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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Running title: Pesticide-pathogen interactions in honey bees

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 sub-lethal 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.

Key words: *Nosema ceranae*, microsporidia, BQCV, virus, thiacloprid, bee decline

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), over-winter 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 behavior (Suchail et al., 2001; Medrzycki et al., 2003; Decourtye et al., 2009; Williamson et al., 2013), foraging success (Yang et al., 2008; Henry et al., 2012; Schneider et al., 2012), learning (Decourtye et al., 2004; Decourtye et al., 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 *Apis 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; Wu et al., 2011; Johnson et al., 2013). 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 sub-lethal 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 *Nosema ceranae*, considered a possible cause of colony decline (Higes et al., 2008; Higes et al., 2009; Bromenshenk et al., 2010), and black queen cell virus (BQCV), a native and widespread bee virus which is known to reduce survival of queen pupae and has historically been associated with another honey bee pathogen, *Nosema 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 sub-lethal 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 (figure 1A). From this dose dependent mortality of larvae, the LD50 (median dose which induces 50% mortality) for BQCV was estimated at 1.53×10^8 genome equivalents (95% confidence intervals: 6.99×10^7/ 1.35×10^9; Supporting information S1). As expected, since it was administrated at sub-lethal levels, thiacloprid fed on its own to larvae did not directly cause mortality, but it did elevate BQCV-induced mortality at all viral dosages (figure 1A).

Survivorship of all treatments was then converted to hazard ratios (instantaneous risk of death compared to 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 (figure 1B). A significant higher mortality was observed in the treatment $BQCV^9$ + thiacloprid compared to 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 ($Z = 1.103$, $P = 0.592$) 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 S2). However no interaction was observed between variables.

147 To test the impact of pesticide ingestion on pathogen growth in honey bee larvae, we
148 quantified the BQCV load in pre-pupae that had been fed as larvae with the medium dose of
149 virus ($BQCV^7$), either with or without thiacloprid, and compare these against control pre-
150 pupae that had not been fed BQCV as larvae (figure 2). Pre-pupae from the $BQCV^7$ +
151 thiacloprid feeding regime showed a significantly higher virus load (6.59×10^{10} genome
152 equivalents; 95% ci: $\pm 8.59 \times 10^{10}$) than pre-pupae from the $BQCV^7$ only regime (56,225
153 genome equivalents; 95% ci: ± 49,946; linear mixed model: $Z = -2.261$, $P = 0.0238$).
154
155 *Experiment 2: Interaction between* N. ceranae *and BQCV in adults*
156 The interaction between the pathogens *N. ceranae* and BQCV was tested in adult
157 honey bees (larval honey bees are not known to suffer from *Nosema* infections). Both
158 pathogens were administrated orally to workers honey bees maintained in small metal cages,
159 alone or in combination, at doses that guaranteed infection of all individuals after five days:
160 10^5 *N. ceranae* spores and 1.4×10^9 BQCV genome equivalents. Nine days post-infection, a
161 synergistic effect (i.e. more than additive) of co-infection was observed on survival: severe
162 mortality of co-infected honey bees (figure 3A). At eleven days post-infection, 50% of the co-
163 infected workers honey bees were dead, while only 20% of *N. ceranae* infected bees and less
164 5% of BQCV infected and control honey bees were dead. Survival analysis revealed a
165 significantly higher mortality of adult honey bees with just *N. ceranae* ($Z = 2.07$, $P = 0.039$)
166 and co-infected honey bees ($Z = 4.05$, $P < 0.001$), compared to non-infected control honey
167 bees. Honey bees infected with BQCV alone did not die significantly faster than control bees
168 ($Z = -0.50$, $P = 0.620$; figure 3B; Supporting information S3). The rate of mortality of co-
169 infected bees was significantly higher than that of bees infected with the two pathogens
170 separately (coefficient contrast adjusted with FDR method; $Z = 4.181$, $P < 0.001$), illustrating
171 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 (figures 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 (figure 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, $P = 0.4541$; figure 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 (figures 6C and 6E) and between *N. ceranae* and thiacloprid (figures 6A and 6E), but less pronounced between BQCV and thiacloprid (figures 6B and 6E). No extensive three ways interaction between the three stressors was observed (figures 6D and 6E). 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 S4).

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 S5). However, using correction for multiple comparisons reduced the statistical power of analysis. Separately analyzing each set of "double" treatments, the co-infection treatment *N*. *ceranae* + BQCV showed a significantly 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 S5).

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 thirdly thiacloprid (table2). 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 to the treatment *N*. *ceranae* + thiacloprid, that itself induced late mortality during the third week (figure 7). The triple treatment (*N*. *ceranae* +

222 BQCV + thiacloprid) also induced early mortality, similar to the co-infection (*N*. *ceranae* +
223 BQCV) treatment (figure 7).

224 Daily records of sugar consumption per treatment (per bee) showed no effect of either
225 pathogen (linear mixed model, BQCV: t = -1.833, df = 18, $P = 0.0834$; *N*. *ceranae*: t = -1.042,
226 df = 18, $P = 0.3114$), while the pesticide thiacloprid mixed into the sugar solution had a
227 significant negative effect on sugar consumption (t = -3.998, df = 18, $P < 0.001$), with a
228 decrease in median sugar consumption of 15% (median values of 40.1 and 34.0 µl/bee/day of
229 sucrose solution for groups fed without and with thiacloprid, respectively; Supporting
230 information S6). On average, adult honey bee workers from treatments including pesticides
231 ingested 185 (±4) ng thiacloprid per bee per day, similar to experiments conducted elsewhere
232 (Vidau et al., 2011).

233 Experiments 2 and 3 both included infections of adult honey bees with *N*. *ceranae* and
234 BQCV alone and in combination, but were conducted during June and July respectively.
235 Since honey bees can respond differently to pathogens at different times of the year, the
236 survival data from Experiment 2 and 3 were incorporated into a single analysis using 'season'
237 as an additional fixed variable, together with the presence or absence of the two pathogens.
238 This analysis revealed an interaction between *N*. *ceranae* and season ($Z = -4.51$, $P < 0.001$),
239 reflecting higher mortality due to *N*. *ceranae* infection during Experiment 2, in June, than
240 during Experiment 3, in July. No significant interaction was observed between BQCV and
241 season ($Z = -0.67$, $P = 0.5$).

242

243 **Discussion**

244 We found that two common pathogens of the honey bee, *N*. *ceranae* and BQCV, act
245 synergistically on adult honey bees and induce rapid mortality. The systemic neonicotinoid
246 insecticide thiacloprid, when fed at a sub-lethal dose, can enhance the mortality of larval and
247 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, since 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*.

Sub-lethal 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 titers 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 defenses (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

274 treatment, compared to non-treated colonies, although this effect was not observed for BQCV
275 and sacbrood virus (SBV) (Locke et al., 2012). In another study, no direct effects of various
276 acaricide treatments applied to honey bee colonies were observed on virus load in adults,
277 including BQCV (Boncristiani et al., 2012). Here, we show the direct effect of the
278 neonicotinoid thiacloprid on BQCV multiplication in individual honey bee larvae, which
279 might explain the observed elevated mortality.
280 The response of adult honey bees to high concentrations of BQCV was very different
281 from that of larvae. While inoculation of 1.4×10^9 BQCV genome equivalents induced very
282 high larval mortality, the same dose did not induce any significant mortality in adult honey
283 bees. This might reflect a physiological difference between larvae and adults in tolerance to
284 BQCV infection. Indeed, most honey bee pathogen have distinct windows of infectivity
285 during the honey bee life cycle, with early brood and newly emerged adults often particularly
286 susceptible (Bailey and Ball, 1991). In adult honey bees, the interaction of thiacloprid with
287 BQCV had a less pronounced effect (figure 6A), which led to a non-significant increased
288 mortality when fed simultaneously. This difference with the observation in larvae might also
289 reflect different tolerance of the two stages to BQCV.
290
291 *Interaction between* N. ceranae *and thiacloprid in adults*
292 Adult honey bee workers infected with *N. ceranae* and additionally exposed to a sub-
293 lethal dose of thiacloprid also showed increased mortality (figure 6B). We observed a late
294 mortality of these honey bees compared to a *N. ceranae* only infection. This delayed effect of
295 the interaction between *N. ceranae* and thiacloprid might reflect an accumulation of the
296 neonicotinoid in the insect body, which eventually interacts with the microsporidia. The late
297 onset of mortality of the bees infected with *N. ceranae* + thiacloprid in our study might also
298 reflect the possible repellent effect of the pesticide, since honey bees fed with a sucrose
299 solution contaminated with thiacloprid consumed significantly less food than bees provided

300 sugar solution without pesticide. This repellent effect of the pesticide, also induced by other
301 neonicotinoid insecticides (Ramirez-Romero et al., 2005), might have delayed the effect of
302 pesticide exposure, thus underestimating the effect of the treatment *N*. *ceranae* + thiacloprid.
303 Alternatively, the lower food intake of honey bees treated with the pesticide might have
304 accelerated their death. Although *N*. *ceranae* is generally thought to induce energetic stress in
305 honey bees and to increase hunger (Mayack and Naug, 2009; Martín-Hernández et al., 2011),
306 *Nosema* infected workers in our experiment did not consumed more sugar that non-infected
307 workers. Nevertheless, a re-designed protocol would be needed to differentiate among these
308 two hypotheses for why the *N*. *ceranae* + thiacloprid treatment showed elevated mortality:
309 though an additive interaction between *N*. *ceranae* and thiacloprid or through reduced sugar
310 consumption. Such a protocol would need to ensure a constant amount of sucrose consumed
311 by different treatment groups with or without pesticide.
312 Vidau et al. (2011), in a similar study to ours, demonstrated that the interaction
313 between *N*. *ceranae* and thiacloprid similarly elevated adult honey bee mortality, and that it
314 was associated with an increase in *Nosema* spore number in the gut (Vidau et al., 2011).
315 Moreover, similar to results of Boncristiani et al. (2012), we found that the two pathogens *N*.
316 *ceranae* and BQCV lead to a different response in the host honey bee when it was exposed to
317 the same pesticide. In our experiment, *N*. *ceranae* seemed to interact more strongly with
318 thiacloprid in adult workers than BQCV did with thiacloprid. This differential response across
319 pathogens when combined with sub-lethal doses of pesticide is an observation that deserves
320 greater attention in pesticide risk assessment.
321
322 *Interaction between* N. ceranae *and BQCV in adults*
323 Among all the combination of stressors tested is our study, the synergistic interaction
324 between the two pathogens *N*. *ceranae* and BQCV in adult honey bees elevated mortality the
325 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 to the control treatment. BQCV has historically been associated with a closely related microsporidia: *Nosema 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 to 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 which 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 six 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 pathogens and an additional late mortality due to the interaction between *N*. *ceranae* and thiacloprid (figure 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 sub-lethal 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, due to 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; Johnson et al., 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 *Apis 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 et al. (2013), including 8 repetitions of a centrifugation step at 28 g for 3 minutes. 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 hours) prior to inoculation. Spores were counted using a Fuchs-Rosenthal haemocytometer. *Nosema* species determination was performed using the multiplex PCR protocol described in Fries et al. (2013). Throughout we use *Nosema 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 10mM phosphate buffer (pH 7.0)/0.02% diethyl dithiocarbamate, as described in de Miranda et al. (2013). The inoculum contained ~1.4 x 10^9 BQCV genome copies per µl extract and had no detectable contamination with ABPV, KBV, CBPV, DWV, VDV-1, LSV-1 and LSV-2; negligible (<0.0001%) contamination with IAPV and SBV, and <1% contamination with SBPV, as determined by RT-qPCR using the

methods of Locke et al. (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 x 10^3 copies/µl) and SBV (~2.7 x 10^8 copies/µl). Primers for virus detection are listed in Supporting information S7, and qPCR conditions are detailed below.

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 sub-lethal dose of 0.1 mg/kg of food was given, which represents a total of 17 ng of thiacloprid per larva over five days of feeding. The sub-lethal 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 S8). 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, personal communication; Smodiš Škerl et al., 2009). For adults, the sub-lethal 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 every day. 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 hours on an empty comb for egg laying. After 24 hours, 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 20µl of food, prepared according to Aupinel et al.'s (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 two 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 x 10^7 BQCV genome equivalents per larva, with or without pesticide, and their controls without virus. We stopped the experiments seven 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 every day 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 every day for 25 days. To retain bees we used metal cages (10 × 10 × 6 cm) containing an 8 cm piece of organic beeswax, each with 30 newly emerged worker bees from the same colony. The two pathogens were administrated orally to two day old bees individually in 10 µl of 50% sucrose solution using a micropipette, without prior anesthesia. *N*. *ceranae* was fed at a concentration of 10^5 spores per bee, and BQCV at a concentration of 1.4 x 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 pre-infection and kept isolated in 1.5 ml Eppendorf tubes for one hour 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 et al. (2013). Three and four replicates where undertaken for each treatment in experiment 2 and 3 respectively, using five different colonies (Supporting information S9). 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 every day 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 twenty 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 RT-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). RNA was extracted from all samples using the RNeasy mini (large sample) kit and a QiaCube robot (Qiagen, Germany). Total cDNA was synthetized using random hexamer primers and M-MLV Revertase (Promega) following manufacturer's instructions. Real-time PCRs were performed on a Bio-Rad C1000 thermal cycler, using SYBRgreen Sensimix (Bioline, Germany) and the primers for *N. ceranae* and BQCV listed in the Supporting information S7. Amplification steps were: 5 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 30 sec 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°C to 95°C at 0.5°C increments per second. Absolute quantification of BQCV was calculated using standards (ten-fold dilutions of a cloned fragment of the virus genome). Quantification data were analyzed 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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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. 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.

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

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). Interactions between variables had low estimates of coefficients and thus are not shown here.

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	

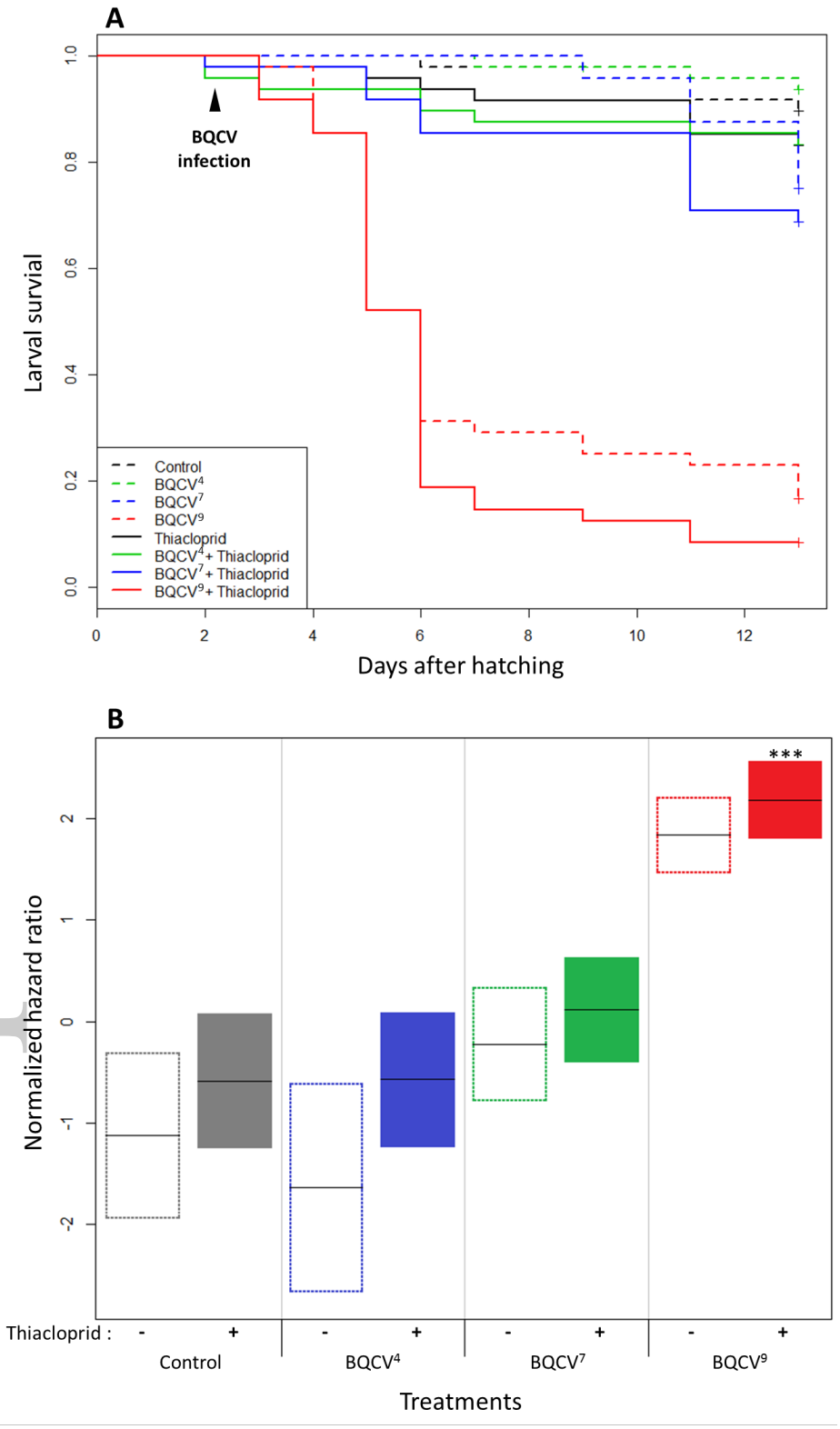
Figure 1: Interaction between BQCV and thiacloprid in larval honey bees (Experiment 1). **A**: Survival curves of larval worker honey bees treated with BQCV and thiacloprid, alone or in combination, and a control treatment. Three concentrations of BQCV were used: low ($BQCV^4$), medium ($BQCV^7$) and 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% c.i.) for larvae in each treatment compared with the model average of 0. Empty boxes represent treatment without pesticide, full boxes represent treatment with pesticide. Grey, blue, green and red colors represent treatments with no virus, low, medium and high doses of virus. *** shows the treatment $BQCV^9$ + thiacloprid, which induced a signicantly higher mortality than with $BQCV^9$ or thiacloprid when each was administrated seperataly.

Figure 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.

Figure 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, ± 95% c.i.) for adult honey bees in each treatment compared with the model average of 0. *** shows the treatment *N*.

838 *ceranae* + BQCV, which induced a significantly higher mortality than the two pathogens
839 separately.
840
841 **Figure 4:** Absolute quantification of BQCV (log10 transformed) in adult honey bee midguts
842 from Experiment 2 (*N*. *ceranae* and BQCV). n.s., no significant difference was observed
843 between the bees from the treatement BQCV only and *N*. *ceranae* + BQCV. N = 18 adults for
844 each treatment.
845
846 **Figure 5:** Absolute quantification of *N*. *ceranae* 16S rRNA gene copies in adult honey bee
847 midguts from Experiment 2 (*N*. *ceranae* and BQCV). n.s., no significant difference was
848 observed between the bees from the treatement *N*. *ceranae* only and *N*. *ceranae* + BQCV. N =
849 18 adults for each treatment.
850
851 **Figure 6:** Interaction between *N*. *ceranae*, BQCV and thiacloprid in adult honey bees
852 (Experiment 3). Honey bees were treated with 10^5 *N*. *ceranae* spores, 1.4 x 10^9 BQCV and
853 thiacloprid (0.1 mg/kg), alone or in combination, or a control solution. Pathogens were given
854 once at day 0, while thiacloprid was fed continously across the experiment. **A**: Survival curve
855 of honey bees treated with BQCV and thiacloprid (alone or in combination). **B**: Survival
856 curve of honey bees treated with *N*. *ceranae* and thiacloprid. **C**: Survival curve of honey bees
857 treated with *N*. *ceranae* and BQCV. **D**: Survival curve of honey bees treated with *N*. *ceranae*,
858 BQCV and thiacloprid, in pariwise combination of two and all three together. **E**:
859 Instantaneous risk of death (hazard ratio, ± 95% c.i.) for adult honey bees in each treatment
860 compared with the model average of 0.
861
862 **Figure 7:** Comparison of adult honey bee mortality (±sem) induced at two time points (16
863 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).



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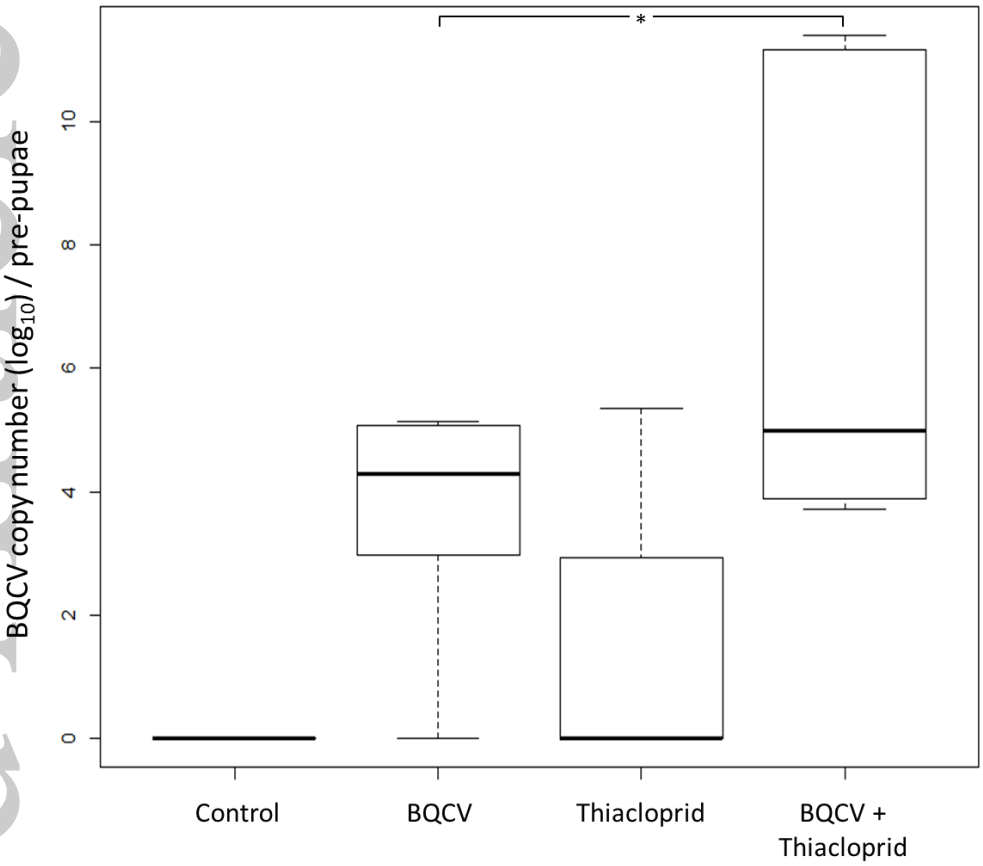

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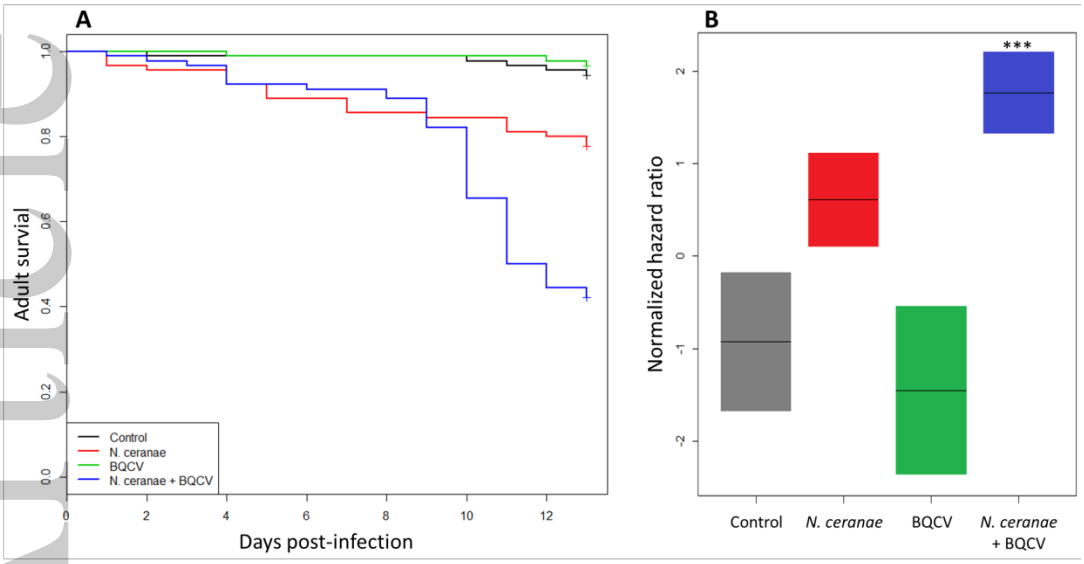

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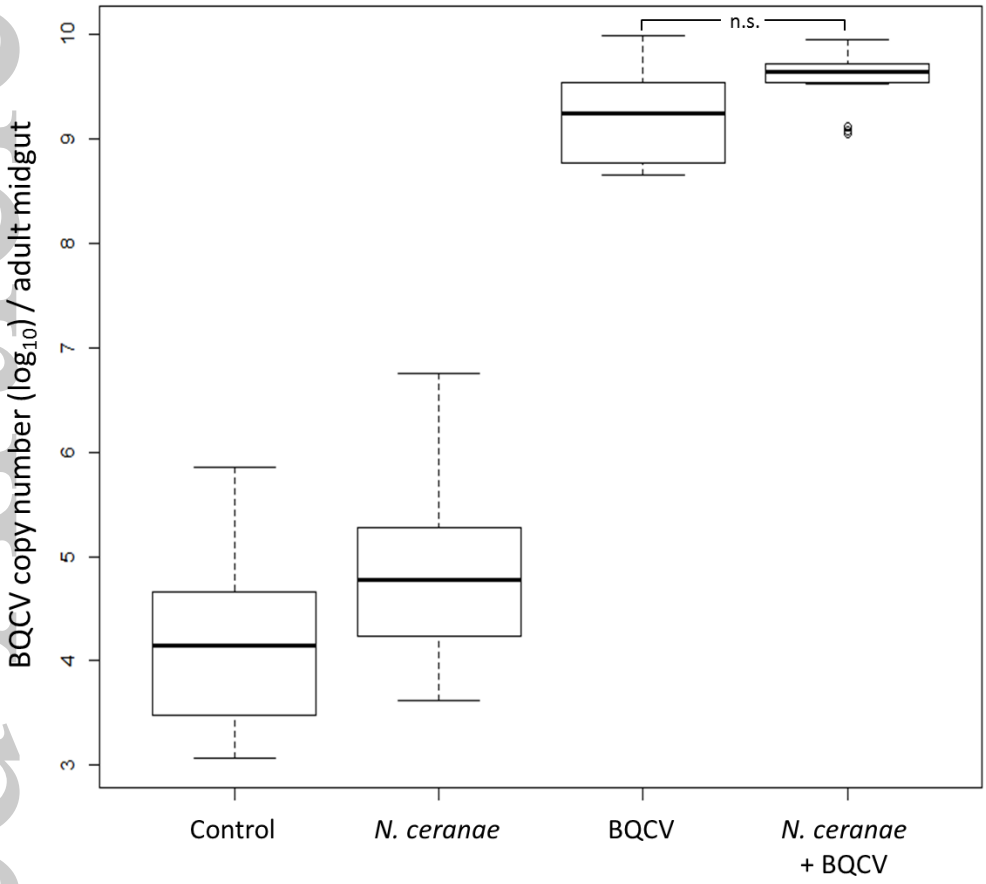

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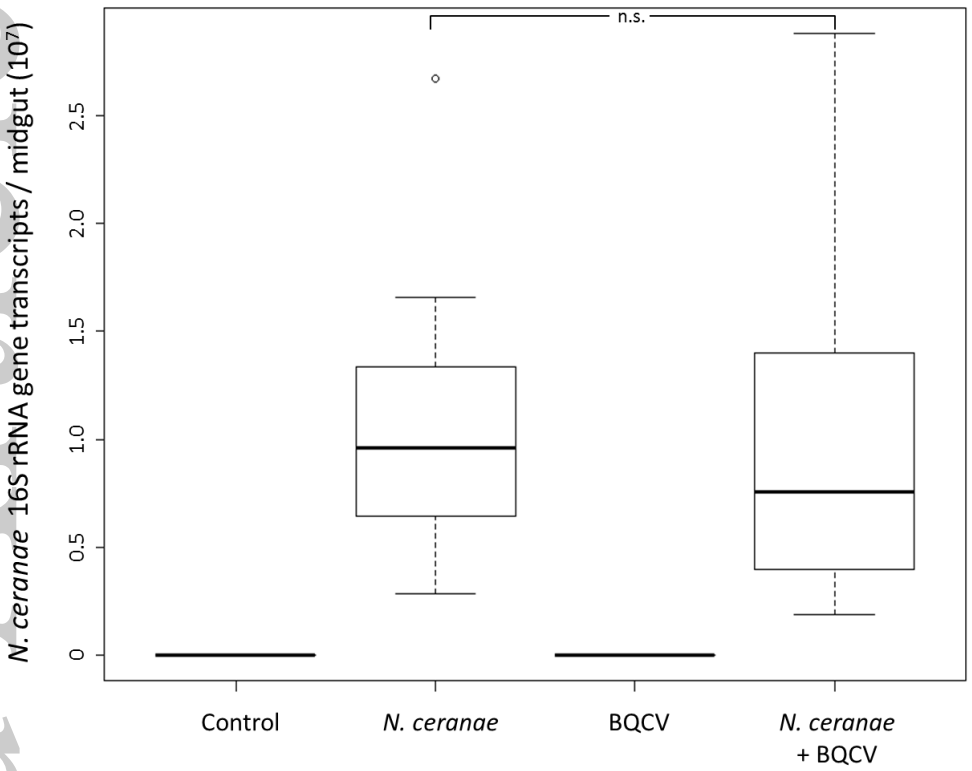

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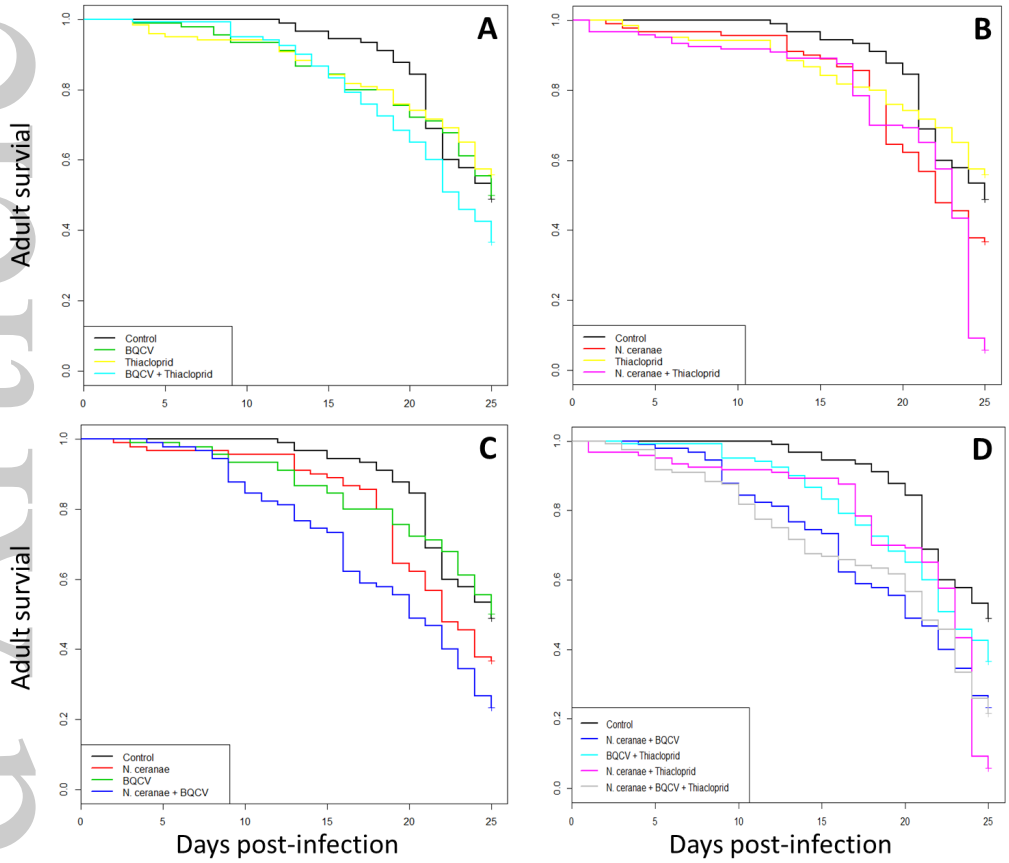

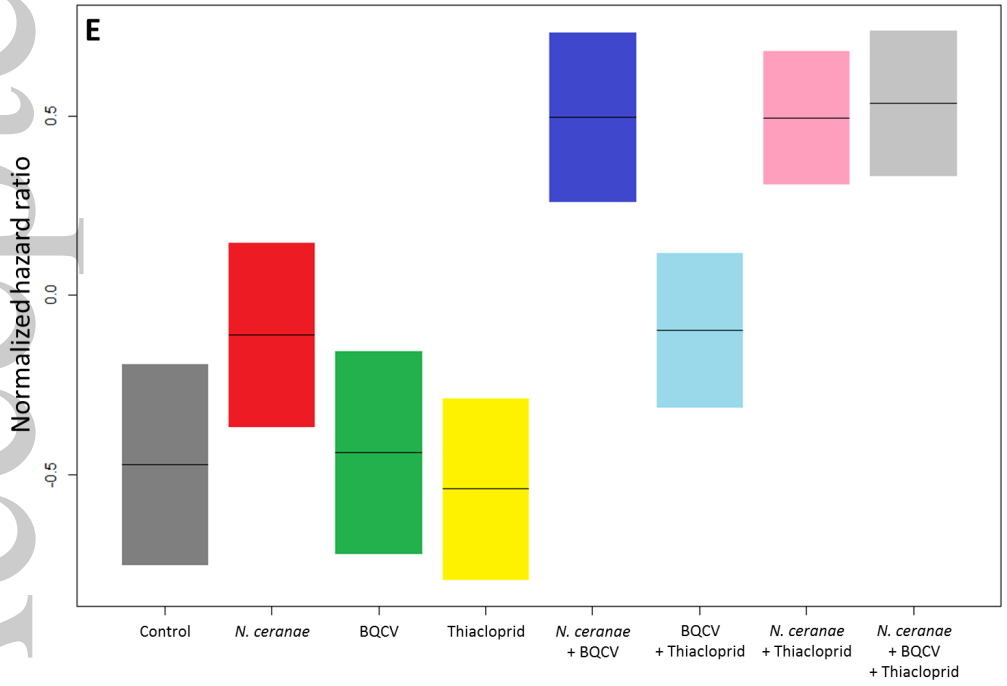

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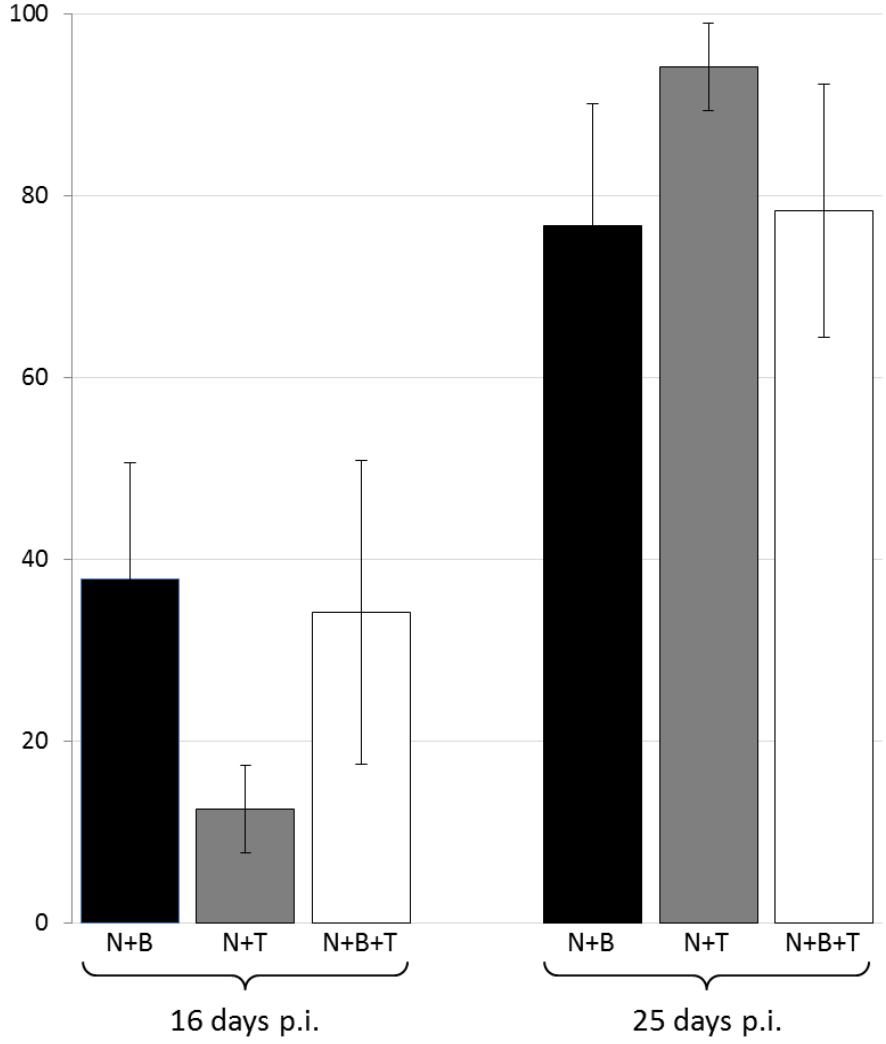

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885 **emi_12426_f7**

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