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Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle

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Summary

Microbial pathogens are thought to have a profound impact on insect populations. Honey bees are suffering from elevated colony losses in the northern hemisphere possibly because of a variety of emergent microbial pathogens, with which pesticides may interact to exacerbate their impacts. To reveal such potential interactions, we administered at sublethal and field realistic doses one neonicotinoid pesticide (thiacloprid) and two common microbial pathogens, the invasive microsporidian *Nosema ceranae* and black queen cell virus (BQCV), individually to larval and adult honey bees in the laboratory. Through fully crossed experiments in which treatments were administered singly or in combination, we found an additive interaction between BQCV and thiacloprid on host larval survival likely because the pesticide significantly elevated viral loads. In adult bees, two synergistic interactions increased individual mortality: between *N. ceranae* and BQCV, and between *N. ceranae* and thiacloprid. The combination of two pathogens had a more profound effect on elevating adult mortality than *N. ceranae* plus thiacloprid. Common microbial pathogens appear to be major threats to honey bees, while sublethal doses of pesticide may enhance their deleterious effects on honey bee larvae and adults. It remains an open question as to whether these interactions can affect colony survival.

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Introduction

The ecosystem service of insect pollination is of great importance both for biodiversity through the pollination of wild plants and for human livelihoods through crop pollination (Potts *et al.*, 2010; Burkle *et al.*, 2013), with the global economic value of pollination estimated at US$215 billion in 2005 (Gallai *et al.*, 2009). Approximately 75% of crop plants are pollinated by insects, of which bees represent by far the most important group (Klein *et al.*, 2007). However, bees have suffered from recent declines in their populations in Europe and North America, particularly in the last decades; bumble bee community diversity has decreased (Cameron *et al.*, 2011; Bommarco *et al.*, 2012) and ranges of solitary bees have shrunken (Biesmeijer *et al.*, 2006; Bartomeus *et al.*, 2013), while honey bees (*Apis mellifera*), the most important commercially managed pollinator, have suffered from high colony mortality, including colony collapse disorder (CCD), overwinter or seasonal colony losses (Neumann and Carreck, 2010; vanEngelsdorp *et al.*, 2012).

Many factors are suspected to have a detrimental impact on pollinator health, including direct anthropogenic pressures (fragmentation of habitats, loss of foraging resources and the use of pesticides) as well as the spread of parasites and pathogens (Ratnieks and Carreck, 2010; González-Varo *et al.*, 2013; Vanbergen and The Insect Pollinators Initiative, 2013). Among these factors, sublethal doses of pesticides have recently been found to affect honey bee behaviour (Suchail *et al.*, 2001; Medrzycki *et al.*, 2003; Decourtye *et al.*, 2009; Williamson and Wright, 2013; Williamson *et al.*, 2013), foraging success (Yang *et al.*, 2008; Henry *et al.*, 2012; Schneider *et al.*, 2012), learning (Decourtye *et al.*, 2004; 2005; Aliouane *et al.*, 2009; Yang *et al.*, 2012; Frost *et al.*, 2013; Palmer *et al.*, 2013; Williamson and Wright, 2013) and colony development (Dai *et al.*, 2010; Wu *et al.*, 2011; Gill *et al.*, 2012; Whitehorn *et al.*, 2012; Elston *et al.*, 2013). Pesticides are now considered of high risk to bees and potentially one of the major causes of honey bee colony losses.

Another type of stressor, pathogens, also represents a major threat to bees. Among the large spectrum of bee pathogens, several are suspected to cause honey bee decline (Evans and Schwarz, 2011). These include multiple viruses (Bromenshenk *et al*., 2010; Evans and Schwarz, 2011; Dainat *et al*., 2012; Francis *et al*., 2013), and the microsporidian *Nosema ceranae* (Paxton, 2010; Higes *et al*., 2013), which infects gut epithelia of adult honey bees and was initially detected in the Asian honey bees *A. cerana* but is now globally distributed and invasive in *A. mellifera* (Klee *et al*., 2007; Paxton *et al*., 2007).

Although these stressors can potentially reduce the development, performance and survival of honey bee colonies, no single factor seems to account for all observed colony declines. Rather, it has been suggested that a combination of several biotic and abiotic stressors might be the cause of global pollinator decline, including honey bee declines and CCD (Potts *et al*., 2010; Ratnieks and Carreck, 2010; Vanbergen and The Insect Pollinators Initiative, 2013). Interactions among stressors remain largely uncharacterized but may be classified as: (i) antagonistic, when the effect of one factor reduces the effect of the second; (ii) additive, when several factors have cumulative effects; or (iii) synergistic, when several factors together have a greater effect than the sum of their individual effects (see also González-Varo *et al*., 2013).

Recent studies on honey bees have identified potential synergistic interactions between different stressors. The synergistic interaction between the parasitic mite *Varroa destructor* and several viruses that it transmits to honey bees has been clearly demonstrated and can increase mortality at the individual honey bee and colony levels (Nazzi *et al*., 2012; Francis *et al*., 2013). Combinations of pesticide have been shown to increase honey bee mortality and development (Pilling and Jepson, 1993; Johnson *et al*., 2009a; 2013; Wu *et al*., 2011). Additionally, pesticides have been suspected to increase pathogen burden in larval or adult honey bees (Locke *et al*., 2012; Pettis *et al*., 2012; Wu *et al*., 2012; Di Prisco *et al*., 2013), or to increase individual honey bee mortality (Alaux *et al*., 2010; Vidau *et al*., 2011). However, many of these studies suffer from being based on field observations in an uncontrolled environment of from using pesticide beyond field-realistic doses, and a direct, causal relationship between these factors and bee health remains uncertain.

Here, using a carefully controlled and fully crossed laboratory experimental design, we tested the combination of three common stressors at sublethal doses, one pesticide and two pathogens, in order to identify their potential interactions as well as their relative impact on individual survival across the life cycle of a honey bee worker from the larval to the adult stage, and their impact on pathogen load. As a pesticide, we used thiacloprid, a common systemic neonicotinoid insecticide applied worldwide on crops, vegetables and ornamental flowers, and considered only slightly toxic to bees (Iwasa *et al*., 2004; Laurino *et al*., 2011). As pathogens, we used the microsporidian *N. ceranae*, considered a possible cause of colony decline (Higes *et al*., 2008; 2009; Bromenshenk *et al*., 2010), and black queen cell virus (BQCV), a native and widespread bee virus that is known to reduce survival of queen pupae and has historically been associated with another honey bee pathogen, *N. apis*, but for which no obvious symptoms of viral disease have been observed when infecting larval and adult honey bee workers (Bailey and Ball, 1991; Chen and Siede, 2007). In addition, BQCV is thought to have increased in prevalence in recent years and has been found in colonies exhibiting CCD (Johnson *et al*., 2009b).

Results

Experiment 1: Interaction between BQCV and thiacloprid in host larvae

To test the interaction between sublethal doses of the insecticide thiacloprid (0.1 mg/kg of larval food) and BQCV [three doses: low (1.4×10^4 genome equivalents/larva), medium (1.4×10^7) and high (1.4×10^9), respectively, named $BQCV^4$, $BQCV^7$ and $BQCV^9$], honey bee larvae were reared artificially in the laboratory, and mortality was recorded on a regular basis.

BQCV fed on its own only caused significant mortality at the highest dosage ($BQCV^9$) at 6 days post-infection. The medium dosage ($BQCV^7$) caused a slight increase in mortality, which was observed much later in development, while the low dosage ($BQCV^4$) had no effect on mortality (Fig. 1A). From this dose-dependent mortality of larvae, the LD50 (median dose that induces 50% mortality) for BQCV was estimated at 1.53×10^8 genome equivalents [95% confidence intervals (CIs): $6.99 \times 10^7/1.35 \times 10^9$; Supporting Information Fig. S1]. As expected, since it was administrated at sublethal levels, thiacloprid fed on its own to larvae did not directly cause mortality, but it did elevate BQCV-induced mortality at all viral dosages (Fig. 1A).

Survivorship of all treatments was then converted to hazard ratios (instantaneous risk of death compared with the model average) for statistical analysis. The effect size of the interaction between the pesticide and the virus also showed dependence on the virus dosage (Fig. 1B). A significant higher mortality was observed in the treatment $BQCV^9$ + thiacloprid compared with the effect of the two stressors separately (coefficient contrast adjusted for multiple comparisons with FDR method; $Z = 6.265$, $P < 0.001$), suggesting a strong interaction between the two treatments, while no difference was observed for the medium ($Z = 1.512$, $P = 0.329$) and the lower ($Z = 1.103$,

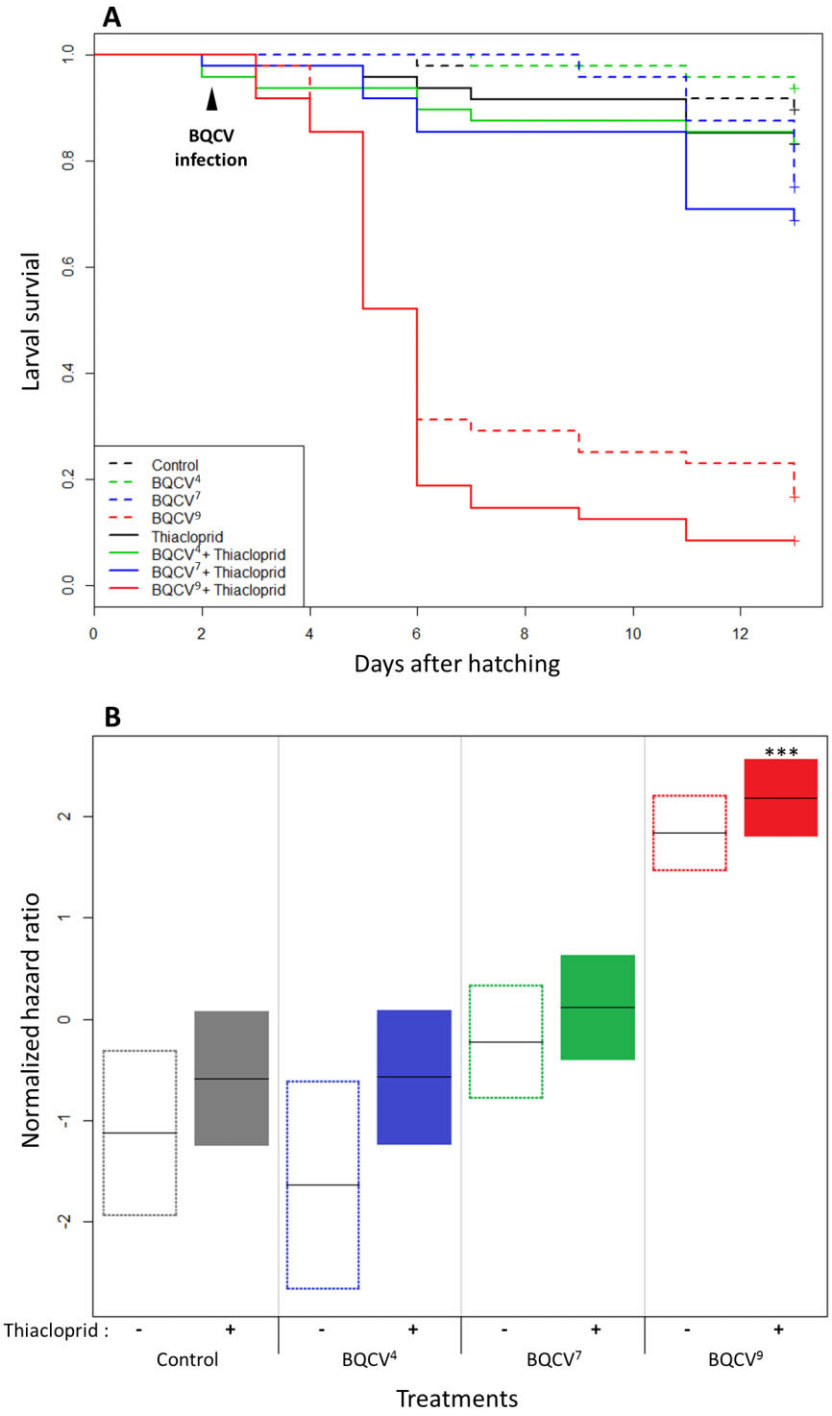

Fig. 1. Interaction between BQCV and thiacloprid in larval honey bees (Experiment 1).
A. Survival curves of larval worker honey bees treated with BQCV and thiacloprid, alone or in combination, and a control treatment. Three concentrations of BQCV were used: low ($BQCV^4$), medium ($BQCV^7$) and high ($BQCV^9$). Thiacloprid was fed continously (0.1 mg/kg) during larval development (first 5 days), while BQCV was fed at day 2 only. Dashed lines represent survival curves of the treatment without thiacloprid and solid lines represent treatments with thiacloprid. At the highest BQCV concentration, there appears to be an additive interaction between BQCV and thiacloprid on larval honey bee survival.
B. Instantaneous risk of death (hazard ratio, ± 95% CI) for larvae in each treatment compared with the model average of 0. Empty boxes represent treatment without pesticide, and full boxes represent treatment with pesticide. Grey, blue, green and red colours represent treatments with no virus, low, medium and high doses of virus. Three asterisks show the treatment $BQCV^9$ + thiacloprid, which induced a signicantly higher mortality than with $BQCV^9$ or thiacloprid when each was administrated seperataly.

$P = 0.592$) dosages of virus when combined with pesticide. No significant effect of colony of origin on mortality was observed (Cox proportional hazard mixed model: $\chi^2 = 0.3834$, df = 1, $P = 0.5358$).

The use of an alternative survival model where BQCV concentration was a four-level variable (null, low, medium and high), and thiacloprid a two-level variable (present or absent) showed that medium and high concentrations of BQCV and thiacloprid had a significant effect on larval mortality (Supporting Information Appendix S1). However, no interaction was observed between variables.

To test the impact of pesticide ingestion on pathogen growth in honey bee larvae, we quantified the BQCV load in pre-pupae that had been fed as larvae with the medium dose of virus ($BQCV^7$), either with or without thiacloprid, and compare these against control pre-pupae that had not

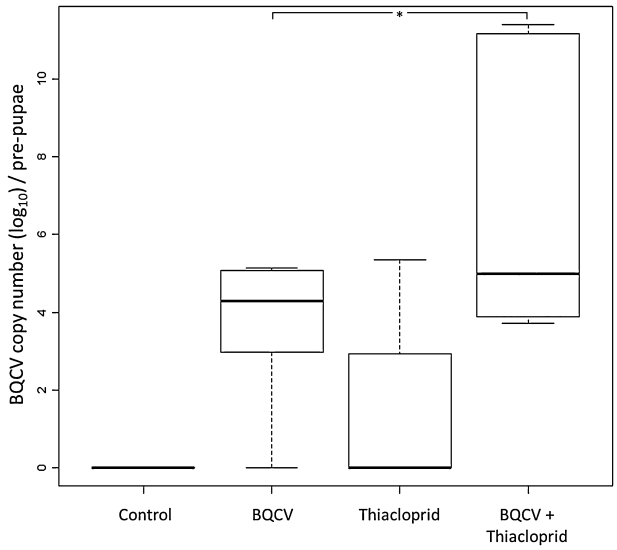

Fig. 2. Absolute quantification of BQCV (log10 transformed) in honey bee pre-pupae treated without virus and without thiacloprid (control), 1.4×10^7 BQCV only, thiacloprid only or both together. $n = 6$ pre-pupae for each treatment.

been fed BQCV as larvae (Fig. 2). Pre-pupae from the BQCV^7 + thiacloprid-feeding regime showed a significantly higher virus load (6.59×10^{10} genome equivalents; 95% CI $\pm$ 8.59×10^{10}) than pre-pupae from the BQCV^7 only regime (56 225 genome equivalents; 95% CI: $\pm$ 49 946; linear mixed model: $Z = -2.261$, $P = 0.0238$).

Experiment 2: Interaction between N. ceranae *and BQCV in adults*

The interaction between the pathogens *N. ceranae* and BQCV was tested in adult honey bees (larval honey bees are not known to suffer from *Nosema* infections). Both pathogens were administrated orally to workers honey bees maintained in small metal cages, alone or in combination, at doses that guaranteed infection of all individuals after 5 days: 10^5 *N. ceranae* spores and 1.4×10^9 BQCV genome equivalents. Nine days post-infection, a synergistic effect (i.e. more than additive) of co-infection was observed on survival: severe mortality of co-infected honey bees (Fig. 3A). At 11 days post-infection, 50% of the co-infected workers honey bees were dead, while only 20% of *N. ceranae*-infected bees and less 5% of BQCV-infected and control honey bees were dead. Survival analysis revealed a significantly higher mortality of adult honey bees with just *N. ceranae* ($Z = 2.07$, $P = 0.039$) and co-infected honey bees ($Z = 4.05$, $P < 0.001$) compared with non-infected control honey bees. Honey bees infected with BQCV alone did not die significantly faster than control bees ($Z = -0.50$, $P = 0.620$; Fig. 3B; Supporting Information Table S1). The rate of mortality of

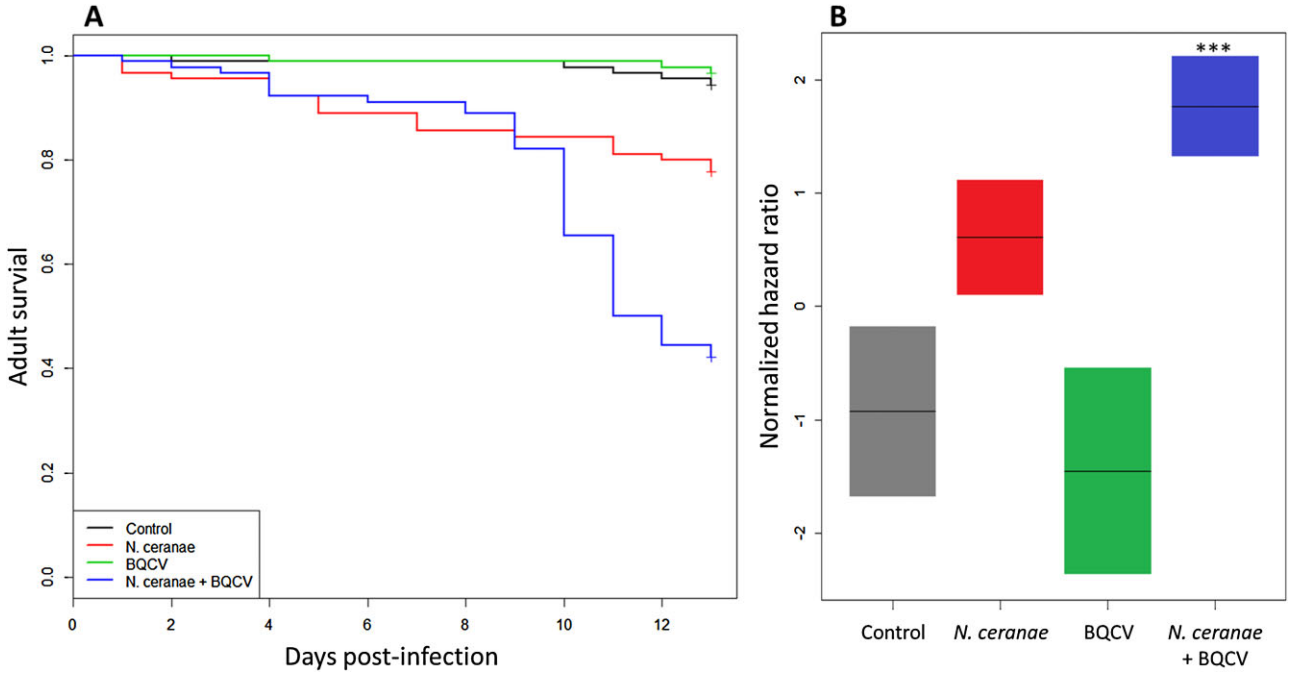

Fig. 3. Interaction between *N. ceranae* and BQCV in adult honey bees (Experiment 2).
A. Survival curves of adult worker honey bees treated with 10^5 *N. ceranae* spores, 1.4×10^9 BQCV or both (*N. ceranae* + BQCV), and a control solution. Pathogens were given once, at day 0. There appears to be a synergistic interaction between *N. ceranae* and BQCV on adult honey bee survival.
B. Instantaneous risk of death (hazard ratio, $\pm$ 95% CI) for adult honey bees in each treatment compared with the model average of 0. Three asterisks show the treatment *N. ceranae* + BQCV, which induced a significantly higher mortality than the two pathogens separately.

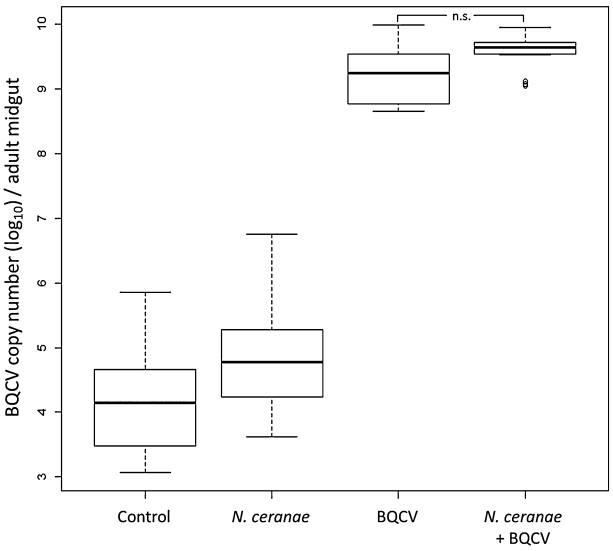

Fig. 4. Absolute quantification of BQCV (log10-transformed) in adult honey bee midguts from Experiment 2 (*N. ceranae* and BQCV). n.s., no significant difference was observed between the bees from the treatment BQCV only and *N. ceranae* + BQCV. $n = 18$ adults for each treatment.

co-infected bees was significantly higher than that of bees infected with the two pathogens separately (coefficient contrast adjusted with FDR method; $Z = 4.181$, $P < 0.001$), illustrating the synergistic interaction between the two pathogens when co-infecting a host.

No difference in the number of pathogens (copy number) was observed in the midgut between singly infected and co-infected honey bees at 13 days post-infection (Figs. 4 and 5). Although almost all bees (including controls) had BQCV in their midgut, there was a significant difference in the absolute quantification of the virus in the midgut across treatments (Fig. 4); both treatments incorporating a BQCV inoculation had a significantly higher virus load than control (linear mixed model; BQCV only: $t = 12.68$, $P < 0.001$; *N. ceranae* and BQCV: $t = 13.33$, $P < 0.001$), suggesting successful inoculation by the virus. Although co-infected bees had a higher BQCV load then bees treated with BQCV only, the difference was not significant ($Z = -0.838$, $P = 0.402$), with 4.59×10^9 (95% CI $\pm 1.1 \times 10^9$) and 2.84×10^9 (95% CI $\pm 1.5 \times 10^9$) BQCV genome equivalents respectively. Likewise, no difference was found in *N. ceranae* load between co-infected bees and bees infected with *N. ceranae* only (linear mixed model, overall effect of treatment: $\chi^2 = 0.5604$, df = 1, $P = 0.4541$; Fig. 5). Midguts of bees from the control and the BQCV only treatments were devoid of *Nosema* spores, indicating that our controls were uncontaminated.

Experiment 3: Interaction between N. ceranae*, BQCV and thiacloprid in adults*

The same design as in Experiment 2 was employed in Experiment 3 but with an additional experimental factor: the neonicotinoid insecticide thiacloprid. The pesticide was mixed at a concentration of 5 mg/kg in the 50% sucrose solution available *ad libitum*, thus resulting in chronic exposure across the duration of the experiment. Clear interactions were observed between *N. ceranae* and BQCV (Fig. 6C and E) and between *N. ceranae* and thiacloprid (Fig. 6A and E), but less pronounced between BQCV and thiacloprid (Fig. 6B and E). No extensive three ways interaction between the three stressors was observed (Fig. 6D and E). The survival analysis revealed three treatments with significant higher mortality than control honey bees: *N. ceranae* + BQCV ($Z = 2.50$, $P = 0.012$), *N. ceranae* + thiacloprid ($Z = 2.74$, $P = 0.006$) and *N. ceranae* + BQCV + thiacloprid ($Z = 2.79$, $P = 0.005$; Supporting Information Table S2).

To identify synergistic interactions between stressors, we compare the effect of the stressors in combination ('double treatments', e.g. '*N. ceranae* plus BQCV') with the effect of the stressors separately ('single' treatments, e.g. '*N. ceranae*' and 'BQCV'), as well as the effect of the three stressors in combination ('triple' treatment '*N. ceranae* + BQCV + thiacloprid') with the 'double' treatments, combining two stressors. No significant differences were observed (Supporting Information Table S3). However, using correction for multiple comparisons reduced the statistical power of analysis. Separately analysing each set of 'double' treatments, the co-infection treatment *N. ceranae* + BQCV showed a significantly

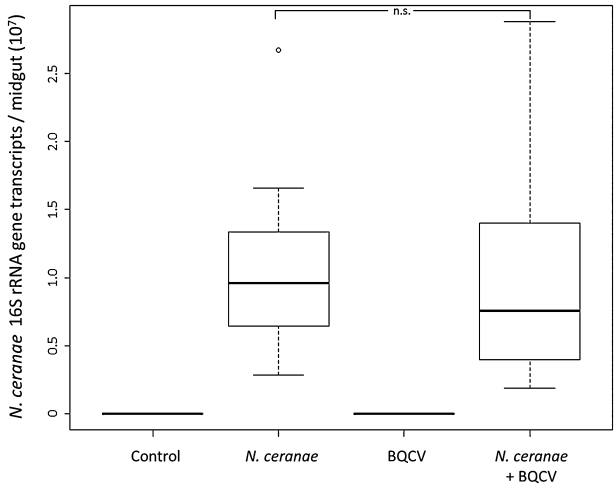

Fig. 5. Absolute quantification of *N. ceranae* 16S rRNA gene copies in adult honey bee midguts from Experiment 2 (*N. ceranae* and BQCV). n.s., no significant difference was observed between the bees from the treatment *N. ceranae* only and *N. ceranae* + BQCV. $n = 18$ adults for each treatment.

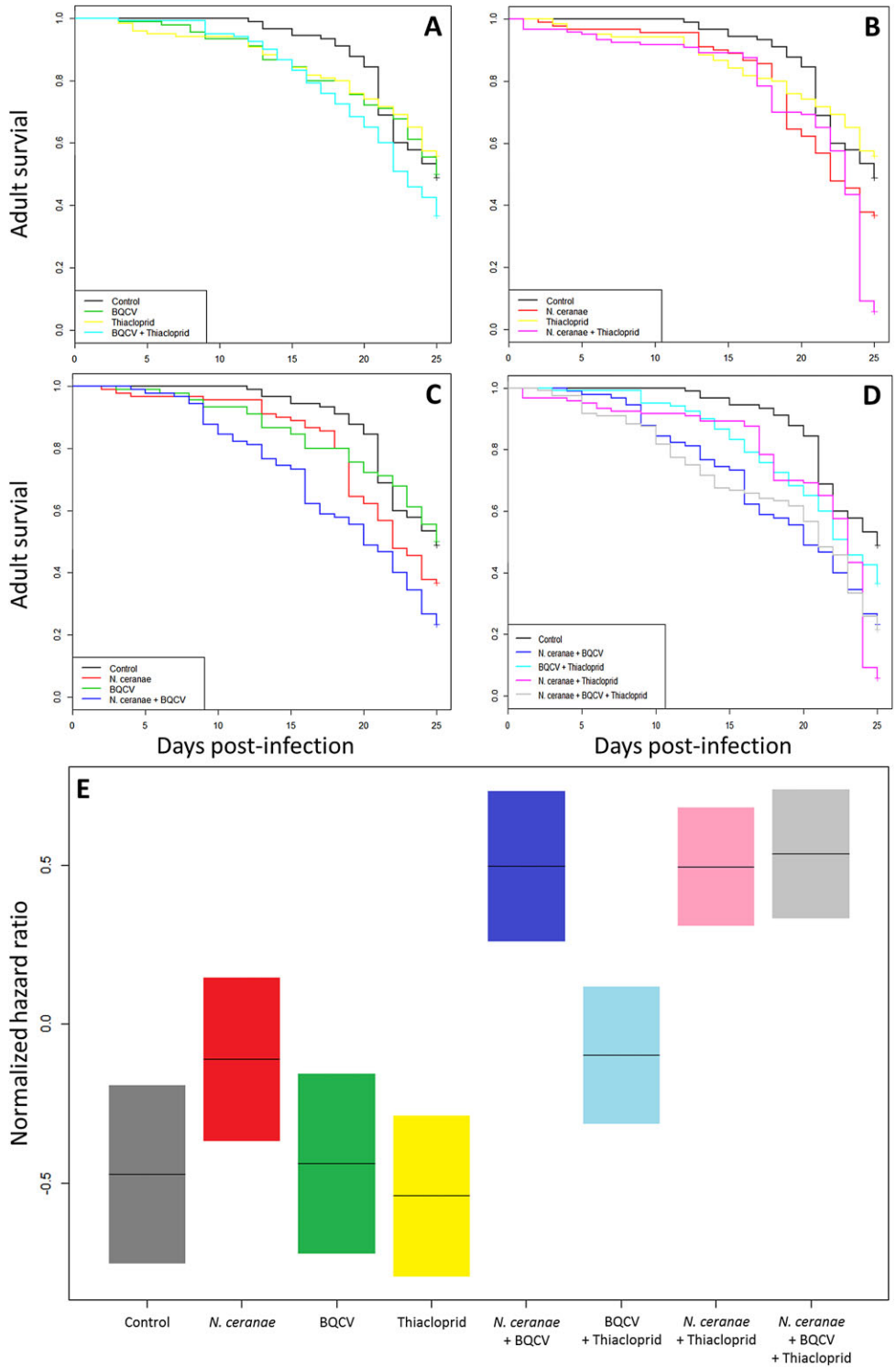

Fig. 6. Interaction between *N. ceranae*, BQCV and thiacloprid in adult honey bees (Experiment 3). Honey bees were treated with 10^5 *N. ceranae* spores, 1.4×10^9 BQCV and thiacloprid (0.1 mg/kg), alone or in combination, or a control solution. Pathogens were given once at day 0, while thiacloprid was fed continously across the experiment.
A. Survival curve of honey bees treated with BQCV and thiacloprid (alone or in combination).
B. Survival curve of honey bees treated with *N. ceranae* and thiacloprid.
C. Survival curve of honey bees treated with *N. ceranae* and BQCV.
D. Survival curve of honey bees treated with *N. ceranae*, BQCV and thiacloprid, in pariwise combination of two and all three together.
E. Instantaneous risk of death (hazard ratio, ± 95% CI) for adult honey bees in each treatment compared with the model average of 0.

Table 1. Top Cox proportional hazard models explaining individual adult honey bee mortality from Experiment 3 (*N. ceranae*, BQCV and thiacloprid), obtained from model selection.

Rank	Models	df	AIC	ΔAIC	Weight
1	*N. ceranae* + BQCV + (1|colony/cage)	4	6789.96	0.00	0.18
2	*N. ceranae* + (1|colony/cage)	3	6789.98	0.02	0.18
3	*N. ceranae* + thiacloprid + BQCV + (1|colony/cage)	5	6790.49	0.52	0.14
4	*N. ceranae* + thiacloprid + (1|colony/cage)	4	6790.65	0.68	0.13

Treatment/non-treatment with *N. ceranae*, BQCV and thiacloprid were used as fixed variables, while colony and cage were used as random variables. Models are ranked with increasing AIC. ΔAIC presents the difference between model 1 and the following models. Models with a weight less than half that of model 1 are excluded.

higher mortality than the two pathogens fed singly to adult honey bees ($Z = 2.247$, $P = 0.0246$), which is consistent with what we found in our Experiment 2. The other comparisons of 'double' versus 'single' treatments remained non-significant (Supporting Information Table S3).

Using the three stressors as a binary variable (present or absent) and applying model selection from the full model (the three stressors alone and all interactions), four models were identified as explaining adult honey bee mortality equally well (delta AIC < 1; Table 1). No interaction between presence and absence of stressors are in these four models, and all include the presence of *N. ceranae*, either alone or in combination with the other stressors. Thus, by ranking the three stressors in term of their impact on mortality, the most important is *N. ceranae*, then BQCV and third, thiacloprid (Table 2). The impacts of BQCV and thiacloprid on adult honey bee mortality appeared similar and highly variable, while *N. ceranae* had a more pronounced effect, which is significant on its own (Table 2).

The co-infection treatment *N. ceranae* + BQCV induced earlier mortality during the second week post-infection compared with the treatment *N. ceranae* + thiacloprid, which itself induced late mortality during the third week (Fig. 7). The triple treatment (*N. ceranae* + BQCV + thiacloprid) also induced early mortality, similar to the co-infection (*N. ceranae* + BQCV) treatment (Fig. 7).

Daily records of sugar consumption per treatment (per bee) showed no effect of either pathogen (linear mixed model, BQCV: $t = -1.833$, df = 18, $P = 0.0834$; *N. ceranae*: $t = -1.042$, df = 18, $P = 0.3114$), while the pesticide thiacloprid mixed into the sugar solution had a significant negative effect on sugar consumption ($t = -3.998$, df = 18, $P < 0.001$), with a decrease in median sugar consumption of 15% (median values of 40.1 and 34.0 µl/bee/day of sucrose solution for groups fed without and with thiacloprid respectively; Supporting Information Fig. S2). On average, adult honey bee workers from treatments including pesticides ingested 185 (± 4) ng thiacloprid per bee per day, similar to experiments conducted elsewhere (Vidau *et al*., 2011).

Experiments 2 and 3 both included infections of adult honey bees with *N. ceranae* and BQCV alone and in combination but were conducted during June and July

Table 2. Model-averaged coefficients of the three variables *N. ceranae*, BQCV and thiacloprid obtained from the model selection of the Cox proportional hazard models (see Table 1).

Variables	Estimates	SE (+/–)	*Z*	*P*
N. ceranae	0.77223	0.23117	3.341	< **0.001**
BQCV	0.28690	0.23726	1.209	0.2266
Thiacloprid	0.21586	0.24701	0.874	0.3822

P-value below 0.05 shown in bold.
Interactions between variables had low estimates of coefficients and thus are not shown here.
SE, standard error.

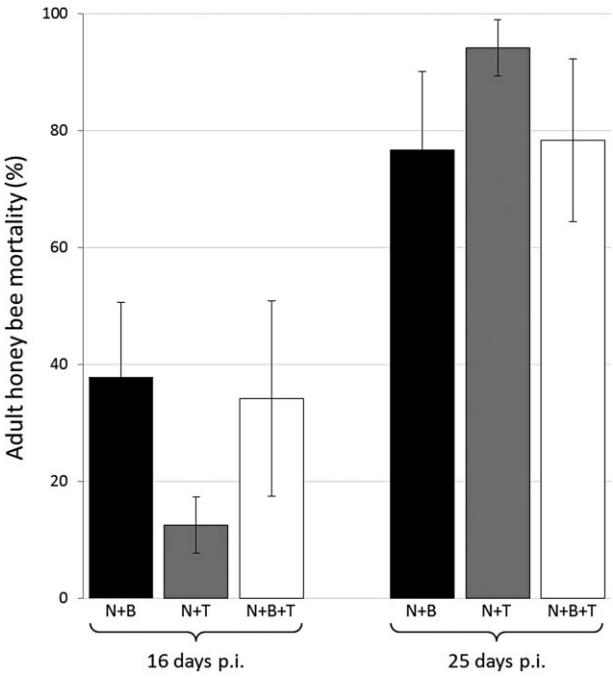

Fig. 7. Comparison of adult honey bee mortality (± SEM) induced at two time points (16 days and 25 days post-infection) of Experiment 3 by the three interaction treatments: *N. ceranae* + BQCV (N + B, in black), *N. ceranae* + thiacloprid (N + T, in grey) and *N. ceranae* + BQCV + thiacloprid (N + B + T, in white).

respectively. Because honey bees can respond differently to pathogens at different times of the year, the survival data from Experiments 2 and 3 were incorporated into a single analysis using 'season' as an additional fixed variable, together with the presence or absence of the two pathogens. This analysis revealed an interaction between *N. ceranae* and season ($Z = -4.51$, $P < 0.001$), reflecting higher mortality because of *N. ceranae* infection during Experiment 2, in June, than during Experiment 3, in July. No significant interaction was observed between BQCV and season ($Z = -0.67$, $P = 0.5$).

Discussion

We found that two common pathogens of the honey bee, *N. ceranae* and BQCV, act synergistically on adult honey bees and induce rapid mortality. The systemic neonicotinoid insecticide thiacloprid, when fed at a sublethal dose, can enhance the mortality of larval and adult honey bees induced by pathogens.

Interaction between BQCV and thiacloprid in larvae and adults

The infection of honey bee larvae with different quantities of BQCV *per os* led to a dose-dependent response on host mortality. Previous studies have reported asymptomatic inoculation of BQCV *per os* to larvae (Bailey and Woods, 1977). Our results confirm that honey bee larvae are resistant to relatively low doses of this virus but susceptible to high doses. Although a dose of 1.4×10^9 BQCV per larva may seem rather high, the number of RNA copies of a virus is most likely an overestimate of the number of infectious virus particles, as most of the viral RNA present at any one time in a cell (or an extract) will be unpackaged. Furthermore, given the amount of virus that can be detected in pollen and royal jelly (Chen *et al*., 2006; Cox-Foster *et al*., 2007; Singh *et al*., 2010), it is entirely possible for honey bee larvae to acquire such high doses of BQCV through cumulative chronic ingestion of virus *in vivo*.

Sublethal exposure to thiacloprid combined with BQCV infection revealed a significant impact of thiacloprid on larval mortality, suggesting an additive effect of thiacloprid over and beyond the effect of BQCV. The effect of thiacloprid was moreover greater when the virus dosage was higher. The interaction between the virus and the pesticide was particularly perceptible for the dose of 1.4×10^9 BQCV. One explanation for the increased mortality induced by the combination of the virus and the pesticide could be the higher virus titres induced by the pesticide observed in pre-pupae infected with the medium dose of virus and exposed to thiacloprid. According to a recent study, exposure to neonicotinoid insecticides as clothianidin or imidaclorpid decreases the expression of the regulation factor NF-κB, which control the honey bee antiviral defences (Di Prisco *et al*., 2013). At the colony level, a similar effect has recently been reported for deformed wing virus (DWV) infection of pupae from colonies exposed to tau-fluvalinate (an acaricide used in-hive to control Varroa mites), where the DWV levels increased briefly immediately following treatment compared with non-treated colonies, although this effect was not observed for BQCV and sacbrood virus (SBV) (Locke *et al*., 2012). In another study, no direct effects of various acaricide treatments applied to honey bee colonies were observed on virus load in adults, including BQCV (Boncristiani *et al*., 2012). Here, we show the direct effect of the neonicotinoid thiacloprid on BQCV multiplication in individual honey bee larvae, which might explain the observed elevated mortality.

The response of adult honey bees to high concentrations of BQCV was very different from that of larvae. While inoculation of 1.4×10^9 BQCV genome equivalents induced very high larval mortality, the same dose did not induce any significant mortality in adult honey bees. This might reflect a physiological difference between larvae and adults in tolerance to BQCV infection. Indeed, most honey bee pathogen have distinct windows of infectivity during the honey bee life cycle, with early brood and newly emerged adults often particularly susceptible (Bailey and Ball, 1991). In adult honey bees, the interaction of thiacloprid with BQCV had a less pronounced effect (Fig. 6A), which led to a non-significant increased mortality when fed simultaneously. This difference with the observation in larvae might also reflect different tolerance of the two stages to BQCV.

Interaction between N. ceranae *and thiacloprid in adults*

Adult honey bee workers infected with *N. ceranae* and additionally exposed to a sublethal dose of thiacloprid also showed increased mortality (Fig. 6B). We observed a late mortality of these honey bees compared with a *N. ceranae* only infection. This delayed effect of the interaction between *N. ceranae* and thiacloprid might reflect an accumulation of the neonicotinoid in the insect body, which eventually interacts with the microsporidia. The late onset of mortality of the bees infected with *N. ceranae* + thiacloprid in our study might also reflect the possible repellent effect of the pesticide, as honey bees fed with a sucrose solution contaminated with thiacloprid consumed significantly less food than bees provided sugar solution without pesticide. This repellent effect of the pesticide, also induced by other neonicotinoid insecticides (Ramirez-Romero *et al*., 2005), might have delayed the effect of pesticide exposure, thus underestimating the effect of the treatment *N. ceranae* + thiacloprid. Alternatively, the lower

food intake of honey bees treated with the pesticide might have accelerated their death. Although *N. ceranae* is generally thought to induce energetic stress in honey bees and to increase hunger (Mayack and Naug, 2009; Martín-Hernández *et al*., 2011), *Nosema*-infected workers in our experiment did not consumed more sugar than non-infected workers. Nevertheless, a re-designed protocol would be needed to differentiate among these two hypotheses for why the *N. ceranae* + thiacloprid treatment showed elevated mortality, although an additive interaction between *N. ceranae* and thiacloprid, or through reduced sugar consumption. Such a protocol would need to ensure a constant amount of sucrose consumed by different treatment groups with or without pesticide.

Vidau and colleagues (2011), in a similar study to ours, demonstrated that the interaction between *N. ceranae* and thiacloprid similarly elevated adult honey bee mortality and that it was associated with an increase in *Nosema* spore number in the gut (Vidau *et al*., 2011). Moreover, similar to results of Boncristiani and colleagues (2012), we found that the two pathogens *N. ceranae* and BQCV lead to a different response in the host honey bee when it was exposed to the same pesticide. In our experiment, *N. ceranae* seemed to interact more strongly with thiacloprid in adult workers than BQCV did with thiacloprid. This differential response across pathogens when combined with sublethal doses of pesticide is an observation that deserves greater attention in pesticide risk assessment.

Interaction between N. ceranae *and BQCV in adults*

Among all the combination of stressors tested is our study, the synergistic interaction between the two pathogens *N. ceranae* and BQCV in adult honey bees elevated mortality the most. This interaction appears particularly strong since the inoculation of a high dose of BQCV on its own did not induce significant mortality in comparison with the control treatment. BQCV has historically been associated with a closely related microsporidia: *N. apis* (Bailey *et al*., 1983; Bailey and Ball, 1991). This association was based on the occurrence of both pathogens in colonies that collapsed overwinter in the UK, and an increased BQCV load in the presence of *N. apis*, suggesting that infection by the microsporidia facilitates BQCV replication in its host (Bailey *et al*., 1983). Such a synergy was also observed for the chronic bee paralysis virus (CBPV) when co-infecting honey bees with *N. ceranae* (Toplak *et al*., 2013). In our experiments, however, no differences in virus and *N. ceranae* load per bee were observed in singly infected and co-infected honey bees at 13 days post-infection. Retrospectively, our sampling of infected honey bees at 13 days post-infection might have been slightly too late to see any difference, as the increased mortality of co-infected bees started at day 9 post-infection. Thus, we cannot rule out the idea that a difference in pathogen load could have generated an elevated mortality of the co-infected honey bees in our experiments. Interestingly, gypsy moth *Lymantria dispar* larvae also show increased mortality as a result of a synergistic interaction between a virus and a *Nosema* (Bauer *et al*., 1998), while surprisingly, the microsporidia has a negative impact on virus multiplication. A synergistic interaction between two pathogens leading to higher host mortality does not necessarily induce increased virulence (within-host multiplication) of pathogens.

We observed a significantly higher effect of *N. ceranae*-BQCV co-infection on mortality in Experiment 2 compared with Experiment 3. This difference may be due to the variable response of the honey bee to *N. ceranae* infection across the season. Indeed, *N. ceranae* appeared more virulent in Experiment 2 (performed in June), significantly elevating mortality on its own. These data support the view that *N. ceranae* is a serious pathogen of the honey bee, a view that has been debated recently (Fries, 2010; Higes *et al*., 2013).

Seasonal variation in response to *N. ceranae* is probably due to a shift in the physiology of honey bees that emerge in spring versus summer, resulting in a difference in innate immunity and susceptibility to *N. ceranae* infection. A recent survey of *N. ceranae* infection rates across season also showed similar variation, with spring honey bees carrying many more spores than summer bees (Traver *et al*., 2012). Physiological variation across the seasons is well known in honey bees (Harris and Woodring, 1992; Huang and Robinson, 1995; Ray and Ferneyhough, 1997; Hoover *et al*., 2006) and might be due to changes in diet, which then might directly or indirectly affect resistance to pathogens. In addition, 'winter bees', workers eclosing later in the season, have greater investment in fat bodies and other physiological differences, allowing them to overwinter in the hive for up to 6 months (Fluri *et al*., 1982; Crailsheim, 1990); such differences may include greater investment in innate immunity and resistance to pathogens. Interestingly, we did not observe seasonal difference in resistance to BQCV; mortality induced by this virus was equally low in Experiments 2 and 3.

Multiple stressor interactions in honey bees

Although we identified strong interactions between BQCV and thiacloprid in larvae and between *N. ceranae* and BQCV, as well as between *N. ceranae* and thiacloprid in adult honey bees, there was no additional mortality of adult honey bees treated with the three factors in combination. Despite this, the 'triple' treatment showed early mortality due to the interaction between the two patho-

gens and an additional late mortality due to the interaction between *N. ceranae* and thiacloprid (Fig. 6). Overall, we found that co-infection with two common pathogens of honey bees, *N. ceranae* and BQCV, has a relatively high impact on host survival, while pesticide can enhance significantly their effect.

Insofar as honey bees represent a good model for solitary bees, our results suggest that sublethal doses of pesticide may cause rates of mortality elevated beyond those induced directly by pathogens. However, the impact of pathogen-pesticide interactions on honey bees at the colony level remains unknown. The few studies that have been conducted on honey bee colonies suggest interactions in which pesticide treatments elevate pathogen loads, but no increase in colony mortality was reported (Locke *et al*., 2012; Pettis *et al*., 2012; Wu *et al*., 2012). As virulent pathogens at the individual level might not be highly virulent at the colony level (Schmid-Hempel, 1998; Fries and Camazine, 2001), it is likely that interaction between a pathogen and an additional stressor is buffered at the colony level, for example, because of the massive production of brood during spring. To understand further the role of the synergistic interactions identified by us at the individual level, between widespread pathogens (*N. ceranae* and BQCV) and the pesticide thiacloprid, experimentation at the colony level is necessary.

Conclusions

Recent studies have highlighted pathogens as potential risk factors causing individual honey bee mortality and colony collapse (Cox-Foster *et al*., 2007; Evans and Schwarz, 2011; Cornman *et al*., 2012; Dainat *et al*., 2012; Ravoet *et al*., 2013; Vanbergen and The Insect Pollinators Initiative, 2013). In addition, several synergistic interactions between stressors have been shown to increase the mortality of individual honey bees. These encompass very diverse types of interaction, including between pathogens (this study), between the parasitic Varroa mite and several viruses (Nazzi *et al*., 2012; Francis *et al*., 2013), between pathogens and pesticides (Alaux *et al*., 2010; Vidau *et al*., 2011; and this study) and among pesticides (Pilling and Jepson, 1993; Johnson *et al*., 2009a; 2013). These indicate that combined exposure to individually non-lethal stressors can have a detrimental effect on honey bees at the level of the individual insect. By mixing two pathogens and one pesticide in the same experimental design, we have demonstrated that a synergistic interaction between two pathogens induces very high mortality in individual adult honey bees and that pesticide accentuates rates of individual mortality. We strongly suggest considering common honey bee pathogens as the most serious threat to honey bees, not only due to their high prevalence, but also to their high potential to interact with multiple other factors. How these disease and pesticide impacts on individual honey bees play out at the level of the colony remains an open question.

Experimental procedures

Honey bees

Colonies of *A. mellifera carnica* were used from May to July 2012, located in Halle (Saale), Germany. They had been treated to control Varroa mites with Varidol® (Amitraz, TolnAgro, Hungary) in November 2011.

Isolation of pathogens and pesticide preparation

Nosema spores used for infections were isolated after propagation in otherwise clean honey bees kept in the laboratory. Fresh spore suspensions were filtrated through cotton wool and then purified prior to infection following a triangulation method modified by Fries and colleagues (2013), including eight repetitions of a centrifugation step at 28 *g* for 3 min. This triangulation process helps to remove remaining host tissue and microbial contaminants that may confound the experimental treatment. Purified spore suspensions were kept at room temperature (max. 24 h) prior to inoculation. Spores were counted using a Fuchs–Rosenthal haemocytometer. *Nosema* species determination was performed using the multiplex polymerase chain reaction (PCR) protocol described in Fries and colleagues (2013). Throughout, we use *N. ceranae* only.

The BQCV inoculum was prepared by propagating a 10^{-4} dilution of a BQCV reference isolate (Bailey and Woods, 1977) in 150 white-eyed honey bee pupae and preparing a chloroform-clarified extract in 10 mM phosphate buffer (pH 7.0)/0.02% diethyl dithiocarbamate, as described in de Miranda and colleagues (2013). The inoculum contained ~1.4×10^9 BQCV genome copies per µl extract and had no detectable contamination with acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), chronic bee paralysis virus (CBPV), deformed wing virus (DWV), Varroa destrustor virus-1 (VDV-1), Lake Sinai virus-1 (LSV-1) and Lake Sinai virus-2 (LSV-2); negligible (< 0.0001%) contamination with Isreali acute paralysis virus (IAPV) and sacbrood virus (SBV), and < 1% contamination with slow bee paralysis virus (SBPV), as determined by reverse transcription quantitative PCR (qPCR) using the methods of Locke and colleagues (2012). A control extract was prepared from non-inoculated pupae. None of the viruses could be detected in this control extract, except BQCV (~1.5×10^3 copies/µl) and SBV (~2.7×10^8 copies/µl). Primers for virus detection are listed in Supporting Information Table S4, and qPCR conditions are detailed later.

To simulate natural exposure to insecticide, thiacloprid was given chronically *per os* to both larvae and adults via food using a stock solution of 5 g/l of thiacloprid in acetone. For brood, the sublethal dose of 0.1 mg/kg of food was given, which represents a total of 17 ng of thiacloprid per larva over 5 days of feeding. The sublethal dose of 0.1 mg/kg of food was defined after several trials of different dilutions and is approximately 1/100th of the LD50, estimated at 76.9 mg/kg

(Supporting Information Fig. S3). The dose of 0.1 mg/kg administrated to larvae falls in the upper range of what is naturally observed in exposed pollen collected by honey bees (German Bee Monitoring Project, pers. comm.; Smodiš Škerl *et al*., 2009). For adults, the sublethal dose of 5 mg/l was chosen from a previous experiment (Vidau *et al*., 2011), which also represent of approximately 1/100th of the LD50 concentration. This concentration is also within those observed in nectar of thiacloprid-treated plants in the field (Smodiš Škerl *et al*., 2009).

Interaction between BQCV and thiacloprid in larvae

In experiment 1 (July 2012), worker honey bee larvae were fed with the pesticide thiacloprid and three doses of BQCV, alone or in combination, and mortality was recorded everyday. A total of 384 larvae was used: 48 larvae per treatment, from three different colonies. To obtain first instar larvae of identical age, honey bee queens were caged for 24 h on an empty comb for egg laying. After 24 h, queens were released, and the combs were isolated from the queens using an excluder. Three days later, first instar larvae were grafted with a soft brush from the comb into 48-well polyethylene plates containing 20 μl of food prepared according to Aupinel and colleagues' (2005) standard protocol (see also Crailsheim *et al*., 2013). From day 1 to day 7, plates with larvae were kept in an incubator at 34°C ± 1 and 96% relative humidity (RH) (using a potassium sulfate saturated solution) and were taken out once a day to record mortality and for feeding, except on day 1. After day 7, pre-pupae were moved to a second incubator at 35°C ± 1 and 80% RH (using a sodium chloride saturated solution), whereupon mortality was recorded every 2 days. Both pesticide and virus were mixed in the larval food. Thiacloprid (0.1 mg/kg; 0.1% acetone) was fed chronically across larval development. Control treatments without pesticides were fed with food containing 0.1% acetone. BQCV was fed only once to larvae, at day 2 after grafting. Treatments without virus were fed with an extract from non-infected pupae prepared in the same way as virus-treated pupae.

To test the effect of thiacloprid on the replication of BQCV in larvae, we repeated the treatments for the median concentration of 1.4×10^7 BQCV genome equivalents per larva, with or without pesticide, and their controls without virus. We stopped the experiments 7 days post-infection and froze six pre-pupae per treatment at –80°C prior to further analysis (quantification of BQCV copy number).

Interaction between BQCV, N. ceranae *and thiacloprid in adults*

In experiment 2 (June 2012), *N. ceranae* and BQCV were fed individually or in combination to adult worker honey bees, and mortality was recorded everyday for 13 days. In Experiment 3 (July 2012), *N. ceranae*, BQCV and pesticide were fed individually or in combination to adult worker honey bees, and mortality was recorded everyday for 25 days. To retain bees, we used metal cages ($10 \times 10 \times 6$ cm) containing an 8 cm^2 piece of organic beeswax, each with 30 newly emerged worker bees from the same colony. The two pathogens were administrated orally to 2 day old bees individually in 10 μl of 50% sucrose solution using a micropipette, without prior anaesthesia. *Nosema ceranae* was fed at a concentration of 10^5 spores per bee and BQCV at a concentration of 1.4×10^9 genome equivalents per bee. For co-infection, both pathogens were mixed in the same inoculum at the same concentrations. Treatments without BQCV were fed pupal extract devoid of virus in the same buffer as used for the BQCV inoculum. Bees were starved half an hour preinfection and kept isolated in 1.5 ml Eppendorf tubes for 1 h post-infection to avoid trophallaxis and pathogen exchange with other individuals, ensuring that each bee received its complete treatment. In Experiment 3, thiacloprid was mixed daily into the sucrose solution and given *ad libitum* at a concentration of 5 mg/L. Treatments without pesticide were given a 50% sucrose solution containing 0.1% acetone. As for pathogen inoculation, pesticide treatment started at day 2 of the worker honey bee's life.

Cages were placed into incubators at 30°C ± 1 and 50% RH. Bees were fed 50% sucrose solution *ad libitum* following guidelines in Williams and colleagues (2013). Three and four replicates where undertaken for each treatment in Experiments 2 and 3, respectively, using five different colonies (Supporting Information Fig. S4). In total, 360 and 840 adult workers bees were used for Experiment 2 and Experiment 3 respectively. After Experiment 2, honey bees from all cages were frozen at –80°C at 13 days post-infection prior to further molecular quantification of pathogens.

Sugar consumption was recorded everyday for each cage, as was bee mortality. The effect of each of the three stressors on the quantity of sugar ingested per bee per day was then calculated for the first 20 days of the experiment (there were not enough bees in cages for days 20–25 post-infection to estimate reliably the sugar consumption per bee) using a linear mixed model to account for the repeated measures nature of the data.

Survival analyses

All statistical analyses were undertaken using R (R Development Core Team, 2008). Survival analysis were performed using Cox proportional hazard models using 'cage' as random effect for larval survivorship, and 'cage' within 'colony' as nested random effect for adults, to take into account the variability across colonies and replicates (Williams *et al*., 2013). The R packages *coxme* was used to include mixed effects to the Cox regression models (Therneau, 2012), and *frailtyHL* for the graphical representation of hazard ratio (Ha *et al*., 2012). Coefficient contrasts were performed using the *multcomp* package (Hothorn *et al*., 2013). Model selection was undertaken using the *dredge* function of the R package *MuMIn* (Bartoń, 2013).

RNA extraction and real-time reverse transcription PCR

For pathogen quantification, six pre-pupae (Experiment 1) and 18 adult honey bees (Experiment 2) were sampled per treatment. Pre-pupae were crushed in 1 ml RNAse free water, and 1/10 was used for RNA isolation. Adult honey bee midguts were dissected and tissue preserved in RNAlater (Invitrogen, San Diego, CA, USA). RNA was extracted from all samples using the RNeasy mini (large sample) kit and a

QiaCube robot (Qiagen, Hilden, Germany). Total cDNA was synthetized using random hexamer primers and M-MLV Revertase (Promega, Madison, WI, USA) following manufacturer's instructions. Real-time PCRs were performed on a Bio-Rad C1000 thermal cycler, using SYBRgreen Sensimix (Bioline, Luckenwalde, Germany) and the primers for *N. ceranae* and BQCV listed in the Supporting Information Table S4. Amplification steps were: 5 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 57°C (including a read at each cycle). Following the real-time PCR, DNA was denaturated 1 min at 95°C then cooled to 55°C in 1 min, and a melting profile was obtain from 55 to 95°C at 0.5°C increments per second. Absolute quantification of BQCV was calculated using standards (10-fold dilutions of a cloned fragment of the virus genome). Quantification data were analysed with linear mixed models using the R package *MASS*; values were log10-transformed, 'treatment' was considered a fixed effect, and 'colony' and 'cage' were random effects. Treatment comparisons were performed using the R package *multcomp* (Hothorn *et al*., 2013).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Estimated LD50 of BQCV concentration on larval honey bees calculated from a linear mixed model using colony as random effect. The estimated LD50 is 1.53×10^8 genome equivalents (95% CI = 6.99×10^7/ 1.35×10^9) at 11 days post-infection. Linear regression: $R^2 = 0.714$.
Fig. S2. Mean sugar consumption per bee and per day (± SEM) for treatments with and without pesticide during Experiment 3 (*N. ceranae*, BQCV and thiacloprid). Presence of thiacloprid had a negative effect on sugar consumption (linear mixed model using colony as random effect; $t = -3.998$, df = 18, $P < 0.001$), while infection with pathogens did not: *N. ceranae* ($t = -1.042$, df = 18, $P = 0.3114$) and BQCV ($t = -1.833$, df = 18, $P = 0.0834$). The decrease in sugar consumption due to the presence of thiacloprid in the food is of the order of 15% (calculated from the median values of sugar consumption for both groups, without (40.1 μl/bee/day) and with thiacloprid (34.0 μl/bee/day). The green line represents the sugar consumption of honey bees from the treatments without thiacloprid, and the blue line represents the mean consumption of honey bees from the treatments with thiacloprid. Thiacloprid was mixed into the 50% sucrose solution (0.1% acetone) provided *ab libitum* to honey bee workers across the whole experiment.
Fig. S3. Estimated LD50 following chronic exposure of larval honey bees to thiacloprid, calculated from a linear mixed model using colony as random effect. The estimated LD50 is 76.9 mg/kg thiacloprid (95% CI 60.5/99.8), 7 days after the first ingestion. Linear regression: $R^2 = 0.913$.
Fig. S4. Design of the three experiments.
Table S1. Instantaneous risk of death (hazard ratio, ± 95% CI) for adult honey bees in each treatment of Experiment 2 (*N. ceranae* and BQCV) compared with the control treatment, calculated from a Cox proportional hazard mixed model using treatment as fixed effect and colony and cages as nested random effects. In bold are the treatments with a hazard ratio statistically different to the control treatment.
Table S2. Instantaneous risk of death (hazard ratio, ± 95% CI) for adult honey bees in each treatment of Experiment 3 (*N. ceranae*, BQCV and thiacloprid) compared with the control treatment, calculated from a Cox proportional hazard mixed model using treatment as fixed effect and colony and cages as nested random effects. In bold are the treatments with a hazard ratio statistically different to the control treatment.
Table S3. Coefficient contrast comparisons, adjusted (or not) for multiple comparisons with FDR method based on the hazard ratio from each treatment in Experiment 3 (*N. ceranae*, BQCV and thiacloprid). Double treatments were compared with single treatments, while the triple treatment (*N. ceranae* + BQCV + thiacloprid) was compared with the three doublet treatments. In bold is the comparison that appeared significant without correction for multiple analyses.
Table S4. List of primers used for quantification of viruses after propagation, using RT-qPCR (and efficiency of qPCR). The qPCR efficiency for BQCV quantification after experimental infections in adults and larvae was of 103.3%.
Appendix S1. Effect of chronic exposure to a sublethal dose of thiacloprid (0.1 mg/kg) and three different doses of BQCV, alone or in combination, on larval honey bee mortality.