Neonicotinoids override a parasite exposure impact on hibernation success of a key bumblebee pollinator

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Abstract. 1. Seasonal adaptations enabling the bridging of periodic challenging abiotic conditions are taxonomically widespread. However, sensitivity to other environmental stresses can be heightened during these periods.

2. Several temperate insects with over-wintering strategies play key ecosystem and economic roles, including wild bee pollinators. For example, hibernation survival in temperate bumblebees is crucial, as only new queens of future social colonies over-winter. These bees are also faced with other abiotic and biotic stressors, some of which have been linked to recent pollinator declines, such as exposure to pesticides and parasites.

3. Using a fully crossed experiment, the influence of dietary exposure to neonicotinoid insecticides (thiamethoxam and clothianidin) and/or the prevalent bumblebee parasite *Crithidia bombi* on hibernation survival and hibernation weight change of *Bombus terrestris* bumblebee queens was investigated.

4. Both neonicotinoid and *C. bombi* exposures reduced hibernation success individually, but no additive or synergistic effects between the stressors were found. Further, effects were asynchronous, with early neonicotinoid effects on hibernation mortality overriding later parasite effects under combined exposures. Neonicotinoid exposure also increased hibernation weight loss of surviving queens.

5. Diapause periods, employed by numerous temperate organisms, are likely to be especially vulnerable to environmental stresses, besides the seasonal challenge for which these periods are an adaptation. Thus, diapause requires inclusion during the consideration of the impacts of such stresses. Accordingly, it is demonstrated here that naturally relevant exposures of pesticides and parasites have important detrimental effects on bumblebees during a critical hibernation period, with potential consequences for populations of these key wild pollinators.

Key words. Diapause, hymenoptera, multiple stresses, pollinator health, trypanosome, virulence.

Introduction

The vast majority of habitats experience seasonal fluctuations in numerous abiotic factors, creating a challenge to the survival of numerous organisms that live in these environments. Many species have evolved a plethora of adaptations that enable them to overcome difficult seasonal periods (e.g. Arctic winters; Danks, 2004). However, frequently these periods are still a considerable hurdle and represent a time of heightened susceptibility to detrimental effects that result from exposure to other stressful environmental factors. For example, diapause periods (including hibernation), exhibited by the majority of temperate insect species (Tauber & Tauber, 1976), place organisms under a tight energy budget, where they are at the mercy of perturbations by external stressors (Hahn & Denlinger, 2011).

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Winter hibernation is an especially sensitive time for temperate bumblebee species (Beekman *et al.*, 1998). Although they are social insects, thus displaying colony-level superorganism resilience (Straub *et al.*, 2015), most temperate bumblebee colonies have an annual life cycle, and only mated daughter queens of these colonies persist in isolation over winter and found new colonies alone the next season (Goulson, 2003). This represents a precarious bottleneck, with the diapause resistance of these individual queens being critical to population viability (Straub *et al.*, 2015). Large stored fat reserves are built up by queens prior to hibernation and, as the queens do not feed over hibernation, are required to sustain metabolic processes (Beekman *et al.*, 1998), with the amount of reserves present likely associated with the probability of hibernation survival (Holm, 1972).

Pollinating insects, such as bumblebees, contribute significantly to agricultural productivity (Klein *et al.*, 2007; Aizen *et al.*, 2008; Garibaldi *et al.*, 2013) and ecosystem maintenance (Bascompte *et al.*, 2006; Fontaine *et al.*, 2006), and are thus crucial for food security and biodiversity. Recent reports on worldwide losses of managed colonies and declines of wild pollinators are therefore alarming (Neumann & Carreck, 2010; Potts *et al.*, 2010; Cameron *et al.*, 2011; Vanbergen *et al.*, 2013; Goulson *et al.*, 2015). Although they have very different life histories, various wild and managed pollinators are experiencing similar population declines (Potts *et al.*, 2010), suggesting common causal factors, even if sensitivities may vary by species (Arena & Sgolastra, 2014; Cresswell *et al.*, 2014; Lundin *et al.*, 2015). A variety of factors have been proposed to be related to population declines, including climate change, fragmentation and decline in habitat quality, pesticides in agroecosystems, and invasive or native parasites (Vanbergen *et al.*, 2013; Goulson *et al.*, 2015). Colony losses of managed honeybees and population losses of bumblebees and other important pollinating insects have been linked to parasites (Moritz *et al.*, 2005; Cameron *et al.*, 2011; Dainat *et al.*, 2012; Fürst *et al.*, 2014; Manley *et al.*, 2015). Parasites themselves can present a substantial threat, especially after host shifts (Woolhouse *et al.*, 2005; Fürst *et al.*, 2014), but the detrimental effects they precipitate on host individuals and populations may be exacerbated in the presence of other environmental stressors (Vanbergen *et al.*, 2013). Indeed, in parasites like the bumblebee infecting trypanosome *Crithidia bombi* (Lipa & Triggiani, 1988), observed virulence effects (Brown *et al.*, 2000; Brown *et al.*, 2003; Gegear *et al.*, 2006) can be greater under heightened additional stress, such as food deprivation. Therefore, other factors imposing stress on infected individuals, including during periods of predicted heightened vulnerability to mortality or reduction in condition (e.g. hibernation), may be particularly relevant to understanding the effects of parasites on individual fitness and population viability. Moreover, with these parasites not infecting every single individual within a population (Tognazzo *et al.*, 2012), additional stresses may increase detrimental effects seen in populations as a whole. Certain individuals may suffer from parasite infection, while others that are parasite-free may succumb to the effects of exposure to further environmental stresses.

A relevant additional factor touted as a major threat to pollinators is pesticide use for crop protection in agroecosystems (Desneux *et al.*, 2007; van der Sluijs *et al.*, 2013; Simon-Delso *et al.*, 2015). Non-target organisms can be exposed through multiple routes, but pollinators are particularly vulnerable to exposure during foraging on crops and other nearby flowering plants where trace residues of pesticides can be found (Desneux *et al.*, 2007; Blacquière *et al.*, 2012; Bonmatin *et al.*, 2015). Neonicotinoid insecticides, especially the widely used imidacloprid, thiamethoxam and clothianidin, are of particular concern for pollinators (Sanchez-Bayo & Goka, 2014; Bonmatin *et al.*, 2015). Exposure can be in the range of parts per billion in nectar and pollen (Sanchez-Bayo & Goka, 2014) and presents a season-long threat in temperate agroecosystems (Long & Krupke, 2016). Exposure of bumblebees to neonicotinoid trace residues have shown detrimental effects on numerous key traits of these social insects both in the laboratory and under semi-field conditions (Gill *et al.*, 2012; Whitehorn *et al.*, 2012; Larson *et al.*, 2013; Fauser-Misslin *et al.*, 2014; Gill & Raine, 2014; Scholer & Krischik, 2014; Goulson, 2015; Rundlöf *et al.*, 2015; Stanley *et al.*, 2015, 2016). As mentioned earlier, when multiple stresses (such as pesticides and parasites) are combined, the outcome for individual bees, colonies and populations is predicted to be negative (Bryden *et al.*, 2013; Vanbergen *et al.*, 2013; Goulson *et al.*, 2015). Indeed, studies supporting this idea are on the increase (Alaux *et al.*, 2010; Vidau *et al.*, 2011; Fauser-Misslin *et al.*, 2014; Retschnig *et al.*, 2014; Doublet *et al.*, 2015; but see Retschnig *et al.*, 2015), but significant knowledge gaps relating the effects of potential ecologically relevant threats to diverse pollinators persist (Lundin *et al.*, 2015).

The negative effects of individual stresses or combinations of these stresses may prevail particularly during periods of increased vulnerability, such as winter hibernation in temperate bumblebees. Therefore, the investigation of stresses, such as pesticides and parasites, during these periods is a worthy inclusion in assessment of the impacts of naturally relevant ecological stresses encountered by these bees. Survival of hibernation by bumblebee queens is fundamental to the maintenance of local populations, but, although this trait has been investigated with regard to parasite infection (Brown *et al.*, 2003), it has been overlooked with regard to studies of the multiple stresses of pesticides and parasites in concert. Hibernation for bumblebee queens represents a persistent stress of several months and any effects of pesticides and/or parasites could be amplified under such conditions. We conducted laboratory hibernation experiments with the bumblebee *Bombus terrestris* (Linnaeus, 1758) to investigate the impacts of sub-lethal doses of neonicotinoids (thiamethoxam and clothianidin) and exposure to an infective dose of the prevalent bumblebee parasite *C. bombi*. In addition to its primary application, clothianidin is a bioactive metabolite of thiamethoxam (Simon-Delso *et al.*, 2015). Both have been routinely used in agricultural settings, and in some regions, such as Britain, have exceeded imidacloprid in their application in more recent years (Simon-Delso *et al.*, 2015). Prior work on *B. terrestris* has demonstrated detrimental effects on the colony level of these two neonicotinoids (Fauser-Misslin *et al.*, 2014). We ask if and when these two important stresses of neonicotinoids and parasites influence laboratory hibernation survival and weight

loss, and whether any detrimental effects are altered, either positively or negatively, when the exposures are combined.

Materials and methods

Insects and pesticides

Bombus terrestris colonies were kept under red light at $28 \pm 2\,^{\circ}C$, with pollen and sugar water provided *ad libitum*. Both the sugar water and honeybee collected pollen sources were previously shown to be free of detectable levels of neonicotinoid pesticides (Fauser-Misslin *et al.*, 2014). The colonies had been initiated in the laboratory in spring using wild caught queens collected in northern Switzerland. Parasite-free status, concerning common gut-infecting parasites of bumblebees (e.g. *Nosema* and *Crithidia*), was confirmed through microscopic faecal checks of the original queen and subsequently produced workers upon colony foundation, at the stage of eight workers, and before experimental microcolony formation. Upon initiation of sexual production, taken as either the eclosion of the first adult male or the observable presence of gyne pupae, colonies were divided into two queen-less microcolonies, each containing 30 workers and equal amounts of brood, with remaining workers and the mother queen kept in the original colony with a small amount of brood. This design allowed for all treatments to be performed within each genotypic background unit of a colony. On a weekly basis, freshly produced brood in the original colony was distributed equally to the microcolonies. One of the microcolonies was randomly assigned to the neonicotinoid control group, receiving non-spiked nutrition, and the other one was provided with both the neonicotinoid thiamethoxam ($4\,\mu g\,kg^{-1}$, 4 ppb) and its major metabolite clothianidin ($1.5\,\mu g\,kg^{-1}$, 1.5 ppb) in sugar water and pollen patties following methods used previously (Fauser-Misslin *et al.*, 2014). To ensure that queens had been exposed to the neonicotinoid treatment for at least part of their larval development, queens emerging within 15 days of the onset of the microcolony and neonicotinoid treatment were discarded. With a pupal stage of approximately 15 days during development of bumblebee queens (Cnaani *et al.*, 2002), we could calculate the neonicotinoid exposure time in the colony of each individual. The median colony exposure time prior to pupation was 21 days (range 10–26 days). As queen larval development is in the range of 14–17 days for bumblebees (Cnaani *et al.*, 1997; Cnaani *et al.*, 2002), all individuals within the neonicotinoid exposure group were exposed in their microcolonies for the majority of their larval development. In addition, checks of subsequent downstream measurements of hibernation survival and pre-hibernation weight showed no significant effect of length of within-colony exposure (likelihood ratio tests: hibernation survival, $\chi_1^2 = 0.022$, $P = 0.883$; weight loss, $\chi_1^2 = 0.063$, $P = 0.803$), and thus individuals from microcolonies were considered as only exposed or unexposed to neonicotinoids in further analyses. Newly emerged adult queens were removed daily, separated in sister groups and fed *ad libitum*, including receiving the same neonicotinoid treatment as in their natal microcolonies. After adult emergence, queens typically stay within the natal colony before leaving to mate (Alford, 1975), thus making continued exposure to colony conditions a realistic scenario.

Matings and parasite infections

At 10 days after adult eclosion, queens were individually isolated and mated to unrelated males. Following mating, all queens were deprived of sugar water for 5–6 h and then presented with $10\,\mu l$ of sugar water alone (=parasite exposure control) or $10\,\mu l$ of sugar water containing *C. bombi* cells [20 000 cells from four different strains of equal proportions (5000 cells per strain), previously collected from northern Switzerland and cultured in the laboratory (Salathé *et al.*, 2012) (=parasite exposure treatment)]. The queens were observed to ensure that the sugar water was imbibed. After mating, young queens will forage before entering hibernation (Alford, 1975), an act that will put them at risk of parasite infection (Durrer & Schmid-Hempel, 1994). Measures of parasite infection were not taken due to logistical constraints relating to queens being hibernated before infections are typically seen in the faeces, and the additional stress that would have been placed on the queens as a result of faeces collection to check for transmitting parasites. Therefore, we subsequently refer to parasite exposure treatment rather than to infection. However, based on prior observations, it is likely that infections were established in the majority of *C. bombi*-exposed queens given the dose used (Brown *et al.*, 2003; B. M. Sadd, pers. obs.).

Queen hibernation

Five days after mating, queens were weighed to obtain their pre-hibernation weight and hibernated individually in plain cardboard matchboxes at $4\,^{\circ}C$ and 50% RH in continuous darkness for 4 months. The boxes of queens hibernated on the same day were placed together in containers in the hibernation chamber. At monthly intervals throughout hibernation, the containers were removed briefly (<5 min) and the queens checked for survival. Queens were considered dead if no movement of appendages was observed upon inspection. Individuals categorised as dead were left at room temperature to confirm this status. The status was confirmed in all cases. After the 4 months of hibernation, the same approach was used to assess survival to the end of the hibernation period, and surviving queens were immediately weighed to enable weight loss over hibernation to be calculated.

Analyses

Analyses were performed in R version 3.2.4 (R Development Core Team, 2016) using the lme4 package (Bates *et al.*, 2015). Queens were derived from colonies from two blocks (2011, 2012), with 96 and 268 queens from each block, respectively. Queens were only included from colonies where all treatments could be achieved, with seven and six original colony origins for 2011 and 2012, respectively, with a median of 25 queens per colony (range 4–60). Three queens died prior to pre-hibernation weighing and were thus removed from further analyses, leaving 361 queens. Generalised linear mixed models with binomial responses and logit link functions were fitted for overall hibernation survival and monthly survival throughout hibernation. Full

models were fitted with weight at hibernation ($\log_{10}$-transformed to meet assumptions), neonicotinoid exposure and parasite exposure, with interactions as fixed effects and queen colony of origin as a random effect. Adaptive Gauss–Hermite approximation (nAGQ = 15) was used for evaluating the log-likelihood (Lesaffre & Spiessens, 2001). Maximal models were simplified by sequentially eliminating non-significant terms through likelihood ratio tests (LRTs), and best-fitting models were chosen based on the Akaike information criterion (AIC) and explanatory power (Somers' D_{xy}). Explanatory variable significance was assessed using LRTs and a χ^2 distribution. Significant χ^2 values in the text are from minimal models, while non-significant values correspond to the value of the factor before removal. Confidence intervals for fixed effects were determined by bootstrapping (replicates = 1000). Proportions presented in the text are average marginal predicted probabilities across all samples in the data for the predictor of interest. To assess individual effects on overall hibernation survival as well, models constructed in a similar manner to those described earlier, and including parasite exposure or neonicotinoid exposure, were fitted to data excluding neonicotinoid exposure and parasite exposure groups, respectively. A linear mixed model was fitted by maximum likelihood for weight change over hibernation, with the response variable $\log_{10}$-transformed to meet assumptions, and model terms tested and removed as described earlier in the paper.

Results

Queen survival through hibernation

Over the 4-month hibernation period, 247 queens (68.42%) survived hibernation, with 25, 25, 24 and 40 queen deaths at the mortality checkpoints of 1, 2, 3 and 4 months of hibernation, respectively. A model including weight prior to hibernation ($\log_{10}$-transformed), *C. bombi* exposure, neonicotinoid exposure, and the interaction between *C. bombi* exposure and neonicotinoid exposure, with colony of origin as a random effect, was the best fit for the data of the survival of queens to the end of hibernation (Table 1). $\log_{10}$-transformed weight prior to hibernation had a significant effect on survival through hibernation (LRT: $\chi_1^2 = 29.88$, $P < 0.001$), with the probability of survival positively associated with weight on the initiation of hibernation. The interaction between parasite exposure and neonicotinoid exposure also had a significant effect (LRT: $\chi_1^2 = 7.58$, $P = 0.006$; Fig. 1). Average marginal predicted probabilities show that under control conditions, queens in the experimental population had a 0.84 probability of survival. This survival probability was reduced under *C. bombi* exposure (0.67) and neonicotinoid exposure (0.50), but the combination of the two does not lead to a further reduction (0.58) (Fig. 1).

Assessing the influence of parasite exposure in the absence of neonicotinoids, there was a significant effect of weight prior to hibernation ($\log_{10}$-transformed, LRT: $\chi_1^2 = 35.92$, $P < 0.001$) and *Crithidia* exposure (LRT: $\chi_1^2 = 8.77$, $P = 0.003$). Parasite exposure reduced survival probability by 0.15. In the non-parasite-exposed queens, once again weight before hibernation ($\log_{10}$-transformed) increased the probability of hibernation survival (LRT: $\chi_1^2 = 24.44$, $P < 0.001$), while exposure to neonicotinoids (LRT: $\chi_1^2 = 21.00$, $P < 0.001$) decreased it by 0.31.

Breaking down survival for each month of the 4-month hibernation period shows that the influences of parasite and neonicotinoid exposures are not synchronous (Table 1, Fig. 1). In all cases, greater weight prior to entering hibernation ($\log_{10}$-transformed) significantly or nearly significantly increased the probability of survival (LRT: month 1, $\chi_1^2 = 3.64$, $P = 0.056$; month 2, $\chi_1^2 = 31.28$, $P < 0.001$; month 3, $\chi_1^2 = 37.39$, $P < 0.001$). Month 1 and 2 survivals were reduced by neonicotinoid exposure (LRT: month 1, $\chi_1^2 = 5.20$, $P = 0.023$; month 2,

Table 1. Original (SE) and bootstrapped parameter estimates with confidence intervals (CIs) of fixed effects on survival in generalised linear models with binomial error distributions and logit link functions.

Response[†]	Parameter	Estimate (SE)	Bootstrap estimate	Lower 95% CI	Upper 95% CI
Full hibernation	Intercept	−25.26 (5.34)	−28.80	−46.58	−13.27
	$\log_{10}$(weight)	9.50 (1.88)	10.80	5.23	17.02
	Parasite	−1.08 (0.35)	−1.18	−2.31	−0.10
	Neonicotinoids	−1.91 (0.41)	−2.10	−3.32	−1.00
	Parasite × neonicotinoid	1.48 (0.54)	1.60	−0.15	3.48
Month 1	Intercept	−11.45 (7.75)	−9.67	−36.60	19.01
	$\log_{10}$(weight)	5.21 (2.72)	4.81	−3.85	14.44
	Neonicotinoids	−1.00 (0.44)	−1.04	−2.90	0.43
Month 2	Intercept	−31.24 (6.48)	−36.34	−66.91	−16.25
	$\log_{10}$(weight)	11.94 (2.32)	13.88	6.84	25.13
	Neonicotinoids	−1.29 (0.35)	−1.48	−3.11	−0.28
Month 3	Intercept	−30.69 (5.90)	−36.20	−64.88	−17.71
	$\log_{10}$(weight)	11.78 (2.10)	13.88	7.42	24.44
	Parasite	−1.22 (0.46)	−1.44	−3.01	−0.06
	Neonicotinoids	−2.22 (0.48)	−2.61	−4.73	−1.15
	Parasite × neonicotinoids	1.70 (0.63)	2.07	0.15	4.40

Queen colony of origin was included as a random effect in all analyses.
†Effects are presented for survival to the end of experimental hibernation (full hibernation), and for each prior month throughout hibernation. Only effects from the best-fitting models are given.
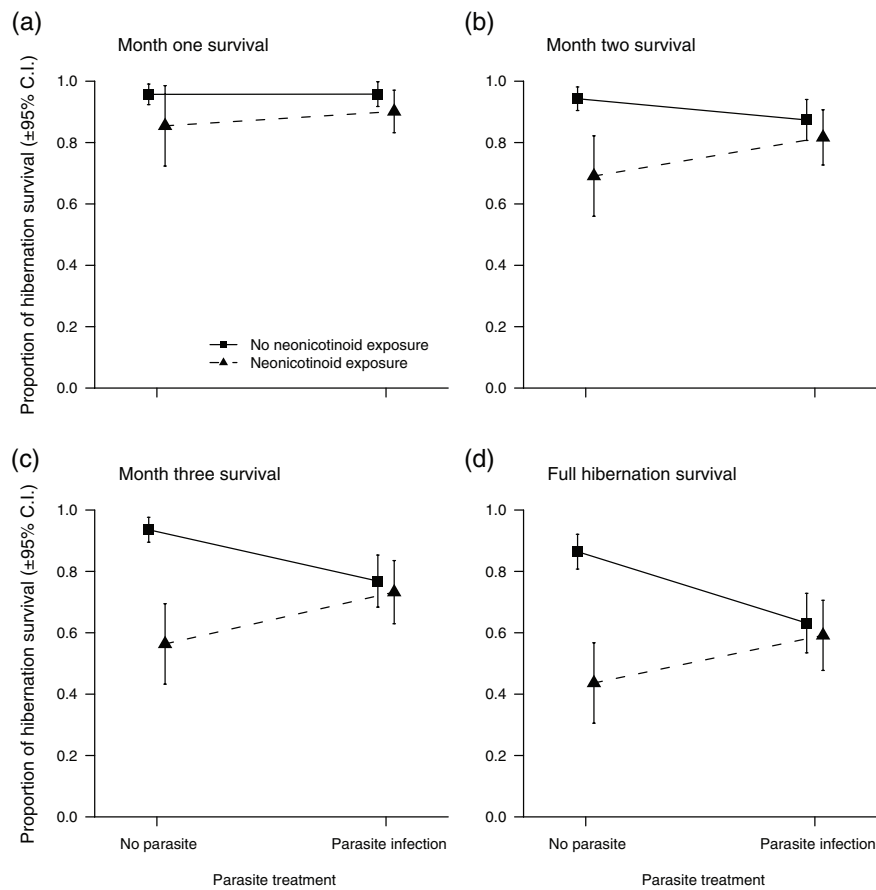
Fig. 1. Proportion of surviving *Bombus terrestris* queens after 1 (a), 2 (b), 3 (c) and 4 months (d) of experimental hibernation depending on infection with the trypanosome parasite *Crithidia bombi* and/or exposure to neonicotinoid pesticides (clothianidin and thiamethoxam) prior to hibernation. Plotted values are from the raw data (controls, $n = 140$; parasite-exposed, $n = 95$; neonicotinoid-exposed, $n = 55$; double-exposed, $n = 71$), although average marginal predicted probabilities from the fitted models (see text) show close agreement. Error bars represent 95% confidence intervals (CIs) based on a binomial distribution.

$\chi_1^2 = 14.03$, $P < 0.001$), with neonicotinoid exposure reducing survival probability from 0.96 to 0.89 at month 1 and from 0.92 to 0.78 at month 2. Parasite exposure, however, was not significant in predicting survival at month 1 or month 2 (LRT: month 1, $\chi_1^2 = 0.27$, $P = 0.605$; month 2, $\chi_1^2 = 0.00$, $P = 0.999$), nor was the interaction between parasite and neonicotinoid exposures (LRT: month 1, $\chi_1^2 = 0.01$, $P = 0.941$; month 2, $\chi_1^2 = 1.65$, $P = 0.200$). Survival at month 3 mirrored the pattern for overall hibernation survival, with the interaction between parasite and neonicotinoid exposures significantly influencing survival probability (LRT: $\chi_1^2 = 7.52$, $P = 0.006$). At month 3, marginal predicted survival probabilities were 0.92, 0.80, 0.64 and 0.72 for control, parasite exposed, neonicotinoid exposed and double-exposed queens, respectively.

Weight loss over hibernation of surviving queens

Post-hibernation mean ($\pm$SE) weights were 628 ($\pm$12), 577 ($\pm$17), 514 ($\pm$30) and 550 ($\pm$21) mg for control, parasite-exposed, neonicotinoid-exposed and double-exposed queens, respectively. These post-hibernation weights followed mean ($\pm$SE) weight losses over hibernation of 162 ($\pm$4), 171 ($\pm$8), 191 ($\pm$14) and 180 ($\pm$9) mg, for the respective groups. The best-fitting model for weight loss over hibernation included weight prior to hibernation (LRT: $\chi_1^2 = 4.47$, $P = 0.035$) and neonicotinoid exposure (LRT: $\chi_1^2 = 5.19$, $P = 0.023$). Weight loss was significantly greater for queens that were heavier on entry to hibernation and for those individuals exposed to neonicotinoids (Fig. 2). Neither parasite exposure (LRT: $\chi_1^2 = 0.13$, $P = 0.717$) nor the interaction between parasite and neonicotinoid exposures (LRT: $\chi_1^2 = 0.77$, $P = 0.380$) significantly affected the weight loss over hibernation of surviving queens.

Discussion

Numerous organisms will undergo periods in their life when they show increased vulnerability to external stresses. Perturbations from the norm may compromise not only individual survival, but also population viability if this period is ubiquitous and crucial for the life cycle. This is the case for hibernation in temperate
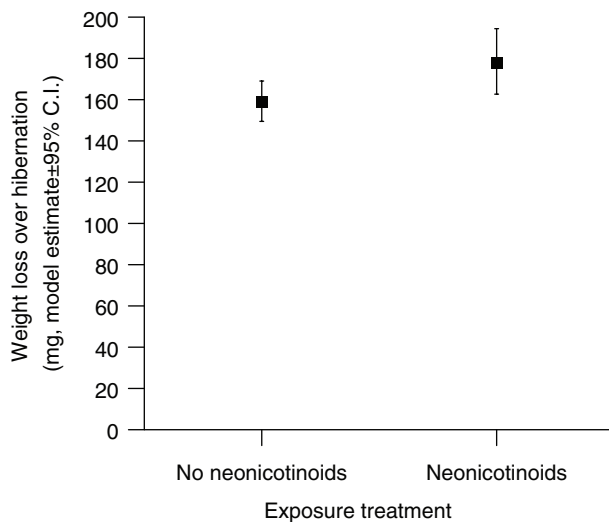
Fig. 2. The effect of neonicotinoid exposure on the weight loss over hibernation of surviving queens. Presented values are back-transformed effect estimates based on a linear mixed model fitted to $\log_{10}$(weight change) with $\log_{10}$(weight prior to hibernation) and neonicotinoid exposure as fixed effects and queen colony of origin as a random effect. CI, confidence interval.

bumblebee species. Exposure to the neonicotinoid pesticides thiamethoxam and clothianidin and exposure to the prevalent parasite *C. bombi* both significantly reduced hibernation survival of *B. terrestris* queens. However, effects were not synchronous, with an earlier neonicotinoid exposure effect in months 1 and 2 of hibernation, while a parasite exposure effect only became apparent later, in months 3 and 4 of hibernation. In the combined exposure treatment, there are no significant additive or synergistic effects of the exposures, with hibernation survival not reduced further relative to reductions resulting from the individual treatments. Therefore, under combined exposure, it appears that the earlier effects of neonicotinoid exposure override the later parasite effects. In addition, neonicotinoid exposure prior to hibernation increased weight loss in those queens that survived the hibernation period.

The effects shown here of neonicotinoid exposure prior to hibernation on bumblebee queens during hibernation are the consequence of a relatively short and realistic pesticide exposure and will compound with other individual and colony-level effects (Gill *et al.*, 2012; Whitehorn *et al.*, 2012; Larson *et al.*, 2013; Fauser-Misslin *et al.*, 2014; Gill & Raine, 2014; Scholer & Krischik, 2014; Goulson, 2015; Rundlöf *et al.*, 2015; Stanley *et al.*, 2015, 2016). There is a real potential for bumblebees to be exposed to widespread neonicotinoid pesticides under field conditions at the doses used, with concentrations in nectar and pollen measured in flowers and in storage within honeybee and bumblebee colonies around these levels or higher (Krupke *et al.*, 2012; Stoner & Eitzer, 2012; Sanchez-Bayo & Goka, 2014; David *et al.*, 2016). Although some authors have argued that neonicotinoid exposures will occur in very short pulses (Carreck & Ratnieks, 2014), studies of field levels suggest that bees may be chronically exposed throughout their lives (Sanchez-Bayo & Goka, 2014), with exposure being a season-long threat

(Long & Krupke, 2016). Queen bumblebees developing within colonies in agricultural areas are likely to be minimally exposed for periods consistent with the exposure in this study due to late-season seed-dressing applications leading to drift of neonicotinoids (David *et al.*, 2016), systemic neonicotinoids in certain late-flowering crop plants (e.g. winter squash and pumpkin; Stoner & Eitzer, 2012), and exposure when foraging on contaminated non-cultivated plants (Long & Krupke, 2016). The neonicotinoid pesticide effects on hibernation shown here are in agreement with results for solitary bee hibernation (Sandrock *et al.*, 2013) and over-wintering of honeybee colonies (Lu *et al.*, 2014), and support the idea that the proposed threat of pesticides to pollinators may be particularly acute during the passing of challenging environmental periods, such as hibernation.

Parasite exposure prior to queen hibernation had a detrimental impact on their survival; however, due to a lack of information on the infection status of the queens, it is not possible to accurately attribute this to parasite exposure alone or to an established parasite infection. However, given the dosage of *C. bombi* used, the establishment of an infection would be likely in the majority of individuals (>95%, B. M. Sadd, pers. obs.), especially given that a mixed parasite strain cocktail was given to the bees, which reduces the possibility of non-establishment due to specific host–parasite genotype × genotype interactions (Sadd & Barribeau, 2013). Parasite impacts on host survival and fitness under stressful conditions have been demonstrated in this and other systems (Schaub & Losch, 1989; Jaenike *et al.*, 1995; Brown *et al.*, 2000; Bedhomme *et al.*, 2005; Ryan & Kohler, 2010), but this case of reduced hibernation survival of *C. bombi*-exposed individuals is perhaps surprising. A previous study in the same host species showed no impact of infection on hibernation survival, only on colony founding (Brown *et al.*, 2003). Additionally, infection with the microsporidian parasite *Nosema bombi* had no effect on hibernation survival in *B. terrestris* (van der Steen, 2008). However, due to strong host–parasite genotype × genotype interactions in the bumblebee–*Crithidia* system (Schmid-Hempel, 2001; Mallon *et al.*, 2003; Schmid-Hempel & Reber Funk, 2004; Sadd & Barribeau, 2013), identities of interacting host and parasite populations will probably influence timing and magnitude of effects. The virulence effect shown in this particular study will intensify the overall effect of infection on bumblebee fitness together with previously reported effects on colony foundation (Brown *et al.*, 2003), worker foraging (Gegear *et al.*, 2006) and worker longevity (Brown *et al.*, 2000). In the same way as for pesticides, these effects will accumulate and contribute detrimentally to overall pollinator health and population viability.

The non-synchrony of the effects of the neonicotinoid pesticides and the parasite exposure suggests that their effects may have different underlying causes. The early hibernation effects of neonicotinoid exposure may be most parsimoniously explained by toxicity that is not necessarily linked to the energy reserves of the hibernating queens, although increased weight loss in neonicotinoid-exposed queens surviving hibernation suggests the potential for chronic resource-based costs over the longer term. These costs could be related to energy-sapping detoxification processes. The manifestation of parasite effects

later on during the hibernation period may be coupled with energy budgets of the queens, which are likely to be tight under situations such as diapause (Hahn & Denlinger, 2011). It is feasible that the decreased probability of hibernation survival of parasite-exposed queens was a result of a reduction in necessary resources. This could have resulted from the direct use of host resources by parasites (Brown *et al.*, 2003) or indirectly through a physiological cost of a host immune response directed towards the parasite. It is well established that immune responses are energetically costly (Sadd & Schmid-Hempel, 2009), and exposure to *C. bombi* has been shown to result in an up-regulation of immunity in bumblebee hosts (Barribeau *et al.*, 2014). Either the direct use of resources or a costly immune response could disturb the delicate balance of the host energy budget during hibernation. Alternatively, this temporal disparity could result from the fact that neonicotinoid pesticide exposure was initiated earlier in the lifetime of the queens than the parasite exposure.

That weight prior to hibernation influences survival in a positive manner is not unexpected and has been shown previously in this bumblebee species (Beekman *et al.*, 1998). However, we also demonstrate that, in addition to its influence on the survival of hibernating queens, exposure to neonicotinoids prior to hibernation results in greater weight loss over the hibernation period. This is probably due to a reduction in vital fat reserves of the queens and will place the queens in an increasingly vulnerable position. Under laboratory conditions, weight loss over hibernation and post-hibernation weight were not found to affect the initiation of colonies in *B. terrestris* (Beekman *et al.*, 1998), but under field conditions it is reasonable to expect that weight and fat reserves will be an important determinant of queen success, including survival and nest establishment post-hibernation. Additionally, the experimental hibernation period used in this study is shorter than a hibernation period of 6–9 months that many temperate bumblebee queens will experience (Alford, 1975; Goulson, 2003). Continuation of the weight loss pattern shown in this study will mean that neonicotinoid-exposed queens would sooner drop below the weight threshold that they need to remain above to survive (Beekman *et al.*, 1998).

The interaction of pesticides and parasites is of grave concern in relation to pollinator health (Vanbergen *et al.*, 2013). On an individual level, it appears that combined exposure to pesticides and parasites does not exacerbate detrimental impacts resulting from exposure to these factors alone. However, to gain a complete picture of the influence of multiple ecological stresses in nature, it is necessary to combine the effects seen on the individual level with the prevalence of exposure in the field to either one or both of these stresses. It is feasible that, on a population level, exposure to neonicotinoid pesticides could elevate losses of queens through reduced hibernation survival, above those losses that are attributed to parasites, if exposures to the pesticides and parasites are not ubiquitous or fully corresponding on the individual level. Although prevalent and ubiquitous on a population level, parasite infection is not pervasive across all individuals within populations. *Crithidia bombi*, for example, is found in 5–10% of spring queens in central Europe (Tognazzo *et al.*, 2012). The parasite exposure-imposed reduction in the probability of survival, as shown here, means this proportion is probably higher pre-hibernation, for which there are no data

available, but it will still not be universal. Thus, queens that would otherwise be free of the burden of the detrimental effects of parasite exposure could have hibernation survival reduced by exposure to neonicotinoids. We speculate that this will impose a greater cost for the population as a whole when neonicotinoids are present than if the threat is from parasite exposure alone.

In conclusion, parasite exposure, shown here for the trypanosome *C. bombi*, can reduce hibernation survival of bumblebee queens, and widespread pesticide use for crop protection will add greater pressure during this sensitive time of the bumblebee life cycle. The results further emphasise the vulnerability of organisms to perturbation by external abiotic and biotic stresses during strategic life-stage adaptions that are utilised by many organisms in response to the challenges imposed by seasonality.

Acknowledgements

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