec.					
Live	r_s	−0.303	0.376	0.213	−0.425
	P value	***0.092***	**0.010**	0.159	0.253
Dead	r_s	0.104	0.372	0.234	−0.335
	P value	0.425	**0.003**	***0.068***	***0.064***
Jan.					
Live	r_s	−0.207	0.536	0.371	−0.398
	P value	0.399	**<0.001**	**0.023**	**0.032**
Dead	r_s	0.020	−0.028	−0.024	−0.145
	P value	0.958	0.922	0.934	0.669
Feb.					
Live	r_s	−0.548	0.710	0.528	0.146
	P value	0.151	**<0.001**	**0.005**	0.484
Dead	r_s	0.417	−0.454	0.078	−0.200
	P value	0.249	0.186	0.828	0.704

[a] Grouped per month, according to the fate of the colony: surviving (live) or collapsing (dead). Vg, *vitellogenin* mRNA expression. Significant *P* values ($P < 0.05$) are in bold; borderline case values ($P < 0.1$) are in bold and italics.

variables on survival. It showed that among all the variables analyzed, only DWV (estimate $E = 0.112354$; $P < 0.001$; 95% CI, 0.070603, 0.154105) and *V. destructor* (estimate $E = 0.001080$; $P < 0.05$; 95% CI, 0.000077, 0.002084) were significantly tied to reduced life expectancy.

DISCUSSION

The data show a clear reduction in worker life span in the group of colonies that did not survive winter, as well as a higher proportion of DWV-infected bees during the period when colonies died. Both *V. destructor* loads in colonies and individual worker DWV loads were tied to significantly lower winter bee life spans, suggesting that they play a key role in this phenomenon. However, no significant correlation could be established between DWV loads and *vitellogenin* mRNA titers, an indicator of bee ageing. Furthermore, neither ABPV nor *N. ceranae* was associated with a reduction in worker longevity. The difference in life span observed between the two groups was particularly high between mid-November and the end of December, corresponding to the start of the period where most of the colonies collapsed. It has been previously shown that a minimum of 5,000 workers is required to survive winter in central and northern Europe (40, 44) in order to maintain the brood temperature around ~34°C, or above 18°C in broodless bee clusters (51, 64). Then, it can be assumed that the reduction in the winter bee life span progressively led to a number of bees in the colony that was below a critical threshold, resulting in colony collapse, confirmed by regular estimations of colony size in the performed experiment.

The question of whether honeybee pathogens detected in colonies in fall and winter can be responsible for such a reduction in the honeybee life span was investigated. Parasite loads from individual bees collected in the front of the hive were recorded; additionally, the *V. destructor* infestation at the colony level was measured. The data were compared with the expression of the *vitellogenin* gene, a marker previously shown to be associated with bee ageing and physiological health (3, 4). The analyses were focused on three honeybee parasites, which were previously identified as candidates responsible for colony collapse, the viruses DWV and ABPV (8, 16, 28, 33) and the microsporidian *N. ceranae* (31, 32). Israeli acute paralysis virus (IAPV), another virus suspected to be involved in colony losses (13), was not detected in the samples. The gathered data showed that DWV was the most prevalent among the parasites examined, thereby confirming findings of earlier studies (59). In October, the prevalence and load of DWV recorded from individual bees were equivalent between colonies which survived and those which collapsed during winter. Previous observations showed similarly that DWV prevalence and loads in bee colonies increases to reach a peak at the end of the warm season (27, 65). However, a distinct pattern was observed later on. A gradual decrease in DWV prevalence and titers in surviving colonies from October to March was observed, arguably as expected from previous studies showing the impact of acaricide treatments on DWV loads in bee colonies (46). Conversely, in colonies which died during winter, both DWV prevalence and DWV loads recorded in individual bees remained significantly higher than those in the group of colonies which survived. This was particularly true in December, when the majority of colonies died, suggesting a causal relationship between DWV infection and winter mortalities. This assumption is reinforced when looking at the decrease in the proportion of DWV-positive bees from October to January in the surviving group of colonies, suggesting that only DWV-negative bees were able to survive and consequently highlighting the pathogenic effect of DWV on bee physiology.

Curiously, no positive correlation was observed between mite loads and ABPV, despite its known association with *V. destructor* (16), which might be due to the low prevalence of this virus in this study. Likewise, no significant negative relationship was observed between colony mortality and the presence or levels of *N. ceranae*. In sharp contrast, the data show that bees from colonies that survived winter in fact had significantly higher loads of *N. ceranae* in November than bees from failing colonies ($P < 0.001$), strongly suggesting that this parasite is not responsible for colony collapse in winter, at least in certain parts of Europe. It is conceivable that *Nosema* loads were higher in surviving colonies because bees from these colonies lived significantly longer (10 to 15% longer) and older bees tend to accumulate more *Nosema* spores (30). Nevertheless, this effect would not be strong enough to mask a positive trend between *Nosema* load and mortality. While the data are in agreement with those of a recent study from Germany (28), they conflict with findings of other studies from Spain suggesting that *N. ceranae* might be a central pathogen for honeybee colony losses (30–32, 47, 48). The underlying reasons for this discrepancy remain unclear but could potentially be due to differential host sus-

ceptibility (*Apis mellifera iberica* in Spain versus *Apis mellifera carnica* or *Apis mellifera mellifera* in Germany and Switzerland) and/or differential pathogen transmission as well as virulence. At present, the latter point seems to be more likely to explain the obvious differences, because the viability of *N. ceranae* decreases after freezing and spores maintained at +4°C rapidly degenerate (20). Such low temperatures are more common in temperate regions than in the Mediterranean region.

Statistical analysis performed showed that DWV and *V. destructor* were both associated with a reduction of the honeybee life span. Honeybee ageing has been studied previously in detail and has been shown to be associated with the egg-yolk precursor protein vitellogenin, which plays diverse roles in the physiology of *A. mellifera* (54). In particular, it has been shown that winter bees have elevated titers of vitellogenin, comparable with those of nurse bees, which use this protein as a building block for synthesizing royal jelly proteins (18). In contrast, as soon as workers start foraging, levels of vitellogenin drop abruptly, as does their life expectancy (23, 34, 41, 60). In a previous study, the DWV genome was identified in fat body cells, the main site of vitellogenin synthesis (9, 54), suggesting that viral replication might impair the function of this tissue, thereby reducing the expression of *vitellogenin* mRNA. Despite that, however, a negative correlation between *vitellogenin* gene expression and DWV loads was not observed in the study. This absence of a relationship between DWV and *vitellogenin* expression was recently described for honeybee queens as well (26). Conversely, ABPV seems to have an impact on the expression of this gene, since strong positive correlations from November through March were identified. This suggests that DWV and ABPV infections might lead to distinct effects on the bee physiology, therefore requiring further studies. It might be interesting to emphasize again that this study focused only on *vitellogenin* gene expression and not the level of protein in the hemolymph. Therefore, how the infection of these viruses impacts the level of protein produced by the fat body and released into the circulation was not investigated in this study. More research would be required to better understand virus-protein interactions in hemolymph.

The data presented in this study, which are supported by an extended number of analyses of individual bees, agree with the consensus that *V. destructor* is the major driver of colony mortalities observed during winter in Europe and that consistent treatments to reduce mite levels can improve winter survival. Strong evidence that these mortalities are related to a chronic reduction in the honeybee life span, arguably due to the replication of DWV in bee tissues when winter bees are produced, is provided. Experiments can now be carried out to test a direct role for DWV in honeybee life span and winter survival, using controlled inoculations and such methods as RNA interference targeting DWV.

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Use of commercial names in this paper is for information only.

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