RESEARCH ARTICLE

WILEY **JEZ-A** ECOLOGICAL AND INTEGRATIVE PHYSIOLOGY

Detrimental interactions of neonicotinoid pesticide exposure and bumblebee immunity

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Abstract

Pesticides are well known to have a number of ecological effects. However, it is only now becoming understood that sublethal exposures may have effects on nontarget insects of conservation concern through interactions with immunity, thus increasing detrimental impacts in the presence of pathogens. Pesticides and pathogens are suggested to have played a role in recent declines of several wild bee pollinators. Compromised immunity from exposure to widely used neonicotinoids has been demonstrated in honeybees, but further research on interactions between neonicotinoids and immunity in other important bees is lacking. In this study, adult workers of the bumblebee *Bombus impatiens* received 6-day pulses of either low (0.7 ppb) or high (7 ppb) field realistic doses of the neonicotinoid imidacloprid prior to assaying immunity and survival following a nonpathogenic immune challenge. High-dose imidacloprid exposure reduces constitutive levels of phenoloxidase, an enzyme involved in melanization. Hemolymph antimicrobial activity initially increases in all groups following an immune challenge, but while heightened activity is maintained in unexposed and low imidacloprid dose groups, it is not maintained in the high exposure dose bees, even though exposure had ceased 6 days prior. Additionally, imidacloprid exposure followed by an immune challenge significantly decreased survival probability relative to control bees and those only immune challenged or imidacloprid exposed. A temporal lag for immune modulation and combinatorial effects on survival suggest that resource-based trade-offs may, in part, contribute to the detrimental interactions. These interactions could have health consequences for pollinators facing multiple stresses of sublethal neonicotinoid exposure and pathogens.

1 | INTRODUCTION

Variation in immune responses, at the heart of the study of ecological immunology (Demas & Nelson, 2012; Rolff & Siva-Jothy, 2003; Sadd & Schmid-Hempel, 2009), can result from variation in the abiotic environment. This can arise due to differential selection pressures shaping investment into immunity under different environments or proximate mechanisms constraining current immune investment below optimal levels. This variation and suboptimality in immunity will permeate to determine individual and population level susceptibility to infection. While pesticides used in agricultural and other systems have been long accepted as having a wide array of critical ecologically relevant effects (Brown, 1978), the ecological relevance of sublethal exposure of organisms to pesticides influencing immune function and disease resistance are only recently becoming appreciated. While interactions between pesticide exposure, immunity, and pathogens may be beneficial when considering control of pests (Paula, Carolino, Paula, & Samuels, 2011; James & Xu, 2012), for species of conservation concern and with key beneficial ecosystem roles (e.g., pollinators, parasitoids, and predatory

arthropods), these interactions may exacerbate the individual negative effects of pesticide and pathogen exposure (James & Xu, 2012; Mason, Tennekes, Sánchez-Bayo, & Jepsen, 2013).

As pollinators, bees provide critical ecosystem services in many natural and human dominated terrestrial environments (Fontaine, Dajoz, Meriguet, & Loreau, 2006; Klein et al., 2007; Ollerton, Winfree, & Tarrant, 2011). However, there is growing concern about the security of these services, with global patterns of decline in many managed and wild bee species (Biesmeijer et al., 2006; Goulson, Lye, & Darvill, 2008; Potts et al., 2010; Cameron et al., 2011; Brown et al., 2016). A large investment of research effort over the last decade has sought to understand the causes and consequences of these disturbing declines. It has been proposed that bees in general may be both ecologically and environmentally predisposed to be particularly vulnerable to multiple environmental stresses to which they are exposed (Klein, Cabirol, Devaud, Barron, & Lihoreau, 2017). Factors that have been implicated in declines include climate change (Kerr et al., 2015), habitat loss and fragmentation (Kennedy et al., 2013), exposure to pesticides (Godfray et al., 2015; Rundlöf et al., 2015; Woodcock et al., 2017),

and pathogens and parasites (Furst, McMahon, Osborne, Paxton, & Brown, 2014; Manley, Boots, & Wilfert, 2015; Nazzi & Le Conte, 2016). It is clear that these multiple stresses on bee health will not be in isolation, but rather as a suite of potentially interacting factors, with the possibility of synergistic effects enhancing negative impacts (Vanbergen et al., 2013; Goulson, Nicholls, Botías, & Rotheray, 2015). Two local-scale factors that have been touted for their potential interactive capacity are exposure to pathogens and pesticides, with the bee immune system proposed to mediate the outcome of coexposure (James & Xu, 2012; Goulson et al., 2015).

Bees, particularly those foraging in agroecosystems, will be exposed to a diverse suite of pesticides (Mullin et al., 2010; Sanchez-Bayo & Goka, 2014; David et al., 2016; Long and Krupke, 2016). A group of pesticides receiving considerable attention, due to a proposed link with insect pollinator declines, are neonicotinoid insecticides (Blacquière, Smagghe, van Gestel, & Mommaerts, 2012; Bonmatin et al., 2015; Lundin et al., 2015). Neonicotinoids, including commonly used imidacloprid, thiamethoxam, and clothianidin, specifically target the nervous system of insects through blocking of the nicotinic acetylcholine receptors (nAChRs) (Simon-Delso et al., 2015). This specificity, resulting from fundamental differences between nAChRs of insects and mammals (Tomizawa & Casida, 2003), has led to their widespread and effective use against insect pests, but nontarget insects, such as bees, can be exposed when foraging on agricultural crops and nearby flowering plants following foliar applications or neonicotinoid seed treatments (Bonmatin et al., 2015). While there has been some debate over the doses that bees are exposed to (Carreck & Ratnieks, 2014), several studies have now demonstrated that bee exposure to trace residues of neonicotinoid insecticides can be pervasive and persistent (Blacquière et al., 2012; David et al., 2016; Long and Krupke, 2016; Zimmermann & Stout, 2016). Important ecological and fitness-related effects of exposure to these trace residues have been shown for honeybees (Lu, Warchol, & Callahan, 2014; Williams et al., 2015), bumblebees (Whitehorn, O'Connor, Wackers, & Goulson, 2012; Fauser-Misslin, Sadd, Neumann, & Sandrock, 2014; Gill and Raine, 2014; Scholer & Krischik, 2014; Stanley, Russell, Morrison, Rogers, & Raine, 2016; Baron, Raine, & Brown, 2017; Ellis et al., 2017; Fauser, Sandrock, Neumann, & Sadd, 2017), solitary bees (Sandrock et al., 2014), and bee communities (Rundlöf et al., 2015; Woodcock et al., 2017).

Infection by pathogens will have detrimental consequences alone (Dainat, Evans, Chen, Gauthier, & Neumann, 2012; Manley et al., 2015), but it has been proposed that the additional stress of pesticide exposure may heighten susceptibility to infection and increase negative infection-related effects on individual bees and populations (Goulson et al., 2015; Collison, Hird, Cresswell, & Tyler, 2016; Sánchez-Bayo et al., 2016). It has been shown that neonicotinoid exposure increases pathogen spore load and mortality in honeybees infected with the microsporidian *Nosema ceranae* (Alaux et al., 2010; Vidau et al., 2011; Pettis, van Engelsdorp, Johnson, & Dively, 2012; Retschnig, Neumann, & Williams, 2014). Similar results have been shown for honeybee viral (Di Prisco et al., 2013; Doublet, Labarussias, de Miranda, Moritz, & Paxton, 2015) and bacterial infection (López et al., 2017). In bumblebees, exposure to clothianidin and thiomethoxam and a trypanosome parasite reduce mother queen longevity in a greater than additive

manner (Fauser-Misslin et al., 2014), although effects on overwintering were less than additive, with strong early pesticide exposure effects masking later parasite effects (Fauser et al., 2017).

An obvious candidate linking neonicotinoid exposure to heightened susceptibility to infection and the consequences of infection is the immune system, with neonicotinoid exposure having the potential to compromise the immune system via a number of routes. In honeybees, exposure to neonicotinoids has been associated with decreased cellular responses on bacterial infection (López et al., 2017), reduced hemocyte numbers, encapsulation, and antibacterial responses (Brandt, Gorenflo, Siede, Meixner, & Büchler, 2016; Siede et al., 2017), and reduced activity of an enzyme involved in social immune sterilization of food (Alaux et al., 2010). NF-κB signaling, with a central role in immunity, has also been shown to be inhibited by neonicotinoid exposure in honeybees (Di Prisco et al., 2013). It is possible that immunity is compromised due to direct or indirect toxic effects (Collison et al., 2016), or regulatory cross-talk between the nervous system, which is targeted by the neonicotinoids, and the insect immune system (Demas, Adamo, & French, 2011; Di Prisco et al., 2013). An alternative, but nonmutually exclusive possibility is that compromised immunity results from resource-based trade-offs that are a core concept in the field of ecological immunology (Rolff & Siva-Jothy, 2003; Sadd & Schmid-Hempel, 2009; Demas & Nelson, 2012). Bees can clear neonicotinoids and their metabolites, which may be equally as toxic, from their bodies relatively rapidly via detoxification processes (Suchail, Debrauwer, & Belzunces, 2004a; Suchail, De Sousa, Rahmani, & Belzunces, 2004b; Simon-Delso et al., 2015), but these processes clearing xenobiotics in insects will be metabolically expensive (Berenbaum & Zangerl, 1994; du Rand et al., 2015). Therefore, even without any direct negative effects on immunity, neonicotinoid exposure could compromise immunity due to costly detoxification consuming resources that would otherwise be invested into immunity.

Independent of the underlying mechanisms, we lack studies of the effects of realistic neonicotinoid exposures on immunity in non-*Apis* bees (Collison et al., 2016), which will enable us to understand the generality of neonicotinoid exposure related immune-mediated disease susceptibility. Interestingly, it has recently been shown that bumblebees from neonicotinoid exposed colonies may exhibit increased expression of antimicrobial peptides (AMPs; Simmons & Angelli, 2017). However, age-related effects between treatments cannot be discounted in this case, and additionally expression of these immune peptides, usually considered to be largely induced (Barribeau et al., 2015), was only measured under naïve conditions. Thus, more studies are required to form a general conceptual understanding linking neonicotinoids, immunity, and disease that will subsequently allow us to make predictions about landscape level patterns of threats to bees posed by combinatorial stresses.

The aim of this study was to investigate interactions between pesticide exposure, in the form of the neonicotinoid insecticide imidacloprid, and immune responses in the Common Eastern Bumblebee *Bombus impatiens*. Risk assessment based on field concentrations has identified imidacloprid as one of the greatest exposure risks for pollinator insects (Sanchez-Bayo & Goka, 2014). While *B. impatiens* is itself not declining (Lozier, Strange, Stewart, & Cameron, 2011),

it represents an excellent model for laboratory-based studies, not possible for rarer declining species, that can be extrapolated to bumblebees as a whole. Without excluding alternative routes by which neonicotinoid exposure may influence immunity, it is hypothesized that resource-based trade-offs between detoxification of pesticides, immune investment, and somatic maintenance will lead to detrimental interactions between pesticide exposure and immunity for individuals. First, it is tested if prior exposure to imidacloprid may constrain immune investment, measured as the activity of phenoloxidase, a key enzyme in the production of cytotoxins and the melanization response in invertebrate immunity (Gillespie, Kanost, & Trenczek, 1997; Söderhäll & Cerenius, 1998; González-Santoyo & Córdoba-Aguilar, 2012), and humoral antimicrobial activity of the hemolymph, mediated mostly by AMPs and responsible for the targeted elimination of microbes (Gillespie et al., 1997; Bulet, Hetru, Dimarcq, & Hoffmann, 1999). Second, it is tested if a nonpathogenic immune challenge subsequent to imidacloprid exposure leads to a reduction in longevity, as would be predicted by the hypothesis that pesticide detoxification and mounting an immune response are both costly processes that deprive resources from somatic maintenance.

2 | MATERIALS AND METHODS

2.1 | Bumblebee maintenance

Colonies of the Common Eastern Bumblebee, *B. impatiens*, sourced from Koppert Biological Systems were transferred to plaster nests (adapted design of Pomeroy & Plowright, 1980) for maintenance throughout the experiments. At colony transfer, and every subsequent 2 weeks, parasite-free status, concerning common gut infecting parasites of bumblebees (e.g., *Nosema bombi* and *Crithidia bombi*), was confirmed through microscopic fecal checks of the mother queen and samples of workers. Colonies were kept under red light at $24 \pm 2°C$, with pollen (Brushy Mountain Bee Farm) and sugar water provided ad libitum. Sugar water from cane sugar dissolved in water (55% w/v) was partially inverted by the addition of cream of tartar (0.001% w/v) followed by 15 min of heating the solution to boiling. Bees from five colonies were used to investigate immune responses under pesticide exposure, and bees from an additional six colonies used to investigate survival under immune challenge and pesticide exposure. Colonies were maintained under standard laboratory conditions for 3 weeks to acclimatize before workers started to be removed for experiments. To control for worker age, adult workers were removed from colonies on the day of eclosion and maintained in individual isolation for 2 days, with all other conditions as standard, before being uniformly allocated within blocks of colony of origin to the relevant treatments for the immune response or survival experiments.

2.2 | Imidacloprid pesticide exposure preparation

Imidacloprid (37894, Sigma-Aldrich, St. Louis, MO, USA) was used as the neonicotinoid exposure for the adult bees. Imidacloprid was prepared in sugar water at two doses representing low

(0.7 ppb/μg L^{-1}) and high (7 ppb/μg L^{-1}) field realistic doses. These preparations were produced immediately before use in the experiments from a 100 ppb stock kept at 2°C. The doses were chosen based on reported concentrations to which bumblebees could be exposed to in nature. While concentrations up to 1,000 ppb have been detected in pollen and nectar (Bonmatin et al., 2015), detected levels have been more usually between <1 and 15 ppb (Pohorecka et al., 2012; Stoner and Eitzer, 2012; David et al., 2016; Long and Krupke, 2016). However, even the high-field realistic dose may be an underestimate of exposure for certain times of the year, especially as pesticides, including imidacloprid, can become more concentrated in stored resources of social honeybees and bumblebees (David et al., 2016). It has been argued that bees in the field will be only exposed intermittently to neonicotinoids (Carreck & Ratnieks, 2014), and thus, we use a conservative 6-day pulse of exposure. However, it is becoming increasingly clear that exposure of bees to a cocktail of neonicotinoids and other pesticides has the potential to occur for several months throughout the year (Long & Krupke, 2016), and our exposure period is shorter than that advocated for in environmental risk assessment trials (Cabrera et al., 2016). In preliminary trials, there was no difference in the time taken to consume 20 μL of sugar water with 0, 0.7, 7 or 70 ppb of imidacloprid (data not shown). Additionally, a lack of aversion has previously been noted for these concentrations in bumblebees and honeybees (Kessler et al., 2015; Raine & Gill, 2015).

2.3 | Bacteria-based immune challenge preparation and inoculation

Immune challenges were carried out using a solution of heat-killed *Arthrobacter globiformis* bacteria (ATCC 8010). This challenge has been used previously in bumblebees to study the effects of an immune response without the confounding pathogenic effects of a live infection, and has been demonstrated to lead to induction of antimicrobial immune pathways and expression of other immune-related genes (e.g., Sadd, Kleinlogel, Schmid-Hempel, & Schmid-Hempel, 2005; Sadd & Schmid-Hempel, 2007; Barribeau et al., 2015; Barribeau, Schmid-Hempel, & Sadd, 2016). Preparation and inoculation was carried out as in these previous studies. Briefly, bacteria were cultured at 30°C in medium (10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl in 1,000 mL of distilled water, pH 7.0). To prepare bacterial suspensions for challenge injections, 1 mL of an overnight culture was centrifuged (3,000 rpm, 4°C, 10 min) and the supernatant was discarded and replaced with sterile insect ringer saline. This procedure was repeated three times and the concentration of cells adjusted to 10^8/mL. The bacteria were then heat killed (90°C, 5 min). Efficiency of killing was confirmed by plating out samples of the suspension on agar medium. Immune challenge inoculations took place following immobilization of isolated bumblebee workers on ice for 20–30 min. The immune challenge treatment consisted of a 2 μL injection of the aforementioned heat-killed bacteria solution using a sterile pulled glass microcapillary inserted between the first and second abdominal tergites. Sham control treatment bees received the same treatment but were injected with 2 μL of sterile ringer saline solution. Following injection, workers were taken off ice and returned to their individual boxes.

2.4 | Immune responses following imidacloprid exposure treatments

The first experiment tested the effects of imidacloprid exposure on two measures of immunity, phenoloxidase activity and humoral antimicrobial activity, in naïve and immune-challenged bees. Two days after emergence, isolated workers were exposed to either one of three imidacloprid treatments administered in sugar water for 6 days: (1) no imidacloprid, (2) low-field realistic dose (0.7 ppb) or (3) high-field realistic dose (7 ppb). On day 8 following adult exposure, after the 6-day pulse of imidacloprid, all bees were given imidacloprid-free fresh sugar water. Within each imidacloprid treatment, bees were split into two groups and either (1) left naïve or (2) given an immune challenge of heat-killed bacteria, as outlined above. The bees were destructively sampled at 6, 48, or 144 hr after the immune treatment to assess the temporal dynamics of the measured immune responses for the different groups.

At the time of sampling, bees were immobilized by being placed on ice for 20–30 min, then pierced with a sterilized 26G needle between the fifth and sixth sternite of the abdomen, and 5 μL of hemolymph was collected using a prechilled graduated glass microcapillary tube. Extracted hemolymph was added to 20 μL of sodium cacodylate buffer (0.01 M Na-Cac, 0.005 M CaCl$_2$). Hemolymph samples were split for assaying phenoloxidase activity, diluted 1:20, and antimicrobial activity, diluted 1:5 in a tube prelined with 1-phenyl-2-thiourea (PTU, Sigma-Aldrich, St. Louis, MO, USA) to inhibit melanization. Samples were snap frozen in liquid nitrogen and stored at −80°C until assayed. The lengths of the forewing radial cells were measured as a surrogate for body size (Muller, Blackburn, & Schmid-Hempel, 1996; Schmid-Hempel & Schmid-Hempel, 1996), with the average radial cell length calculated for each bee. A total of 381 bees from five unrelated colonies were used in the immune assays.

Activity of phenoloxidase was measured using a spectrophotometric assay (after Moret & Schmid-Hempel, 2009). Thawed individual samples, measured in duplicate, were added to wells of a flat-bottomed 96-well plate being held on ice. Wells also contained 20 μL of phosphate buffered saline (PBS: 8.74 g NaCl; 1.78 g Na$_2$HPO$_4$, 2H$_2$O; 1,000 mL nanopure water; pH 6.5) mixed with 140 μL of nanopure water. Sample blanks, with 20 μL cacodylate buffer instead of a sample, were included to account for nonenzymatic changes in optical density. Immediately before starting spectrophotometric readings, 20 μL of L-Dopa substrate (4 mg/mL of nanopure water, Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The reaction was allowed to proceed at 30°C in a Multiskan GO microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min. Optical density readings were taken every 15 sec at 490 nm. Enzyme activity was measured as the slope (V_{max} value) during the linear phase of the reaction, between 10 and 20 min after the reaction start. Measurements were taken and checked until 30 min to ensure that the reaction curve was plateauing and the maximum rate of the reaction was captured during the expected period between 10 and 20 min.

Antimicrobial activity was assayed from zones of inhibition produced by samples on petri dishes with agar seeded with *A. globiformis* (see Sadd & Schmid-Hempel, 2007 with modifications). Briefly, *A. globiformis* from a single colony on a streak plate were incubated overnight

at 30°C in 7 mL of the previously described media. From this culture, bacteria were added to liquid media containing 1% agar held at 45 °C to achieve a final density of 10^5 cells/mL. Six milliliters of seeded medium was poured into a 100 mm diameter petri dish to solidify. Sample wells were made using a Pasteur pipette (Volac D810) fitted with a ball pump, 2 μL of sample solution thawed on ice was added to each well, and additionally negative (cacodylate buffer and PTU) and positive (Tetracycline, Sigma-Aldrich, St. Louis, MO, USA) control wells were included on each plate. Plates were inverted, incubated for 28 hr at 30°C, and then the diameter of inhibition zones were measured for each sample. Two diameter measurements perpendicular to one another were taken for each sample and averaged. Because zone of inhibition diameter does not increase linearly with increasing AMP activity, measured zone diameters were converted, based on a standard curve, to units (μg/mL) of the antibiotic tetracycline. Each bee sample was tested in duplicate, with the mean of the duplicates being used in subsequent analyses. Bacterial growth failed on plates for one technical replicate in an assay block representing 21% of all samples, resulting in only one usable technical replicate for these samples. However, these single value samples are included with confidence because of the high repeatability across the other technical replicates that were measured ($F = 431.5$, df = 1, 67, $P < 0.001$, $R^2 = 0.87$).

2.5 | Survival following imidacloprid exposure and subsequent immune challenge treatments

The second experiment tested the effects of imidacloprid exposure and/or a later immune challenge on bumblebee worker survival. Two days after emergence, isolated workers were exposed to one of three imidacloprid treatments administered in sugar water for 6 days: (1) no imidacloprid, (2) low-field realistic dose (0.7 ppb), or (3) high-field realistic dose (7 ppb). On day 8 following adult eclosion, after the 6-day pulse of imidacloprid, all bees were given imidacloprid-free fresh sugar water, which was replaced as necessary on a weekly basis. At this time, the bees from each treatment were also given an immune treatment, being either (1) left naïve, given a (2) sham injection of 2 μL of ringer saline, or (3) immune challenge treatment injection of 2 μL of the heat-killed bacteria solution described above. This produced a fully crossed design of imidacloprid exposure treatment and immune challenge treatment. Survival was recorded daily, and when the bees died the date was recorded. Average radial cell measurements were again taken as a surrogate for bee body size. Bees that did not survive 24 hr after the immune treatment were removed from the study, as it is likely that these deaths were the result of mishandling during treatment. A total of 169 bees were assayed for survival across six unrelated colonies.

2.6 | Data analyses

Analyses were performed in R version 3.2.4 (R Development Core Team, 2016) using the *survival* (Therneau, 2015) and *lme4* (Bates, Mächler, Bolker, & Walker, 2015) packages. Immune responses were analyzed separately for naive and immune-challenged groups of bees.

TABLE 1 Summary of linear mixed models for phenoloxidase activity (square root transformed) in naive (A) and immune-challenged (B) bumblebee workers

Model term	F	df	P
A) Naive			
Sampling time	4.71	2	0.01
Imidacloprid treatment	11.34	2	<0.001
B) Immune challenged			
Sampling time	0.25	2	0.778
Imidacloprid treatment	3.01	2	0.052

Colony was included as a random effect in the models. Only effects from the best fitting models are reported here.

The data for phenoloxidase activity in both groups were analyzed using linear mixed effects models, with the response variable square root transformed to meet model assumptions. Induced antimicrobial activity in the immune-challenged group was also analyzed using a linear mixed effects model. The models included imidacloprid treatment, sampling time, and the interaction between the two factors. The adult emergence date of the bee and its size, given by the length of the forewing radial cell, were included as covariates. Differences between colonies were accounted for by including colony as a random effect. As only 17.5% of bees in the naïve group assayed for antimicrobial activity had any measurable zones, they were analyzed with a generalized linear mixed model, with the same factors as above, but with a binomial response (zone/no zone). Maximal models were simplified by sequentially eliminating nonsignificant terms through likelihood ratio tests (LRTs), and best-fitting models were chosen based on the Akaike information criterion. For factor level comparisons, the package *lsmeans* (Lenth, 2016) was used to extract predicted marginal means for treatment levels from the best fitting models. The data for the survival experiment were analyzed using a Cox proportional hazards model. The model included imidacloprid treatment, immune challenge treatment, and the interaction between the two. The adult emergence date of the bee and its size were included as covariates. Differences between colonies were accounted for by including colony as a random effect using the frailty function in the *coxph* model of the *survival* package (Therneau, 2015).

3 | RESULTS

3.1 | Measures of immunity in naïve and immune challenged bees following imidacloprid exposure

Imidacloprid exposure treatment has a significant effect on constitutive levels of phenoloxidase activity in naïve bees (Table 1, panel A). Phenoloxidase activity is significantly lower in the high (7 ppb) imidacloprid exposed group compared to both the unexposed and low (0.7 ppb) exposed groups (Fig. 1). There is an identical but marginally nonsignificant trend in the immune-challenged group (Table 1, panel B). For the naïve group, there is also a significant effect of sampling time (Table 1, panel A), with back-transformed estimated marginal means (−1 SE,
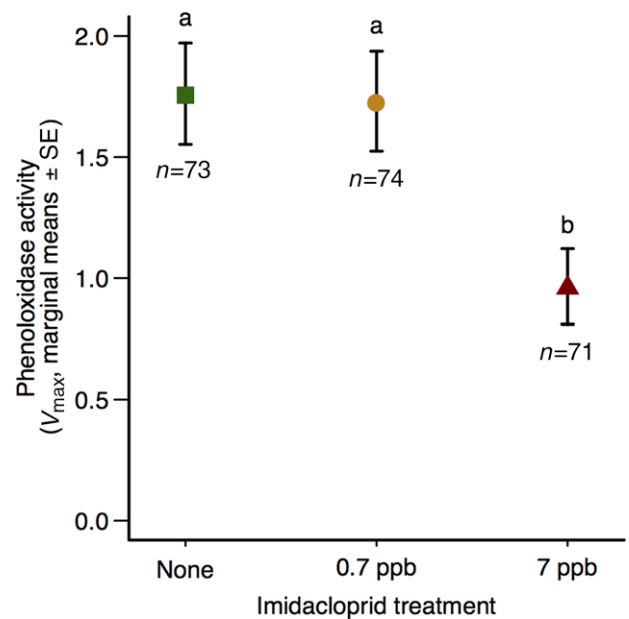

FIGURE 1 Phenoloxidase activity in naïve worker bumblebees following imidacloprid exposure treatments. Back-transformed predicted marginal means and standard errors estimated from the fitted model for phenoloxidase activity across imidacloprid treatments. Different letters above bars indicate significant differences between groups (Sequential Bonferroni corrected pairwise *t*-tests, P < 0.05) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Summary of linear mixed model for induced antibacterial activity post immune challenge in bumblebee workers

Model term	F	df	P
Sampling time	19.81	2	<0.001
Imidacloprid treatment	0.31	2	0.73
Imidacloprid treatment × sampling time	2.68	4	0.03

Colony was also included as a random effect. Only effects from the best fitting models are reported here.

+1 SE) of 1.81 (1.61, 2.02), 1.25 (1.08, 1.44), and 1.33 (1.14, 1.52) for sampling at 6, 48, and 144 hr, respectively. However, the interaction between sampling time and imidacloprid treatment is not significant ($F = 1.41$, df = 4, $P = 0.231$), and is not included in the final model.

Neither prior imidacloprid exposure (LRT: $X^2 = 2.17$, df = 2, $P = 0.338$) nor sampling time (LRT: $X^2 = 0.79$, df = 2, $P = 0.673$) has a significant influence on the production of inhibition zones, indicating detectable antibacterial activity, in naïve worker bees. In the immune-challenged group, there is a significant interaction between the imidacloprid exposure treatment and sampling time on antibacterial activity (Table 2, Fig. 2). Imidacloprid treatments do not significantly differ in their effect on antibacterial activity at 6 and 48 hr following the immune challenge. However, at 144 hr bees previously exposed to the high 7 ppb dose of imidacloprid have significantly reduced antibacterial activity relative to the unexposed and low (0.7 ppb) exposed groups (Fig. 2).
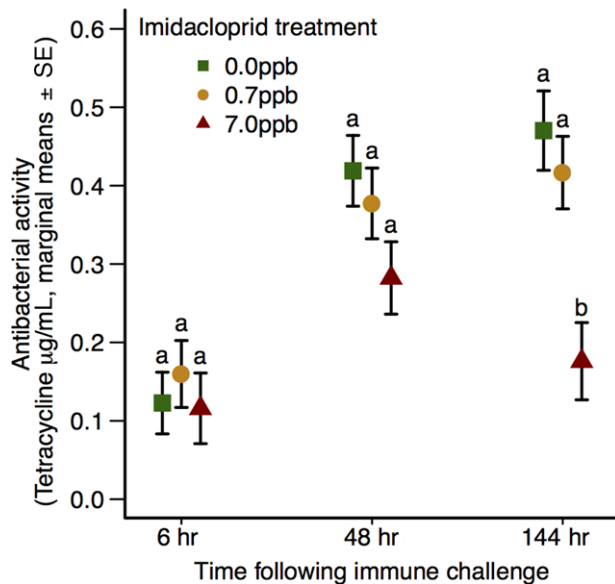
FIGURE 2 Antibacterial activity following a nonpathogenic immune challenge in worker bumblebees that had previously received one of three imidacloprid exposure treatments. Predicted marginal means and standard errors estimated from the fitted model for antibacterial activity (standardized to units of the antibiotic tetracycline) across imidacloprid treatments at 6, 48, and 144 hours following a bacterially based immune challenge. Different letters above bars indicate significant differences between imidacloprid groups within sampling times (Sequential Bonferroni corrected pairwise t-tests, $P < 0.05$) [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Analysis of deviance for factors influencing bumblebee worker survival based on log-likelihood as they are added into the Cox proportional hazards model

Factor	X^2	df	P
Date emerged	6.10	6	0.413
Wing size	0.72	1	0.395
Imidacloprid treatment	5.09	2	0.079
Immune treatment	9.02	2	0.011
Imidacloprid treatment × Immune treatment	18.04	4	0.001

Colony of origin included as a random effect using a frailty function.

3.2 | Survival to an immune challenge following imidacloprid exposure

There is a significant interaction between imidacloprid exposure treatment and immune challenge treatment on survival (Table 3). The predicted hazard ratios from the fitted Cox proportional hazards model, showing the relative mortality rates to the reference of imidacloprid-unexposed and naïve unchallenged bees, indicate that this interaction is driven by decreased survival in groups that are both exposed to imidacloprid and immune challenged (Fig. 3). Significantly elevated hazard ratios, demonstrating decreased probability of survival, are present for both the low (0.7 ppb, $P = 0.005$) and high (7 ppb, $P = 0.026$) imidacloprid-exposed bees that also received the bacterially based immune challenge. In all other groups of bees, whether exposed to imidacloprid alone or only immune challenged, there is an overlap of
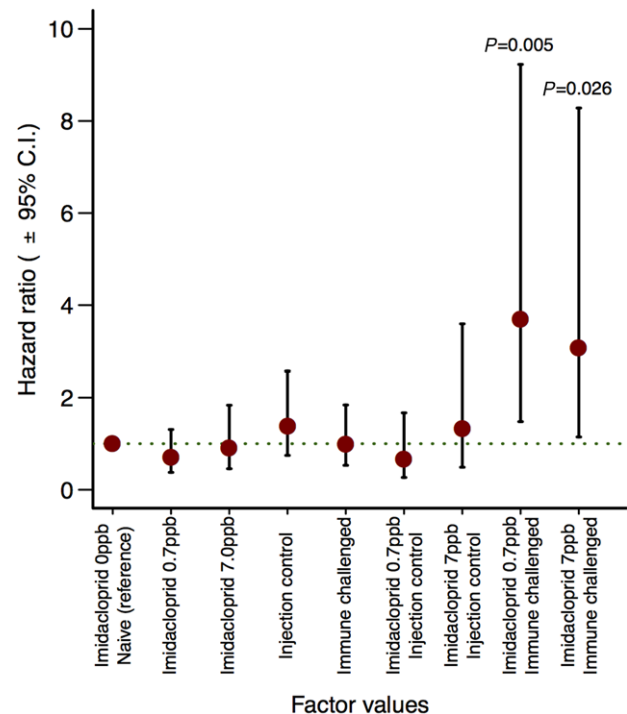

FIGURE 3 Worker bumblebee survival is reduced significantly on combined exposure to imidacloprid and a nonpathogenic immune challenge. Presented hazard ratios are from the Cox proportional hazards model relative to the reference bee group (horizontal dashed line) that was not exposed to imidacloprid (imidacloprid, 0 ppb) and did not receive an immune challenge (naïve) [Color figure can be viewed at wileyonlinelibrary.com]

estimated 95% confidence intervals of predicted hazard ratios with the reference category.

4 | DISCUSSION

The results of this study demonstrate important interactions between exposure to the neonicotinoid imidacloprid at field relevant levels and certain components of bumblebee immunity, with consequences for understanding variability in immunity, pathogen resistance, and bee health. A 6-day exposure to the high, but still field realistic, dose of 7 ppb of imidacloprid subsequently compromised both one component of constitutive immunity, as measured by phenoloxidase activity, and one component of induced immunity, as measured by humoral antimicrobial activity. These effects could make the bees more susceptible to the negative consequences associated with pathogen infection. However, even in the absence of a live pathogen infection, investment into an immune response following imidacloprid exposure has a cost for bee longevity, which is not apparent following either an immune challenge or imidacloprid exposure alone. This represents an extra burden for bumblebees that are coexposed to the multiple stresses of pesticides and pathogens in natural environments.

The results demonstrated here in bumblebees are congruent with the outcomes of prior studies investigating interactions between neonicotinoid exposure and immunity in honeybees (Alaux et al., 2010;

Di Prisco et al., 2013; Brandt et al., 2016; López et al., 2017). This is not a trivial extension, as honeybees and bumblebees, while both highly susceptible, have been suggested to differ in their detoxification of neonicotinoids (Cresswell, Robert, Florance, & Smirnoff, 2014), and neonicotinoid exposure and parasite stress has been shown to have differential effects in the two groups of bees (Piiroinen & Goulson, 2016). The reduction in antimicrobial activity in *B. impatiens* is in contrast to a recent study showing elevated AMP expression following imidacloprid exposure (Simmons & Angelli, 2017). However, this gene expression study only measured constitutive expression of an immune component that is generally considered to be induced (Barribeau et al., 2015).

The reduction in constitutive phenoloxidase activity in neonicotinoid-exposed individuals could limit the capacity of these bees to mount a rapid nonspecific response, which is a key component of the invertebrate immune response (Siva-Jothy, Moret, & Rolff, 2005). However, while phenoloxidase activity is linked to pathogen resistance in some systems (e.g., Ayres & Schneider, 2008; González-Santoyo & Córdoba-Aguilar, 2012), in other systems the link is weak or absent (Adamo, 2004; González-Santoyo & Córdoba-Aguilar, 2012). Additionally, investment into other components of constitutive immunity may not be affected, or phenoloxidase activity could even be inhibited in response to an elevation of an unstudied component such as lysozyme (Rao, Ling, & Yu, 2010). Further studies of a broader suite of immune components are required to make concrete generalizations.

Although the antimicrobial response is induced in all groups following the bacteria-based immune challenge, its waning under neonicotinoid exposure is significant. The humoral antimicrobial response of bumblebees is known to be maintained in an induced state past 7 days (Korner & Schmid-Hempel, 2004), and in other insects it has been shown that such a lasting induced response is important for protection against persistent microbes that are resistant to the initial constitutive response (Haine, Moret, Siva-Jothy, & Rolff, 2008). Expression of insect AMPs is known to be heightened for several days after an immune challenge (Johnston, Makarova, & Rolff, 2014), but without measuring AMP expression directly, it is not possible to differentiate between reduced AMP expression in the neonicotinoid-exposed group and reduced activity due to effects on the standing pool of peptides. Overall, however, the immune deficiencies shown could lead to increased consequences for pathogen infection in bumblebees, just as in honeybees, and suggest that such effects can be extrapolated to more bees that are harder to study experimentally.

The decreased probability of survival when any level of prior exposure to imidacloprid was combined with a nonpathogenic immune challenge suggests that the interaction between neonicotinoid and pathogen exposure will have consequences extending beyond those relating to the current disease state. Even if a pathogen infection is cleared, costs will be imposed on coexposed individuals. Costs of immune activation for longevity are well documented (Sadd & Schmid-Hempel, 2009), but the result here of no increase in mortality following only an immune challenge is in agreement with demonstrations that such costs are only uncovered under nutritionally limited conditions in bumblebees (Moret & Schmid-Hempel, 2000). In contrast to other studies with neonicotinoids and bumblebees (Gill, Ramos-Rodriguez, & Raine, 2012; Fauser-Misslin et al., 2014), there was no reduction in

survival under imidacloprid exposure alone. Exposure in this current study was, however, lower in dose and reduced in time compared to these other experiments. The decreased survival probability following exposure to a 6-day pulse of imidacloprid and subsequent immune challenge is an important demonstration. It shows combinatorial negative effects can arise even when stresses are not faced concurrently, with lifetime effects resulting from a short pulsed field realistic pesticide exposure, even though the active imidacloprid and potentially toxic metabolites can be relatively rapidly cleared from bees (Suchail et al., 2004b).

The survival and immune assay results differed in relation to the influence of the two field realistic doses on the outcomes. In comparison to the survival results, imidacloprid exposure affected immune measures only at the high dose. This dose apparently crossed a threshold that the low-field realistic dose was not sufficient to cross, and short-term negative effects could be countered. It is possible that this was achieved through an allocation of resources away from other bodily functions to maintain immune levels, such as somatic maintenance, which would explain why an effect is detected in the longevity data even for the low dose. The potential for bumblebees to be exposed to widespread neonicotinoid pesticides at the doses used is high, with concentrations in nectar and pollen and in colony storage at these levels or higher (Stoner & Eitzer, 2012; Sanchez-Bayo & Goka, 2014; David et al., 2016; Long & Krupke, 2016). In addition, although short pulsed exposures, as used here, may be considered more realistic on average (Carreck & Ratnieks, 2014), field level studies suggest that exposure to a cocktail of pesticides can be chronic and a season-long threat (Sanchez-Bayo & Goka, 2014; Long & Krupke, 2016). This suggests that the results shown here on the interactions between neonicotinoid exposure and immunity in bumblebees are likely conservative, and depending on natural exposure regimes, these detrimental effects may be greater in some locations.

Although this study does not explicitly investigate mechanisms underlying the effect of pesticide exposure on immunity, some speculative inferences can be made. Mechanisms are not exclusive of one another, and could act in concert to further compromise immunity. Neonicotinoid pesticides bind nAChRs and lead to disruption of neural transmission (Matsuda et al., 2001; Simon-Delso et al., 2015). Crosstalk between the neuroendocrine and immune systems is prevalent in insects and other organisms (Demas et al., 2011), and adaptive reconfiguration can take place across the two systems (Adamo, 2014; Adamo, 2017). Therefore, one possibility is that neurobiological dysfunction imposed by neonicotinoid exposure has knock on effects for immunity (Sánchez-Bayo et al., 2016). Indeed, effects on hemocytes have been shown in honeybees (Brandt et al., 2016; López et al., 2017), and hemocytes are considered to work at the interface between the immune and neuroendocrine systems (Malagoli, Mandrioli, Tascedda, & Ottaviani, 2017). Direct cytotoxic activity of neonicotinoid pesticides or their metabolites on tissues with direct or indirect links to immunity could also play a role. For example, imidacloprid exposure has been shown to degenerate honeybee malpighian tubules (Rossi, Roat, Tavares, Cintra-Socolowski, & Malaspina, 2013), with proposed consequences for osmolarity and potentially immune function (Collison et al., 2016).

In addition to the mechanisms outlined in the previous paragraph, it is also possible that reduced immunity following pesticide exposure in bees could result from a trade-off in using shared resources, for example energy, at the center of the cost-based explanations for immune variation of ecological immunology (Rolff & Siva-Jothy, 2003; Sadd & Schmid-Hempel, 2009; Demas & Nelson, 2012). P450 monooxygenases, glutathione transferases, and carboxylesterases are involved in the detoxification of xenobiotics, including neonicotinoids (Li, Schuler, & Berenbaum, 2007; Berenbaum & Johnson, 2015). Although bees are depauperate in genes for these detoxification enzymes relative to other sequenced insects (Claudianos et al., 2006; Sadd et al., 2015), they are capable of detoxification and have been shown to be able to clear imidacloprid and metabolites after exposure to doses up to an order of magnitude greater than those used in the current study (Suchail et al., 2004a, 2004b). Such detoxification will be energetically expensive (du Rand et al., 2015) and could therefore be at the expense of investment of energy and other resources into immunity and or somatic maintenance. A general energetic trade-off, as suggested previously (Collison et al., 2016), would be parsimonious for the effects on both phenoloxidase and humoral antimicrobial activity, as these responses come from different pathways (Barribeau et al., 2015). In addition, the measured effects on antimicrobial activity are not present in the initial response, as would be expected if it resulted from direct damage or neural-immune cross-talk. Instead, the antimicrobial response is not maintained 6 days following the end of the neonicotinoid exposure, when detoxification is largely completed (Suchail et al., 2004b). Finally, reduced survival when imidacloprid exposure is followed by a nonpathogenic immune response would be consistent with allocation of resources to detoxification and immunity at the cost of somatic maintenance and longevity. However, the lack of a dose–response relationship between survival and the two doses of imidacloprid in immune-challenged bees does not fit perfectly into a model for costly investment into detoxification, immunity, and somatic maintenance being at the heart of these results. It is expected that detoxification of a higher dose would correspond to a greater allocation of energy, and hence reduced survival relative to the lower dose. While this is not seen, it is still possible that the results can be congruent with the trade-off framework when both experiments are considered together. Under the low dose, measured aspects of immunity are not reduced. As stated above, this could be achieved through an allocation of resources away from other bodily functions, such as somatic maintenance, to maintain immune levels, thus explaining the effect in the longevity data even for the low dose. Under the high dose, survival is not reduced further, but the reduction of the measured immune response at this dose suggests that investment in detoxification may be greater at the high dose than the low dose, but now additionally compromises immunity. However, it is clear that considerable further work is required to understand the potentially complex interacting causes of the important phenotypic responses that are demonstrated in this study.

The interaction of pesticides and parasites is of grave concern in relation to pollinator health (Vanbergen et al., 2013; Goulson et al., 2015). Environmental challenges faced by important pollinator insects can be exacerbated due to detrimental interactions between abiotic conditions and immunity. The potential immune compromising effects of exposure to neonicotinoids and the survival related costs of immune activation and neonicotinoid exposure, as shown here, are a plausible means by which sublethal pesticide exposures can synergize with pathogens to elevate detrimental impacts in bees. This highlights the importance of including relevant physiological measures, including of immunity, in recommended studies under semifield and field conditions (Cabrera et al., 2016). While the results presented here alone add to our understanding of how the abiotic environment of the anthropocene (Lewis & Maslin, 2015) may influence immunity and the individual and population health of a key pollinator insect, field extensions of this work will enable a link to be made between heterogeneity in immunity, resulting from interactions between multiple environmental stressors, and disease ecology in natural settings (Hawley & Altizer, 2011).

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