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RESEARCH ARTICLE

A neonicotinoid pesticide alters how nectar chemistry affects bees

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Abstract

1. Neonicotinoid pesticides in the nectar and pollen of managed crops and wildflowers contribute to the global declines of bees. These chemicals can have detrimental effects on bees' physiology, behaviour and reproduction. Floral nectar also contains secondary chemistry with its own effects on bee health. How nectar secondary chemistry may act additively or synergistically with neonicotinoids is unknown.
2. Here, we asked how an acute exposure to a common neonicotinoid, imidacloprid (IMD) affected the longevity, immune function and behaviour of bumble bee *Bombus impatiens* workers maintained on diets enriched with one of three nectar secondary metabolites (NSMs; the alkaloid caffeine, the terpenoid thymol or the cardiac glycoside digoxin). A factorial design allowed us to assess the potential for additive and interactive effects of each NSM and IMD combination on multiple health outcomes.
3. Without IMD exposure, different dietary NSMs each had positive effects on life span (caffeine), immune function (digoxin) and activity levels (caffeine, thymol), although these came with trade-offs. A single sublethal IMD exposure overshadowed these NSM effects, and in two cases, an NSM-enriched diet magnified the negative effects of pesticide exposure.
4. In summary, we show that even a single acute exposure to a pesticide has the potential to reshape interactions between pollinators and plants mediated by nectar secondary chemistry.

KEYWORDS
Bombus impatiens, nectar secondary chemistry, neonicotinoids, pollinators

1 | INTRODUCTION

Identifying the causes of insect population declines is a pressing challenge, given their widespread consequences for ecosystem functioning and human food security (Sánchez-Bayo & Wyckhuys, 2019; Wagner, 2020). Pesticides are among the most serious factors implicated in these declines, as they are ubiquitous in many landscapes (Halsch et al., 2020) and known to have detrimental effects on beneficial insects (Alkassab & Kirchner, 2017; Blacquière et al., 2012; European World Safety Authority, 2013; Goulson, 2013; Woodcock et al., 2016). Floral visitors encounter widely used neonicotinoid pesticides in nectar and pollen of agricultural crops and horticultural

plants, as well as in plants inadvertently exposed through shared water or soil (Blacquière et al., 2012; Herbertsson et al., 2021). Our understanding of how neonicotinoids influence pollinator declines is largely based on two lines of research: real-world observational evidence of pollinator declines in nature correlating with agrochemical usage (i.e. Mallinger et al., 2015; Main et al., 2020), and controlled experiments that measure the behavioural and physiological responses of insect pollinators to neonicotinoids in the laboratory (reviewed in Blacquière et al., 2012; Cresswell, 2011; Siviter, Richman, et al., 2021). Yet, we currently do not know how the effects observed in laboratory experiments scale up to real-world settings.

By offering bees' artificial nectar containing sucrose and systematically varied pesticide dosages, researchers can characterize their effects on behaviour, physiology and survival (EPA, 2016). This approach necessarily sacrifices some realism, namely because it tends to limit exposure to the numerous other chemicals in floral nectar. In field settings, a given pesticide may be encountered amidst a blend of different anthropogenic plant protection chemicals (pesticides, herbicides, fungicides; Zioga et al., 2020) giving rise to potentially interactive effects (Halsch et al., 2020; Siviter, Bailes, et al., 2021). Likewise, natural (i.e. phytochemical) chemicals could also be modulators of pesticide toxicity, as floral nectar often contains amino acids, ions and secondary metabolites such as alkaloids, phenolics, terpenoids and glycosides (Adler, 2000; Palmer-Young et al., 2019). Whether or not these nectar secondary metabolites (NSMs) affect bee performance under neonicotinoid exposure represents a critical gap in our understanding of how pesticides affect bees in natural settings, given NSMs' complex effects on bee physiology and behaviour (Stevenson et al., 2017).

NSMs are chemically diverse and widespread across plant taxa (Palmer-Young et al., 2019). Beyond their ability to attract or deter floral visitors, some NSMs such as caffeine may promote pollen transfer (Thomson et al., 2015) and enhance bees' recall of floral stimuli (Wright et al., 2013). Because many NSMs are also mechanisms of chemical defence against herbivores, they can be toxic (Adler, 2000) potentially filtering the identity of floral visitors (Stevenson et al., 2017). As has been found for neonicotinoids themselves (Alkassab & Kirchner, 2017), the effects of NSMs on pollinator performance often vary with concentration or depend on factors such as bee species and nutritional state (Anthony et al., 2015; Palmer-Young et al., 2019; Stevenson et al., 2017). Some NSMs even benefit bees: The terpenoid thymol, for example, can reduce loads of the bumble bee gut trypanosome parasite *Crithidia bombi* (Richardson et al., 2015) and is widely used as a miticide for honeybee colonies (Gregorc & Planinc, 2013; Imdorf et al., 1995). Whether systemic pesticides interfere or combine with nectar phytochemistry to affect pollinator performance is a critical question for pollination biologists in the Anthropocene.

As a first step towards increasing the ecological realism of pesticide-focused studies, we consider how NSMs combine with a neonicotinoid to affect bees. We conducted a lab-based experiment testing the effects of three focal NSMs present in bumble bee diets combined with a single acute exposure to the popular neonicotinoid imidacloprid (IMD). Given the chemical diversity of NSMs, and their variable effects on different aspects of bee performance, many outcomes were possible. We focused on evaluating the evidence for the following key hypotheses: (H1) Exacerbation: It is possible that bees already contending with phytochemicals in their diet may be less able to cope with neonicotinoid exposure, exhibiting decreased performance. This could be the case both if the chemicals overlap in their mode of action (e.g. both nicotine and imidacloprid target nicotinic acetylcholine receptors; Matsuda et al., 2020) or in the form of additive costs. For instance, an NSM that suppresses appetite could reduce energetic reserves needed for pesticide detoxification (Stuligross & Williams, 2020; Tosi & Nieh, 2017). (H2) Amelioration: An NSM-rich diet could prime bees against the effects of pesticide exposure. In honeybees, for example, the phytochemical quercetin, although toxic at high concentrations, upregulates the expression of cytochrome P450 genes involved in xenobiotic detoxification, offsetting the effects of IMD (Ardalani et al., 2021; Liu et al., 2021; Wong et al., 2018). (H3) Pesticide mediation: While hypotheses 1 and 2 address how NSMs could alter the effects of IMD exposure, we explored the parallel hypothesis that IMD exposure might alter the effects of an NSM-rich diet. Finally, we considered (H4) the null hypotheses that an NSM has no direct or indirect effect on how bees respond to IMD exposure and vice versa. Practically, H4 might bolster confidence in the external validity of research into the sublethal effects of pesticides on bees (EPA, 2016) and/or encourage translating what we know about the beneficial effects of NSMs for bees to settings where pesticide exposure is unavoidable (Arnold et al., 2021).

We used a factorial design to assess the separate and combined effects of a nectar diet enriched with an ecologically realistic concentration of an NSM (caffeine, thymol or digoxin) and a single acute oral exposure to IMD on bumble bees *Bombus impatiens*. We assessed worker longevity, which contributes to colony growth (Crone & Williams, 2016); activity level, which underlies foraging and nest maintenance (Crall et al., 2018); and performance of the constitutive immune system (activity of the phenoloxidase (PO) enzyme). We chose the focal NSMs because they are found in both economically important crops and plants popular with home gardeners, two contexts in which IMD is widely used. Furthermore, each of these NSMs is known to play a role in bee cognition, health or foraging preference (Manson et al., 2012; Richardson et al., 2015; Wright et al., 2013). By assessing multiple performance outcomes, we sought to capture a diversity of ways in which natural and anthropogenic components of nectar chemistry could combine to influence bee health.

2 | MATERIALS AND METHODS

2.1 | General methods

2.1.1 | Colony maintenance and individual chambers

We used the Eastern Bumble Bee, *Bombus impatiens*, as subjects ($N = 960$ individuals, 12 colonies) for this lab-based study. *B.*

impatiens is a common species, native to eastern North America, and is widely used as a commercial pollinator. We used workers from apiary-raised colonies consisting of 50–70 individuals with the natal queen, purchased from Koppert Biological Systems and housed in plastic boxes provided by the supplier. This species does not require ethical approval for animal research. Colonies were maintained on a diet of 30% (w/w) sucrose solution, offered ad libitum from wicked feeders inside 1 m^3 caged arenas, accessible to the colony through plastic tubing. We provided colonies with ~0.5 g honey bee-collected pollen (Koppert Biological Systems) every other day. The colony was kept in darkness, but foraging arenas were illuminated by a combination of natural and fluorescent room lighting. We collected foragers for use in the experiment from the wicked feeder using an insect aspirator (BioQuip Products) and cold-anaesthetized them.

Subjects were transferred to individual experimental chambers: transparent plastic cylindrical tubes with ventilation holes (TAP plastics; L × D 13 × 2.5 cm, wall thickness: 1.6 mm) housed in an incubator (Percival Scientific; 20°C, 70% RH 12:12 light:dark). The experimental chambers were fitted with a rubber cap at one end and a plastic plug at the other end. The rubber cap end contained a feeder constructed from a 1.5-mL Eppendorf microcentrifuge tube. From a small hole at the end of the Eppendorf tube, a tapered cotton swab (Fran Wilson Nail Tees Cotton Tips) extended towards the bee; this design prevented spillage. Depending on dietary treatment (described below), the feeders offered 1 ml of sucrose solution either enriched with a single NSM or not (control). To track the consumption of these diets, we weighed feeders before and after they were offered to bees, at intervals noted in the methods for each of the three experiments.

2.1.2 | Dietary treatments

Bees were randomly assigned to different artificial nectar diet treatments. All diets involved sucrose solutions with a concentration of 30% (w/w sugar, Walmart). We supplemented the sucrose solution with one of three chemicals: caffeine, an alkaloid; thymol, a terpenoid; and digoxin, a cardiac glycoside (HPLC grade, Millipore Sigma). These compounds occur naturally in floral nectar consumed by bumble bees; we used published accounts to select an ecologically relevant concentration for each compound: 98 ppm caffeine, 0.20 ppm thymol and 10 ppm digoxin (as in Manson et al., 2012; Richardson et al., 2015). Digoxin visually dissolved after vortexing in sucrose solution; however, to dissolve caffeine and thymol, we first added a small amount of 100% ethanol (EtOH; 2 ml/L for caffeine solution; 4 μl/L for thymol solution). To ensure the ethanol did not affect any of our response variables, we ran a separate control in which bees were maintained on sucrose solution that contained the same volume of ethanol (Table S7).

2.1.3 | Pesticide exposure

We offered the widely used neonicotinoid pesticide imidacloprid (IMD) to bees in a 50% (w/w) sucrose solution, a higher sugar concentration than the dietary solution, to entice bees to consume it. We prepared the sucrose solution as above, and then dissolved 93 mg of analytical standard PEDESTAL® imidacloprid powder in 93 ml of acetone, resulting in a 1:1 stock solution. An aliquot of 50 μl of this solution was then added to 1 L of 50% (w/w) sucrose solution to reach a concentration of 50 ppb. This value is high compared to the median concentration published in the literature (8 ppb); however, the maximum concentrations found in floral nectar extend upwards almost three orders of magnitude, to 6.5 ppm (Siviter, Richman, et al., 2021; Zioga et al., 2020). For the sham exposure solution (containing no IMD), the same volume of acetone was added to 1 L of the same concentration of sucrose.

After 72 hr with ad libitum access to their sucrose solution diet (either sugar only or sugar + one of the three focal NSMs), we removed the feeder. After 2 hr of starvation, we provided each bee with 75 μl of either the IMD-spiked solution or its sugar-only control (sham dose) in a capillary tube (ID × L: 3.4 × 150 mm, World Precision Instruments) inserted into the experimental chamber. This volume approximates that which a bee might collect on a single foraging bout (Cresswell et al., 2000); for instance, following an initial pesticide spray or the first bloom of plants with treated seeds. Giving bees a single, acute dose allowed us to focus on the short-term effects of neonicotinoid exposure (a common focus of pesticide research: Goulson, 2013) as well as ask whether a single exposure is enough to mediate the effects of NSMs. Bees that did not consume the entire dose within 4 hr were removed from the experiment. After dosing was completed, a fresh sucrose feeder offered the bees' assigned diet (Figure S3).

2.1.4 | Treatment assignments

The diet assignments and dosing treatments gave rise to three fully crossed, two-way designs (Figure S3) where a bee was assigned to one of four diet/pesticide exposure combinations: (a) an NSM-enriched diet combined with an (single) acute dose of IMD-spiked sucrose solution (NSM+IMD treatment), (b) an NSM-enriched diet with a sham pesticide dose (NSM treatment), (c) a sugar-only diet combined with a single acute dose of neonicotinoid-spiked sucrose solution (IMD treatment), or (d) a sugar-only diet with a sham pesticide dose (control). This design was used for three separate experiments exploring the three focal NSMs' effects on longevity (Exp. 1), immune function (Exp. 2) and activity level (Exp. 3). Each NSM was tested singly, and not in combination with other NSMs. Furthermore, each experiment was assigned its own control. Our design allowed us to determine whether effects of NSMs and IMD on bees were additive, synergistic, or antagonistic by assessing the slope differences between single and combined responses (i.e. Figure 2). While reporting calculated measures of synergism, such as the Bliss index (Bliss, 1939), is relatively common in the pharmacological literature (Foucquier & Guedj, 2015), it generally requires measuring a dose response curve across multiple concentrations. We opted to test a range of different response variables across multiple NSMs, and therefore tested single concentrations of each

to preserve statistical power. For all experiments, we used a sample size of 20 bees/treatment. Experiment 1 used two colonies for each NSM assay (caffeine, thymol, digoxin; six colonies total), resulting in 10 bees/treatment/colony. Experiments 2 and 3 used bees across two to six colonies with bees assigned to a given treatment pulled from multiple colonies (Tables S5 and S9). All experiments involved bees from at least two separate colonies. We also measured the effects of NSMs on sucrose solution consumption prior to IMD dosing, and the effects of NSMs and IMD on sucrose solution consumption following IMD dosing. Digoxin and IMD reduced overall consumption; see Figures S1 and S2; Tables S1–S4 for results.

2.2 | Experiment 1 Methods: The combined effects of nectar secondary metabolites and IMD on worker longevity

After dosing, bees were monitored daily for survival; their feeders were regularly weighed to track consumption and refilled every 3 days. Dead bees were removed and stored at −20°C in individual tubes and we weighed their feeders a final time. We continued the experiment until all bees died at which point we measured their intertegular (IT) spans to estimate body size (Cane, 1987).

2.3 | Experiment 2 Methods: The combined effects of nectar secondary metabolites and IMD on the constitutive immune system

Twenty-four hours after dosing, we sampled haemolymph by piercing the bee between the T5 and T6 abdominal segments using a $25^{5/8}$-gauge syringe needle. We collected 4 μl of haemolymph at the piercing site using a micropipette (Drummond). Bees were then euthanized and stored in a −20°C freezer after measuring their IT span using callipers. In preparation for measuring phenoloxidase (PO) activity, haemolymph was added to 16 μl phosphate-buffered saline (PBS) and placed on ice in a 1.5-ml Eppendorf tube. Each PBS-bound sample was combined with 20 μl of 1-DOPA (0.0114 g DOPA mixed with 15-ml deionized water) in a 96-well plate (Smilanich et al., 2018). We measured PO activity using an iMark Microplate Absorbance Reader (Bio-Rad). Measurements were taken every 30 s over a total of 45 min at 490 nM. We measured the maximum rate of activity, calculated as the maximum linear rate of increase PO_{max} over the assay period. A higher rate of activity indicates a stronger immune response (Barthel et al., 2016; Lee et al., 2006).

2.4 | Experiment 3 Methods: The combined effects of nectar secondary metabolites and IMD on bee activity level

Hypo- and hyperactivity are some of the most consistently reported effects of IMD (Cresswell et al., 2014; Tosi & Nieh, 2017). At two time points, 24 and 72 hr after dosing, we ran activity trials, collecting data on four bees simultaneously. We placed bee chambers 15 cm apart on top of a white foamcore board marked with a centre line that visually divided the chambers widthwise. For the next 30 min, we recorded the number of times a bee crossed this line (Muth et al., 2020). After the second activity assay, we weighed feeders, euthanized bees and measured their IT span.

2.5 | Statistical methods

All analyses were performed using R version 4.0.2 (R Core Team, 2018). Mixed-effects models were carried out using the LME4 package (Bates et al., 2015); overall test of treatment effect was assessed using the CAR package (Fox & Weisberg, 2019) and pairwise comparisons were assessed using the PHIA package (Rosario-Martinez, 2015). To test whether nectar additives affected worker longevity, we performed a series of general and generalized linear mixed effects models. We performed a separate model for each NSM (caffeine, thymol, digoxin) within each experiment. Response variables for each experiment were as follows: The number of days a bee survived following dosing (longevity; Poisson error distribution), maximum rate of phenoloxidase enzyme activation (PO_{max}; immune function; linear mixed model), activity level during each of the 30-min observation periods (24 and 72 hr following dosing; activity level; Poisson error distribution). We checked bees daily in the longevity experiment with no gaps or censored data, so we used a GLMM to compare longevity across treatments rather than a Cox Proportional Hazards Model. In all models, we used dietary treatment as the explanatory variable. Body size was included as a covariate (Table S6) and colony as a random effect (random intercept). Colony age was initially included as a covariate, but we removed it after discerning that it did not improve model fit. We assessed the overall effects of dietary treatment using a Wald Type II Chi-square test; pairwise differences between treatment levels were assessed using a Holm test (Table S8).

3 | RESULTS

3.1 | Experiment 1: The combined effects of nectar secondary metabolites and imidacloprid on worker longevity

On their own, individual dietary NSMs had variable effects on worker longevity. When effects were positive, a single acute exposure to IMD eliminated their benefits. When they were negative, IMD exposure increased their costs (see *Thymol*). *Caffeine*: Bees on a caffeine-enriched diet lived approximately 7 days longer than bees in any other treatment group ($\chi^2_3 = 29.0$, $p < 0.001$, Figures 1a and 2a). An acute exposure to IMD while on this diet eliminated its benefit but did not reduce survival beyond what would be expected following exposure to IMD alone: pairwise differences between the three other treatment groups (control, IMD, caffeine+IMD) were negligible and

not significantly different from one another (Table S8). *Thymol*: Bees on a thymol-enriched diet lived 4 days less on average compared to the control group and 6 days less compared to the IMD group (overall effect of treatment on longevity $\chi^2_3 = 55.6$, $p < 0.001$, Figures 1a and 2e). A thymol-enriched diet paired with an acute IMD exposure resulted in the worst treatment outcomes: Bees in the thymol+IMD group lived only an average of 12.4 days, compared to 15.8 days for the thymol group, 21.8 days for the IMD group and 19.8 days for the control group (Figures 1a and 2e; Table S8). *Digoxin*: Similar to the thymol trial, bees on a digoxin-enriched diet died sooner than bees in the control group and the IMD group, by an average of approximately 2 and 6 days, respectively (Figures 1a and 2i; Table S8; overall effect of treatment $\chi^2_3 = 58.8$, $p < 0.001$). The combination of a digoxin-rich diet and an acute IMD dose did not result in survival outcomes distinct from the digoxin-only group (Figure 1a).

3.2 | Experiment 2: The combined effects of nectar secondary metabolites and imidacloprid on the constitutive immune system

Two of the three individual NSMs tested (caffeine and thymol) had no effect on immune function, and in these cases, IMD exposure did not alter outcomes. The digoxin experiment revealed slightly differing results; each chemical (IMD and digoxin) generated an increased immune response on its own, but their combined effects did not differ from the control condition. *Caffeine*: PO_{max} (activity of the phenoloxidase enzyme) remained relatively consistent across bees on control or caffeine-enriched diets, whether or not they received an acute IMD dose. However, bees in the IMD-only group exhibited the reduced rates of enzyme activation. Although the difference was not statistically significant, the effect size ±std. error was negative (overall effect of treatment $\chi^2_3 = 2.4$, $p = 0.49$; Figures 1b and 2b). *Thymol*: PO_{max} remained relatively consistent across control bees, as well as bees in the IMD and thymol+IMD treatment groups (overall effect of treatment $\chi^2_3 = 1.6$, $p = 0.65$; Figures 1b and 2f). *Digoxin*: Dietary digoxin and an acute IMD exposure each increased PO_{max} to 60% higher than control group bees and 25% higher than bees in the digoxin+IMD treatment (overall effect of treatment $\chi^2_3 = 10.7$, $p = 0.01$; Figures 1b and 2j; Table S8). In contrast, the combination of a digoxin-enriched diet and acute IMD exposure eliminated their individual effects: PO_{max} for these bees did not differ from bees in the control group (Figures 1b and 2j).

3.3 | Experiment 3: The combined effects of nectar secondary metabolites and imidacloprid on activity

At both time points tested (24 and 72 hr following dosing), IMD caused hypoactivity in workers, both on its own and in combination

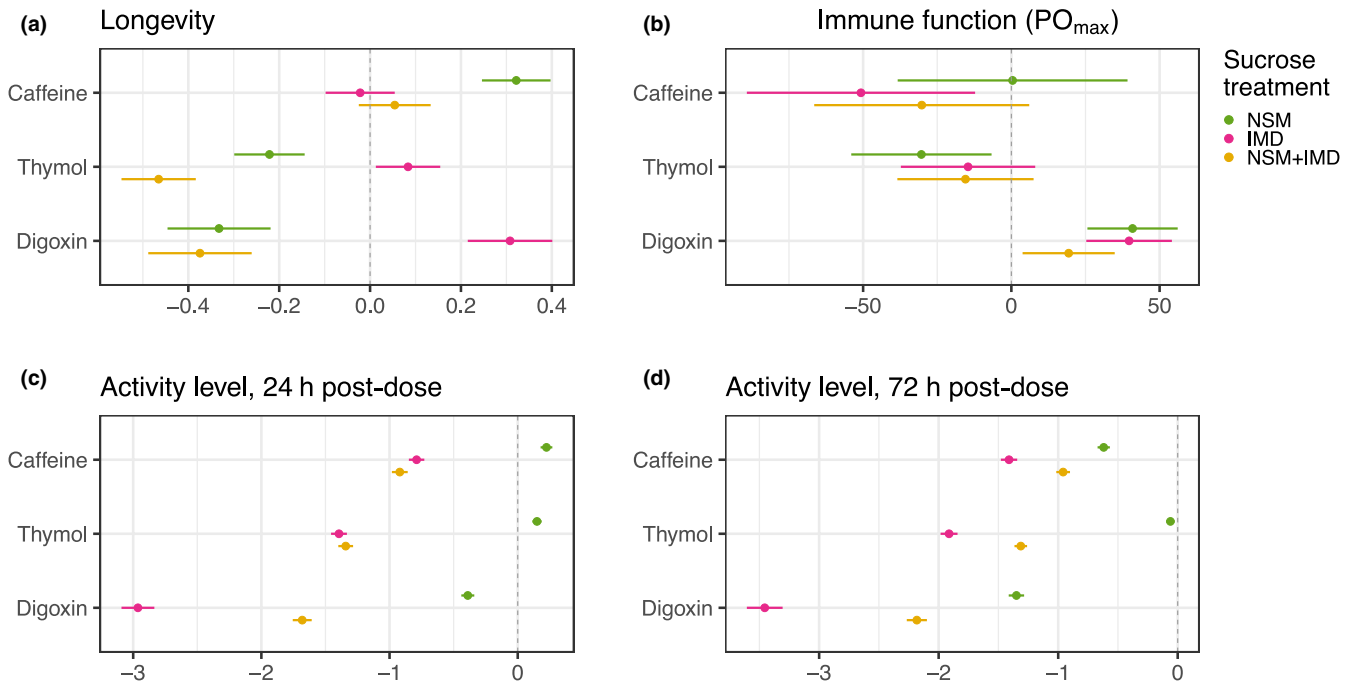

FIGURE 1 Results of all statistical models. Colours correspond to model effect sizes ±*SE* (*x*-axis) for each nectar dietary treatment relative to the control condition of no dietary NSM and no IMD exposure: NSM, nectar secondary metabolite; IMD, acute imidacloprid exposure; NSM+IMD, nectar secondary metabolite and acute imidacloprid exposure. *Y*-axis tick marks refer to different NSMs tested. Different panels correspond to different experiments and different statistical models within experiments: (a) Experiment 1, Longevity (Poisson error distribution; log scale); (b) Experiment 2, Immune Function (phenoloxidase activity, linear distribution); (c) Experiment 3, Activity Level, 24 hr following IMD dosing; (d) Experiment 4, Activity Level, 72 hr following IMD dosing (c and d both Poisson error distribution; log scale). Note that all estimates are shown with std. errors, although some values are small enough to appear to be zero

with NSMs, regardless of how a given NSM affected activity level when consumed on its own. *Caffeine*: Exposure to IMD (IMD and caffeine+IMD treatments) significantly reduced bee activity level at 24 and 72 hr (24 hr: $\chi^2_3 = 567.8$, $p < 0.001$; 72 hr: $\chi^2_3 = 583.3$, $p < 0.001$; Figures 1c,d and 2c,d). Dietary caffeine without IMD caused hyperactivity compared to the control group at 24 hr, but it caused hypoactivity at 72 hr (Figures 1c,d and 2c,d); IMD exposure eliminated these effects. *Thymol*: Exposure to IMD (IMD and thymol+IMD treatments) significantly reduced bee activity at 24 and 72 hr (24 hr: $\chi^2_3 = 1{,}200.8$, $p < 0.001$; 72 hr: $\chi^2_3 = 1{,}287.5$, $p < 0.001$; Figures 1c,d, and 2g,h). Dietary thymol caused hyperactivity compared to the control group at 24 hr; at 72 hr, there was no activity difference among bees in these groups (Figures 1c,d and 2g,h; Table S8). *Digoxin*: IMD exposure (IMD and digoxin+IMD treatments) significantly reduced activity at 24 and 72 hr (24 hr: $\chi^2_3 = 963.7$, $p < 0.001$; 72 hr: $\chi^2_3 = 1{,}379.5$, $p < 0.001$; Figures 1c,d and 2k,l). Dietary digoxin caused hypoactivity compared to the control group at 24 and 72 hr, although bees from this treatment were more active than bees from the IMD and digoxin+IMD groups (Figures 1c,d and 2k,l; Table S8).

4 | DISCUSSION

We asked how consumption of nectar secondary metabolites (NSMs) combined with an acute exposure to the common neonicotinoid imidacloprid (IMD) to affect bumble bee life span, physiology and behaviour. In keeping with the results of others (Richardson et al., 2015; Stevenson et al., 2017), the consumption of NSMs provides a mixture of physiological benefits and costs. Notably, however, the addition of a single IMD dose to the diet in certain cases offset or exacerbated the effects of NSMs on their own. Our results suggest that systemic pesticides can alter the ecological costs and benefits of nectar chemistry, potentially disrupting plant–pollinator interactions.

Consistent with Hypothesis 1 (exacerbation), thymol on its own decreased bee life spans. Although IMD alone did not affect longevity in this experiment, IMD+thymol led to significantly shorter life spans than thymol alone (Figure 2e). Acute exposure to IMD lowered immune function but not when bees were fed a caffeine-enriched diet (Figure 2b). These results are consistent with the hypothesis that NSM's buffer bees against the toxicity of IMD (Hypothesis 2, amelioration). The caffeine longevity experiment suggested that IMD exposure can eliminate the positive effects of NSMs on pollinators (Hypothesis 3, pesticide mediation). Although caffeine increased life span, a single dose of IMD eliminated that effect (Figure 2a). Similarly, hypoactivity caused by IMD 24 hr post-dose overshadowed any stand-alone effects of caffeine and thymol on activity level (Figure 2c,g). The null hypothesis (H4), of no effect of NSM presence on IMD exposure outcomes (and vice versa), also found support: for example, the effect of IMD on activity level 72 hr post-dose was similar regardless of thymol diet status (Figure 2h), and the effect of this diet on immune performance was similar regardless of IMD exposure (Figure 2f).

4.1 | Potential for systemic pesticides to disrupt plant–pollinator interactions

Human activities that alter floral chemistry may not only have proximate consequences on pollinator behaviour and physiology, as demonstrated here, but also longer term consequences on the ecological and evolutionary trajectory of plant–pollinator interactions. Nectar secondary metabolites can affect bees in a multitude of ways (Adler, 2000), giving rise to costs and benefits that depend on ecological context (Stevenson et al., 2017). For example, the nectar alkaloid gelsemine, found in *Gelsemium*, reduces parasitic infection in bumble bees, but may also limit oocyte development (Manson et al., 2012; Manson & Thomson, 2009). Similarly here, the NSMs we assessed all provided some form of benefit to pollinators, albeit not without costs. Digoxin increased immune response at the expense of a shorter life span; caffeine increased life span although bees eventually became less active over time; and thymol increased activity level but shortened life span. Our findings represent the first evidence that pesticides may alter the form of these trade-offs, usually eliminating beneficial aspects of NSMs while preserving or exacerbating their costs. Pesticide-mediated disruption of these processes has the potential to drive novel foraging preferences and pollinator trait evolution in response to floral chemistry. There is evidence that bees are finely tuned to the trade-offs of consuming floral rewards; for example, *Bombus impatiens* will only accept alkaloid-rich nectar or pollen when nectar sugar content is high (Francis et al., 2019; Gegear et al., 2007). Furthermore, across taxa, bees can vary in their ability to cope with NSMs, perhaps as a result of evolutionary history with specific plant taxa (Tiedeken et al., 2016). As interest grows in understanding the benefits of nectar and pollen secondary chemistry in agroecosystems (Adler et al., 2021; Folly et al., 2021; Fowler et al., 2020) or use in promoting pollination service (Arnold et al., 2021), our results suggest an important caveat, which is that systemic pesticides may alter these dynamics.

Pesticides in nectar may also be ecologically consequential for plants, silently altering the eco-evolutionary fine-tuning of plant–pollinator interactions. Caffeine, for example, in the nectar of plants such as *Citrus*, *Coffea* and *Onobrychis* (sainfoin) increases bee activity (e.g. Figure 2c), reduces parasite load (Folly et al., 2021) and promotes pollen transfer (Thomson et al., 2015). The addition of IMD, which *B. impatiens* workers do not show any preference for on its own (Muth et al., 2020), and which limits activity (Figure 2c,d), would likely result in lowered visitation rate with potential negative impacts on plant fitness. Additionally, plant reproduction relies on a healthy pollinator workforce. Thymol is one of the phytochemicals most well studied for promoting bee health (Costa et al., 2010) and is used as a miticide treatment inside honey bee colonies (Gregorc & Planinc, 2013; Imdorf et al., 1995). However, the treatments come at a cost: A recent study revealed *Apis mellifera* simultaneously treated with thymol and IMD performed worse on visual learning tests than bees exposed to either chemical alone (Colin et al., 2020). Thus, beyond the shortened life spans reported here, doubly exposed bees run the risk of less efficient pollination (Gegear et al., 2021).

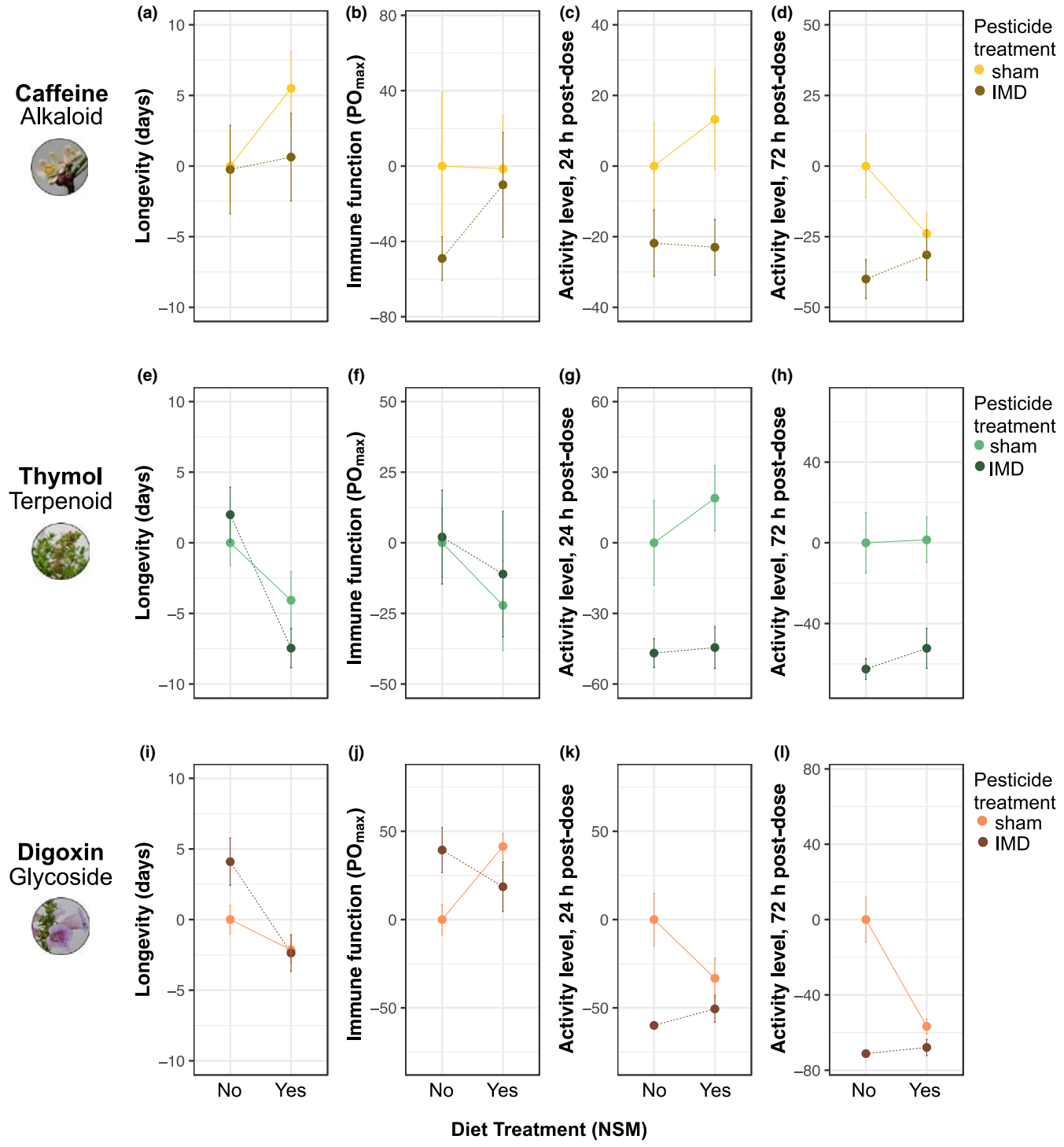

FIGURE 2 Focal nectar secondary metabolites (NSMs) and interactions with pesticide exposure on bee performance. (a–d) Effects of caffeine and imidacloprid (IMD) on all experimental response variables: longevity, immune function, activity level (two time points: 24 and 72 hr after IMD dosing); (e–h) Effects of thymol and IMD on all experimental response variables; (i–l) effects of digoxin and IMD on all experimental response variables. Values standardized to the mean of the control condition (no NSM, no IMD). Points and error bars represent mean ± *SE* values. Diet treatment (no NSM/NSM) indicated on the *x*-axis; colours and line type represent pesticide treatment (sham dose/IMD dose). Photos are of representative genera containing each NSM (*Citrus*, *Thymus*, *Digitalis*)

Some plants, however, benefit from offering NSMs that promote bee health: parasitized *B. impatiens* individuals forage more heavily on aucubin- and catalpol-enriched *Chelone glabra* nectar than non-infected individuals, increasing pollen receipt (Richardson et al., 2016). Three-way plant–pollinator–parasite interactions such as these (i.e. Manson & Thomson, 2009, Palmer-Young et al., 2019) represent cases where pesticide exposure can influence a range of outcomes for all species involved.

4.2 | Consideration of nectar chemistry in studies of systemic pesticides

Nectar secondary metabolites are common constituents of the nectars of cultivated crops, horticultural plants and wildflowers alike. Palmer-Young et al. (2019) recently analysed floral reward chemistry of 15 crop species and detected at least one NSM in the majority of them. When bumble bees visit IMD-laced plants, does NSM presence matter? Our findings indicate little difference between IMD-only and NSM+IMD treatments (in contrast to NSM-only vs. NSM+IMD discussed above; Figures 1 and 2), adding support to the external validity of acute exposure assays run on bumble bees with IMD-spiked sucrose solutions. We only detected one instance in which (a) the effects between the IMD and the NSM+IMD treatments differed and (b) the difference did not appear to be due to stand-alone effects of the NSM: IMD and digoxin had separate positive effects on immune function, whereas their combination did not differ from the control (Figure 2j). A positive effect of digoxin on the insect immune system is not without some precedence. Different cardenolides found in *Asclepias* nectar improve Lepidopteran immune performance (Gowler et al., 2015). We are the first, however, to report this effect in insects that do not specialize on cardenolide-producing plants. Given the popularity of cardenolide-rich plants (e.g. *Digitalis*, *Asclepias*) in pollinator gardens, and the likely exposure of these plants to neonicotinoids in gardens, agroecosystems and conservation areas (Halsch et al., 2020), these combinatorial effects warrant further investigation. Although studies on the effects of neonicotinoids on bumble bee immune systems are still somewhat limited, most report negative (Czerwinski & Sadd, 2017) or neutral (Collison, 2015) effects on the enzymatic (constitutive) immune system. Dosing schedule is one major difference between these studies (involving pulsed or chronic doses) and ours (a single acute dose). The positive response bees showed may be the early stage of a biphasic reaction, whereby additional exposure would become harmful (e.g. Anthony et al., 2015). The fact that the addition of digoxin to IMD led to suppressed immune performance supports this idea. However, it is important to note that the effects of IMD alone on bee immune systems varied across individual NSM assays (Figures 1b and 2b,f,j). We discuss these findings below.

4.3 | Caveats

While we detected clear effects of neonics, NSMs and their combination across our three experiments, caveats exist that point to the need for further research in this area. First, in each experiment, an NSM diet treatment was paired with its own control group, which in some cases showed unexpected differences. For example, in the absence of an NSM-enriched diet, IMD-exposed bees lived longer than control bees in the digoxin group, while the same treatment comparisons of longevity did not differ in the caffeine or thymol groups (Figure 2a,e,i). More strikingly, the effects of IMD (absent NSM exposure) on immune function ranged from positive to neutral to negative, (Figure 2b,f,j). We can think of a few possible reasons for these results. First, while we assigned colonies equally across treatments with a given experiment, colonies used across experiments did differ (Tables S5 and S9). Therefore, it may be that there were factors intrinsic to the groups of colonies used for each experiment that contributed to the varied results across experiments. Another factor we were unable to control for was individual worker age, which can influence bee immune function (Whitehorn et al., 2010). The age of the colony, based on its delivery date, was initially included as a covariate in our models, but removed as it was not a statistically significant variable, nor did it improve model fit ($\Delta AIC < 2$).

Implementation of additional pesticide exposure regimens would further speak to the external validity of these results. Like much work on the sublethal effects of neonicotinoids on bees (Muth & Leonard, 2019; Samuelson et al., 2016; Tosi & Nieh, 2017), we opted for an acute exposure scenario. Given that many bees in real-world settings likely face longer term exposure, our results may be conservative estimates, as some effects of pesticide exposure emerge only after chronic exposure (Stanley et al., 2015). Comparing the results we report to long-term pesticide exposure would be an obvious next question. Although we used concentrations of NSMs based on previous research (Manson et al., 2012; Richardson et al., 2015), and an IMD concentration that is field-relevant (Blacquière et al., 2012) and known to have effects on bee behaviour (Muth et al., 2020), our values for IMD and caffeine are high (Wright et al., 2013). Studies that systematically vary concentrations of multiple nectar chemicals and/or compare different exposure regimens could further expand upon our findings. This approach would also work towards filling the gap of lab (and field) studies that manipulate a single nectar chemical (such as purely IMD-focused work) or of only two nectar chemicals (as done here within a given experiment). Designs inspired by the separate literature on drug combinations (Foucquier & Guedj, 2015; Yadav et al., 2015) could be an especially powerful approach for characterizing complex phytochemical–pesticide interactions.

5 | CONCLUSIONS

Pollinators are facing unprecedented challenges, some of which, such as habitat loss and climate change, are more obvious to human observers than others, such as altered nectar chemistry. Our findings suggest that mitigation efforts, such as putting in pollinator-friendly plants in or near areas where systemic pesticides are used, may come with ecological complications. By expanding the dietary realism involved in studies of agrochemicals, we can better identify plants suitable to a particular mitigation effort, or recommend plants to avoid when a particular pesticide is in use. More broadly, if a pesticide can mediate the costs and benefits of nectar phytochemicals, it has the potential to influence the eco-evolutionary dynamics of plant–pollinator interactions. Exploring the cascading effects of human-altered nectar chemistry may help us understand

the consequences of pesticide exposure for the performance of pollinators and plants alike.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

A.S.L. conceived of the study; S.K.R., I.M.M., A.M.S. and A.S.L. designed the experiments; S.K.R., I.M.M., D.M.S. and S.Z.M. collected data; S.K.R. performed statistical analyses and wrote the first draft of the manuscript; all authors contributed to subsequent drafts.

DATA AVAILABILITY STATEMENT

All data and code associated with this manuscript are available in Figshare:https://figshare.com/articles/dataset/Data_and_code_for_A_neonicotinoid_pesticide_alt.ers_how_nectar_chemistry_affects_bees/16543620 (Richman, 2021).

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