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Effects, but no interactions, of ubiquitous pesticide and parasite stressors on honey bee (*Apis mellifera*) lifespan and behaviour in a colony environment

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Summary

Interactions between pesticides and parasites are believed to be responsible for increased mortality of honey bee (*Apis mellifera*) colonies in the northern hemisphere. Previous efforts have employed experimental approaches using small groups under laboratory conditions to investigate influence of these stressors on honey bee physiology and behaviour, although both the colony level and field conditions play a key role for eusocial honey bees. Here, we challenged honey bee workers under *in vivo* colony conditions with sublethal doses of the neonicotinoid thiacloprid, the miticide tau-fluvalinate and the endoparasite *Nosema ceranae*, to investigate potential effects on longevity and behaviour using observation hives. In contrast to previous laboratory studies, our results do not suggest interactions among stressors, but rather lone effects of pesticides and the parasite on mortality and behaviour, respectively. These effects appear to be weak due to different outcomes at the two study sites, thereby suggesting that the role of thiacloprid, tau-fluvalinate and *N. ceranae* and interactions among them may have been overemphasized. In the future, investigations into the effects of honey bee stressors should prioritize the use of colonies maintained under a variety of environmental conditions in order to obtain more biologically relevant data.

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Introduction

All living organisms are exposed to a broad array of environmental stressors, including pests, parasites and contaminants. Mortality represents the strongest and most defined index of effect (i.e. death); however, sublethal impacts affecting behaviour and physiology can also be measured (e.g. Marcogliese and Pietrock, 2011; Pettis *et al.*, 2012; Schneider *et al.*, 2012b). To obtain a thorough understanding of the effects of a particular stressor or combination of stressors, it is therefore crucial to examine multiple potential indices of effect.

The Western honey bee (*Apis mellifera*; hereafter honey bee) is a eusocial insect that can be used to investigate the environmental effects of parasites and pesticides due to its well-described natural history and ease of maintenance in an experimental setting. Additional interest in honey bee health has been stimulated by severe colony mortalities reported recently (Neumann and Carreck, 2010). The widely distributed ectoparasitic mite *Varroa destructor* has been identified as one important driver for colony losses (Genersch *et al.*, 2010; Le Conte *et al.*, 2010; Dietemann *et al.*, 2012); however, it appears that concurrent assaults by multiple other stressors likely have a large influence on colony survival (Potts *et al.*, 2010). While the detrimental consequences of stressor-driven mortality are apparent, the dimensions of the impact of sublethal effects on honey bee colonies are often less visible. Sublethal effects can comprise various parameters ranging from anatomical and physiological impairments to more complex processes such as orientation or foraging behaviour (e.g. Desneux *et al.*, 2007). The functioning of the colony superorganism as a unit depends heavily on the social behaviours among the individuals in the hive because the coordination of fundamental tasks in a colony (e.g. brood care, cleaning, foraging,

attending etc.) requires the transfer of relevant information among the members of the colony (Moritz and Southwick, 1992). Even though social in-hive behaviours are key for colony functioning, few studies have investigated potential stressor effects on social behaviour, despite data suggesting that stressors can influence other behaviours (e.g. foraging) (Schneider *et al*., 2012a; Dussaubat *et al*., 2013a).

The microsporidian *Nosema ceranae* is an obligatory intracellular midgut parasite that host-switched from the Eastern honey bee (*Apis cerana*) to the Western honey bee more than a decade ago (Paxton *et al*., 2007). It has since developed a nearly ubiquitous distribution worldwide (e.g. Klee *et al*., 2007; Williams *et al*., 2008; Giersch *et al*., 2009; Higes *et al*., 2009a; Invernizzi *et al*., 2009; Yoshiyama and Kimura, 2011). Despite numerous investigations of the impact of the parasite, its role in honey bee mortalities is highly debated (Fries, 2010; Higes *et al*., 2013). Whereas some studies did not detect increased individual bee or colony mortality (e.g. Invernizzi *et al*., 2009; Genersch *et al*., 2010; Williams *et al*., 2011; Dainat *et al*., 2012; Martin *et al*., 2013), others have reported lethal effects in the laboratory (Higes *et al*., 2007) as well as colony deaths (Martín-Hernández *et al*., 2007; Higes *et al*., 2008; 2009b). Observed sublethal effects of *N. ceranae* on individuals include host immune suppression (Antúnez *et al*., 2009), energetic stress (Mayack and Naug, 2009; 2010; Naug and Gibbs, 2009), as well as altered flight behaviour (Kralj and Fuchs, 2010; Dussaubat *et al*., 2013a) and pheromone production (Dussaubat *et al*., 2010). Numerous studies have demonstrated that parasites can alter the behaviour of infested honey bees (e.g. Wang and Mofller, 1970; Delfinado-Baker *et al*., 1992); however, none have investigated if *N. ceranae* affects social behaviour within a colony setting.

Pesticides, acting singly or in combination, can also affect non-target organisms such as solitary bees (Sandrock *et al*., 2014a), bumble bees (Fauser-Misslin *et al*., 2014) and honey bees (Bortolotti *et al*., 2003; Desneux *et al*., 2007; Aliouane *et al*., 2009; Wu *et al*., 2011; Henry *et al*., 2012; Sandrock *et al*., 2014b). Doses of pesticides that exceed a certain threshold level (depending on substance and type of exposure) affect the survival of exposed honey bees, while sublethal doses of pesticides can exhibit various effects on individual honey bees, including development, learning performance and orientation (Desneux *et al*., 2007; Blacquière *et al*., 2012). While many studies have investigated this kind of pesticide effects on honey bees, similar to *N. ceranae*, little is known about the potential impact of pesticides on honey bee social behaviour at the colony level. The neonicotinoid crop protection insecticide thiacloprid and the pyrethroid tau-fluvalinate are two pesticides widely applied to combat pest insects (Elbert *et al*., 2008) and *V. destructor* (Tsigouri *et al*., 2001), respectively. Residues of both substances are common in bee hive matrices; thiacloprid in honey (Tanner and Czerwenka, 2011), bee bread (Genersch *et al*., 2010), nectar and pollen (Pohorecka *et al*., 2012), and tau-fluvalinate in beeswax and pollen (Chauzat and Faucon, 2007; Mullin *et al*., 2010). Thiacloprid is of relatively low toxicity to bees (oral $LD_{50} = 17.32\ \mu g\ bee^{-1}$) versus other neonicotinoids, and can act synergistically with *N. ceranae* to kill honey bees in the laboratory (Vidau *et al*., 2011; Retschnig *et al*., 2014a). Tau-fluvalinate has an acute contact toxicity of $0.2\ g\ \mu g\ bee^{-1}$, but was reported to have no lethal effect at daily oral doses of 5 or $10\ \mu g\ bee^{-1}$ (Decourtye *et al*., 2005). However, it was shown to promote honey bee mortality in the presence of the miticide coumaphos (Johnson *et al*., 2009) as well as influence honey bee locomotion (Teeters *et al*., 2012). Although combined effects of tau-fluvalinate and any neonicotinoid have not yet been investigated in honey bees, exposure of bumble bees to a similar combination of pesticides (i.e. a neonicotinoid and a pyrethroid) increased worker mortality and impaired foraging behaviour (Gill *et al*., 2012).

The simultaneous exposure to a combination of parasites and pesticides can lead to interactions between the stressors in the host and can cause increased host mortality or various sublethal effects (Marcogliese and Pietrock, 2011). For example, in honey bees, concurrent exposure to *N. ceranae* and certain neonicotinoid insecticides caused both lethal and sublethal effects (e.g. Alaux *et al*., 2010; Vidau *et al*., 2011; Aufauvre *et al*., 2012; Pettis *et al*., 2012). In the past, the investigation of specific mechanisms of stressor effects often took place in laboratory studies under standardized conditions (e.g. Alaux *et al*., 2010; Forsgren and Fries, 2010; Aufauvre *et al*., 2012), which allowed for the control of potentially interferring factors (Williams *et al*., 2013). However, it remains unclear to what extent such findings can be extrapolated to honey bees in the field. As demonstrated in previous investigations, the study arena (laboratory versus field) can have a strong influence on the physiological development (Maleszka *et al*., 2009) as well as measured stressor effects in individual bees, including interactive effects of pesticides on honey bee mortality (Schmuck *et al*., 2003). Naturally, laboratory studies focus on parameters that can be tested reliably in this particular study arena, including worker longevity and parasite intensity (e.g. Alaux *et al*., 2010; Vidau *et al*., 2011). However, some traits that are crucial for the functioning of the honey bee colony, such as the social in-hive behaviour of the workers, have received too little attention so far. Therefore, the primary objective of this study was to look at potential stressor effects on honey bee worker

longevity, the ultimate measure of stress impact, as well as on important behaviours among workers including antennation (communication), grooming (hygiene) and trophallaxis (nutrition), as well as flight activity (nutrition and hygiene) (Moritz and Southwick, 1992). Using observation hives in two locations, we investigated the lethal and sublethal effects of the widely applied pesticides thiacloprid and tau-fluvalinate, as well as the ubiquitous parasite *N. ceranae*, on individual honey bees that faced natural conditions. Experimental individuals were allocated to one of four treatment groups (control, pesticides, *N. ceranae*, and *N. ceranae* and pesticides); pesticide and *N. ceranae* exposure occurred during development and post-emergence, respectively. Due to previous reports of the effects of *N. ceranae* and pesticides on honey bee survival and behaviour (e.g. Alaux *et al.*, 2010; Kralj and Fuchs, 2010; Aufauvre *et al.*, 2012), we expected to observe a similar impact of these stressors and anticipated to find stronger effects on individuals that were exposed to the combination of both *N. ceranae* and pesticides due to potential synergistic interactions (Alaux *et al.*, 2010; Vidau *et al.*, 2011; Aufauvre *et al.*, 2012; Pettis *et al.*, 2012).

Results

Mortality

Location A. Honey bee workers exposed to pesticides during development showed significantly higher mortality than did control individuals during the 14 day trial (Kaplan–Meier, log-rank test with Bonferroni correction, both *P*s = 0.0006, Fig. 1). No such significant difference

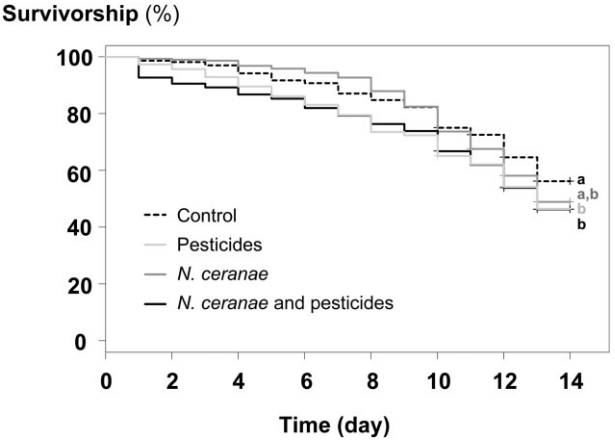

Fig. 1. Kaplan–Meier survival curve of the experimental honey bee (*Apis mellifera*) workers at location A (Switzerland). Workers that were exposed to pesticides (thiacloprid and tau-fluvalinate) during development showed significantly higher mortality than the control group (log-rank test with Bonferroni correction, both *P*s = 0.0006). Significant differences between treatments are marked with different letters (a, b).

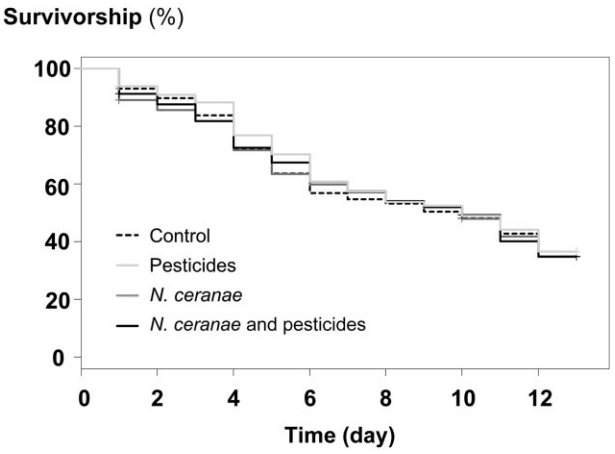

Fig. 2. Kaplan–Meier survival curve of the experimental honey bee workers at location B (Germany). No differences in mortality were observed between the investigated treatment groups (log-rank test with Bonferroni correction, all *P*s =1).

was observed between control workers and those belonging to the *N. ceranae*-only treatment group (Kaplan–Meier, log-rank test with Bonferroni correction, $P = 0.3$). Similarly, no significant difference in mortality occurred among the non-control treatment workers (pesticides versus *N. ceranae*, pesticides versus *N. ceranae* and pesticides, and *N. ceranae* versus *N. ceranae* and pesticides, Kaplan–Meier, log-rank test with Bonferroni correction, $P = 0.19$; 1; 0.23). Mortality, when compared using only data at termination day, was similar to survival analyses that incorporated daily mortality; workers exposed to pesticides showed significantly higher mortality compared with control individuals, and no significant difference was observed among non-control treatment individuals (binary logistic regression with Bonferroni correction, pesticides groups versus control, both *P*s < 0.012, for all other comparisons *P*s > 0.186).

Location B. No significant difference in mortality was observed among treatments when daily deaths (Kaplan–Meier, log-rank test with Bonferroni correction, all *P*s = 1, Fig. 2), or total death number at experiment termination (binary logistic regression with Bonferroni correction, all *P*s = 1) were considered.

Comparison of mortality between locations A and B. In all treatment groups, the workers showed significantly higher mortality in location B compared with location A (log-rank test, all *P*s < 0.001, Table 1, Figs. 1 and 2).

Behaviour

Location A – In-hive behaviour. A total of 22147 individual behaviours were observed during 14 days (Fig. 3),

Table 1. Overview and comparison of the stressor impacts on honey bees (*Apis mellifera*) in locations A (Switzerland) and B (Germany).

	Mortality			Behaviour	
Treatments	Location A	Location B	Location A versus B	Location A	Location B
Control	–	–	Higher mortality in B ($P < 0.001$)	–	–
Pesticides	Higher mortality ($P < 0.001$)	–	Higher mortality in B ($P < 0.001$)	–	–
N. ceranae	–	–	Higher mortality in B ($P < 0.001$)	Higher inactivity ($P < 0.001$)	–
N. ceranae and pesticides	Higher mortality ($P < 0.001$)	–	Higher mortality in B ($P < 0.001$)	Higher inactivity ($P < 0.05$)	–

The absence of significant effects is marked as '–' in the table.

with frequency of observations of the three behavioural categories consistent for each treatment: other (including all behaviours except for social behaviours and motionlessness, such as walking, feeding, brood care, cleaning etc.) was observed most (total: 16280 events, 71.41–75.18% events per treatment), followed by motionless (total 3250 events, 13.09–16.37% events per treatment), and social (antennation total: 1458 events, 6.21–6.94% events per treatment; grooming total: 696 events, 3.0–3.29% events per treatment; trophallaxis total: 463 events, 1.99–2.26% events per treatment). For all possible combinations of behaviour comparisons ($n = 24$), only three showed significant differences (all Ps < 0.05); all others had P-values greater than 0.23 (multinomial logistic regression with False discovery rate (FDR) correction, Fig. 3). Workers inoculated with *N. ceranae* (*N. ceranae*, and *N. ceranae* and Pesticides), regardless of pesticide exposure, were motionless more than control individuals (multinomial logistic regression with FDR correction, both $P < 0.016$). Additionally, workers exposed to *N. ceranae* only were motionless more than those exposed to pesticides only (multinomial logistic regression with FDR correction, $P = 0.0024$, Table 2 and Fig. 3).

Location B – Flight activity. There were no significant differences in flight activity, measured as number of flights per minute, among the three treatment groups (pesticides, *N. ceranae* or the combination of both) and the controls [analysis of variance (ANOVA) and Tukey–Kramer test, $P < 0.05$]. However, the *N. ceranae* treatment group showed significantly higher flight activity compared with the pesticides treatment group (ANOVA and Tukey–Kramer test, $P < 0.05$).

Treatment confirmation

Pesticides. Pesticide application to the donor colonies was confirmed by residue analyses of the respective chemical substances in the feeding solutions as well as of different hive matrices. Sucrose feed contained an average level of 611.5 ppb of thiacloprid in the treatment and no detectable thiacloprid residues in the control solutions. In the pesticide-treated colonies, thiacloprid residues were detected in honey (190 ppb), wax (147 ppb) and pollen (68 ppb), whereas tau-fluvalinate was found in wax (8280 ppb) and pollen (105 ppb). In the control colonies, traces of thiacloprid (7.7 ppb in honey, 34.2 ppb in wax and 3.6 ppb in pollen), but not tau-fluvalinate, was detected.

Nosema ceranae

Location A. Workers inoculated with *N. ceranae* showed mean spore amounts of 14.32×10^6 [standard deviation (SD): 5.73×10^6] for the *N. ceranae* only and 14.56×10^6 (SD: 6.31×10^6) for the *N. ceranae* and pesticides treatment group. There was no significant difference between the spore amounts of these two treatment groups (Wilcoxon rank-sum test, $P > 0.05$). Workers that were not inoculated with *N. ceranae* showed median spore

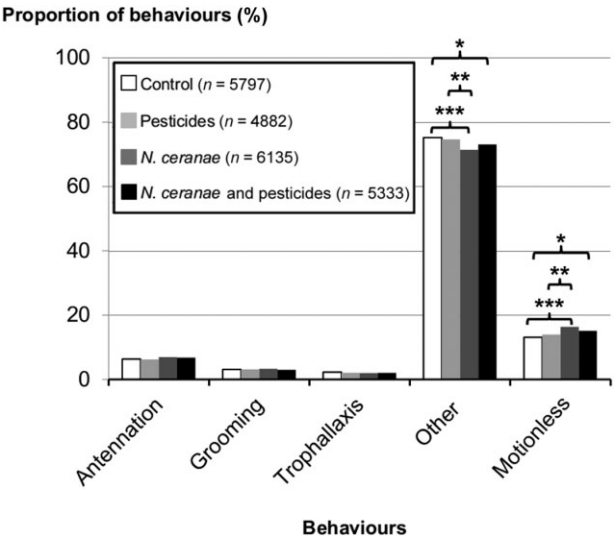

Fig. 3. Frequency of honey bee behaviours in the different treatments at location A (Switzerland). Significant differences among treatments were detected only between the behavioural categories being idle and other behaviours and are indicated with asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Table 2. Comparisons of behaviour ratios (reference: category 'other behaviours') in honey bees (*Apis mellifera*) among pairs of treatments.

Compared treatments	Behaviour (Reference: other behaviours)	*P*-value with FDR correction
Control versus pesticides	Motionless	0.5573
	Antennation	0.9698
	Grooming	0.9698
	Trophallaxis	0.8414
Control versus *N. ceranae*	Motionless	0.0000[a]
	Antennation	0.2304
	Grooming	0.6352
	Trophallaxis	0.8238
Control versus *N. ceranae* and pesticides	Motionless	0.0152[b]
	Antennation	0.5573
	Trophallaxis	0.8238
	Grooming	0.9698
Pesticides versus *N. ceranae* and pesticides	Motionless	0.2619
	Antennation	0.5573
	Grooming	0.9698
	Trophallaxis	0.9698
N. ceranae versus Pesticides	Motionless	0.0024[c]
	Antennation	0.2304
	Grooming	0.7272
	Trophallaxis	0.9698
N. ceranae versus *N. ceranae* and pesticides	Motionless	0.2304
	Antennation	0.8238
	Grooming	0.6155
	Trophallaxis	0.9698

a. Motionless was more frequent in the *N. ceranae* treatment group.
b. Motionless was more frequent in the *N. ceranae* and pesticides treatment group.
c. Motionless was more frequent in the *N. ceranae* treatment group.
Higher motionlessness was observed in three out of 24 treatment comparisons (in grey). Footnotes at the end of the table give more details about the direction of the outcome.

amounts (not normally distributed) of 0 spores per bee. However, 21 (35%) and 24 (40%) of the 60 workers analysed from the control and pesticides-only treatment groups were infected with *N. ceranae* at day 14. Mean spore counts in these workers were 8.73×10^6 spores per bee in the control and 10.8×10^6 spores per bee in the pesticides treatment group. Compared with inoculated workers, mean infection level in the non-inoculated individuals was significantly lower (ANOVA and Tukey–Kramer test, $P < 0.001$).

Location B. Mean spore counts of the inoculated workers were 2.93×10^6 (SD: 4.54×10^6) for the *N. ceranae* only and 2.33×10^6 (SD: 3.19×10^6) for the *N. ceranae* and pesticides treatment group. There was no significant difference between spore counts of these treatment groups (Wilcoxon rank-sum test, $P > 0.05$). Workers not inoculated with *N. ceranae* showed mean spore counts of 0.06×10^6 (SD: 0.25×10^6) spores per bee in the control and 0.014×10^6 (SD: 0.07×10^6) in the pesticides treatment group. Five control workers (8.62%) and three (5.45%) individuals of the pesticides-only treatment group were infected with *N. ceranae* at the end of the study with mean spore counts of 0.67×10^6 and 0.26×10^6, respectively. Compared with the *N. ceranae* inoculated workers, the mean infection level in those not fed *N. ceranae* was significantly lower (ANOVA and Tukey–Kramer test, $P < 0.01$).

Discussion

The data consistently revealed for both study locations no evidence of any interactions between parasite and pesticide stressors, as well as no effect of *N. ceranae* on worker mortality. However, overall worker mortality and the effect of pesticide exposure on mortality differed between the two locations. *Nosema ceranae* influenced in-hive activities by increasing frequency of motionless behaviour, but did not show an effect on flight activity.

The field-realistic approach of this study allowed for stressor exposure and collection of mortality and behavioural data under colony conditions. The vast majority of stressor-specific investigations are performed in the laboratory (e.g. Alaux *et al.*, 2010; Forsgren and Fries, 2010; Aufauvre *et al.*, 2012). Although this promotes a relatively controlled environment whereby potentially confounding factors can be more easily excluded (e.g. temperature, humidity, nutrition, etc.) (Williams *et al.*, 2013), results may not always reflect natural conditions because important features to honey bees, like eusociality, are not well represented (e.g. Mattila and Otis, 2006; Maleszka *et al.*, 2009; Retschnig *et al.*, 2014b). Alternatively, incidental exposure of experimental workers to *N. ceranae* and pesticides in colony-level studies is typically much greater than those used for laboratory assays. Similar to Wu and colleagues (2011), traces of pesticide residues were detected in control hives, possibly due to drifting bees or environmental contamination (e.g. Mullin *et al.*, 2010). Likewise, some control workers were infected with *N. ceranae*; this is not surprising as contaminated hive materials are believed to be major sources of *N. ceranae* infection (Higes *et al.*, 2008; Giersch *et al.*, 2009). The mean *N. ceranae* spore amounts of the respective treatment groups were in line with other studies that applied similar methods (e.g. Paxton *et al.*, 2007; Alaux *et al.*, 2010; Pettis *et al.*, 2012).

For both parasite and pesticide stressors, the effects on worker mortality were not consistent at the two study locations. Strong effects on honey bee health are usually highly reproducible, such as the considerable damage due to *V. destructor* parasitism (e.g. Liebig, 2001; Fries *et al.*, 2003; Rosenkranz *et al.*, 2010; Schäfer *et al.*, 2010). Inconsistencies of stressor effects in both locations suggest that they are rather weak. Regardless, pesticide exposure of immature workers increased mortality at the

adult stage in one study location, thereby supporting previous work that showed increased mortality of adults can occur when larvae are exposed to pesticides (Wu *et al*., 2011; Pettis *et al*., 2012; Berry *et al*., 2013).

Sublethal application of either tau-fluvalinate (Berry *et al*., 2013) or thiacloprid (Siede *et al*., 2014) on honey bee colonies did not reveal measurable effects on the population dynamics of bees or brood. Here we present the first approach to measure the combined application of these two pesticides at the colony level. In bumblebees, the combination of a neonicotinoid and pyrethroid was demonstrated to increase worker mortality (Gill *et al*., 2012); our study also observed this effect in honey bees in one location. In contrast to mortality, the data showed no evidence for an impact of the pesticides on the observed behaviours as has been shown in bumblebees (Gill *et al*., 2012). This could be explained because previous studies that demonstrated sublethal pesticide effects typically applied similar doses of pesticides that have a comparatively higher toxicity, such as clothianidin or imidacloprid (Schneider *et al*., 2012a; Teeters *et al*., 2012; Yang *et al*., 2012).

Nosema ceranae showed no effect on honey bee mortality at both locations. This is in line with a growing number of studies (e.g. Invernizzi *et al*., 2009; Genersch *et al*., 2010; Williams *et al*., 2011; Martin *et al*., 2013), but contrary to others (e.g. Higes *et al*., 2007; Martín-Hernández *et al*., 2007; Higes *et al*., 2008; Higes *et al*., 2009b; Williams *et al*. 2014). This may be explained by variable strains of *N. ceranae* exhibiting a different virulence or differential susceptibility of bees in different geographic regions (Dussaubat *et al*., 2013b). A further reason for the different outcomes may be that the effect of *N. ceranae* on individual honey bee mortality has so far been tested in laboratory studies only, where the bees might have been influenced by more stressful conditions compared with a natural colony environment (e.g. Retschnig *et al*., 2014b). Although *N. ceranae* appeared to not influence flight activity at one location, the parasite reduced the overall activity of bees at the other location. This might be explained by the energetic stress caused by *N. ceranae* (Mayack and Naug, 2009; 2010; Naug and Gibbs, 2009).

In contrast to previously reported synergistic effects between neonicotinoid pesticides and *N. ceranae* (Alaux *et al*., 2010; Vidau *et al*., 2011; Aufauvre *et al*., 2012; Pettis *et al*., 2012), our data provided no such evidence. A potential explanation for this difference may be that previous studies were carried out under laboratory conditions. It is known that influence of stressors may differ depending on test arena (e.g. laboratory versus field) (Schmuck *et al*., 2003; Mattila and Otis, 2006), which may potentially be a consequence of a higher sensitivity due to the artificial conditions in the laboratory (e.g. Huang *et al*., 2014; Retschnig *et al*., 2014b). Experimental workers in the present study lived in a colony environment (i.e. natural hive composition including queen, workers and drones) where they could feed (pollen, bee bread, honey), socially interact and exit the hive.

The mortality of the experimental workers in the two study locations showed clear differences, and the significantly greater worker mortality at one location compared with the other was consistent for all treatments. The workers that remained geographically closer to their donor colonies showed an overall better survival. Although it is difficult to determine mechanisms for these differences due to experimental methods, potential reasons for the higher mortality in the second location may include factors such as genotype–environment interactions (e.g. Costa *et al*., 2012) or the transportation of the bees in the pupal stage (300 km) (Oldroyd, 2007; Pettis and Delaplane, 2010; Pirk *et al*., 2014). Such potential impacts should be considered in future studies and closely investigated to improve the investigation of honey bee stressors in natural conditions.

Experimental procedures

Study set-up

The study was performed in summer 2012 at two locations: location A: Bern, Switzerland; and location B: Stuttgart, Germany. Both locations employed experimental honey bee workers from the same donor colonies located in Bern, Switzerland. Four treatment groups: (i) control, (ii) pesticides (thiacloprid and tau-fluvalinate), (iii) *N. ceranae* and (iv) *N. ceranae* and pesticides were investigated for differences in survivorship (both locations), in-hive behaviour (location A) and flight activity (location B).

At location A, eight local European honey bee colonies (*A. mellifera*) headed by sister queens (hereafter called donor colonies for the experimental workers) were randomly assigned to either the pesticide or the non-pesticide treatment ($n = 4$ per group). For the pesticide treatments, thiacloprid and tau-fluvalinate were applied for 6 weeks to encompass two complete brood cycles prior to removal of workers for the experiments. Thiacloprid was administered weekly by supplying colonies with 1 kg of 1000 ppb of 98.0% thiacloprid (Dr. Ehrenstorfer GmbH, Germany) sucrose solution (72–73% Hostettler® syrup, Hostettler Spezialzucker AG, Switzerland) using an in-hive feeder; control workers were fed with sucrose solution only. Tau-fluvalinate was applied using two Apistan® strips (Vita [Europe] LTD, UK), each 0.8 g active substance, placed in the lower brood chamber of each colony according to the manufacturer's recommendations. To confirm exposure, the thiacloprid and control solutions, as well as honey, wax and pollen samples were collected and analysed for pesticide residues at the United States Department of Agriculture (USDA) National Science Laboratory, Gastonia, USA, using routine liquid chromatographic procedures (Mullin *et al*., 2010).

Two weeks prior to the start of data collection, three observation hives were set up using standard approaches

(Scheiner *et al*., 2013) in both locations A and B. Briefly, each observation hive was equipped with a mated egg-laying local queen of the same year and two stacked Zander frames containing ~ 2000 bees: one frame contained brood in various developmental stages and the other consisted of stored honey and empty cells.

To obtain age cohorts of workers for experiments, queens from the eight donor colonies were caged on an empty brood frame for 48 h. Prior to emergence, brood frames were transferred to the laboratory and maintained in frame holders in the dark at 34.5°C and ≥ 50% relative humidity in an incubator (Williams *et al*., 2013). For transport to location B, brood frames (1–2 per donor colony) containing age cohorts of workers within capped brood cells (i.e. pupae) were carefully added to the brood chambers of a full-size colony for the ~ 300 km journey by car. Frames were kept under the same conditions as described above upon arrival at the new site.

After emergence, workers (4752 in total) at both locations were randomly assigned to the appropriate treatment group, marked on the thorax using coloured number plates and paints (Marabu Brillant, Gerstaecker, Switzerland) and inoculated with either *N. ceranae* or control suspension using a group feeding approach (Fries *et al*., 2013). For this, workers were starved for approximately 2 h in disposable plastic cages (20 individuals per cage). For the *N. ceranae* inoculum, fresh spores were obtained from naturally infected foragers that were collected at the hive entrance of local colonies in both sites the day prior. Midguts were carefully extracted from the workers using forceps, crushed in water and then purified by multiple centrifugation runs at 5000 *g* (Fries *et al*., 2013). Spores were then quantified using light microscopy and a haemocytometer (Cantwell, 1970). Dilution of the suspension using 50% (w/v) sucrose solution yielded a final concentration of 2 000 000 spores per 1.5 ml, whereas the control solution consisted of only freshly prepared 50% (w/v) sucrose solution. Each disposable plastic cage was supplied with either 1.5 ml *N. ceranae* or control inoculum using a 2 ml microcentrifuge tube with a 2 mm diameter hole in bottom tip to allow feeding, thus providing each of the 20 workers per cage with ~ 100 000 spores. Feeding devices were filled with 50% (w/v) sucrose solution when the entire suspension was consumed during frequent checks; after 48 h, all devices were refilled completely. After the inoculation process, a total of 792 workers, 198 individuals per treatment per observation hive, were sprayed with sucrose solution and carefully inserted into the appropriate observation hive at night.

Location A

Mortality and behaviours of experimental honey bee workers was assessed by examining the observation hives twice daily, between 09h00–12h00 and 14h00–17h00, during 14 consecutive days. Order of observation hive viewing was rotated daily to avoid a potential bias of time. Observed behaviours were allocated to the following categories: (i) social interactions between adults (i.e. antennation, trophallaxis and grooming), (ii) motionless (i.e. individual is not moving) and (iii) other (i.e. performing any task not included in the previous categories, see Scheiner *et al*., 2013). Social behaviours of experimental workers with two or more other individuals were defined as the following: antennation (contact of the moving antennae), trophallaxis (exchange of food) and grooming (cleaning manipulation using the mouthparts and antennae).

At day 14, all surviving workers were carefully collected using forceps from observation hive frames and immediately frozen at –20°C. To ensure maximum recovery of marked workers, multiple collection attempts occurred during day and night. A subsample of 20 collected workers per treatment group per observation hive was used to determine *N. ceranae* infection levels. This was achieved by homogenizing each individual in a 2 ml Eppendorf tube using a bead mill homogenizer (MM300 Retsch), one metal bead and 1 ml of nuclease-free water. *Nosema ceranae* quantification was performed according previously mentioned techniques.

Location B

Similar to location A, mortality at location B was determined daily by recording all of the marked workers. Flight activity observations occurred between day 7 post-insertion of the marked workers until day 13, when the experiment was terminated. Departing and returning workers were viewed through a 10 cm long transparent plastic tube connecting the colony to the outdoors. Workers surviving to day 13 were collected according to previously discussed methods for location A. Similarly, a subsample of 16–28 workers, depending on number of available bees after collection, per treatment group and observation hive was used to determine *N. ceranae* infection levels. This was achieved by pressing out the midgut content by gently squeezing the abdomen of each individual. The gut suspension was viewed using light microscopy and a haemocytometer according to Cantwell (1970).

Statistics

Differences in survival of experimental workers during the study were tested using Kaplan–Meier survival statistics with the log-rank test (Mantel–Haenszel test) and Bonferroni correction, whereas survival at experiment termination was tested using binary logistic regression using tests that are based on the standard normal z-statistic (Wald statistic). For these analyses, workers collected at the end of the experiment were considered censored, as were those observed but not collected on the final day. Furthermore, workers that disappeared during the experiment were considered dead on the last day they were seen. Differences in survival of the workers between the two locations were analysed using the log-rank test. Comparison of social interactions between adults, motionless and other behaviours among treatments were performed using multinomial logistic regression with *P*-values deduced from Wald statistics using the category 'other behaviours' as a reference. Thus, the ratio of one specific behaviour versus other was compared between two treatment groups for each case. FDR correction was applied to compensate for multiple comparisons (Benjamini and Hochberg, 1995). Flight activity was compared using repeated measures ANOVA. *Nosema ceranae* data were

analysed using Kruskal–Wallis one-way ANOVA, because of non-normal data distribution, followed by the Tukey–Kramer multiple comparison tests. All statistical analyses were carried out using the programmes SYSTAT 13 (Systat Software, USA), R (version 3.0.0., The R Foundation for statistical computing platform) and NCSS (version 8, NCSS LLC, USA).

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