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## **A Neonicotinoid Affects the Mating Behavior of *Spalangia endius* (Hymenoptera: Pteromalidae), a Biological Control Agent of Filth Flies**

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**Abstract** *Spalangia endius* Walker (Hymenoptera: Pteromalidae) is a parasitoid wasp that is commercially-available as a biological control agent for filth fly pests in livestock-rearing operations. Imidacloprid is often used to control these flies. The present study examined the sublethal effect of field-realistic concentrations of imidacloprid on mating behavior, offspring sex ratios, and male preference for virgin females. After exposure to imidacloprid, *S. endius* females that survived were less likely to mate than control females, which will result in male-biased sex ratios because only mated females can produce daughters. Males did not avoid exposed females, but exposed females were almost always unreceptive. Males that survived exposure to imidacloprid exhibited reduced mating competitiveness against unexposed males. However, if an exposed male mated, his mate's sex ratio and ability to control flies was unaffected. Exposed males were also still able to discriminate against mated, and thus usually unreceptive, females. Together with previous studies, these results suggest that not only does imidacloprid reduce the ability of *S. endius* females to survive and parasitize hosts, but when an exposed female does survive and parasitize hosts, she is likely to produce just sons, because of her lack of receptivity. More-male-biased populations of *S. endius* will decrease their efficacy for fly control. Thus, the use of imidacloprid along with this parasitoid may be financially inefficient for pest management.

**Key words:** beneficial insects, imidacloprid, parasitoid, sex ratio, sublethal effects

Filth flies like the house fly, *Musca domestica* Linnaeus (Diptera: Muscidae), and the stable fly, *Stomoxys calcitrans* Linnaeus (Diptera: Muscidae), are common pests at livestock rearing facilities. The flies irritate humans and livestock, decrease livestock feeding efficiency and milk production, spread pathogens, and can lead to lawsuits from neighbors (Thomas and Skoda 1993, Campbell et al. 2001, Nayduch and Burrus 2017). This can result in significant economic loss for animal rearing operations (Geden and Hogsette 2001, Taylor et al. 2012, Baldacchino et al. 2013). Efforts to reduce these pest populations and mitigate loss have focused on an Integrated Pest Management (IPM) approach, a practice that uses some combination of sanitation practices, pesticides, and inundative release of biological control agents to optimize pest control, while minimizing economic and environmental costs.

*Spalangia endius* Walker (Hymenoptera: Pteromalidae) is a geographically-widespread and common solitary pupal parasitoid of filth flies (Rueda and Axtell 1985, Romero et al. 2010). Naturally occurring populations of parasitoids and other natural enemies are not sufficient in suppressing filth fly populations, but can be augmented with weekly or biweekly mass releases of laboratory-reared individuals that are commercially available (Crespo et al. 2002, Floate 2003, LeBeck and Leppla 2015). When the effectiveness of such mass

releases has been formally evaluated, some studies find a significant reduction relative to control farm(s), and some do not (reviewed in Geden 2006).

Imidacloprid is a widely used neonicotinoid pesticide with a high selectivity for insects and low toxicity to mammals, birds, and fish (Elbert et al. 2008, Deacutis et al. 2006, Simon-Delso et al. 2015). Imidacloprid that is used against house flies is formulated as spot sprays and granular baits. The baits are scattered or placed in bait stations, or mixed with water and painted or sprayed on walls and other surfaces (Pospischil et al. 2005). Imidacloprid is highly toxic to *S. endius* at recommended application rates (Burgess and King 2015). Because of dissemination and breakdown, the concentration that *S. endius* and other related parasitoids of filth flies encounter on livestock facilities will sometimes be less than this application amount (Mandeville et al. 1990, Burgess et al. 2018). Individual parasitoids that survive exposure to imidacloprid may nevertheless produce fewer offspring, with more adult flies resulting (Burgess et al. 2017).

Wasps inadvertently contacting imidacloprid is plausible. Wasps may disperse in search of mates and hosts, and have been frequently found along windowsills and doorways, where some scatter bait labels recommend application (Smith et al. 1989, Skovgård 2002). Many labels do not discourage applying bait in locations where natural enemies commonly occur (Bayer Healthcare LLC 2018).

Imidacloprid acts on the insect nervous system, influencing multisensory integration, learning, and memory (Heisenberg 1998, 2003, Zars 2000, Moffat et al. 2016). Decreased mating behavior after pesticide exposure has been observed in other parasitoid wasp species (Delpuech et al. 1998a, b, Alix et al. 2001, Tappert et al. 2017). Females of many parasitoids, including *S. endius*, rarely mate more than once (Ridley 1993, King et al. 2005), so ineffective mating is highly consequential for females. However, ineffective matings may be less common if males and/or females discriminate against potential mates that have been harmed by pesticide exposure. Such discrimination is plausible because both male and female *S. endius* exhibit mate choice in relation to other variables, such as mating status (King et al. 2005).

*S. endius* males are attracted to female extract (King 2006, Nichols et al. 2010). The male fans his wings and runs toward the female (King and Dickenson 2008). She may slow down or stop locomoting, and the male dorsally mounts her and begins vibrating (King 2008). The female then may become receptive (opens her genital orifice), the male backs up, extends his aedeagus, and they copulate. Females rarely copulate more than once, but males readily do so (King and Fischer 2010). Males preferentially mount and copulate with virgin females in both choice and no choice assays, although males exhibit no preference prior to mounting (King et al. 2005).

The present study examines whether imidacloprid, one of the most widely used neonicotinoids (Elbert et al. 2008), affects mating and sex ratio in *S. endius*, a widely used parasitoid of filth flies (Olkowski et al. 2003). In parasitoid wasps, without fertilization, eggs develop parthenogenetically into male offspring; whereas daughters develop from fertilized eggs (Chapman 1971). If an insecticide interferes with mating, insemination or fertilization, a more male-biased sex ratio may result, which may slow population growth. Only females parasitize hosts; thus fewer females in a population will result in lower parasitization rates, reducing control of the pest host. Therefore, understanding how insecticides affect mating behavior can help predict the utility of insecticides in the presence of biological control agents. Sublethal effects are effects on surviving individuals (Desneux et al. 2007). Sublethal effects of imidacloprid on parasitization have been examined in already-mated *S. endius* (Burgess et al. 2017). Parasitization by such females is reduced when hosts are readily accessible, but not when they are buried. The present study examined sublethal effects of imidacloprid on male and female mating behavior, specifically, contact, mounting and copulation.

## Materials and Methods

### Laboratory Colonies

The *S. endius* strain used in the present study originated from parasitized fly pupae collected in fall 2016 from manure at a dairy farm in northern DeKalb County, Illinois. Neonicotinoids had never been applied to this farm. The farm had 450 cows, 650 hectares, a double-8 parallel parlor, sand-bedded free stalls and freedom stalls.

Vouchers of this strain of *S. endius* are catalog numbers 833640 - 833651 in the Insect Collection at the Illinois Natural History Survey Center for Biodiversity.

The parasitoids were reared at approximately 24°C with a 12:12 light dark cycle. The hosts were pupae from the NIU strain of *M. domestica*, which was reared on a mixture of fly larva medium (Lab Diet, St. Louis, MO, USA), pine shavings, fish meal, and water (King et al. 2014, Burgess and King 2015). The *M. domestica* colony had not been exposed to any pesticides for more than 20 years.

For experiments, parasitized fly pupae were isolated individually in test tubes to obtain virgin parasitoids that were 0-2 d old. Within a replicate, the control parasitoid and the parasitoid that would be exposed to pesticide were matched visually for approximate size and matched by age to the nearest day. No parasitoids were used more than once.

### **Parasitoid Exposure to Imidacloprid**

Exposure was by contact because the parasitoids do not appear to eat the fly pesticide formulation (Burgess and King 2015). Parasitoids were exposed to 0.01792 ug cm<sup>-2</sup> of imidacloprid for 48 h, which is the LC<sub>50</sub> for a Florida strain of *S. endius* used in earlier studies (Burgess and King 2015, Burgess et al. 2017). For our Illinois strain of *S. endius*, approximately 10% of females and no males died within 48 h (n = 123 females and 121 males). This concentration is much less than the 0.91535 ug of imidacloprid per cm<sup>2</sup> that will result from application of house fly bait at its recommended coverage (Burgess and King 2015). However, because the pesticide disseminates and degrades over time (Rouchaud et al. 1996, Burgess et al. 2018), the concentration that we used is one that parasitoids of house fly pupae might plausibly encounter.

To expose parasitoids to imidacloprid, ten individuals were placed in an imidacloprid-coated glass vial (Burgess and King 2015, Burgess et al. 2017). To create control parasitoids, other vials were coated with acetone alone. Before being added to the vials, the imidacloprid (99.5% purity, Chem Service West Chester, PA) had been dissolved in pesticide grade acetone (Fisher Scientific, Hampton, NH). To ensure uniform coating, the vials were placed on their side on a hotdog roller for at least 1.5 h to allow the acetone to completely evaporate. After the parasitoids were added to a vial, the vial was sealed with a cotton plug to which 50% (v/v) clover honey solution had been applied *ab libitum*. After 48 h in the vial, under a 12:12 light dark photoperiod at 28 °C ± 0.2 °C, parasitoids that were still alive and walking were collected and used in the experiments detailed below. In all mate choice experiments, the parasitoid that was given a choice is referred to as unexposed because it was taken directly from its original test tube rather than being exposed to a pesticide or control vial.

### **Experiments**

**Male Choice.** This experiment and the next male choice experiment were to determine if female pesticide exposure affects mating behavior. This first male choice experiment was with live females. An unexposed male was presented with a pesticide female and a control female in a sand dish (n = 32 total, with 8 trials run on one day and 24 trials run on another day). The dish was polystyrene (3.5 diameter, 1 cm deep) and was three-fourths full of sand that had been wetted to reduce static electricity. The two females were placed at opposite sides of the dish, and the male was placed about equidistant from the two females. Then the dish was covered with a glass lid. Observations began when the male was placed in the dish and lasted 5 min, or until copulation was completed, whichever came first. One observer followed each female, alternating which observer followed which treatment. We recorded which female was first contacted, first mounted, and first copulated, and whether each mounted female was receptive.

We then repeated this procedure with dead females to test male choice while controlling for effects of female behavior. When *S. endius* males encounter dead females, they readily mount, court, and attempt copulation (King et al. 2005). Trials (n = 33 total, with 6 – 10 trials run on each of 4 d) were conducted as described above; but before presentation to males, females had been freeze-killed at -80°C for at least 24 h after exposure to pesticide or control, and then kept at room temperature (about 23°C) for at least 10 min before being used in the choice assay.

To examine how a male's pesticide exposure affects his response to the mating status of unexposed females, pesticide males and control males were each separately given a choice between a mated female and a

virgin female (n = 28 total of each type of male, 2 – 7 of each type of male run on each of 6 d). Mated females had been generated by adding a virgin male to a test tube with a virgin female and observing copulation under a dissecting scope. After mating was complete as evidenced by copulation and dismount, the male was removed, and mated females were used within 5 min. For choice, the behaviors that were recorded were which female the male first contacted, first mounted, and first attempted copulation with. Copulation attempt was defined as the male backing up and extending his aedeagus as if to copulate. Within each replicate, the virgin and mated female were the same age to the nearest day.

**Female Choice.** Female *S. endius* (n = 37, with 9 -19 trials run on each of 3 d) were subject to a behavior choice assay in the same way as males, but with the choice being between a live control male and a live pesticide male. Female *S. endius* show no evidence of attempting to mate with dead males, so choice of dead males was not tested.

**Parasitism.** This experiment was to examine the effect of male pesticide-exposure on his mate's parasitism and offspring sex ratio. Unexposed females were each paired with a pesticide male (n = 21 total, with wasps from at least 5 different days) or a control male (n = 25 total, with wasps from at least 5 different days). All males were virgin. Mating was observed. Then each female was given 15 hosts daily for 7 consecutive days in a glass vial (7 cm length; 2 cm diameter) at ~24°C, 12:12 light dark cycle. Hosts were kept in those conditions for at least 8 weeks to ensure all emergence (flies and parasitoids) was complete. Number of flies, number of successfully parasitized hosts (hosts with parasitoid emergence holes), and the sex of the parasitoid offspring within each vial were recorded. Hosts with emergence holes were opened to include any parasitoid offspring that had crawled back in.

## Statistical Analyses

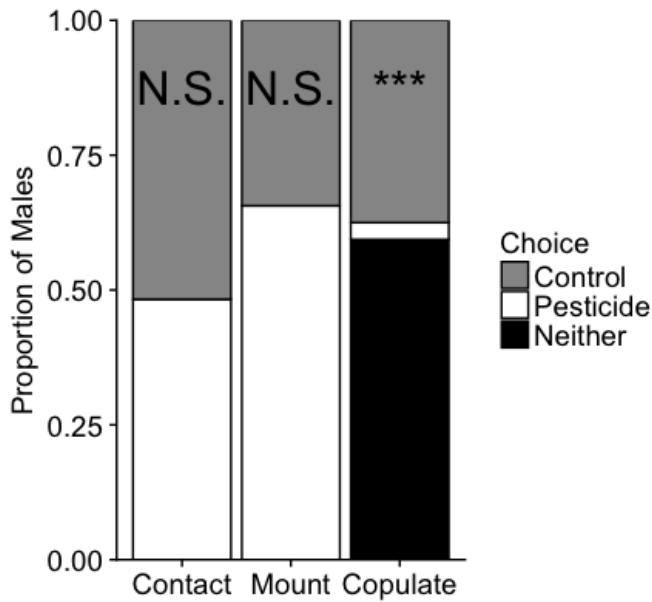
Analyses were with R version 3.2.2 (R Core Team 2015). For all four of the mate choice experiments, alpha was set at 0.05 for each of the mating behaviors within each experiment because patterns can differ among behaviors (King et al. 2005). For each behavior in each of the choice experiments, a chi-square goodness of fit test was used to determine whether the number of responses differed from no preference, i.e., no preference for the pesticide versus control mate in the first two male choice and the female choice experiments and no preference for the mated female versus the virgin female in the third male choice experiment. For each behavior in the third male choice experiment, a chi-square test of independence was used to test whether any preference for the mated versus the virgin female differed between pesticide and control males. Yate's correction for continuity was used in all of the chi-square tests. For all experiments, the number of males that did not respond to either female is presented but was not included in the chi-squares, i.e., choice was examined among males that made a choice.

For the Parasitism experiment, number of emerged flies, parasitized hosts, sons, and daughters were tallied across all 7 d of hosts before comparisons between pesticide and control treatments with independent t-tests. Sex ratios are omitted for four mothers with offspring that escaped from a vial.

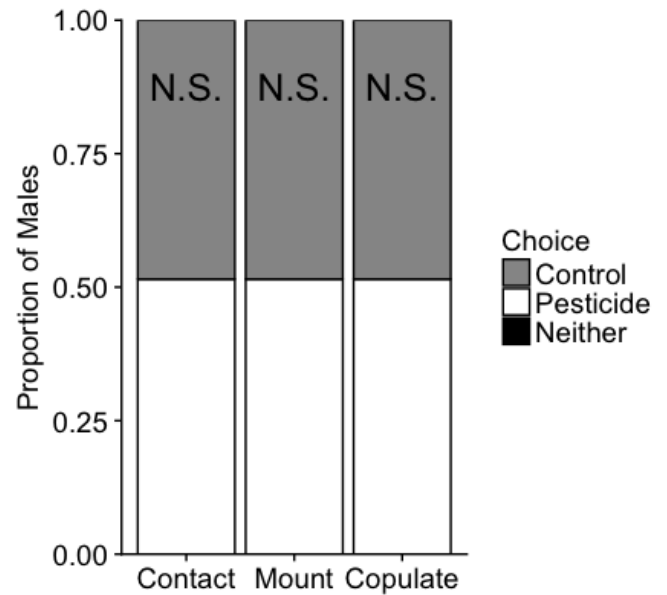
## Results

**Male Choice.** When the female was alive, her treatment had no significant effect on whether she was the first contacted or the first mounted (Fig 1;  $\chi^2 = 0.50$ , df = 1, p = 0.48;  $\chi^2 = 3.13$ , df = 1, p = 0.08); however, significantly more males copulated with control females ( $\chi^2 = 9.31$ , df = 1, p = 0.002). All of the males contacted or mounted at least one female, whereas 59% (19 of 32) did not copulate with either female. Among females that had been mounted by a male, receptivity was exhibited by significantly more control females (91%, 10 of 11) than pesticide females (5%, 1 of 21) ( $\chi^2 = 8.33$ , df = 1, p = 0.003). When the pesticide female was unreceptive, some males then mounted the control female, but not all copulated within what was left of the 5 min trial.

When the female was dead, the first female contacted was always the first mounted and the first that he attempted copulation with. Whether males first responded to the pesticide female versus the control was not dependent on treatment (Fig 2;  $\chi^2 = 0.03$ ,  $df = 1$ ,  $p = 0.86$ ).



**Fig 1.** With live females, the proportion of unexposed males that first contacted, first mounted, and first copulated with each type of female (pesticide, control) or neither, within 5 min. P values are for males that made a choice. N.S. =  $P > 0.05$ ; \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ .

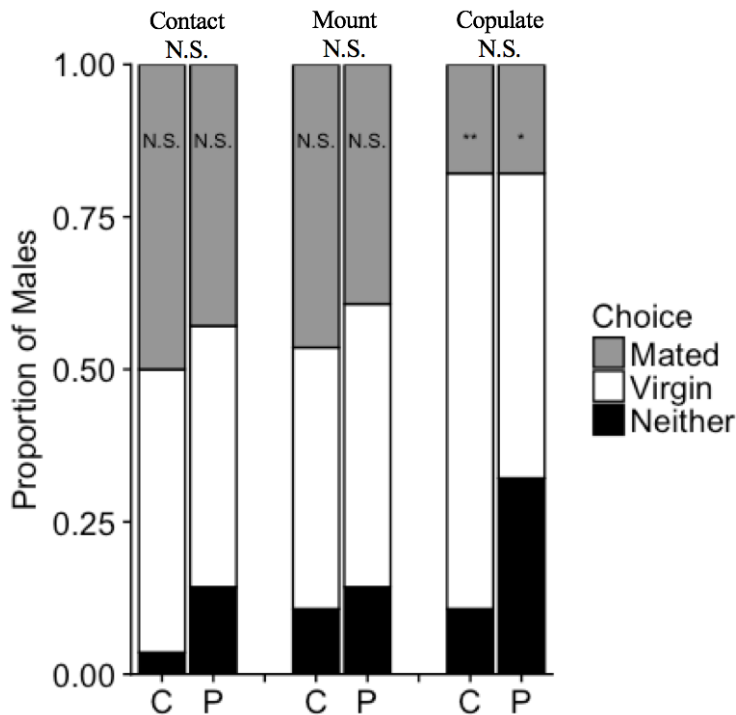


**Fig 2.** With dead females, the proportion of unexposed males that first contacted, first mounted, and first attempted copulation with each type of female (pesticide, control) or neither, within 5 min. N.S. =  $P > 0.05$ ; \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ .

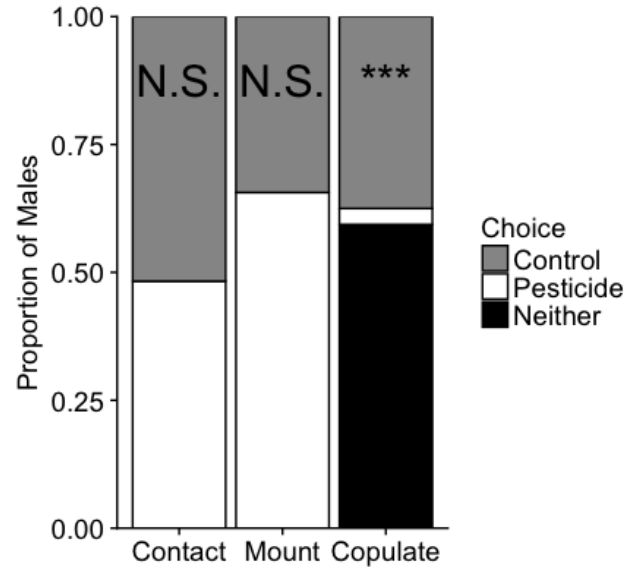
When a pesticide male or a control male was presented with a virgin female and a mated female, whether he responded first to the virgin or mated female did not differ between pesticide and control males in terms of first contact, first mount, and first copulation attempt (Fig 3;  $\chi^2 = 0$ ,  $df = 1$ ,  $p = 1.00$ ;  $\chi^2 = 0.02$ ,  $df = 1$ ,  $p = 0.88$ ;  $\chi^2 = 0.017$ ,  $df = 1$ ,  $p = 0.89$ ). Both control males and pesticide males did not preferentially contact (control:  $\chi^2 = 0$ ,  $df = 1$ ,  $p = 1.00$ , pesticide:  $\chi^2 = 0.037$ ,  $df = 1$ ,  $p = 0.85$ ) or mount (control:  $\chi^2 = 0.04$ ,  $df = 1$ ,  $p = 0.84$ , pesticide:  $\chi^2 = 0.39$ ,  $df = 1$ ,  $p = 0.53$ ) the virgin female versus the mated female, but both attempted copulation significantly more often with the virgin female (control:  $\chi^2 = 9.00$ ,  $df = 1$ ,  $p = 0.003$ , pesticide:  $\chi^2 = 4.26$ ,  $df = 1$ ,  $p = 0.04$ ). Copulation was never completed with mated females.

**Female Choice.** When a female was presented with a pesticide and control male, significantly more control males contacted, mounted, and copulated with the female (Fig 4;  $\chi^2 = 19.70$ ,  $df = 1$ ,  $p < 0.0001$ ;  $\chi^2 = 33.11$ ,  $df = 1$ ,  $p < 0.0001$ ;  $\chi^2 = 27.13$ ,  $df = 1$ ,  $p < 0.0001$ ).

**Parasitism.** There was no significant treatment effect on fly emergence, parasitism or parasitoid sex ratio over 7 d when the female mated with a pesticide or control male (Table 1).



**Fig 3.** Proportion of males that first contacted, mounted, and attempted copulation with the female that was virgin versus with the female that had mated, or neither, within 5 min, for males that had not been exposed to pesticide (C) and for males that had been exposed to pesticide (P). N.S. =  $P > 0.05$ ; \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ . Statistical significance is for males that made a choice. Significance within each bar corresponds to each type of male's preference of female among males that made a choice. Significance above each bar pair corresponds to the difference in preference between control and pesticide males.



**Fig 4.** Proportion of females (all unexposed) for which the first contact, first mount, and first copulation were with each type of male (pesticide, control) or neither, within 5 min. P values are for females that made a choice. N.S. =  $P > 0.05$ ; \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ .

**Table 1.** Mean  $\pm$  SE (range) number of flies emerged, number of hosts successfully parasitized, and parasitoid sex ratio over 7 d of hosts when a mother had mated with either a control or a pesticide male, n = number of mothers

	Fly Emergence	Hosts Parasitized	n	Proportion of Daughters	n
<b>Control Male</b>	38.28 $\pm$ 2.85 (19-77)	52.04 $\pm$ 3.16 (20-82)	25	0.82 $\pm$ 0.019 (0.48-0.95)	24
<b>Pesticide Male</b>	38.61 $\pm$ 4.05 (11-71)	52.76 $\pm$ 4.27 (22-84)	21	0.82 $\pm$ 0.018 (0.65-0.97)	18
	t = 0.07 df = 44 p = 0.94	t = 0.14 df = 44 p = 0.89		t = 0.31 df = 40 p = 0.76	

## Discussion

These results suggest that exposure of adult *Spalangia endius* to imidacloprid is likely to result in more females going unmated and thus more male-biased sex ratios in subsequent generations. Exposure to imidacloprid affected multiple aspects of mating behavior. Female exposure affected male choice of live females only. Specifically, there was no detectable effect on mating until the female receptivity stage, at which point males copulated significantly more often with unexposed females. When females were dead, males did not preferentially contact, mount, or attempt to copulate with the control (or the pesticide) female. Thus, the effect of female pesticide exposure on copulation depends on female behavior. Exposure of the opposite sex to imidacloprid had an effect not only in male choice but also in the female choice experiment. Specifically, contact, mounting and copulation were more often by control males than by pesticide males in the female choice experiment. In contrast, whether or not a male was exposed did not alter his ability to distinguish between mated and virgin females. When pesticide males did mate, parasitism, fly emergence, and parasitoid sex ratios did not depend on whether a mother's mate had been exposed to pesticide or not, suggesting that male exposure did not damage his sperm and did not affect his ability to copulate and transfer sperm successfully.

It is likely that the reason males copulated more often with unexposed females was because almost all exposed females were no longer receptive, whereas most unexposed females were still receptive. Imidacloprid may have hindered female receptivity by acting on the nerves and muscles involved in her opening her genital cavity (Tomizawa and Casida 2003, 2005, Wu et al. 2017). Sublethal pesticide exposure also causes abnormalities of the ovaries, at least in a reduviid (George and Ambrose 2004). Ovary abnormality may affect gonadal hormone release, which at least in some insects influences receptivity (Goltzené et al. 1978, Bownes 1989, Ganter et al. 2012).

Imidacloprid may also hinder female receptivity by preventing females from detecting male mating cues. In *S. endius*, the vibratory courtship that a male begins after mounting triggers the female's receptivity (King and Dickenson 2008), and effects of imidacloprid on female nerve cells may interfere with her perception of such male behavioral cues. In the confamilial *Nasonia vitripennis* Walker, female perception of male sex pheromone decreases when females have previously been exposed to sublethal doses of imidacloprid (Tappert et al. 2017). Decreased pheromone perception after insecticide exposure has also been demonstrated in other insect species (Delpuech et al. 1998a,b, 1999, 2012, Wei and Du 2004).

The results with both the live and the dead females indicate that males did not detect imidacloprid residues on the female, or did not avoid females upon detecting the pesticide. In a study by Burgess and King (2016), females were attracted to, not repulsed by, more than ten times as much imidacloprid as the male was exposed to in the present study; specifically, each female was attracted to 11.07  $\mu\text{g}$  on a glass coverslip, which was 2.28719  $\mu\text{g cm}^{-2}$  per area. In contrast, the quantity on a female in the present study was less than 0.766976  $\mu\text{g}$ , which per surface area is less than 0.01792  $\mu\text{g cm}^{-2}$  (less because some of the imidacloprid may have spread onto the surface of the 10 females in the vial). This is much less than the 0.91535  $\mu\text{g}$  of imidacloprid per  $\text{cm}^2$  that will be present initially when fly baits are applied at recommended rates (Burgess and King 2015). Male response to imidacloprid alone remains to be examined.

When males mounted the pesticide females, the males were often persistent with attempting copulation for the remainder of the trial, rather than moving to the control female. This persistence may decrease the proportion of females that mate prior to burrowing in search of hosts, leading to more male-biased populations and thus less population growth, unless males move on after 5 min, the length of our trials, or unless unexposed males compensate by mating more females.

Besides affecting sex ratios by leaving females unmated, imidacloprid may also affect sex ratios by preventing mated females from producing optimal sex ratios in response to environmental cues (Whitehorn et al. 2015, Cook et al. 2016). This reduces female fitness.

When males were exposed to imidacloprid, control males contacted, mounted and copulated significantly more often than pesticide males when both types of males were present simultaneously, even though there was little visible difference to us between control and pesticide males. One explanation for the almost complete lack of mating by any pesticide males is that the pesticide exposure hindered the male's ability to detect female cues, including female specific pheromones (Nichols et al. 2010, Mowles et al. 2013). In *N.*



*vitripennis*, males that have absorbed imidacloprid are slower to copulate, mate less, and are less able to detect female pheromone (Tappert et al. 2017). As noted above for females, effects of imidacloprid on males may be related to effects of imidacloprid on nerves and muscles.

In a previously studied Florida strain of *S. endius*, males are more likely to mount and to copulate a virgin female than a mated female, in both choice and no choice experiments (King et al. 2005). However, in the present study with an Illinois strain, males were more likely to copulate, but not more likely to mount, a virgin female than a mated female, regardless of whether the male had been exposed to pesticide or not, and despite similar sample sizes. This suggests a strain difference, although in other respects the mating seemed the same. A greater proportion of pesticide males responded to a female in this experiment than in the female choice experiment. This may be related to 2 females:1 male in this experiment as opposed to 1:2 in the female choice experiment. The typical sex ratio of *S. endius* is approximately 61%-75% in the field (Donaldson and Walter 1984, King 1991).

## Conclusions

Even at a concentration that induced little to no mortality, negative sublethal effects on mating were seen for males and females of an Illinois strain of *S. endius* in mate choice situations. This reduced mating may lead to more male-biased populations, despite pesticide males being capable of effective mating in the absence of female choice. Exposure of both sexes simultaneously was not examined but could compound the negative effects of imidacloprid on mating.

The half-life of imidacloprid is highly dependent on environmental conditions (Wagner 2016). Thus, how long after application of fly baits before imidacloprid levels are low enough not to harm parasitoids of filth flies remains to be seen. However, the present study shows that imidacloprid should be at less than 0.01792  $\mu\text{g cm}^{-2}$  before parasitoids are released, whereas 0.91535  $\mu\text{g cm}^{-2}$  is present upon application of imidacloprid-based fly bait.

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