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Sex allocation theory reveals a hidden cost of neonicotinoid exposure in a parasitoid wasp

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Sex allocation theory has proved to be one the most successful theories in evolutionary ecology. However, its role in more applied aspects of ecology has been limited. Here we show how sex allocation theory helps uncover an otherwise hidden cost of neonicotinoid exposure in the parasitoid wasp *Nasonia vitripennis*. Female *N. vitripennis* allocate the sex of their offspring in line with Local Mate Competition (LMC) theory. Neonicotinoids are an economically important class of insecticides, but their deployment remains controversial, with evidence linking them to the decline of beneficial species. We demonstrate for the first time to our knowledge, that neonicotinoids disrupt the crucial reproductive behaviour of facultative sex allocation at sub-lethal, field-relevant doses in *N. vitripennis*. The quantitative predictions we can make from LMC theory show that females exposed to neonicotinoids are less able to allocate sex optimally and that this failure imposes a significant fitness cost. Our work highlights that understanding the ecological consequences of neonicotinoid deployment requires not just measures of mortality or even fecundity reduction among non-target species, but also measures that capture broader fitness costs, in this case offspring sex allocation. Our work also highlights new avenues for exploring how females obtain information when allocating sex under LMC.

1. Introduction

Sex allocation theory explains the evolution of how organisms allocate investment into male or female offspring [1,2]. Building on key theoretical contributions from, among others Fisher [3], Hamilton [4] and Trivers & Willard [5], theoreticians have put together and tested a formidably successful body of theory, which has explained patterns of sex ratio variation across organisms as diverse as microbes to humans [2]. Perhaps the most successful component of sex allocation theory, in terms of quantitative tests of theory, has been Hamilton's theory of Local Mate Competition (LMC) [4]. Briefly, consider a species of parasitic wasp that lays several eggs on the pupa of another insect, such as a fly or butterfly. The male and female wasps develop and then emerge from the host, mate among each other, with the female offspring then dispersing to find new hosts. If a single female (termed a foundress) lays eggs on a host, Hamilton demonstrated that the optimal sex ratio for that female to produce is a female-biased one, producing the minimal number of sons to fertilize her daughters. This female-biased sex ratio both reduces competition among her sons for mates (i.e. it reduces LMC among kin) and also increases the number of available mates for those sons [6]. When several foundresses lay eggs together though, the extent of LMC among brothers is reduced, favouring less female-biased sex ratios. Females that can facultatively alter their offspring sex ratios with respect to the level of LMC their sons will face are, therefore, favoured by natural selection. LMC theory has been extended to include a variety of factors, such as variation in mating within patches [7]

and alternative patterns of dispersal [8], and has proved remarkably successful in predicting the sex allocation patterns in a wide-variety of organisms, not just parasitoid wasps [2].

While sex allocation theory has provided an important test of evolutionary principles, it has proven more limited in applied contexts (e.g. [9]). Here we explore how sex allocation theory can help us unravel the costs of exposure to controversial neonicotinoid pesticides. Neonicotinoids are the most widely used insecticides in the world, with global annual sales of over US $2.6 billion [10]. They are potent neurotoxins and act by binding to nicotinic acetylcholine receptors in the central nervous system, causing disruption of neural transmission. They are typically highly toxic to invertebrates at extremely low doses, but have the advantage of comparatively lower vertebrate toxicity [11]. However, their use remains highly contentious [12]. In particular, neonicotinoids have been linked to declines in species that provide key ecosystem services, such as pollinating insects [13–15]. Moreover, their deployment has also recently been correlated with declines in farmland birds [16]. The broader ecological significance of these findings remains debated though, and the extent to which observed changes are the effects of a (desired) reduction in arthropods in the agricultural environment (reducing food for other species for example), versus the toxic effects of neonicotinoids in the environment more generally remains unclear.

To date, much of the work on the ecological significance of neonicotinoids has focused on one class of beneficial insect: the pollinating insects, in particular honeybees and species of bumblebee (e.g. [15,17,18]). However, pollinators are not the only beneficial insects in the environment. Parasitoid wasps, which kill their arthropod hosts, are important natural enemies of many agricultural pests, and also contribute to often extensive biological control programmes (taken together parasitoids are estimated to bring ecosystem services worth an estimated US $4.5 billion per year in the USA [19]). Maintaining healthy populations of these natural enemies, or alternatively, viable biocontrol programmes, is a key aim of integrated pest management strategies. Parasitoid wasps lay their eggs on or in arthropod hosts (which may be at the egg, larval, pupal or adult stage, depending on species), with the wasp larvae hatching and then predating on the host [20]. As highlighted above, one of the most important reproductive decisions for female parasitoids to make is the sex of her offspring [20]. As with all Hymenoptera, parasitic wasps are haplodiploid, with males developing from unfertilized (haploid) eggs, and females developing from fertilized (diploid) eggs. Females can, therefore, control sex allocation by controlling whether or not an egg is fertilized before being laid.

Here we consider the effect of the commonly used neonicotinoid imidacloprid on the sex allocation behaviour and general reproduction of the gregarious parasitoid wasp *Nasonia vitripennis*. Female *N. vitripennis* allocate sex closely in accordance with LMC theory [7,21,22]. Importantly, the patterns of sex allocation revealed in laboratory studies have been replicated in the field [23,24]. As with many parasitoids, they take nectar in the wild and commonly feed on sucrose solutions in the laboratory [25]. In our first experiment, we tested the effects of varying doses of imidacloprid on female survival, which provided us with information concerning lethal versus sub-lethal doses. In our second experiment, we considered the effects of (sub-)lethal doses of imidacloprid on facultative sex allocation and oviposition. We also used existing LMC theory to estimate the fitness costs imposed on females by any such disruption to their sex ratio behaviour. While there are a number of possible pathways by which sex allocation may be disrupted by blocking neurotransmission (see §4), we expect facultative sex allocation to be disrupted in some way by exposure to imidacloprid.

2. Material and methods

(a) Study organism and general husbandry

Nasonia vitripennis (Hymenoptera, Chalcidoidea) is a generalist parasitoid of large dipteran pupae, including the Calliphoridae [26]. Females typically oviposit between 20 and 50 eggs in an individual host and limit superparasitism if possible. Male offspring emerge shortly before females (after approx. 14 days at 25°C [27]). Male individuals are brachypterous and are unable to fly, remaining close to the emergence site where they compete with each other for emerging females, including their sisters. Females disperse after mating to locate fresh hosts. The focal females used in these experiments were from the AsymC strain (the genome reference strain [28]). Wasps were maintained on *Calliphora vomitoria* or *Calliphora vicina* hosts at 25°C, 16 L : 8 D light conditions. For some experimental treatments co-foundresses were required. These were taken from the red-eye mutant STDR strain, allowing us to track the offspring of a single AsymC female using eye colour (e.g. [22]). The STDR strain was maintained under identical conditions to the AsymC strain.

To control for possible host and other maternal effects, experimental females were not taken straight from the mass cultures. Instead, 2-day old, mated females were isolated into individual glass vials and given hosts to parasitize. Mated females from the resulting F_1 generation were used in experiments. These experimental females were then 'pre-treated': females were given access to a single host for 24 h, which allows host-feeding and facilitates egg development [29]. This host was removed and discarded and females were given access to honey solution for a further 24 h. At this point females were allocated to their experimental treatments.

(b) Experiment 1: neonicotinoid exposure and longevity

In our first experiment, we exposed mated, 2-day old female *N. vitripennis* to imidacloprid at various concentrations (1, 10, 100 and 200 ppb), in 20% sucrose solution for up to 140 h. Experimental females were isolated into individual glass vials. Females were then allocated to one of five imidacloprid diets: control, 1 ppb, 10 ppb, 100 ppb and 200 ppb ($n = 30$ per treatment). Pure imidacloprid (Sigma Aldrich, Dorset, UK) was dissolved in a known volume of distilled water and then diluted to the appropriate concentration in 20% sucrose solution. The control was composed of sucrose alone. Diets were provided in a 200 µl volume in the lid of a 1.5 ml microcentrifuge tube placed into the bottom of the glass vials. Females were exposed to the solutions continuously for a period of 72 h with mortality checks performed three times daily at 10.00, 14.00 and 18.00 h. Solutions were refreshed on a daily basis during this 72 h period and a final mortality check was made at 140 h.

(c) Experiment 2: neonicotinoid exposure and sex allocation

In our second experiment, we exposed females to imidacloprid in sucrose solution for 48 h (control, 2 ppb, 10 ppb, 100 ppb). We used 2 ppb in this experiment as it is very close to the 1.9 ppb value reported by Godfray *et al.* [15] for the average maximum

neonicotinoid concentration in nectar for seed-treated crops across 20 studies. We then tested the facultative sex allocation responses of treated and control (fed only sucrose solution) females in a standard LMC experiment. We isolated 2-day old mated AsymC females and placed them in individual glass vials, before providing each with a single host for 24 h as a pre-treatment to facilitate egg development as before. Pre-treatment hosts were then discarded and each female was given a piece of filter paper soaked in honey solution for a further 24 h. The filter paper was then removed and 150 females were allocated to each of four imidacloprid diets (control, 2 ppb, 10 ppb or 100 ppb). The lid of a 1.5 ml microcentrifuge tube was again placed in the bottom of each glass vial and 200 µl of the appropriate diet transferred to the lids. Females were allowed access to their imidacloprid diets for 48 h. Fresh solution was provided on the second day.

Females were then allocated to one of 12 treatment combinations from a 3 × 4 factorial design, with females from the four diets placed individually in one of three co-foundress treatments (alone, four co-foundresses or nine co-foundresses, yielding total foundress numbers of 1, 5 and 10, respectively). The appropriate number of STDR cofoundresses was provided and then three hosts were added to each vial. Vials were incubated at 25°C 16 L : 8 D cycle. After 60 min, one-way escape tubes were added to each vial to allow females to disperse away from the hosts, preventing us from forcing females to super-parasitize hosts. After 24 h, all females were removed from the hosts and discarded. Hosts were returned to the incubator and 14 days later emergent offspring were sexed, genotyped by eye colour (red-eyed or wild-type) and counted. Data from females that produced no offspring, produced only male offspring (putative virgins) or produced more than 10 diapause larvae were discarded. This left between 30 and 44 replicates for each of the 12 treatment groups, and a total sample size of $N = 482$ females.

(d) Statistical analysis

All statistical analyses were carried out in SPSS v. 21 using a general linear model framework. To test for significant differences in sex ratio a generalised linear model (GLM) with binomial error structure and a logit link function was used. To correct for over-dispersion, common when analysing binomial data, *F*-tests were used. The main effects 'foundress number' and 'imidacloprid diet' were coded as continuous variables as we expect increasing neonicotinoid dosage to have an increased effect (a directional hypothesis is being tested).

(e) Estimating the fitness costs of disrupting sex allocation

We used an approach similar to Shuker & West [22]. First, we estimated the fitness of a focal female if she was producing the optimal sex ratio predicted by the simplest LMC model for haplodiploids, which assumes all foundress females produce the same clutch size [30]. The fitness equation is as follows:

$$W = \frac{1}{2}\left[\frac{S_2}{S_2 + (N-1)S_1}\right][(N-1)(1-S_1) + (1-S_2)] + \left[\frac{N}{2N-1}\right][1-S_2],$$

where N is the number of foundresses, S_2 is the sex ratio of the focal female and S_1 is the sex ratio of the rest of the brood.

Following Hamilton [30], the optimal sex ratio is given as:

$$S^* = \frac{(N-1)(2N-1)}{[N(4N-1)]}.$$

For 5 foundress patches, the optimal sex ratio is therefore $S^* = 0.38$, while for a 10 foundress patch, the optimal sex ratio is $S^* = 0.438$. We calculated the relative fitness of an average female from each treatment (control, 2 ppb, 10 ppb and 100 ppb) when compared to a hypothetical female that was behaving optimally.

Next, we allowed the clutch sizes of females to vary (as they did in the experiment). This is especially relevant here as treated females appeared to have reduced fecundity at all levels of imidacloprid exposure (see below; figure 2*b*). The exact sex ratio a female is predicted to produce depends on a number of factors, including exactly when in the sequence of parasitism a female oviposits (is she first, last or somewhere in between), if she encountered some or all co-foundresses, if she encountered previous eggs, and whether she has laid on one or more hosts. To disentangle these factors requires specific experiments [7,22]. Here, we make the simplifying assumption that all other brood on a patch are laid first, before our female commences oviposition. We assume that this brood has the sex ratio produced by control single foundress females ($S_1 = 0.08$; see figure 2*a*). We then calculated the relative clutch size of an average focal female from each treatment to give us the parameter T (focal female clutch size/number of other brood). The optimal sex ratio of a focal female is given by:

$$S_2^* = \frac{\mathrm{SQRT}[2(1+F)(1+2F)(1+T)S_1] - 2(1+2F)S_1}{2T(1+2F)},$$

where T is as described above, F is the inbreeding coefficient, S_1 is the sex ratio of the initial brood and S_2 is the sex ratio of the focal female. We use $F = 0.197$ from the most recent and complete study of *N. vitripennis* population genetics in the wild [23]. (With haplodiploidy, we need to take inbreeding explicitly into account if females contribute different numbers of offspring to a patch: for a full review of LMC theory, see West [2].)

We calculated the optimal sex ratio for females in the 5 and 10 foundress treatments, and then used the following equation to calculate the fitness of females that behaved optimally under these conditions, and then calculate the relative fitness of our actual control and treated females:

$$W = \left[\frac{TS_2}{S_1 + TS_2}\right][1 - S_1 + T(1-S_2)]\left[\frac{1+F}{2}\right] + T(1-S_2)\left[\frac{1+3F}{2}\right].$$

3. Results

(a) Experiment 1

As expected, imidacloprid reduced female longevity in a dose-dependent manner, with concentrations of 100 ppb and 200 ppb leading to significantly increased mortality after 140 h (log-rank test: $\chi^2_4 = 41.63$, $p < 0.0001$; figure 1). These latter concentrations are typically higher than that to which wasps would be exposed in the wild, with 1 ppb and 10 ppb being sub-lethal and within the more field-realistic range for non-target invertebrate exposure.

(b) Experiment 2

Imidacloprid disrupts facultative sex allocation in female *Nasonia* (figure 2*a*). As expected under LMC, offspring sex ratios varied with foundress number, with single foundresses producing the most female-biased sex ratios (binomial GLM: $F_{1,478} = 72.86$, $p < 0.0001$). However, there was also a significant interaction between foundress number and neonicotinoid treatment ($F_{1,478} = 6.34$, $p = 0.012$). When females oviposited alone (as single foundresses), they produced

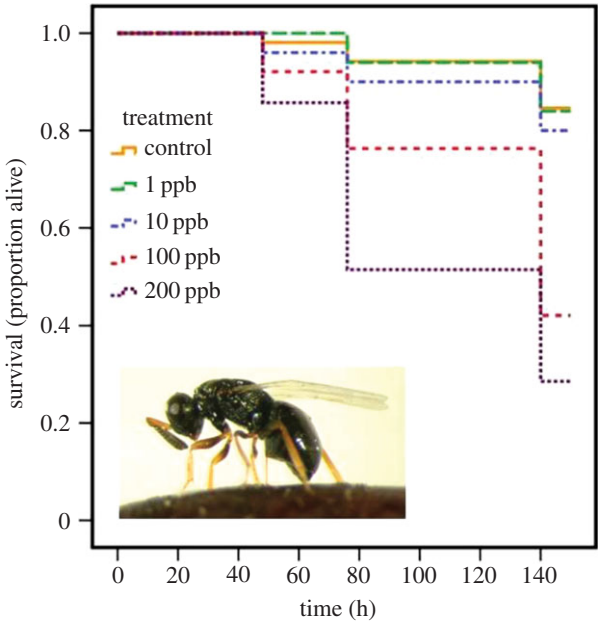

Figure 1. Imidacloprid reduces longevity in a dose-dependent manner. The survival curve shows reduced survival for female *Nasonia vitripennis* fed 100 ppb or 200 ppb imidacloprid, while females fed 1 ppb or 10 ppb had survival similar to controls fed only 20% sucrose solution. Inset: a female *N. vitripennis* ovipositing on a blowfly host.

roughly the same sex ratios regardless of exposure. However, neonicotinoid-exposed females responded less strongly to reduced LMC in the multi-foundress treatments, producing more female-biased sex ratios in the 5 and 10 foundress condition than the controls. Moreover, the effect was dose-dependent, and failure to respond adaptively in the 10 foundress treatment occurred even at the lowest, sub-lethal concentration (2 ppb; figure 2*a*).

Imidacloprid exposure also reduced offspring production by approximately 20–25%, including at the very lowest concentration ($F_{1,478} = 15.93$, $p < 0.0001$; figure 2*b*). Indeed, the reduction in offspring production did not appear to be dose-dependent, but manifested at all exposure levels. Females also produced less offspring when ovipositing with co-foundresses (this was expected as shared hosts provide less resources: $F_{1,478} = 148.85$, $p < 0.0001$; figure 2*b*), and this reduction did not vary with imidacloprid treatment (interaction: $F_{1,478} = 0.57$, $p = 0.45$).

Our results show that neonicotinoid exposure, even at sub-lethal, field-relevant levels, can change reproductive behaviour in a parasitoid wasp. However, by producing more females than expected under multi-foundress conditions, short-term benefits might have pertained in a biological control context (i.e. by producing more daughters, which would then go on to attack more pest individuals). Unfortunately, the reduction in fecundity at even sub-lethal exposure meant that female offspring production was still higher in the controls ($F_{1,478} = 14.28$, $p < 0.0001$; figure 2*c*).

(c) Sex allocation costs of imidacloprid exposure

Thanks to the well-developed LMC theory base, we were also able to estimate the evolutionary fitness costs that the reduction in facultative sex allocation, imposed by

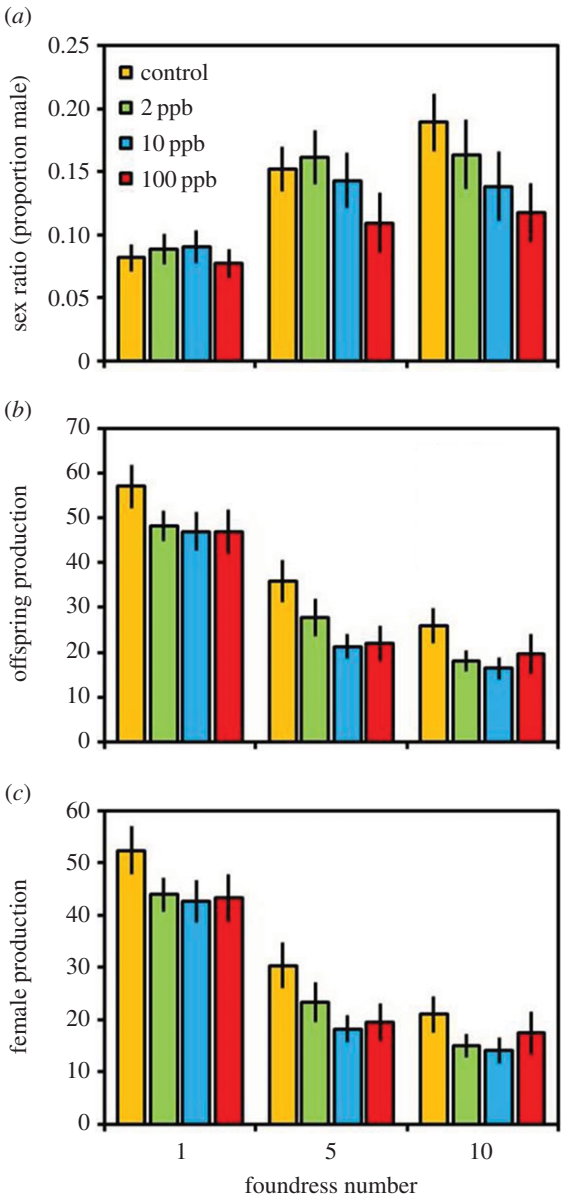

Figure 2. Imidacloprid influences the reproductive output of female *Nasonia vitripennis*. Females were fed either 20% sucrose solution (controls) or imidacloprid at concentrations of 2 ppb, 10 ppb or 100 ppb. (*a*) Sex ratio (proportion male) varies with exposure to both co-foundresses and imidacloprid, with increasing dose reducing the sex ratio response to increasing co-foundress number. (*b*) Total offspring production is reduced by imidacloprid in a dose-independent manner. (*c*) Female offspring production is likewise reduced by imidacloprid across all dose levels and across all foundress numbers; the maintenance of more female-biased sex ratios regardless of foundress number when exposed to imidacloprid therefore does not lead to greater female offspring production. Error bars indicate binomial standard errors.

imidacloprid exposure, resulted in. Under the simplest sex ratio model, that assumes equal clutch sizes for each foundress, neonicotinoid exposure reduced female fitness by up to 4.5% for 10 foundress groups, and up to 7.1% for 5 foundress groups (i.e. selection differentials of $s = 0.045$ and 0.071, respectively). However, if we included the differences in clutch sizes between control and treated females, the fitness costs rose to in excess of 40% (i.e. $s > 0.4$; figure 3). Imidacloprid exposure can therefore result in large fitness costs by disrupting sex allocation.

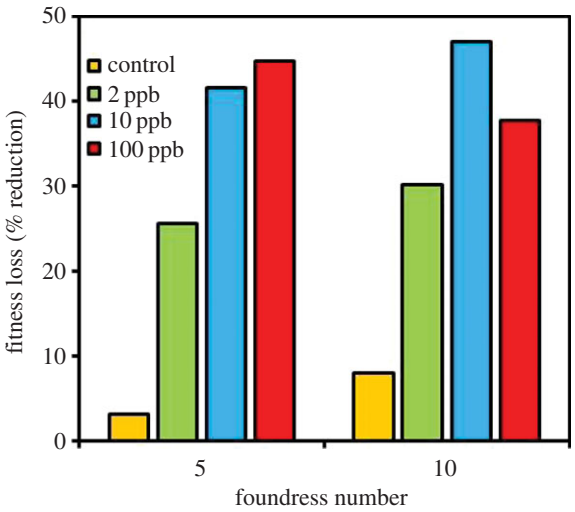

Figure 3. Disruption of facultative sex allocation leads to reduced fitness when ovipositing with co-foundresses. Females were fed either 20% sucrose solution (controls) or imidacloprid at concentrations of 2 ppb, 10 ppb or 100 ppb. Fitness was calculated relative to a hypothetical female producing the evolutionary stable sex ratio.

4. Discussion

Exposure to the neonicotinoid imidacloprid disrupts the ability of female *N. vitripennis* to facultatively allocate sex. This effect is apparent thanks to our knowledge of the theory of sex allocation under LCM [2,4]. The effects we see occur at sub-lethal doses, especially in the highest foundress-number treatment. These concentrations are within the range of 'field-relevant' concentrations reported in the literature, although we recognize that the measurement of 'field-relevant' is both highly contentious and likely to be context-dependent, varying with crop species, application procedure, local environmental conditions and so forth [14,31]. Nonetheless, our data suggest that exposure to a neonicotinoid damages in some way the machinery females use to allocate sex adaptively in the presence of varying numbers of co-foundresses. Importantly, this disruption to sex allocation imposes a significant cost to female *N. vitripennis*, a cost that would be hidden by just considering the mortality costs of imidacloprid exposure, and a cost that is also not fully encapsulated by the reduction in fecundity seen here and in other parasitoids [32,33].

As well as identifying this cryptic cost of neonicotinoids, our results also suggest new insights into the mechanism of adaptive sex allocation in *Nasonia* wasps. The similar sex ratios produced by single foundresses, regardless of exposure, suggests that the fertilization ability of females is not in itself disrupted, nor is the general process of sex determination disrupted. Instead, it is the response to co-foundresses that appears to be affected. This is an important observation, strongly suggesting that the neuromuscular control of sperm release—central to the selective fertilization of eggs that is at the heart of hymenoptera sex allocation [34]—is not damaged by neonicotinoid exposure.

In *N. vitripennis*, much work has explored the range of cues that females use to estimate the extent of LMC their offspring will experience when allocating sex under LMC [7,18,35–38]. For instance, we know that the co-foundress response is associated (at least phenotypically) with the number of times a female touches or bumps into another female [34]. Interestingly, mechanoreceptors in insects use acetylcholine as their neurotransmitter [39]. This means that mechanoreceptors are exactly the type of receptors that we might expect neonicotinoids to disrupt, and with it the ability to perceive through touch the presence of co-foundress females. This also then suggests a new methodological route for experiments to explore further how females assess foundress number. Moreover, these findings offer clues towards solving one of the key problems of breeders of gregarious parasitoid wasps for biological control programmes, namely maximizing female production under mass-rearing conditions [9]. A chemical that disrupts facultative sex allocation at high foundress numbers, but that did not also have an associated fecundity cost, would solve this problem. Our work here suggests that such a chemical might be within reach. However, as mentioned above, female *N. vitripennis* also respond to a variety of other cues when assessing the likely level of LMC that their sons will experience (such as the presence of eggs on hosts [22]), and the next task will be to explore whether neonicotinoids also influence the perception and use of those cues.

These results highlight how important it is to broaden the discussion about the ecologically relevant effects of neonicotinoids, at low, field-applicable doses. 'Quality' of reproduction is as important as quantitative effects such as fecundity; here we have measured an offspring trait known to be very relevant for fitness, i.e. its sex, and shown that sex allocation is disrupted by neonicotinoids. Moreover, sex allocation is a key trait for many other beneficial insects, including both parasitic and pollinating Hymenoptera [20,40]. Social and solitary pollinating Hymenoptera have to allocate sex in response to various aspects of the environment, and disruption of sex allocation may lead to long-term fitness consequences for these insects (and other components of natural and agricultural ecosystems). Therefore, the costs of sex allocation need to join the growing list of costs of neonicotinoids in terms of the functioning and health of beneficial insects and other non-target organisms [13,41]. We also need to test whether exposure to neonicotinoids across multiple generations will select for changes in sex allocation behaviour, including a change in how or what cues are perceived by females when they are making sex ratio decisions. The costs that the disruption to sex allocation can impose are apparent thanks to the development and testing of sex allocation theory across the last five decades [2], clearly showing the relevance of what might seem rather arcane evolutionary theory. Finally, our work emphasizes that subtle behavioural effects are apparent even if life-history traits such as longevity are unaffected. The potential scale of these effects needs to enter our discussion of the economic and ecological costs and benefits of neonicotinoid use.

Data accessibility. Data available on Dryad (doi:10.5061/dryad.22vs3).

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Conflict of interests. We have no competing interests.

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