

Exposure to the neonicotinoid imidacloprid disrupts sex allocation cue use during superparasitism in the parasitoid wasp *Nasonia vitripennis*

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Abstract. 1. Neonicotinoid insecticides are potent neurotoxins of significant economic importance. However, it is clear that their use can adversely impact beneficial insects in the environment, even at low, sub-lethal doses.

2. It has recently been shown that the neonicotinoid imidacloprid disrupts adaptive sex allocation in the parasitoid wasp *Nasonia vitripennis* (Walker) by limiting their ability to respond to the presence of other females on oviposition patches. In the present study, that work was extended to explore whether sex allocation when superparasitising – laying eggs on a host that has already been parasitised – is also disrupted by imidacloprid.

3. Under superparasitism, sex allocation theory predicts that females should vary their offspring sex ratio in relation to their relative clutch size. It was found that sex allocation under superparasitism in *Nasonia* is disrupted in a dose-dependent manner, with exposed females producing more daughters.

4. Importantly, imidacloprid does not appear to influence the ability of females to estimate the number of eggs already present on a host, suggesting a disassociation between the sex ratio and clutch size cues.

5. The present work highlights the fitness costs to beneficial insects of exposure to neonicotinoids, but also provides clues as to how female *Nasonia* use information when allocating sex.

Key words. Neonicotinoids, parasitoid, sex allocation, sex ratio, superparasitism.

Introduction

Sex allocation theory explains how organisms partition resources into either male or female offspring (Charnov, 1982; West, 2009). There is a rich theoretical framework, spanning several decades, predicting how organisms should allocate sex (Fisher, 1930; Hamilton, 1967; Charnov, 1982; West, 2009), and parasitoid wasps have been at the forefront of this research (Godfray, 1994; West, 2009). For instance, females of the parasitoid wasp *Nasonia vitripennis* (Walker) allocate offspring sex in line with Local Mate Competition (LMC) theory (Hamilton, 1967); when mating occurs amongst kin, natural selection favours mothers who bias the sex ratio towards their daughters, thereby reducing the level of LMC experienced by their sons. A single female (termed a foundress) laying eggs on a host alone

should produce just enough sons to fertilise all of her daughters. As the number of other females ('co-foundresses') contributing eggs to a patch increases, the level of competition amongst related males for mates declines, and less female-biased sex ratios are favoured. *Nasonia vitripennis* females alter both clutch size and brood sex ratio (proportion male) in response to the number of co-foundresses present ('social cues') and whether a host contains eggs already laid by another female ('host cues') (Werren, 1980, 1983, 1984). The latter scenario is known as superparasitism, and the optimal sex ratio a female should produce when superparasitising is determined by her relative clutch size: if she lays a large number of eggs relative to the previous female(s), she should produce female-biased sex ratios as again LMC amongst her sons will arise; however, if she lays only a few eggs, she should lay less female-biased, or even male-biased, sex ratios, as the benefit in terms of grand-offspring production of producing sons begins to outweigh the benefits of producing daughters (Suzuki & Iwasa, 1980; Werren, 1980).

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Sex allocation under LMC in *N. vitripennis* is well characterised at the phenotypic level (Burton-Chellew *et al.*, 2008), as it is across a range of other parasitoids (Waage & Lane, 1984; Werren & Simbolotti, 1989; Godfray, 1994; King, 2002). However, research into the mechanisms involved in sex allocation in *N. vitripennis* is much less advanced, both in terms of the genetic basis of sex ratio and in terms of the underlying neuroscience. Quantitative genetic studies have revealed QTL associated with sex ratio variation (Pannebakker *et al.*, 2011), transcriptomics studies have investigated gene expression patterns associated with sex allocation (Cook *et al.*, 2015a), and a role for epigenetics, specifically DNA methylation, has also been inferred (Cook *et al.*, 2015b). However, we still know virtually nothing of the neural substrates of sex allocation.

Recently, we have shown that female *N. vitripennis* exposed to field-relevant doses of the neonicotinoid insecticide imidacloprid exhibited a reduced ability to allocate sex optimally in the presence of co-foundresses (Whitehorn *et al.*, 2015). Neonicotinoids are neurotoxins that bind to nicotinic acetylcholine receptors (nAChRs), thereby disrupting neurotransmission (Tomizawa & Casida, 2005). Interestingly, insect mechanoreceptors use acetylcholine as their neurotransmitter (Parker & Newland, 1995). Female *N. vitripennis* are known to perceive co-foundress females through touch (King *et al.*, 1995) and Whitehorn *et al.* (2015) hypothesised that imidacloprid treatment impaired this detection ability. However, cholinergic neurons influence a broad range of sensory function in insects (Breer, 1987), and indeed neonicotinoids appear to disrupt a range of functions in insects at sub-lethal doses including reduced foraging efficiency in bees (Gill *et al.*, 2012; Henry *et al.*, 2012), feeding, digging, and foraging behaviour in ants (Wang *et al.*, 2015) and fecundity in spider mites (Barati & Hejazi, 2015).

Here, we build on our previous experiment to determine whether imidacloprid-exposed *N. vitripennis* females allocate sex optimally when responding to host cues (from a previously parasitised host) rather than a social cue. Previous work has shown that host and social cues influence sex allocation independently (King & Skinner, 1991; Shuker & West, 2004), with host cues perceived by the ovipositor during oviposition. It is also known that clutch size and sex ratio decisions can be made independently of each other (Werren, 1984; Ivens *et al.*, 2009) but we do not yet know how neonicotinoids influence the use of information for these related, but distinct decisions. If neonicotinoids disrupt sex allocation under superparasitism, they may do so by disrupting the ability of females to discern their relative clutch size, and so both clutch size and sex allocation should be affected by exposure to neonicotinoids. In contrast, the obtaining of clutch size information may not itself be disrupted, but rather how that information is then used when allocating sex, in which case we should only see an effect on sex ratio and not clutch size.

Materials and methods

We used focal females drawn from the AsymC genome reference strain (Werren *et al.*, 2010), maintained on *Calliphora*

vicina hosts at 25 °C, LD 16:8 h conditions. Co-foundresses were drawn from the red-eye STDR strain, allowing us to track the offspring of the focal, black-eyed, females.

To control for possible host and other maternal effects, 2-day old, mated females were isolated into individual glass vials and pre-treated with a single host for 24 h to allow host-feeding (Rivero & West, 2005). This host was removed and females then given access to 50% honey solution for a further 24 h. Females were then provided with two hosts to parasitise and the resulting offspring (14 days later) left for 48 h to mate. These mated females were individually allowed access to a single host for 24 h and then allocated to their experimental treatments.

Focal females were individually exposed to the neonicotinoid Imidacloprid (Sigma-Aldrich, Dorset, U.K.) at a concentration of 0, 2, 10 or 100 ppb in 20% sucrose solution for 48 h. The exact concentrations of imidacloprid to which *Nasonia* will be exposed in the field are not known. However, both 2 and 10 ppb are field-relevant and sub-lethal to *N. vitripennis*; Godfray *et al.*, 2014; Whitehorn *et al.*, 2015). Diets were provided in a 200 µl volume in the lid of a 1.5-ml microcentrifuge tube in the bottom of the glass vials ($n = 100$ per treatment). Solutions were stained green with food colouring and 10 females from each treatment group dissected for traces of green in the gut region after 48 h exposure. All dissected females were confirmed to have fed. The remaining females were then presented with a single host, previously exposed to an STDR female for 24 h, and allowed to oviposit for a period of 24 h. Hosts were incubated at 25 °C LD 16:8 h and the emergent offspring were sexed, genotyped by eye-colour, and counted. Not all females choose to superparasitise, including under control conditions, and we also excluded broods with 10 or more diapause larvae (that cannot be genotyped), putative virgins, or the eight cases where no initial STDR brood was present, yielding $N = 28-35$ females per treatment, total $N = 127$.

Sex ratio data were analysed using generalised linear models with binomial errors and logit link function. In addition to the main effects of ‘treatment’ and ‘relative clutch size’ (focal female brood/STDR brood), the quadratic term (relative clutch size)² was fitted to account for the curvilinear relationship between sex ratio and relative clutch size predicted by theory (Werren, 1980; see below). *F*-tests were used to correct for over-dispersion.

Under superparasitism, the optimal sex ratio for the second female depends on the relative clutch size, with female-biased sex ratios predicted when the second female lays more eggs, and male-biased sex ratios (including only a single male) when the second female lays very few eggs relative to the first (Werren, 1980). The fitness (W) of a theoretical female producing the optimal sex ratio (S_2^*) was calculated for each treatment according to Whitehorn *et al.* (2015):

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

where F is the inbreeding coefficient of *N. vitripennis* in the wild [$F = 0.197$, (Grillenberger *et al.*, 2008)], T is the relative

clutch size of a focal female and (S_1) is the sex ratio of the initial (STDR) brood. The optimal sex ratio (S_2^*) is given by:

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

By calculating W for theoretically optimal females, and then for focal females in this experiment (using empirically-derived sex ratios), we estimated the reduction in fitness for females in each of the four treatment groups.

Results

Imidacloprid exposure disrupted sex allocation during superparasitism, in a dose-dependent manner, but the relative clutch sizes of our treated females were not significantly affected. Experimental females exposed to imidacloprid produced significantly less male-biased sex ratios ($F_{1,126} = 11.84$, $P < 0.001$; Fig. 1). Focal female sex ratios were significantly affected by relative clutch size as predicted by theory (linear term: $F_{1,126} = 4.03$, $P = 0.045$, quadratic term: $F_{1,126} = 5.27$, $P = 0.022$), independently of treatment (interactions: linear $F_{1,126} = 0.96$, $P = 0.326$, quadratic $F_{1,126} = 1.77$, $P = 0.184$; Fig. 1). In terms of relative clutch size, focal females produced clutch sizes that were negatively correlated with the size of the first female's clutch (GLM: $\beta = -0.65 \pm 0.16$, $F_{1,123} = 84.265$, $P < 0.0001$) and the relationship between first and second female clutch sizes did not differ with imidacloprid treatment (interaction: $F_{1,123} = 2.79$, $P = 0.098$; Fig. 2). Treatment females, therefore, responded in the same way to the first female's clutch size, regardless of imidacloprid exposure. Imidacloprid exposure did not appear to affect the production of diapause larvae with very few females overall producing diapause larvae ($n = 7$ across all treatments). Also, the number of experimental females producing no offspring, i.e. propensity and ability to superparasitise, did not vary with imidacloprid treatment ($\chi^2_{3,223} = 1.843$, $P = 0.606$).

In terms of the reduction in fitness imposed by imidacloprid exposure, it was found that the average female exposed to 100 ppb imidacloprid experienced an 11.88% reduction in fitness as compared to a female producing the optimum sex ratio. At the more field-realistic dose of 2 ppb there was a fitness loss of 4.07%.

Discussion

In this study, we have extended previous research to investigate sex allocation disruption by imidacloprid in a situation where 'host cues' as opposed to 'social cues' are used by females (i.e. superparasitism). Here, superparasitising *N. vitripennis* females adjusted the size of their brood with first-foundress clutch size as expected, avoiding excess larval competition for host resources, regardless of their exposure to imidacloprid. This meant that the pattern of relative clutch sizes for imidacloprid-exposed females did not differ from unexposed controls. However, exposure to the neonicotinoid did disrupt how relative clutch size mapped to sex allocation, with treatment females producing more females than expected, in a dose-dependent manner. Given

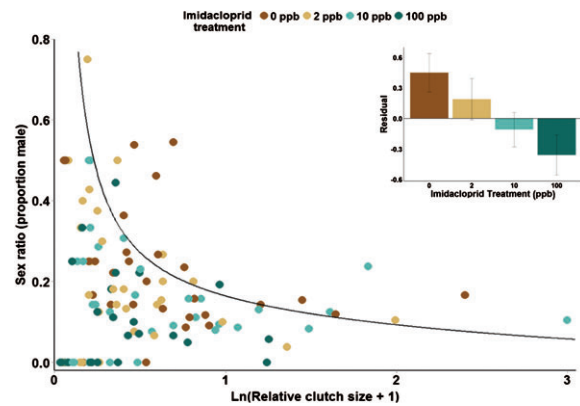


Fig. 1. Second-foundress females produce increasingly female-biased sex ratios as their relative clutch size increases. The black line represents the optimum sex ratio (eqn 2) a female should produce across the range of relative clutch sizes. Inset: females exposed to imidacloprid produce fewer males, data presented as residuals after controlling for relative clutch size; errors are standard errors.

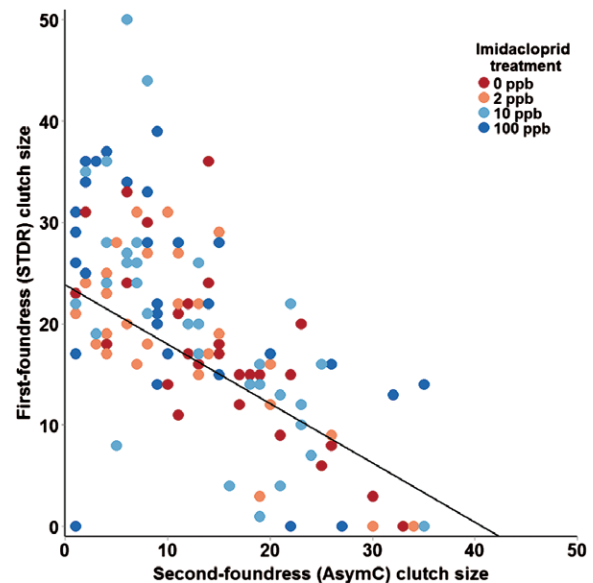


Fig. 2. Second foundress clutch size is negatively correlated with first-foundress clutch size. This relationship is not affected by treatment.

our understanding of the fitness consequences of sex allocation under LMC, we can estimate the fitness costs imposed by the disruption to sex allocation, and these costs are large. For instance, even the smallest effect on sex allocation seen with exposure to 2 ppb imidacloprid represents a fitness cost of 4.8% (i.e. in terms of the differential selection S , $S = 0.048$), which represents a significant evolutionary cost. As such, we would expect strong selection in the wild on *Nasonia* that faced incidental exposure to imidacloprid to evolve resistance to its effects, but the work on the evolution of resistance to neonicotinoids in beneficial insects is still in its infancy (for a review of resistance to neonicotinoids more generally, see Bass *et al.*, 2015).

In our previous research, we demonstrated that exposure to imidacloprid disrupted facultative sex allocation in response to a 'social' cue – the presence of co-foundresses (Whitehorn *et al.*, 2015). That initial work showed two important facts about the action of imidacloprid in female *N. vitripennis*. First, it showed that imidacloprid does not itself disrupt the basic mechanics of sex allocation (i.e. the fertilisation or not of eggs), as single foundress female *Nasonia* laying eggs alone produced the same sex ratios regardless of imidacloprid dose (see Fig. 2a in Whitehorn *et al.*, 2015). This means that imidacloprid does not disrupt the 'fertilisation-end' of sex allocation. Moreover, although we saw in that work that imidacloprid reduced fecundity (regardless of dose), that reduction in fecundity was independent of the effect on sex allocation (again as evidenced by single-foundress sex ratios). This means that imidacloprid was not having its effect by increasing the larval mortality of one sex or another. Instead, given that imidacloprid acts by binding to acetylcholine receptors, and that insect mechanoreceptors use acetylcholine as their neurotransmitter, we hypothesised that females could not accurately perceive the presence of their co-foundresses (Parker & Newland, 1995; Whitehorn *et al.*, 2015), i.e. imidacloprid disrupted the 'information-gathering' process. However, in the present study, females were able to gain information about the number of eggs already present on a host and act upon it in terms of plastically altering their clutch size. Instead, at least in the context of superparasitism, imidacloprid is here disrupting how information is used specifically for sex allocation and not for clutch size decisions after that information has been perceived.

A female's response to a previously-parasitised host is not influenced by cues associated with the host puparium (King & Skinner, 1991). Therefore, it can reasonably be assumed that most or all information relating to both clutch size and sex allocation decisions when superparasitising is obtained via the ovipositor. Recent research has confirmed that gustatory receptors in the ovipositor of the parasitoid *Leptopilina heterotoma* produce distinct electrophysiological activity patterns in response to parasitised or fresh hosts (Ruschioni *et al.*, 2015). Given that focal females, in our study, varied their relative clutch size in line with LMC theory in a dose-independent manner, it seems unlikely that imidacloprid has generally interfered with information-gain via the gustatory receptors in the ovipositor. However, the production of sub-optimal sex ratios suggests that although females responded appropriately to clutch size cues, they either could not or did not use sex ratio cues adequately when allocating sex. Disassociation between clutch size and sex allocation in terms of information use has been observed previously in *Nasonia* in the context of super-parasitism (King & Skinner, 1991). In a series of experiments involving limiting the access of the second female to certain regions of the host, the authors showed that although both clutch size and sex ratio responses were based on cues internal to the host, the cues were not identical. Interestingly, their results suggest that the sex ratio cue may travel more slowly through the host or travel to a lesser extent than the clutch size cue. Similarly, Ivens *et al.* (2009) showed that female *Nasonia* responded to whether their co-foundresses were con-specifics or hetero-specifics in terms of their oviposition decisions, but they did not respond in terms

of their sex allocation decisions (see also Grillenberger *et al.*, 2009).

In terms of how imidacloprid has affected cue use, assuming that (i) clutch size and sex ratio cues are distinct, and (ii) imidacloprid does not disrupt clutch size cue use, there are two possible scenarios that could explain our observed results. First, imidacloprid has interfered with the detection of sex ratio cues from the host but not with the detection of clutch size cues. This interpretation argues that the cues themselves are different, even although sex allocation under superparasitism requires (and appears to respond to) knowledge of the relative clutch size (Werren, 1980; Burton-Chellew *et al.*, 2008). Second, imidacloprid did not interfere with the detection of the relevant cues (which might be the same for both clutch size and sex allocation) but did disrupt the neural processing of the sex ratio cue. At this stage, it is not possible to distinguish which of these scenarios represents the true mode of action by which imidacloprid affects the superparasitism sex allocation response. However, our results confirm the disassociation between clutch size and sex ratio decisions and suggest that the manner in which these cues are processed by female *N. vitripennis* is distinct.

In summary, imidacloprid disrupts the use of both host (this paper) and social (Whitehorn *et al.*, 2015) LMC cues in *Nasonia*. Neonicotinoids are important insecticides, but their effects on off-target insects such as bees (Whitehorn *et al.*, 2012; Fischer *et al.*, 2014; Kessler *et al.*, 2015), butterflies (Gilburn *et al.*, 2015), and parasitic wasps is being increasingly recognised. Here, we have highlighted the complexities of how the neurotoxin imidacloprid interferes with decision-making processes and reduces reproductive fitness. Neonicotinoid effects on reproductive success, encompassing effects on sex ratio, have been noted in other species, such as the solitary bee *Osmia bicornis* (Linnaeus) (Sandrock *et al.*, 2014), and future research will determine whether the mode of action is similar. The present work illustrates how sub-lethal doses can impact important reproductive allocation decisions, adding to our understanding of the effects of neonicotinoids on beneficial insects.

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