

RESEARCH ARTICLE

Parasitic and immune modulation of flight activity in honey bees tracked with optical counters

Cédric Alaux^{*,†}, Didier Crauser^{*}, Maryline Pioz, Cyril Saulnier and Yves Le Conte**ABSTRACT**

Host–parasite interactions are often characterized by changes in the host behaviour, which are beneficial to either the parasite or the host, or are a non-adaptive byproduct of parasitism. These interactions are further complicated in animal society because individual fitness is associated with group performance. However, a better understanding of host–parasite interaction in animal society first requires the identification of individual host behavioural modification. Therefore, we challenged honey bee (*Apis mellifera*) workers with the parasite *Nosema ceranae* or an immune stimulation and tracked their flight activity over their lifetime with an optic counter. We found that bees responded differently to each stress: both *Nosema*-infected and immune-challenged bees performed a lower number of daily flights compared with control bees, but the duration of their flights increased and decreased over time, respectively. Overall, parasitized bees spent more time in the field each day than control bees, and the inverse was true for immune-challenged bees. Despite the stress of immune challenge, bees had a survival similar to that of control bees likely because of their restricted activity. We discuss how those different behavioural modifications could be adaptive phenotypes. This study provides new insights into how biological stress can affect the behaviour of individuals living in society and how host responses have evolved.

KEY WORDS: *Apis mellifera*, Parasites, *Nosema*, Immunity, Energetic stress, Foraging behaviour

INTRODUCTION

For many decades, ecologists and evolutionary biologists showed a great interest in the influence of parasitism on host's life history traits (Clayton and Moore, 1997). Great progress has been made in uncovering the mechanisms of host–parasite interactions from the gene to behavioural levels. Notably, one major finding has been that parasites often induce behavioural alteration of their hosts (Dobson, 1988). For example, infected animals can exhibit key adaptive behavioural responses to improve their survival or the fitness of group members when living in a group (Hart, 1992; Cremer et al., 2007), but in some cases behavioural modifications are the result of the parasite's ability to manipulate the host for increasing its transmission (Lefèvre et al., 2009; Libersat et al., 2009; Adamo, 2012; Biron and Loxdale, 2013). Finally, behavioural changes can simply be a non-adaptive byproduct of infection (Holmes and Zohar, 1990).

Those changes in host behaviour can be directly due to the parasite and its multiplication, but also to the associated immune

response. Indeed, if immune defences are of great benefit to the host by reducing the impact of the parasites, mounting an immune response often implies a direct cost. For instance, in vertebrates, an increase in metabolic rates has been observed during immune activation (Lochmiller and Deerenberg, 2000; Martin et al., 2008). Similarly, a 'relatively simple' immune response such as an encapsulation response can raise the metabolic rate by up to 28% in different insect species (Freitak et al., 2003; Ardia et al., 2012). This suggests a strong cost of immune response and possibly behaviour-related changes in hosts, who might need to adapt to this increase in energy expenditure. Indeed, vertebrates can develop adaptive 'sickness' behaviour by directing energy to immune responses (Hart, 1988; Adelman and Martin, 2009). Other adaptive modifications of behaviours for protecting healthy conspecifics from infectious agents have also been described in immune-challenged individuals [e.g. mammals (see Hart, 1992; Dantzer, 2004) and insects (see Aubert and Richard, 2008; Richard et al., 2008; Alaux et al., 2012)].

In sum, a parasitic modification of host behaviour could be the reflection of different adaptive or non-adaptive traits. However, a better understanding of the host response, the underlying mechanisms and how they evolved is often hindered by the lack of background information on the 'normal' and 'modified' host behaviours (Adamo and Webster, 2013). Therefore, a key point of such studies is to first obtain a clear characterization of the behavioural modifications (Adamo and Webster, 2013). Social insects are valuable for studying parasite modification of host behaviour because they have developed highly evolved behaviours enabling homeostasis, growth, defense and reproduction of the colony in which the presence of numerous individuals and resources is highly attractive to parasites (Schmid-Hempel, 1998). In addition, how the parasite can change host behaviour in the colony has consequences not only for individual fitness, but also at the group level (Cremer et al., 2007; Wilson-Rich et al., 2009). Identifying the behavioural modification is then a first step toward a comprehensive understanding of the effects of parasites in social groups. In this context, the honey bee is an important model as several studies have reported the influence of parasites on bee behaviours. For example, parasites can induce an increase in resin collection at the colony level for potential self-medication (Simone-Finstrom and Spivak, 2012; Popova et al., 2014) and an alteration of pheromone production important in social communication (Dussaubat et al., 2010; Alaux et al., 2011). Immune stimulation alone (without the negative effect of parasites) can also modify social interaction (Richard et al., 2008; Richard et al., 2012). However, precocious foraging, i.e. the transition from nurse to forager behaviour, appears to be a general response of young bees to parasitism [e.g. by *Varroa destructor* (Downey et al., 2000; Janmaat and Winston, 2000), *Nosema apis* (Wang and Moeller, 1970) and *Nosema ceranae* (Dussaubat et al., 2013; Goblirsch et al., 2013)] and immune challenge (Alaux et al., 2012). Such behavioural changes seem adaptive as performing outside activities would limit contact in the

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List of abbreviations

AICc	Akaike's information criterion
GLMM	generalized linear mixed model
INRA	Institut National de la Recherche Agronomique
JH	juvenile hormone
JHE	JH esterase
JHEH	JH epoxide hydrolase
HR	hazard ratio

hive with nestmates of great importance (queen, brood and young nurse) and the spread of parasites within the colony (Cremer et al., 2007). However, less is known about the actual foraging behaviour of the parasitized bees and whether mounting an immune response would also affect flight activity.

To test the behavioural response of bees to parasitism and immune challenge, we analysed the individual flight activity of honey bees (*Apis mellifera* Linnaeus 1761) either (1) parasitized by the microsporidium *Nosema ceranae*, an intracellular spore-forming fungal parasite that develops in the epithelial cell layer of the midgut of adults (Higes et al., 2013), (2) or exposed to a non-pathogenic immune-challenge (pin prick) (Siede et al., 2012). Before the behavioural experiments, we determined whether the parasitic and immune challenges actually induced a precocious development of forager physiology by measuring the expression of vitellogenin and some components of the juvenile hormone (JH) pathway; both vitellogenin and JH are involved in the regulation of bee behavioural development (Sullivan et al., 2000; Nelson et al., 2007). For the JH pathway, we quantified the gene expression levels of *JH esterase* (*JHE*), involved in JH degradation (Mackert et al., 2008; Mackert et al., 2010). The expression of *JH epoxide hydrolase* (*JHEH*), which has a negligible role in JH degradation (Mackert et al., 2010), was also assessed as its function might be related to dietary lipid catabolism (Mackert et al., 2010), a major component of bee behavioural development (Toth and Robinson, 2005). Then, the individual flight behaviour was recorded continuously and automatically during the lifetime of the bees with a newly developed counter that allows tracking 'individual' bees. In Dussaubat et al. (Dussaubat et al., 2013), the flight behaviour of bee 'cohorts' infected with *N. ceranae* was similarly recorded, but tracking 'individual' bees promises to provide more accurate measurements of behavioural changes and data on the survival of each bee. Therefore, we also determined under field conditions the effects of *Nosema* parasitism and mounting an immune response on bee survival. Finally, in the literature, almost all assays developed to test the effect of a stressor on bee survival were performed under laboratory conditions (Williams et al., 2013). However, it is not known whether these results can be extrapolated to field conditions. We thus compared the survival of stressed bees under laboratory and field conditions.

RESULTS

Parasitic and immune challenge effects on *JHE*, *JHEH* and *Vitellogenin* gene expression

Control, parasitized and immune-challenged bees were reared separately in groups of 30 bees. After 8 days, both immune reaction (pin prick) and *Nosema* parasitism induced the decrease of *JHE* and *JHEH* expression (ANOVA: *JHE*: $F_{2,27}=14.51$, $P<0.001$ and *JHEH*: $F_{2,27}=7.79$, $P<0.005$; Tukey's HSD *post hoc* test: *JHE*: $P<0.001$, *JHEH*: $P<0.05$ for both pin prick and *Nosema*; Fig. 1). *Vitellogenin* expression was also reduced in challenged bees ($F_{2,27}=19.29$, $P<0.001$; Fig. 1). However, there was no difference in gene expression between the immune and parasitic challenges (Tukey's

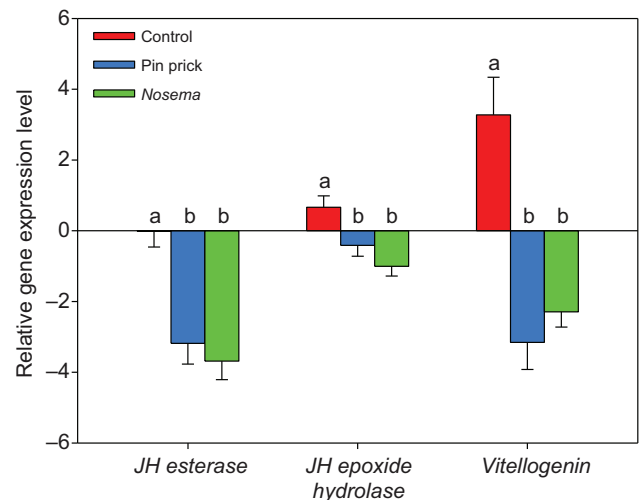


Fig. 1. Gene expression levels of components of the JH signalling pathway and *Vitellogenin* in responses to parasitic and immune challenges. Differences between control (red bars), immune-challenged (pin prick, blue bars) and parasitized (*Nosema ceranae*, green bars) honey bees were determined using ANOVA tests followed by Tukey's HSD *post hoc* tests. Means \pm s.e.m. are shown for 10 pools of three abdomens per treatments. Different letters indicate significant differences ($P<0.05$).

HSD *post hoc* test: *JHE*: $P=0.77$, *JHEH*: $P=0.36$, *Vitellogenin*: $P=0.72$; Fig. 1).

Parasitic and immune challenge effects on individual flight activity

The flight behaviour of 49 marked bees per treatment group (control, pin prick and *Nosema*) and per colony ($N=3$) was recorded with an automatic bee counter over 35 days (Fig. 2). Of the 147 observed bees per treatment, we obtained data for 103 control bees, 96 pin prick bees and 113 *Nosema*-infected bees (colony 1: 39, 38 and 39 control, pin prick and *Nosema*-infected bees, respectively; colony 2: 36, 30 and 39; colony 3: 28, 28 and 35). The observed losses of bees at the beginning of the experiment could be due to the loss of the tag number, the ejection of some tagged bees by nestmates or an early death of individuals.



Fig. 2. Optic bee counters in the field measuring the activity of honey bees with coloured tag numbers. The optic bee counter (at the entrance of the hive) and the power supply (next to the hive) were protected from climatic events by a wood box covered with a metal top. Bees identified with coloured tag numbers are shown in the inset.

At 14 days old, *Nosema*-infected bees had a high load of spores in the gut [3.02 ± 0.7 million spores (mean \pm s.d.), pools of 10 bees per colony] and pin prick and control bees were either uninfected or poorly infected (pin prick: 0.23 million spores in colony 3 and none detected in colonies 1 and 2, control: 0.11 million spores in colony 1 and none detected in colonies 2 and 3).

Data on flight activity were sorted and for each bee we filtered out all exits that lasted less than 2 min to minimize the risk of recording non-flight activity. Indeed, some bees go back and forth between the landing board and the inside hive (e.g. guarding bees).

The results of model selection regarding the effect of treatments on flight activity parameters are presented in Table 1 [generalized linear mixed model (GLMM) analysis]. Overall, six dependent variables were investigated, leading to six sets of models. For each dependent variable of the selected models, values of the coefficient of the fixed terms, their standard error and associated *P*-values are presented in Table 2. The parasitic and immune challenges did not affect the age of bees at the first flight (Fig. 3A). However, we found a significant treatment effect on the duration of flight activity over the bee lifetime (number of days from the first to the last exit) (Fig. 3B). *Nosema*-infected bees had a shorter flight activity than control and pin prick bees (mean observed values of flight parameters with 95% confidence interval: 9.0 days [8.09; 9.83] versus 13.5 [12.12; 14.96] and 12.5 days [10.87; 14.10], respectively). However, immune challenge did not affect the duration of flight activity as compared with the control group (Table 2). Moreover, the parasitic and immune challenges decreased the total number of bee exits over the 35 days (Fig. 3C). Compared with control bees, which had a mean total number of exits of 32 [26.9; 37.9], the mean total number of flights of pin prick and *Nosema*-infected bees was 24 [19.2; 29.4] and 19 [16.8; 22.1], respectively. The number of flights in parasitized bees was even lower than in immune-challenged bees (Table 2).

Regarding bee daily activity, we obtained data for 2341 exits: 878 of control bees, 753 of pin prick bees and 710 of *Nosema*-infected

bees (colony 1: 337, 379 and 231 control, pin prick and *Nosema*-infected bees, respectively; colony 2: 298, 194 and 226; colony 3: 243, 180 and 253). There was a significant effect of the treatment by age interaction on the number of flights per day. The number of exits increased with bee age, but this increase was modified by treatment (Fig. 4A, Table 2). In the youngest bees, the number of daily exits did not differ between treatments (e.g. at 10 days old, the number of daily exits of control, pin prick and *Nosema*-infected bees was 2 [1.98; 2.09], 2.1 [2.01; 2.11] and 2 [1.9; 2.0], respectively). When considering older bees, the number of daily flights decreased in treated bees (e.g. at 20 days old the number of daily exits was 4.8 [4.70; 4.86], 3.6 [3.51; 3.70] and 3.7 [3.62; 3.77] for control, pin prick and *Nosema*-infected bees, respectively). Immune-challenged bees exhibited even lower number of daily flights as compared with parasitized bees (supplementary material Table S1). Regarding the duration of each flight, we also found a significant treatment by age interaction. The duration of daily flight increases with bee age regardless of treatment (Fig. 4B, Table 2). However, the difference in the duration of flights according to treatment was higher in older bees in comparison to young bees. Hence, at 15 days old, pin prick and *Nosema*-infected bees performed longer flights than control bees (3493 [3362.4; 3623.3], 4167 [3990.5; 4344.2] and 2517 s [2432.1; 2602.0], respectively). However, when older, the duration of each exit decreased in pin prick bees as compared with *Nosema*-infected and control bees (day 23: 2858 [2715.0; 3000.9], 3363 [3264.4; 3460.9] and 4663 s [4568.5; 4756.9] in immune-challenged, control and *Nosema*-infected bees, respectively; Table 2 and supplementary material Table S1). Finally, the total amount of time spent outside the colony (sum of all flight durations per day) varied also according to the age and treatment, with a significant treatment by age interaction. Immune-challenged and *Nosema*-infected bees spent more time outside the colony than control bees during the first days of activity (day 11: 8371 [8090.2; 8652.0], 7547 [7213.5; 7880.7] and 5058 s [4791.0; 5325.8], respectively; Fig. 5; Table 2), but starting at day 13 the flight activity of immune-

Table 1. Models fitted to investigate the effect of treatment on bee flight activity parameters

Set of models	Dependent variable	Fixed explanatory variable(s)	Random explanatory variable(s)	Number of statistical units	d.f.	AICc
A	Age at first flight (days)	Treatment Null	Colony Colony	312 bees belonging to 3 colonies	4 2	1358.8 1356.5
B	Duration of flight activity over the 35-day period (days)	Treatment Null	Colony Colony	312 bees belonging to 3 colonies	4 2	2629.6 2737.2
C	Total number of flights over the 35-day period	Treatment Null	Colony Colony	312 bees belonging to 3 colonies	4 2	7149.3 7506.4
D	Number of flights per day	Age × treatment Age + treatment Age Treatment Null	Colony\bee Colony\bee Colony\bee Colony\bee Colony\bee	2341 observations on 312 bees belonging to 3 colonies	8 6 4 5 3	9951.4 9983.0 9990.1 10,794.4 10,798.4
E	Duration of each flight (s)	Age × treatment Age + treatment Age Treatment Null	Colony\bee Colony\bee Colony\bee Colony\bee Colony\bee	7862 observations on 312 bees belonging to 3 colonies	9 7 5 6 4	146,062.5 146,130.2 146,144.9 147,359.2 147,359.4
F	Duration of flight activity per day (s)	Age × treatment Age + treatment Age Treatment Null	Colony\bee Colony\bee Colony\bee Colony\bee Colony\bee	2341 observations on 312 bees belonging to 3 colonies	9 7 5 6 4	49,026.4 49,135.0 49,148.4 49,995.4 49,996.4

For each set of models, the best model [with the lowest corrected Akaike's information criterion (AICc)] is in bold.

Table 2. Coefficients, standard errors and associated *P*-values of the fixed terms of the five selected models investigating a treatment effect on bee flight activity parameters (see Table 1 for details)

Dependent variable	Covariate	Class	Coefficient	s.e.m.	<i>P</i> -value
Duration of flight activity over the 35-day period (days)	Intercept		2.606	0.0435	<0.001
	Treatment	Pin prick	−0.081	0.0393	0.04
		<i>Nosema</i>	−0.413	0.0413	<0.001
Total number of flights activity over the 35-day period	Intercept		3.479	0.0532	<0.001
	Treatment	Pin prick	−0.289	0.0269	<0.001
		<i>Nosema</i>	−0.511	0.0274	<0.001
Number of flights per day	Intercept		0.009	0.0800	0.91
	Age (day)		0.067	0.0034	<0.001
	Treatment	Pin prick	0.071	0.1191	0.55
		<i>Nosema</i>	−0.342	0.1306	0.009
	Age×treatment	Pin prick	−0.015	0.0053	0.004
		<i>Nosema</i>	0.029	0.0072	<0.001
Duration of each flight (s)	Intercept		−1678.19	348.13	<0.001
	Age (day)		266.22	10.59	<0.001
	Treatment	Pin prick	1400.32	397.34	<0.001
		<i>Nosema</i>	−864.46	420.13	0.04
	Age × treatment	Pin prick	−39.72	16.28	0.01
		<i>Nosema</i>	147.34	21.35	<0.001
Duration of flight activity per day (s)	Intercept		−7927.16	963.61	<0.001
	Age (day)		1144.98	45.02	<0.001
	Treatment	Pin prick	5931.45	13,420.99	<0.001
		<i>Nosema</i>	−3806.14	1470.30	0.009
	Age × treatment	Pin prick	−467.52	67.03	<0.001
		<i>Nosema</i>	475.24	89.35	<0.001

Intercept represents the control bees.

challenged bees rapidly decreased until the end (day 20: immune-challenged: 11,857 [11,460.6; 12,253.5], control: 16,997 [16,578.9; 17,415.0] and *Nosema*-infected: 21,742 s [21,303.8; 22,179.4]). However, besides day-to-day variation, the time spent in the field by parasitized bees remained higher than that of control and immune-challenged bees (Fig. 5, Table 2; supplementary material Table S1).

Parasitic and immune challenge effects on survival

In the field, the survival of *Nosema*-parasitized bees was significantly lower than that of control bees (Cox proportional hazards regression model, $Z=4.54$, $P<0.001$; Fig. 6); however, we did not find any difference between control bees and bees who received a pin prick ($Z=-0.13$, $P=0.894$; Fig. 6).

The experiments were repeated at the same time with bees reared in the laboratory (cages) to assess the influence of rearing conditions (natural versus controlled conditions) on the effect of parasitism and immune challenges. Similarly, *Nosema* parasitism decreased the

survival of bees under laboratory conditions ($Z=11.41$, $P<0.001$; Fig. 6). Immune challenge also reduced the survival of bees under those conditions ($Z=7.95$, $P<0.001$).

The experimental conditions had a pronounced effect on bee survival, with bees reared in cages living significantly longer than bees introduced in colonies ($Z=12.54$, $P<0.001$; Fig. 6). This was the case for all treated bees (control: $Z=12.09$, $P<0.001$, pin prick: $Z=4.8$, $P<0.001$, *Nosema*: $Z=2.87$, $P<0.01$). The risks of death [estimated hazard ratio (HR)] induced by immune and parasitic challenges were absent or highly reduced in the field as compared with laboratory conditions (pin prick: HR field=0.98 versus HR laboratory=2.29, *Nosema*: HR field=1.91 versus HR laboratory=3.16).

DISCUSSION

Honey bees are exposed to numerous parasites and likely immune stress, but very little is known about the potential impacts of these stressors on bee behaviour. In this study, we continuously recorded

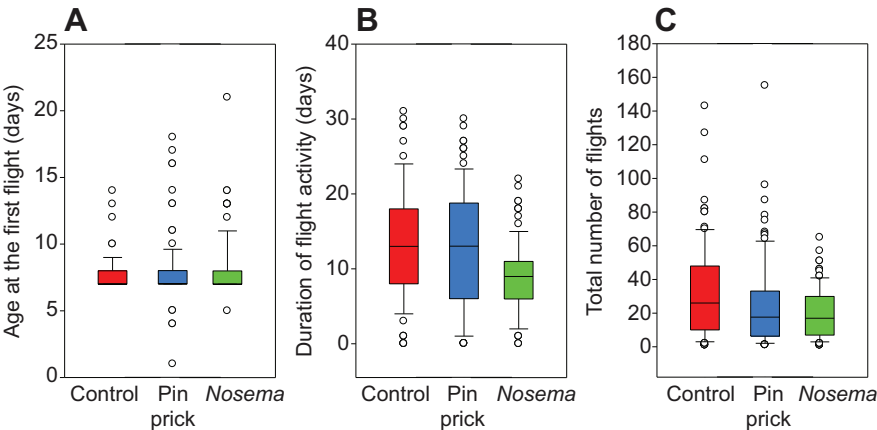


Fig. 3. General flight activity in response to parasitic and immune challenges. (A) Age at first flight, (B) total duration of flight activity and (C) total number of flights over 35 days. Differences between control (red bars), immune-challenged (pin prick, blue bars) and parasitized (*N. ceranae*, green bars) honey bees were determined using GLMM. Box plots show first and third interquartile range with line denoting median. Whiskers encompass 90% of the individuals, beyond which outliers are represented by circles.

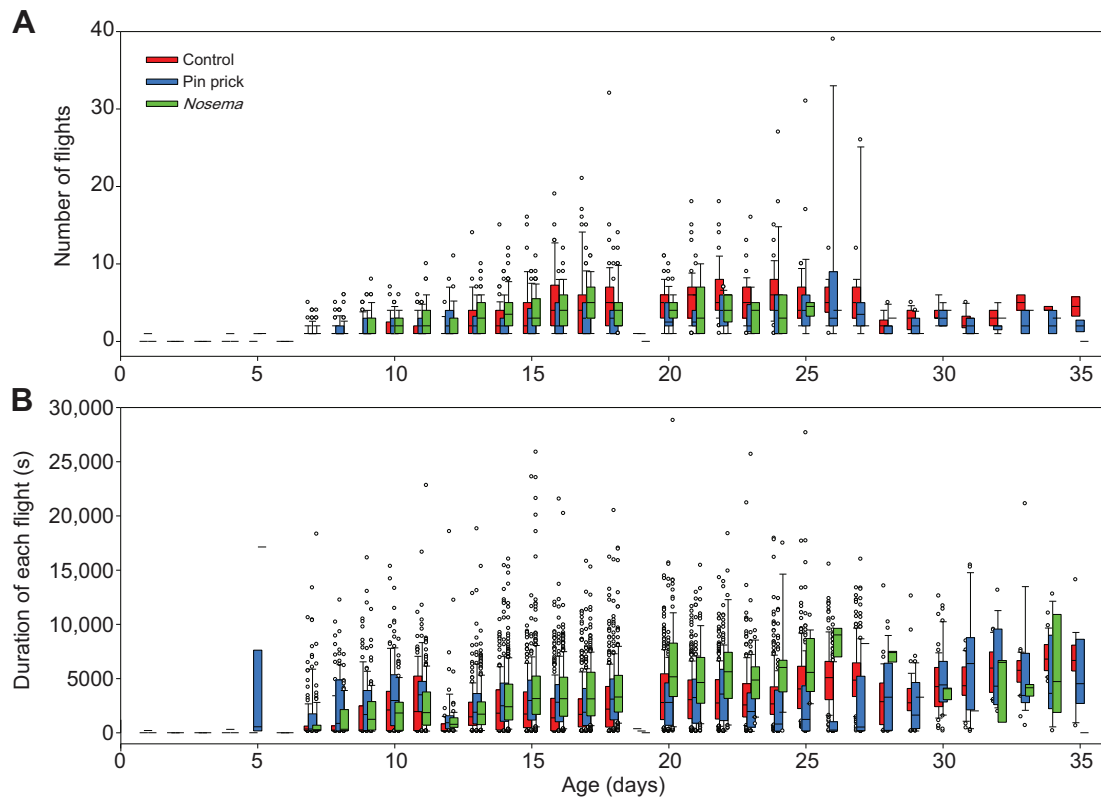


Fig. 4. Daily flight activity in response to parasitic and immune challenges. (A) Number and (B) duration of daily flights over 35 days. Differences between control (red bars), immune-challenged (pin prick, blue bars) and parasitized (*N. ceranae*, green bars) honey bees were determined using GLMM. Box plots show first and third interquartile range with line denoting median. Whiskers encompass 90% of the individuals, beyond which outliers are represented by circles. The absence of activity on day 19 was due to rain showers.

the in-and-out activity of individual bees and showed that parasitic and immune stress can deeply modify their flight activity over their lifetime. While the time spent outside the colony increased in parasitized bees, immune challenged bees significantly reduced their flight activity by staying longer inside the colony.

An early transition from nurse (brood and nest care) to foraging activity has been described in response to parasitism (Wang and Moeller, 1970; Downey et al., 2000; Janmaat and Winston, 2000; Dussaubat et al., 2013; Goblirsch et al., 2013) and immune

challenges (Alaux et al., 2012), and thus seems to be a general sickness response. This precocious foraging is characterized by a dramatic change in bee physiology with a decrease and increase of vitellogenin and JH titers, respectively (Sullivan et al., 2000; Nelson et al., 2007). Our results showed that *N. ceranae* infection triggers such physiological changes as previously found by Goblirsch et al. (Goblirsch et al., 2013). The immune challenge (pin prick) also promoted a forager physiology. The largest difference between control and treated bees was actually found in *Vitellogenin*

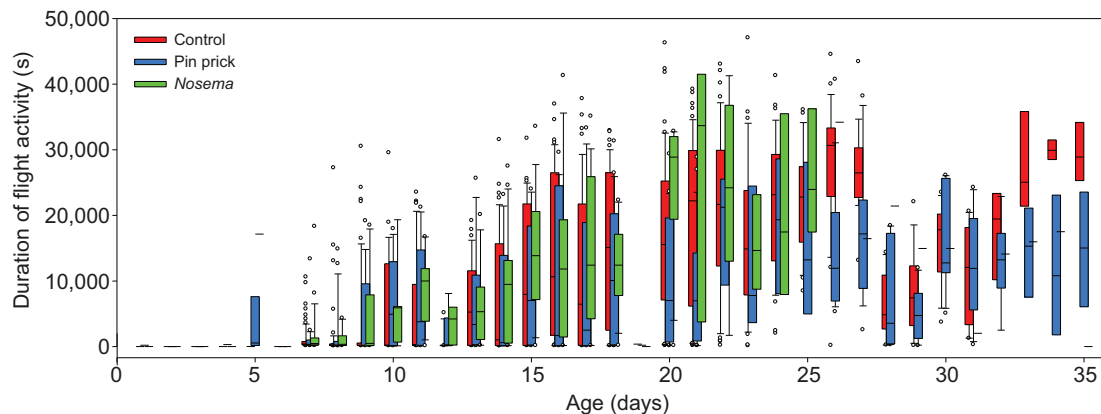


Fig. 5. Daily time spent outside the colony in response to parasitic and immune challenges. Differences between control (red bars), immune-challenged (pin prick, blue bars) and parasitized (*N. ceranae*, green bars) honey bees were determined using GLMM. Box plots show first and third interquartile range with line denoting median. Whiskers encompass 90% of the individuals, beyond which outliers are represented by circles. The absence of activity on day 19 was due to rain showers.

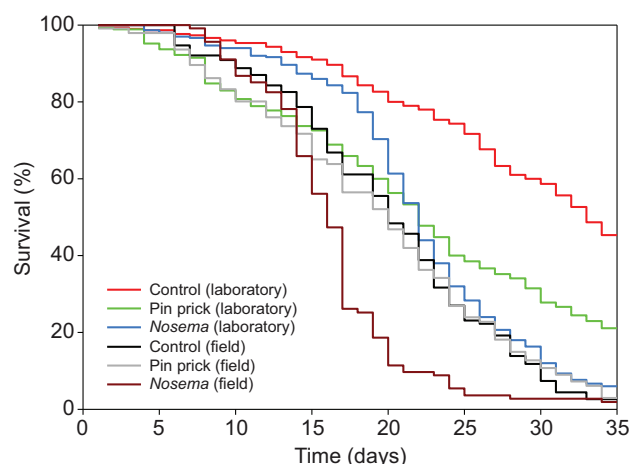


Fig. 6. Effects of parasitic and immune challenges on honey bee survival under field and laboratory conditions. Data show the percentage survival over 35 days for treated bees reared under field ($N=3$ colonies and from 28 to 39 bees per colony and treatment) or laboratory conditions ($N=10$ cages per treatment and 30 bees per cage).

expression. This glucolipoprotein, strongly expressed in nurses, is multifunctional by taking part in the production of brood food and enhancing immunity and lifespan (antioxidant activity) (for a review, see Amdam, 2011). The stress-induced decrease in its expression could thus be involved in the reduction of survival of *Nosema*-infected and immune-challenged bees (see the cage experiment). Interestingly, the modifications in *Vitellogenin*, *JHE* and *JHEH* expressions were not different between the two stressors, which suggests that bees respond similarly to different biological stress in terms of precocious foraging. We thus expected to observe an early flight activity of stressed bees compared with control bees, but there was no difference regarding the age at the first flight between treated bees. Here the flight activity of each treated group started early, when bees were around 1 week old, which might explain why we did not observe any effect of parasitism or immune challenges on the age of first exit. Such early activity might be due to an initial lack of foragers in the colonies.

As mentioned previously, parasites, such as *Nosema*, induce an early foraging activity (Wang and Moeller, 1970; Downey et al., 2000; Janmaat and Winston, 2000; Dussaubat et al., 2013; Goblirsch et al., 2013), but little is known about the actual field activity of the parasitized bees. By following and modelling the activity of infected cohorts, Dussaubat et al. (Dussaubat et al., 2013) found an overall slight increase in the rate of daily flights. However, by following a global activity, it was not possible to filter out short back-and-forth movements at the hive entrance (non-flight activity) and control the mortality during the course of the experiment. This might have biased the estimation of flight activity rate within cohorts. By tracking the number and duration of flights as well as the mortality for each individual, we showed here that infected bees actually perform fewer but longer flights as compared with non-infected bees. It is reasonable to assume that the rate of daily flights decreases because of the extreme duration of each trip. The effect of *Nosema* on the duration of flights could be linked to the disturbance of energetic metabolism caused by the parasite. Indeed, *Nosema* microsporidia depend on the host energy to accomplish their life cycle, which imposes a nutritional and energetic stress on the bees (Mayack and Naug, 2009; Aliferis et al., 2012; Dussaubat et al., 2012). This might be critical for foragers because flight activity

requires high metabolic rates (Harrison and Fewell, 2002). Thus, a decrease in energetic metabolism might impose resting sessions during the flights or the need to constantly seek nectar resources that could compensate for their compromised metabolism. In fact, we recently found that the gene encoding the pheromone biosynthesis-activating neuropeptide was overexpressed in the brain of *Nosema*-infected bees (McDonnell et al., 2013), and this neuropeptide is more highly expressed in nectar foragers compared with pollen foragers (Brockmann et al., 2009). It has also been suggested that *Nosema* might induce cognitive impairment in bees and thus affect their orientation capacities (Kralj and Fuchs, 2010). This was further confirmed by the modifications of some brain functions by the parasites (McDonnell et al., 2013). Such cognitive impairment could also explain the increase in flight duration. However, the energetic and cognitive hypotheses are not mutually exclusive as learning and memory have an energetic cost for bees (Jaumann et al., 2013). Therefore, an energetic stress caused by the parasite would add costs to both orientation and locomotion in the field (Jaumann et al., 2013), leading to a significant increase in flight duration.

Mounting an immune response is important for bee defence, but here we found for the first time that it can also induce a modification of bee activity. This demonstrates that there can be a direct effect of parasite on host behaviour but also an effect because of the immune response associated with the infection. Like parasitism, mounting an immune response is costly (Freitag et al., 2003; Ardia et al., 2012), which can impair learning capacities in bees (Mallon et al., 2003; Alghamdi et al., 2008). We would thus expect an increase in the duration of flight trips. However, even though the duration of flights increased in young bees, it decreased in older bees and this was associated with a consistently lower number of flights per day. Overall, contrary to parasitized bees the time spent outside the colony each day was significantly reduced. The performance of longer flights at the beginning could simply be due to an earlier foraging activity than that of control bees. A reduction of activity (e.g. foraging, mating, parental care) has been often observed following an immune challenge in vertebrates (Aubert et al., 1997; Bonneaud et al., 2003; Owen-Ashley and Wingfield, 2006) and is considered to be an adaptive behaviour (Adelman and Martin, 2009). This sickness behaviour would enable individuals to direct energy to immune responses rather than toward activity irrelevant to surviving infection (Hart, 1988). Such phenomenon could explain why immune-challenged bees drastically reduced their flight activity. Finally, because flight activity starts with orientation flights followed by foraging trips, it is important to note that the differences that are due to *Nosema* and immune challenges could also be due to effects on orientation flights.

Even though the immune challenge caused a chronic stress to the bees, their survival in the field was not significantly different from that of control bees. This absence of cost on the survival of immune-challenged bees was associated with a reduction of flight activity, which is particularly energy demanding (Harrison and Fewell, 2002). Therefore, developing a parsimonious flight activity or entering into 'lethargic' stages might help stressed bees save energy for the immune response, without being at the expense of survival. On the contrary, the survival of *Nosema*-infected bees was significantly reduced likely because of the deleterious effects of spore multiplication in the midgut (Dussaubat et al., 2012; Higes et al., 2013), the energetic stress (Mayack and Naug, 2009; Aliferis et al., 2012; Dussaubat et al., 2012) and the higher amount of time spent outside the colony exposing individuals to predation and other environmental risks. The total duration of flight activity (first day of activity to death) was then lower than control and immune-

challenged bees. Interestingly, when bees were reared in laboratory cages and thus removed from environmental stress and flight activity, they lived longer than their counterparts reared in the field (this was true for each treatment group), showing that environmental conditions and activity performance add to the stress caused by parasitic and immune challenges. However, the risk of death (estimated HR) induced by both stressors either disappeared (immune stimulation) or declined (*Nosema*) in the field as compared with laboratory conditions. The most relevant explanation is that control bees (without any stress challenge) live much longer in artificial than in field conditions. Such an observation indicates that the estimated HR of a stress determined under artificial conditions might not necessarily be the same under field conditions. Therefore, one should be cautious before transposing survival data on stress effects from artificial to natural conditions.

It is often difficult to determine whether behavioural changes induced by parasites are adaptive for the host, a parasite manipulation or a byproduct of host reaction. The location of the parasites in host body can give evidence as to its manipulative potential. Indeed, direct access to the central nervous system might help the parasite to modify the host behaviour (Adamo, 2012); furthermore, the central nervous system might provide protection against the defence machinery of the immune system (Galea et al., 2007). Because *N. ceranae* is an intracellular spore-forming fungal parasite that infects the midgut of adult bees (Higes et al., 2013), it is less likely to directly manipulate the host. The fact that infected bees switch early to foraging tasks (Dussaubat et al., 2013; Goblirsch et al., 2013) and spend a large amount of time in the field (our data) seems to represent adaptive behaviours of the host for minimizing the risk of nestmate infection. This would explain why *Nosema*-infected bees did not enter into a 'lethargic' state or decrease their activity as did immune-challenged bees, despite the perturbation of energy metabolism. Therefore, our results demonstrate that bees can adapt their behavioural responses to the type of stress. It would be interesting then to test how bees would respond to different types and loads of parasites.

The automatic and continuous recording of bee flight activity proved to be an effective tool to characterize bee behavioural modifications induced by different stresses. Further comparative research on 'sickness' behaviour will help to understand better the underlying mechanisms of disease and modelling the spread within and between colonies. Finally, observations in the long-term promise to reveal how parasites can affect colony dynamics and how colonies adapt.

MATERIALS AND METHODS

Bees and experimental setup

Experiments were performed at the Institut National de la Recherche Agronomique (INRA) in Avignon (France) with bees that were a local mixture of *Apis mellifera ligustica* and *Apis mellifera mellifera*. To test the effects of *Nosema* parasites and immune reaction on individual flight activity, 1-day-old bees were challenged in the laboratory, marked and then introduced into hives equipped with an optic bee counter developed at INRA (see below).

Age-matched bees were obtained by placing honeycombs containing late-stage pupae into an incubator at 34°C and 50–70% humidity, and collecting bees that emerged within 10 h. Newly emerged bees originating from three colonies were mixed before challenging them. They were then individually marked with a coloured tag number (3 mm diameter) glued (Sader®) onto the thorax (Fig. 2). Each group (control, parasitic and immune challenges) was marked with a different colour (blue, red and green). In each colony, we introduced 49 bees from each treatment and the experiment was repeated for three colonies. The colonies were established as similarly as possible and

comprised five frames containing brood and food and headed by a 1-year-old queen. Individual flight behaviour was recorded continuously from day 1 (introduction) to day 35.

Parasitic challenge

The microsporidian parasite *Nosema ceranae* was isolated as in Higes et al. (Higes et al., 2007). The *Nosema* species was confirmed by PCR as described in Alaux et al. (Alaux et al., 2010). Newly emerged bees were individually infected with 20,000 spores of *N. ceranae* by feeding them with 2 µl of 50% sucrose solution containing the freshly extracted spores. Before introducing them in the experimental colonies, bees were placed in an incubator at 34°C and 70% relative humidity for 5 h to ensure the solution was ingested and to decrease the chance of having some of the inoculum left in the crop that could later be exchanged with non-infected bees.

Immune challenge

We experimentally activated the bee immune system by a pin prick between the third and fourth tergite. In several studies, mechanical wounding has been shown to activate immune response (Evans et al., 2006; Alaux et al., 2012; Richard et al., 2012; Siede et al., 2012). If a hemolymph drop was released after wounding, the bee was discarded from the experiment. As for the parasitic challenge, bees were held 5 h in an incubator before introduction into the experimental colonies. Activation of the immune system (antimicrobial peptides) 10 h after the pin prick was confirmed in preliminary experiments following the procedure of Siede et al. (Siede et al., 2012) (data not shown). Control bees were handled but did not receive any pin prick or *Nosema* spore solution.

In parallel, 50 bees per treatment and colony were paint-marked with a different colour than the tag number and introduced in the hive to later determine the level of *Nosema* parasitism in each bee group. Those bees were collected at 14 days old.

Parasitic and immune challenge effects on *JHE*, *JHEH* and *Vitellogenin* gene expression

Control, parasitized and immune-challenged bees were reared separately in plastic cages (10.5×7.5×11.5 cm). They originated from three colonies and were mixed before placing them in cages in groups of 30 bees (*N*=3 cages per treatment). They were held in an incubator as above and were provided with honey, pollen and water *ad libitum*. After 8 days, they were flash-frozen in liquid nitrogen and stored at −80°C.

RNA was extracted from pools of three abdomens (three pools per cage) as described in Di Pasquale et al. (Di Pasquale et al., 2013). The gene expression levels of *JH esterase* (*JHE*), *JH epoxide hydrolase* (*JHEH*) and *Vitellogenin* were determined by quantitative PCR using a StepOne-Plus Real-Time PCR System (Applied Biosystems®, Saint Aubin, France) with the SYBR green detection method including the ROX passive reference dye as described in Di Pasquale et al. (Di Pasquale et al., 2013). Cycle threshold (*C_t*) values of selected genes were normalized to two housekeeping genes *Actin* and *eIF3-S8*. Primer sequences (5'–3') were: *JH esterase*: forward: TTCTTTCTTCTTGTGACTTTTGTT, reverse: CCCTTAATTGCA-CCTAAAGGAGTTT (Ament et al., 2011); *JH epoxide hydrolase* forward: TTAACAAAACCTTGGCCTAATCAAA, reverse: GGAGAAACCGTA-TCCAGGAAGTG (Ament et al., 2011); *Vitellogenin* forward: TTGA-CCAAGACAAGCGGAAGT, reverse: AAGGTTCGAATTAACGATGAA (Fischer and Grozinger, 2008); *Actin* forward: TGCCAACACTGTCC-TTTCTG, reverse: AGAATTGACCCACCAATCCA; and *eIF3-S8* forward: TGAGTGTCTGCTATGGATTGCA, reverse: TCGCGGCTCGTGGT-AAA (Richard et al., 2008).

Parasitic and immune challenge effects on individual flight activity and survival

The flight activity of individual bees was recorded by monitoring the in-and-out activity of bees at the entrance of the hive with an optic bee counter, which was developed as a follow-up of the previous counter recording the in-and-out activity of non-marked bees (colony activity) and bees marked with colour paint (bee cohort activity) (patent number: IDN.FR.001.140013.000.R.P.2013.000.31235, INRA, 2010) (Dussaubat

et al., 2013). The system was designed to be minimally intrusive, i.e. bees could freely circulate at the hive entrance, and the tags were flat and of minimal size so bees could still move their body inside cells. The counter, based on image capture of tagged bees and analysis of tag number, is composed of: (1) a modified entrance with eight passages narrow enough so that one bee can circulate in each tunnel and only with the back of the thorax facing the camera, (2) a light-emitting diode lamp, (3) a camera placed at the modified entrance of the hive, (4) a computer for image acquisition and (5) software that processes the images and reports the in-and-out activity (Fig. 2).

Each time a tagged bee is detected, the camera takes a picture of the entrance and the image is saved on the computer. The image was analysed in real time by software designed in our laboratory (patent number: IDDN.FR.001.140013.000.R.P.2013.000.31235, INRA, 2013). Running in LabView (National Instruments, Nanterre, France), the software was programmed to recognize the colour and number of the different tags with maximum sensitivity. The frequency of images captured per second was also adjusted to capture all tagged bees at the entrance. Bee ID (number and colour of the tag), direction (in or out of the hive) and time (day, hours, minutes and seconds) were continuously recorded in EXCEL files until the end of the experiment. If the software could not properly identify a tag, the bee was identified with a question mark in the file. It was then possible to retrieve the corresponding picture and manually assign the bee ID. At the end of the experiment, the data were sorted to retrieve for each bee: the age at the first flight, the total duration of flight activity (number of days from the first to the last exit), the total number of exits over the 35 days, the number and duration of daily flights, and the total amount of time spent outside the colony each day (sum of all flight durations per day).

In order to determine the rate of survival during the 35 days of the experiment, the day of the last exit was considered to be the day of the bee's death. In parallel with the field experiment, control and challenged bees (*Nosema*, pin prick), originating from the same batch used for recording flight activity, were reared in cages (30 bees per cage and $N=10$ cages per treatment). Cages were placed in an incubator (34°C and 50–70% humidity) and bees were provided with honey, pollen and water *ad libitum*. Thus, those bees received exactly the same treatments but different rearing conditions. Dead bees were counted daily and were removed from the cages until day 35.

Statistical analysis

Data were analysed using the statistical software R version 3.0.2 (R Development Core Team, 2009). The influence of parasitic and immune challenges on gene expression was assayed using one-way ANOVA tests followed by Tukey's HSD *post hoc* tests.

Variation in different flight activity parameters due to parasitic and immune challenges were analysed using GLMMs fitted by maximum likelihood using the lme4 package (Bates et al., 2013). Variations in age at the first flight, duration of activity (number of days from the first to the last exit), total number of exits over the 35 days and number of exits per day were fitted with a Poisson error distribution with a log link function using the glmer function. Duration of each flight and total flight activity per day were fitted with a Gaussian error distribution using the lmer function. *P*-values for the *t*-test of fixed terms of the linear mixed model were obtained through the lmer function of the lmerTest package (Kuznetsova et al., 2013). We selected the best model based on the corrected Akaike's information criterion (AICc) (Burnham and Anderson, 2002) using backward model selection from the full model. Variations in age at the first flight, duration of activity and total number of exits were analysed with treatment as a fixed factor and colony replicates as a random factor. Variations in the daily activity of each bee (number of flights, duration of each flight and total flight activity) were analysed with treatment and bee age as fixed factors, and colony replicates and bee identity as random factors.

Differences in survival were determined using a Cox proportional hazards regression model [coxph functions of the survival package (Therneau and Lumley, 2014)]. Data were transformed beforehand in a survival table and the remaining bees were considered alive at day 35. The same procedure was used for laboratory and field experiments.

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Competing interests

The authors declare no competing financial interests.

Author contributions

C.A., D.C. and Y.L.C. conceived the study and its experimental design. C.A., D.C. and S.C. conducted the experiments. C.A., M.P. and S.C. analysed the data, and C.A., D.C., M.P. and Y.L.C. wrote the manuscript. All authors read and approved the final manuscript.

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Supplementary material

Supplementary material available online at
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