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A neonicotinoid affects the mating behavior of the biological control agent *Spalangia endius*

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ABSTRACT

A NEONICOTINOID AFFECTS THE MATING BEHAVIOR OF THE BIOLOGICAL CONTROL AGENT *SPALANGIA ENDIUS*

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Spalangia endius Walker is a parasitoid wasp that oviposits in the pupal stage of certain fly species, killing the fly in the process. Its fly hosts include economic pests in livestock-rearing operations, and it is sold commercially as a biological control agent. Male and female *S. endius* may encounter the commonly used pesticide imidacloprid while walking or resting on treated surfaces, and females may encounter imidacloprid while searching for hosts in contaminated manure. Contact with imidacloprid has been shown to affect survival and subsequent ability to parasitize hosts in *S. endius*. The present study examined the sublethal effect of imidacloprid on mating behavior. How pesticides affect mating in parasitoid wasps of pests is of economic importance. If pesticides suppress mating, wasp populations will be more male biased. A more male-biased sex ratio will slow population growth of parasitoid wasps, reducing numbers available to parasitize hosts. In addition, only females parasitize hosts, so a female-deficient population will result in reduced parasitization rates and ultimately curtail control of pest hosts.

Pesticide treatment of female and male *S. endius* was by exposure to a surface concentration that induces low mortality. First, the effect of pesticide treatment on aspects of mating behavior, offspring sex ratios, and mate choice was examined. In a male mate choice experiment, untreated males were presented with a pesticide-treated female and a control female.

A female's treatment had no significant effect on whether she was the first female to be contacted or mounted, but significantly more males copulated with control females first. Among females that were mounted, receptivity (opening of the female's genital orifice, a behavior necessary for copulation) was observed in 1 of 21 treated females and 10 of 11 control females. Males do not appear to contribute to copulation being more likely with control females than with treated females; when the experiment was repeated but with dead females, there was no difference between treated and untreated females in which was first contacted, mounted and copulation attempted with. Female *S. endius* were subject to a mating choice assay in the same way as males, but with the choice being between a live pesticide-treated male and a live control male. Almost all first contacts, first mounts and first copulations involved the control male. Only one of the 28 pesticide-treated males mounted; he then copulated with the female. In contrast, when pesticide-treated males mated, their ability to produce offspring (daughters, sons lack fathers), as measured by their mate's sex ratio, was unaffected. In addition, whether a male was pesticide-treated had no detectable effect on whether he contacted, mounted or copulated first with a mated female or with a virgin female. Both treated and untreated males were more likely to copulate first with the virgin.

Secondly effects of male and female parasitoids allowed to burrow through used fly-rearing media were examined. Three treatments were tested: for 48 h, a wasp was exposed to pesticide or not and then was exposed to media for 24 h, or a wasp was exposed to pesticide for 48 and then for 24 h to no media. Results suggest that duration until mounting and until copulation, but not until contact, were increased for male wasps that had been exposed to pesticide and then to media relative to wasps that were exposed to just pesticide or just media. Media exposure had no effect on female duration to contact, mounting, and copulation, but

pesticide exposure did affect her time to copulation, suggesting that some deleterious pesticide exposure effects persist at least 24h after exposure.

These experiments with *S. endius* demonstrate that neonicotinoids can suppress mating. This suppression is likely to result in *S. endius* populations that are more male biased if females do not have access to untreated males. Thus, livestock rearing operations may be inefficiently spending money if they use imidacloprid in combination with release of *S. endius* for pest management.

Key words: courtship, mating, parasitoid, Hymenoptera, neonicotinoid, sex ratio

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A NEONICOTINOID AFFECTS THE MATING BEHAVIOR OF THE BIOLOGICAL
CONTROL AGENT *SPALANGIA ENDIUS*

BY

ASPEN KREMER

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Chapter 1

BACKGROUND

Filth Flies

Filth flies such as the house fly, *Musca domestica* Linnaeus (Diptera: Muscidae), and the stable fly, *Stomoxys calcitrans* Linnaeus (Diptera: Muscidae), are pest species because they irritate humans and livestock, decrease livestock feeding efficiency and milk production, spread pathogens, and can lead to lawsuits from neighbors (Thomas & Skoda 1993). This can result in significant economic loss for animal rearing operations (Campbell et al. 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, chemical pesticides, and inundative release of biological control agents to optimize pest control while minimizing economic and environmental costs.

Methods of Controlling Filth Flies

Sanitation is the primary means of controlling filth flies. This involves eliminating potential breeding sites and sources of attraction (e.g., excess animal excrement, used bedding, garbage), which also reduces the chance that flies come into contact with potential pathogens (Keiding 1986). These practices are implemented by expedient removal of waste materials and installation of proper sanitation systems for sewage (Malik et al. 2007). Sanitation is considered the cornerstone of essential control for its long-term effectiveness and low cost relative to

chemical and biological methods. Nevertheless, there are usually times during the summer when additional control is needed.

Chemical control of filth flies is the application of formulated insecticides to areas with large fly populations. Neonicotinoids are a recent class of synthetic insecticides that are widely used for their high selectivity for insects and low toxicity to mammals, birds, and fish (Deacutis et al. 2006; Simon-Delso et al. 2015). Neonicotinoids are similar to nicotine in that they target binding sites on nicotinic acetylcholine receptors (nAChRs) of the insect nervous system and inhibit the break down of acetylcholine (Tomizawa & Casida 2005). Nicotinic acetylcholine receptors are different in insects and vertebrates, allowing for the high selectivity of neonicotinoids. The electronegative tip, unique to neonicotinoids, binds specifically to a cationic subsite of the insect receptor and causes a more potent reaction. This results in overstimulation of the cholinergic receptors, ultimately causing paralysis and death. The overstimulation results from the binding being irreversible. The acetylcholine that usually binds to these receptors is normally broken down by acetylcholinesterase, but acetylcholinesterase does not break down neonicotinoids. Neonicotinoids do not bind (or bind less tightly) to the various vertebrate nAChRs, so the toxicity is less acute in vertebrates (Honda et al. 2006).

Imidacloprid is a widely used neonicotinoid against insect species (Elbert et al. 2008; Simon-Delso et al 2015) and recent studies have shown the effectiveness of imidacloprid in house fly control (Memmi 2010; Nurita & Hassan 2010; Butler et al. 2007). Unfortunately, resistant house fly strains have evolved in some locations (Kaufman et al. 2010; Geden 2012; Scott et al. 2013; Abbas et al. 2015; reviewed in Bass et al. 2015), although resistance is still much lower than to other insecticides that are used against house flies (Kustiati et al. 2016). Across pest systems, including filth flies, these problems of resistance evolving in the target

species (Scott et al. 2000) and of indirect effects on non-target species (Douglas et al. 2015) have resulted in extensive research on the effectiveness of biological control agents in an attempt to minimize these costs.

Biological control agents are natural enemies against pest species that are sometimes used to mitigate the deleterious effects of a pest (Croft 1990). These control agents are often mass released to augment natural populations and can be an environmentally conscious alternative to insecticides or released in addition to chemical control to increase effectiveness (Bale et al. 2008). The major biological control agents that have been used against filth flies on livestock operations are fungal and bacterial pathogens, predators such as the hister beetle *Carcinops pumilio* Erichson (Moore & Kaufmann 2017), parasites such as nematodes, and parasitoid wasps, mostly in the family Pteromalidae (Malik et al. 2007; Table 1). Parasitoid wasps are an especially promising method of biological control because they specialize on filth fly species, so the possibility of impacting non-target arthropod populations is low. A small number of parasitoid wasps are also currently the only commercially available biological control agent for house flies (Olkowski et al. 2004).

Effectiveness of Parasitoid Wasps in Controlling Filth Flies

Spalangia endius Walker (Hymenoptera: Pteromalidae) is a geographically-widespread and common solitary pupal parasitoid of filth flies that is found naturally throughout animal rearing operations in congruence with filth flies (Rueda & Axtell 1985; Romero et al. 2010). Naturally occurring populations of parasitoids and other natural enemies are not sufficient in suppressing filth fly populations, but they are sometimes augmented with weekly or biweekly mass releases of laboratory-reared individuals (Skovgård & Nachman 2004). This parasitoid is

known to burrow below the surface of manure in search of hosts (Legner 1977; Rueda and Axtell 1985; Geden 2002), possibly increasing its ability as a biological control agent (Morgan 1980).

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 (Pitzer et al. 2010; Morgan et al. 1975; Skovgård & Nachman 2004; Petersen et al. 1983; Meyer et al. 1990).

Table 1. Biological agents that have been examined for potential effectiveness in controlling filth flies.

Species	Type	Application Method	Advantages	Disadvantages	References
<i>Spalangia spp.</i>	parasitoid	mass release	found naturally in same areas as filth flies burrow for hosts easy to mass rear	only controls pupal stage existing parasitoid populations may inhibit integration	Morgan & Patterson 1977; Andress & Campbell 1994; Skovgård & Nachman 2004
<i>Muscidifurax spp.</i>	parasitoid	mass release	found naturally in same areas as filth flies easy to mass rear	only controls pupal stage existing parasitoid populations may inhibit integration	Geden et al. 1992; Petersen & Cawthra 1995; Floate et al. 2000

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Table 1. continued.

Species	Type	Application Method	Advantages	Disadvantages	References
<i>Trichomalopsis sarcophagae</i> (Gahan) and <i>Urolepis rufipes</i> Ashmead	parasitoid	mass release	found naturally in same areas as filth flies found in northern climates easy to mass rear	only controls pupal stage existing parasitoid populations may inhibit integration not commercially available	Floate & Skovgård 2004
<i>Nasonia vitripennis</i> Walker	parasitoid	mass release	found naturally in same areas as filth flies gregarious parasitoid easy to mass rear	only controls pupal stage existing parasitoid populations may inhibit integration prefers hosts of less economic concern	Legner 1967; McKay & Galloway 1999; Kaufman et al. 2001; Tomberlin & Bogran 2004
<i>Hydrotaea aenescens</i> Wiedemann	Predatory black dump fly	mass release	commercially available effective at multiple life stages	Difficult to establish populations	Geden 2006

(continued on following page)

Table 1. continued.

Species	Type	Application Method	Advantages	Disadvantages	References
<i>Entomophthora muscae</i> Cohn	fungal pathogen	mass release of infected flies	naturally infects filth flies high natural infection rates	4-6 d to kill high fly population needed to transmit pathogen flies can develop behavioral fever to mitigate effects	Kramer & Steinkraus, 1987; Mullens 1990; Geden et al., 1993; Steinkraus et al. 1993; Six & Mullens 1996; Geden 2012
<i>Beauveria bassiana</i> Balsamo-Crivelli	fungal pathogen	sugar baits with conidia space sprays	compatible with other natural enemies easily cultured on artificial media long shelf life	4-6 d to kill field populations usually have low infection rate	Geden et al., 1995; Kaufman et al. 2005; Nielsen et al. 2005; Geden 2006; Malik et al. 2007;; Cova et al. 2009a,b; Geden 2012
<i>Bacillus thuringiensis</i> Berliner	bacterial pathogen	feeding spores to cattle/birds mixing with breeding substrates	effective at multiple life stages	rapid resistance toxicity to vertebrates	Burns et al. 1961; Miller et al. 1971 Rupes et al. 1987; Choi et al. 2000; Oh et al. 2004 Geden 2012
<i>Carcinops pumilio</i> Erichson	predatory beetle	mass release	effective at multiple life stages		Geden et al. 1988; Geden 1990' Kaufman et al. 2000

Effects of Pesticides on Parasitoid Wasps

S. endius and other related parasitoids of filth flies are likely to encounter a range of concentrations of pesticides, including of imidacloprid, on farms (Burgess et al. in prep). To reduce filth fly populations, imidacloprid is painted or sprayed on walls and other surfaces, or mixed with attractants, sucrose, and inert materials to make granular baits, which are scattered or placed in bait stations (Pospischil et al. 2005). As a result of such applications, imidacloprid is likely to be on some of the same surfaces and substrates where parasitoids occur (Burgess & King 2016). Behavioral studies show that about 40% of *S. endius* females willingly contact filth fly granular baits that contain imidacloprid (Burgess & King 2016). Imidacloprid is highly toxic to *S. endius* at recommended application rates (Burgess & King 2015). Even parasitoids that survive pesticide exposure may produce fewer offspring, with more adult flies resulting (Burgess et al. 2016).

In all of the prior tests of lethal and sublethal effects of pesticide exposure on adult parasitoids of filth flies, the parasitoids had already mated (Scott et al. 1988; Rutz & Scott 1990; Geden et al. 1992; Whitehorn et al. 2015; Burgess & King 2015, 2016; Burgess et al. in prep). The focus of my research is whether one of the most widely used neonicotinoids, imidacloprid (Elbert et al. 2008), affects mating behavior of one of the most widely used parasitoids of filth flies (Olkowski et al. 2004). An effect seems plausible because imidacloprid acts on the nervous system (Chiela & Beer 1997), and the nervous system contributes to insect behavior. Also, in other parasitoid wasps, mating behavior is affected by pesticide exposure (Table 2). 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

harméd by pesticide exposure. Such discrimination is plausible because both males and females exhibit mate choice in *S. endius* in relation to other variables, such as mating status (King et al. 2005).

Table 2. Effect of parasitoid wasps being exposed to insecticide on behaviors associated with mating.

Parasitoid Species	Insecticide	Application Method	Results: compared to control	Reference
<i>Trybliographa rapae</i> Westwood	chlorfenvinphos (CHLF)	topical	decreased mating success	Alix et al. 2001
<i>Nasonia vitripennis</i> Walker	imidacloprid (IMI)	topical	females: decreased response to sex pheromone both: decreased mating success	Tappert et al. 2017
<i>Trichogramma brassicae</i> Bezdenko	chlorpyrifos (CHL)	surface contact	males: decreased response to sex pheromone females: decreased pheromone emission	Delpuech et al. 1998a

Mating is important in parasitoid wasps and other hymenopterans in that uninseminated females, i.e., virgins, are constrained to produce only male offspring; only mated females can produce either sex (Chapman 1971). If an insecticide suppresses mating behavior or otherwise results in a more male-biased sex ratio, population growth may be slowed. It will be slowed because a single male can inseminate multiple females and more males simply compete with each other without necessarily increasing matings, with less per male contribution to the size of the subsequent generation. In addition, only females parasitize hosts, so fewer females in a population will result in reduced parasitization rates and ultimately curtail control of the pest

host. Thus, the examination of how insecticides affect mating behavior is important to determining the utility of using insecticides in the presence of biological control agents.

Sublethal pesticide exposure has been shown to cause abnormalities on both the testes and ovaries of insect species (George & Ambrose 2004). This may affect their ability to store sperm (female and male) and produce sperm (male), resulting in decreased production of daughters. Among parasitoid species, female parasitoids being exposed to insecticide typically decreases the proportion of females in their subsequent offspring (Table 3). This effect could result from insecticide inhibiting the fertilization of the ova or the female's ability to store sperm, both of which would result in a decrease in female offspring (Desneux et al. 2007). One sex could also be more robust to parental exposure to insecticides. Direct exposure of offspring may also have an effect (e.g., Carvalho et al. 2003), but that is not examined here because that will simply be through an effect on differential mortality of the sexes, which also applies to strictly sexual insects. In contrast to many other studies, when female *S. endius* were exposed to imidacloprid, their offspring sex ratios were not significantly different from females that had not been exposed to pesticide (Burgess et al. 2016).

How exposure of male parasitoids to pesticide affects mating and their mates' offspring sex ratios has seldom been examined. In the confamilial parasitoid *Nasonia vitripennis*, exposure to imidacloprid inhibits some sexual communication by decreasing courtship behavior and female responsiveness to the male sex pheromone, resulting in an 80% reduction in mating in exposed individuals (Tappert et al. 2017). Even when males are able to complete copulation, the insecticide may inhibit their muscle action and alter their ability to transfer sperm to the female, resulting in a male-biased sex ratio.

Insecticides may interfere not only with responsiveness to mating-related pheromones but also with the ability to discriminate quantity or quality of pheromone or with sex pheromone production and release. Sublethal doses of the insecticide chloropyrifos decreases both the ability for two male *Trichogramma sp.* to discriminate between conspecific and closely related species' sex pheromones and females ability to emit sex pheromones (Dupont et al. 2010). The ability to detect and discriminate conspecific sex pheromones is important because chemical signals are important for mate detection, and hybrid mating in *Trichogramma*, as well as in many other parasitoids (Gröning & Hochkirch 2008), does not produce viable offspring or it produces fewer offspring. This may result in a reduction in mating, causing female-deficient population and ultimately slow population growth.

Table 3. Effect of parasitoid wasp females being exposed to insecticide on their subsequent offspring sex ratio in the absence of the insecticide.

Parasitoid Species	Insecticide	Application Method	Results: compared to control	Reference
<i>Nasonia vitripennis</i> Walker	imidacloprid (IMI)	oral: dissolved in sucrose solution	higher proportion of daughters	Cook et al. 2016
<i>Spalangia endius</i> Walker	imidacloprid (IMI)	surface contact	no difference	Burgess et al. 2016

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Table 3. continued.

Parasitoid Species	Insecticide	Application Method	Results: compared to control	Reference
<i>Trichogramma galloi</i> Zucchi	fipronil (FIP) lambda-cyhalothrin + thiamethoxam (LCT) spinosad (SPI) thiamethoxam (TH) triflumuron (TR)	sprayed F0 egg/larval, pre-pupa, pupa stages; measured F1	egg-larval F1: lower proportion of daughters for FIP, LCT, SPI, and TH pre-pupa F1: lower proportion of daughters for FIP, LCT, and SPI pupa F1: lower proportion of daughters for FIP, and SPI	Costa et al. 2014
<i>Encarsia inaron</i> Walker	imidacloprid (IMI) buprofezin (BUP)	surface contact	sig. lower proportion daughters for BUP but not IMI	Sohrabi et al. 2012
<i>Diaeretiella rapae</i> McIntosh	pirimicarb (PIR) dimethoate (DIM)	hosts dipped	sig. lower proportion of daughters for PIR but not DIM	Umoru & Powell 2002
<i>Trichogramma brassicae</i> Bezdenko	chloropyrifos (CHL)	surface contact	sig. lower proportion of daughters	Delpuech & Meyet 2003
<i>Habrobracon hebetor</i> Say	indoxacarb (IND) imidacloprid (IMI) deltamethrin (DEL)	pupae treated with aqueous solution	no sig. difference	Rafiee-Dastjerdi et al. 2012

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Table 3. continued.

Parasitoid Species	Insecticide	Application Method	Results: compared to control	Reference
<i>Aphytis melinus</i> DeBach	carbaryl (Car) chlorpyrifos, (CHL) dimethoate (DIM) malathion (MAL) methidathion (MET)	surface contact	sig. lower proportion of daughters for CHL but not the other insecticide treatments	Rosenheim & Hoy 1988

Mating Behavior of *Spalangia endius*

Among parasitoid wasps, the mating behavior of *S. endius* has been particularly well-studied (King et al. 2005; King & Fischer 2005, 2010; King 2006, 2008, 2010; Fischer & King 2008, 2012; King & Dickenson 2008a, b; Mowles et al. 2013). Parasitoids are sexually mature at emergence and typically mate almost immediately after eclosion (King 2000). This immediate mating behavior may be attributed to males emerging one day prior to females and not dispersing but rather staying among parasitized hosts near where he emerged (King 2006). Solid-phase microextraction reveals two female-specific compounds and no male-specific compounds; however, males and females differ in the quantity and relative quantity of compounds that they both share (Nichols et al. 2010). Males, but not females, arrest to extracts of the opposite sex (Nichols et al. 2010) and to hosts from which the opposite sex has emerged (King 2006). Most contacts before mating are initiated by males, not females (King 2008).

Mating begins with the male fanning his wings and running in the direction of a female (King & Dickenson 2008b). She may slow down or stop locomoting, and the male dorsally mounts her and begins vibrating (King 2008). The female then becomes receptive (opens her genital orifice), as she folds her antennae against her head. Once the female opens her genital orifice, the male backs up, extends his aedeagus, and they copulate. Males do not appear to use the female's antennal folding as a signal to know when to back up, rather the folding is thought to be a vestigial trait. After copulation, the male continues to vibrate, the female's genital orifice begins to close, and she begins to sweep her hind legs across her back, which hastens the male dismounting (King 2010). Females rarely re-mate after the initial copulation event, but males mate multiple times, although there is a temporary decrease in male sexual responsiveness post-mating (Fischer & King 2008).

Males, especially mated males, preferentially mount virgin females (King et al. 2005). Males will often retreat, sometimes abruptly, upon contact with a mated female (King 2008; Fischer & King 2012). This lack of mounting may be beneficial to males because a mated female is typically unreceptive, i.e., fails to open her genital orifice, and may be beneficial to females because being mounted interferes with drilling into hosts and ovipositing. Thus, a second mating would not increase fecundity or daughter production (King 2010). Several behavioral and chemical factors may be contributing to this male retreat behavior in *S. endius*. Preventing male post copulatory courtship by removing the male from a female increased the probability that a subsequent male would mount her and that she would become receptive (King & Fischer 2005; King 2010). Females also actively release the pheromone methyl 6-methylsalicylate throughout mating activities, but higher concentrations have been associated with mated females and post-copulatory behaviors, suggesting that the pheromone may also play a role in some males

abruptly retreating from mated females (Mowles et al. 2013). This pheromone may be emitted from the female's head or thorax because removal of the abdomen does not alter female unattractiveness (King & Dickenson 2008a). The present study examines many aspects of mating behavior after pesticide exposure in both male and female parasitoid wasps in an attempt to understand sublethal effects more fully. Mating is a crucial component of a parasitoid's ability to act as an effective biological control, but how pesticides affect mating has been much less studied than effects on exposure after mating.

Chapter 2

MATING BEHAVIORS ARE ALTERED BY SUBLETHAL EXPOSURE TO IMIDICLOPRID

Introduction

S. endius is likely to encounter a range of types and concentrations of pesticides while searching for pest species (Burgess et al. in prep). In addition to being lethal at field-relevant concentrations, sublethal exposure alters many behaviors that aid in an insects ability to act as an effective biological control agent, such as host location (Stapel et al. 2000), parasitization (Burgess et al. 2016), and offspring sex ratios (see Table 3). Few studies on how sublethal pesticide exposure affects mating behaviors have been executed (see Table 2), but they are crucial to determining the effectiveness of biological control agents, especially when considering long-term control and population stability. If mating is suppressed or altered, populations may be male biased because unfertilized females produce only sons. Male biased populations may slow population growth in addition to directly hindering *S. endius* ability to act as a biological control because only females parasitize hosts. In the present study, I examine how *S. endius* behaviors associated with reproduction are altered by sublethal concentrations of the neonicotinoid imidacloprid to shed light on an understudied effect of pesticide exposure.

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 at the Illinois Natural History Survey Center for Biodiversity.

The parasitoids were reared in a chamber at approximately 24°C with a 12:12 light/dark cycle. The host was pupae from a 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 & 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

In all experiments (described below), female or male parasitoids were exposed to imidacloprid or an acetone (solvent) control. Exposure was by contact because the parasitoids do not appear to eat the fly pesticide formulation (Burgess & King 2015). Parasitoids were exposed to 17.92 ng cm⁻¹ of imidacloprid for 48 h, which is the LC₅₀ for a Florida strain of *S. endius* used

in earlier studies (Burgess and King 2015; Burgess et al. in prep). For our Illinois strain of *S. endius*, approximately 10% of females and 0% of males died within 48 h (n = 123 females and 121 males). This concentration is more than ten to a hundred times less than in house fly baits but is a concentration that parasitoids of house fly pupae might plausibly encounter because the baits disseminate and degrade over time (Burgess et al. in prep).

To expose parasitoids to imidacloprid, ten individuals were placed in a glass vial coated with 17.92 ng cm^{-1} of imidacloprid for 48 h (Burgess & King 2015; Burgess et al. in prep). To create control parasitoids, other vials were coated with acetone alone. To obtain coated vials, the imidacloprid (99.5% purity, Chem Service, West Chester, PA) was first dissolved in pesticide grade acetone (Chem Service, West Chester, PA). To ensure uniform coating, the vials were placed on their side on a hotdog roller until the acetone completely evaporated, following Burgess & King (2015). Previous experiments showed that acetone has no effect on parasitoid mortality (Burgess et al. 2016). After the parasitoids were added to a vial, the vial was sealed with a cotton plug to which a drop of diluted honey (food) had been applied and placed in an environmental chamber with a 12:12 light/dark photoperiod at $28^\circ\text{C} \pm 0.2^\circ\text{C}$. After 48 h, 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 had not been exposed to pesticide, i.e., was unexposed.

Mate Choice

Male Choice of Pesticide versus Control Live-Females. This experiment and the next male choice experiment were to determine if female pesticide exposure affects mating behavior (Fig 1). The 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). The sand dish was a petri dish (3.5 diameter, 1 cm deep) that 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. One observer followed each female, alternating which observer followed which treatment. I recorded which female was first contacted, first mounted, first copulated, and duration until that mount and copulation. Observations began when the male was placed in the dish and lasted 5 min or until copulation was completed, whichever came first.

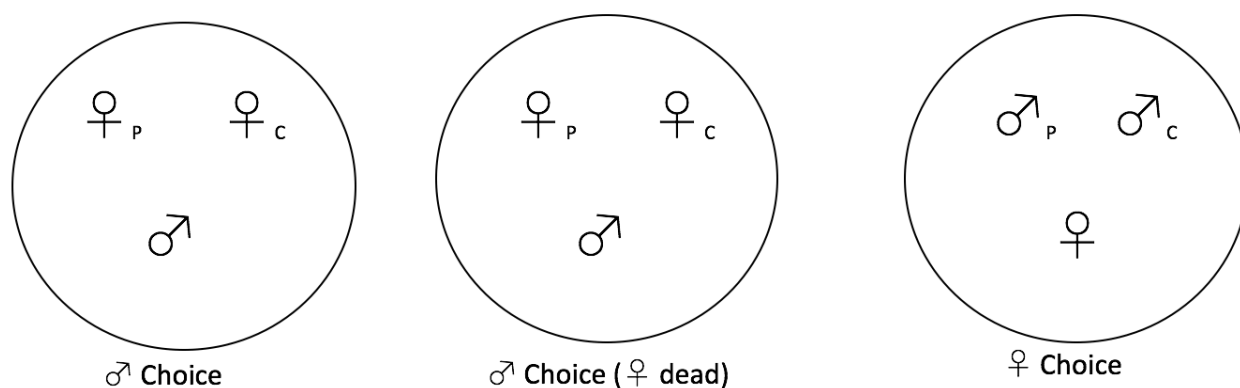


Fig 1. Choice arena for male choice and female choice experiments. 'P' and 'C' correspond to 'M' and 'V' in the *Choice of Virgin versus Mated Females by Pesticide versus Control Males* experiment

Male Choice of Pesticide versus Control Dead-Females. The second male choice experiment was 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) 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, then allowed to thaw before being used in the choice assay.

Female Choice of Pesticide versus Control Males. Female *S. endius* (n = 37) 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.

Choice of Virgin versus Mated Females by Pesticide versus Control Males. Male *S. endius* preferentially mount virgin females over mated females (King et al. 2005). This may have evolved because females rarely copulate more than once. This experiment examined how a male's pesticide exposure affects his response to the mating status of unexposed females. Pesticide males (n = 28) and control males (n = 28) were each given a choice between a mated female and a virgin female. 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. The male was removed after post copulatory behavior had finished, and mated females were used within 5 min post copulation. I recorded which female the male first contacted, first mounted, first attempted copulation with, and duration until that contact, mount, and copulation attempt. Copulation attempt was defined as the male backing up and extending his aedeagus as if to copulate. Within each replicate, the virgin and mated females were the same age.

Indirect Effects

This experiment was to examine effects of imidacloprid on a male's mate, specifically on her ability to parasitize hosts and produce offspring, particularly daughters. Unexposed females were each paired with a pesticide male (n = 18) or a control male (n = 24). All males were virgin. Immediately after observation of a completed mating, the female was removed by placing a test tube over her for her to walk up. This was to minimize disturbance. She was then tapped into a

glass vial (7 cm length; 2 cm diameter), in which she was given 15 hosts daily for 7 consecutive days. Vials were left in an environmental chamber (~24°C 12:12 light/dark cycle) for at least 8 weeks to ensure all emergence (flies and parasitoids) was complete. Number of flies, number of parasitoid offspring, and the sex of the parasitoid offspring within each vial were recorded. All hosts with emergence holes were opened to include any parasitoid offspring that had crawled back in.

Statistical Analyses

Alpha was set at 0.05 for each response variable (contact, mount and copulation) within each experiment because patterns can differ among response variables (King et al. 2005).

Response variables were first to contact (for males) or to be contacted (for females), first to mount, first copulation (or copulation attempt for dead female and mated vs virgin tests), and female receptivity (opening orifice) for male choice. For all choice experiments, whether the number of first responses differed by treatment (pesticide versus control; mated versus virgin) was compared to 50% using chi-square tests of independence with Yates correction for continuity. Lack of response is presented but not included in the chi-square; i.e., choice was examined among males that made a choice because few males failed to choose, except where noted. A separate chi-square test was used for each response variable within each experiment.

Duration until first contact, duration from contact to mount and duration from mount to copulation were compared for males using log-rank tests on Kaplan-Meier survival curves generated with the “survival” package (Therneau 2015) in R version 3.1.2 (R Core Team 2015). Survival analysis accounts for the possibility that nonresponders might have responded if the duration of observation had been longer. The duration of behaviors was not compared between

treatments in the female choice experiment because few pesticide males exhibited sexual behavior.

For the Indirect Effects experiment, number of offspring and offspring sex ratio were tallied across all 7 d of hosts and then compared between pesticide and control treatments with independent *t* tests. In the total offspring analysis, number of emergence holes was used if there were fewer wasps than number of emergence holes, indicating some offspring had escaped from the vial before counting. Number of wasps is preferable because occasionally two offspring emerge from a single host. Any mother with large discrepancies between number of wasps and number of emergence holes on any day was omitted from the sex ratio analysis (three mothers).

Results

Mate Choice

Male Choice of Pesticide versus Control Live-Females. In the first male choice experiment, i.e., when the female was alive, treatment had no significant effect of pesticide exposure on which female was first contacted or first mounted (Fig 2; $\chi^2 = 0.50$, $df = 1$, $p = 0.48$; $\chi^2 = 3.13$, $df = 1$, $p = 0.08$); however, significantly more males copulated with control females (Fig 2; $\chi^2 = 9.31$, $df = 1$, $p = 0.002$). All of the males contacted or mounted at least one female, whereas 59% (19 of 32) did not copulate with either female within 5 min. Receptivity, i.e., the female opening her genital orifice, was exhibited by 91% (10 of 11) of the control females that

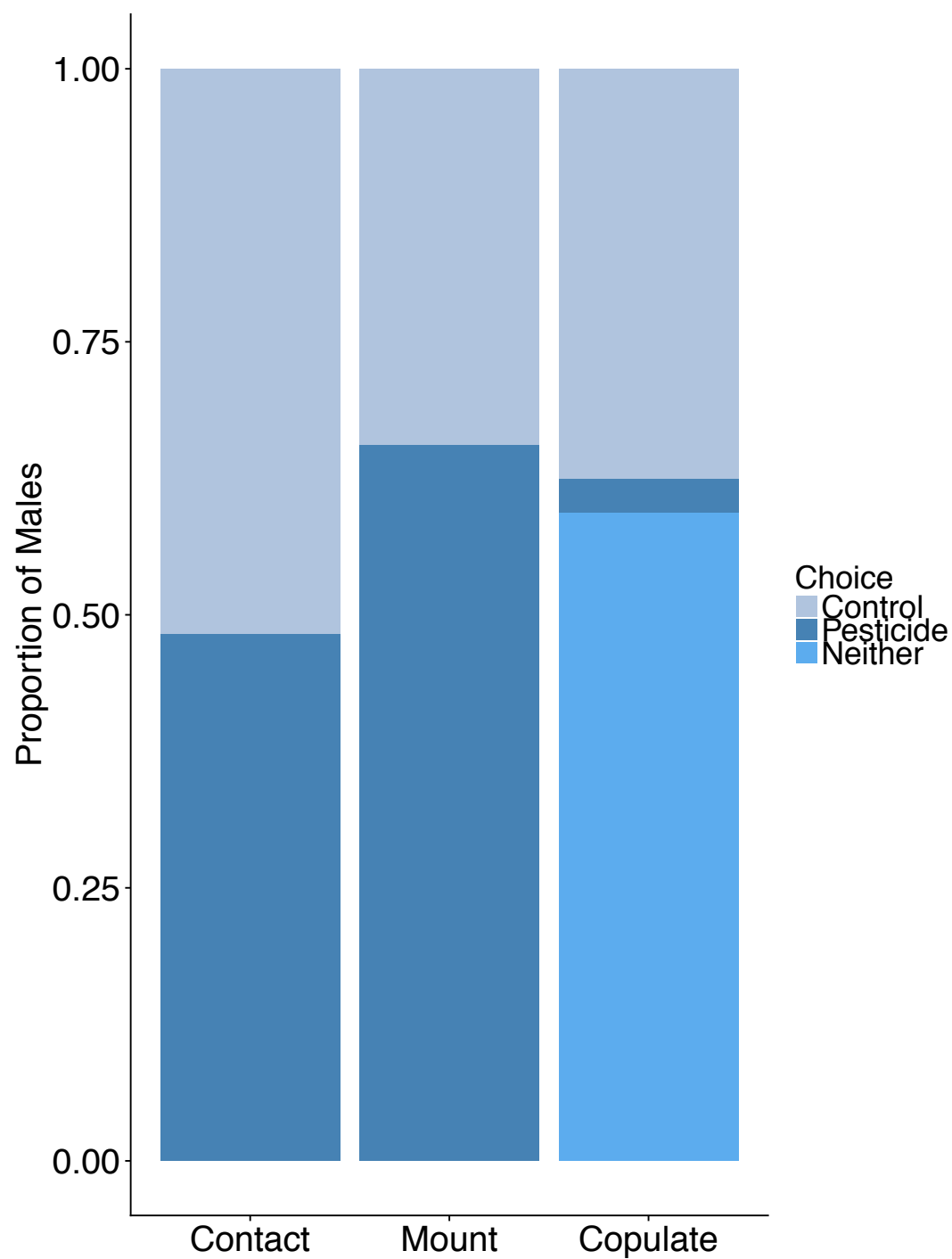


Fig 2. Male choice: pesticide vs. control live-female. The proportion of unexposed males that first contacted, first mounted, and first copulated with each type of female (pesticide, control) or neither within 5 min.

had been mounted and by 0.05% (1 of 21) of the pesticide females that had been mounted ($\chi^2 = 8.33$, $df = 1$, $p = 0.003$). Four of 21 males moved from the initial mount with the pesticide female to the control female and completed copulation and two control females were receptive, but the male did not complete copulation within 5 min. Among the 21 of 32 males that mounted the same female as was initially contacted, treatment had no significant effect on duration to contact (Fig 3; $\chi^2 = 0.10$, $df = 1$, $p = 0.74$; n contacted the pesticide female = 11 males; n contacted the control female = 9 males), duration from contact to mount (Fig 4; $\chi^2 = 0.9$, $df = 1$, $p = 0.35$), and between contact and mount (Fig 5; $\chi^2 = 0.00$, $df = 1$, $p = 1.00$). In contrast, across all 32 males, because pesticide females were rarely receptive, duration from a male's first mount to first copulation was longer for pesticide females compared to controls ($\chi^2 = 20.00$, $df = 1$, $p < 0.001$; n attempted copulation with the pesticide female = 17 males; n attempted copulation with the control female = 15 males).

Male Choice of Pesticide versus Control Dead-Females. With dead females, whether the males first responded to the pesticide female versus the control was not dependent on treatment for which female was first contacted, first mounted, or first attempted copulation with (Fig 6; $\chi^2 = 0.03$, $df = 1$, $p = 0.86$ for all three behaviors).

Choice of Virgin versus Mated Females by Pesticide versus Control Males. Whether male responded first to virgin or mated female was not dependent on exposure method (pesticide or control) for contact (Fig 7; $\chi^2 = 0$, $df = 1$, $p = 1.00$), mount (Fig 8; $\chi^2 = 0.02$, $df = 1$, $p = 0.88$), and copulation attempt (Fig 9; $\chi^2 = 0.017$, $df = 1$, $p = 0.89$). Neither control males nor pesticide males preferentially contacted or mounted with either the virgin or mated female but both

attempted copulation significantly more often with the virgin female (Table 4). Copulation was never completed with mated females.

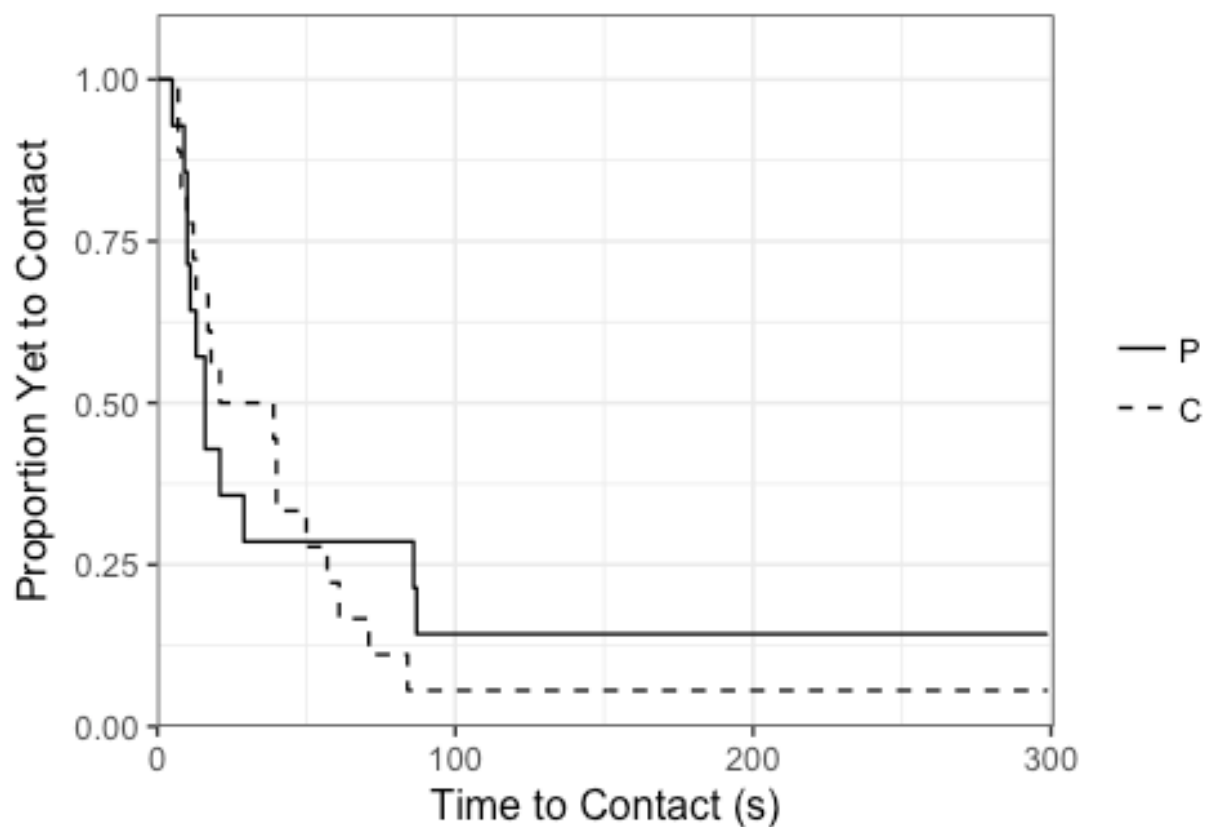


Fig 3. Male choice: time to contact. The proportion of males (all unexposed) that had not yet contacted a female at different times, for males whose first contact was with the pesticide female (P) and for males whose first contact was with the control females (C).

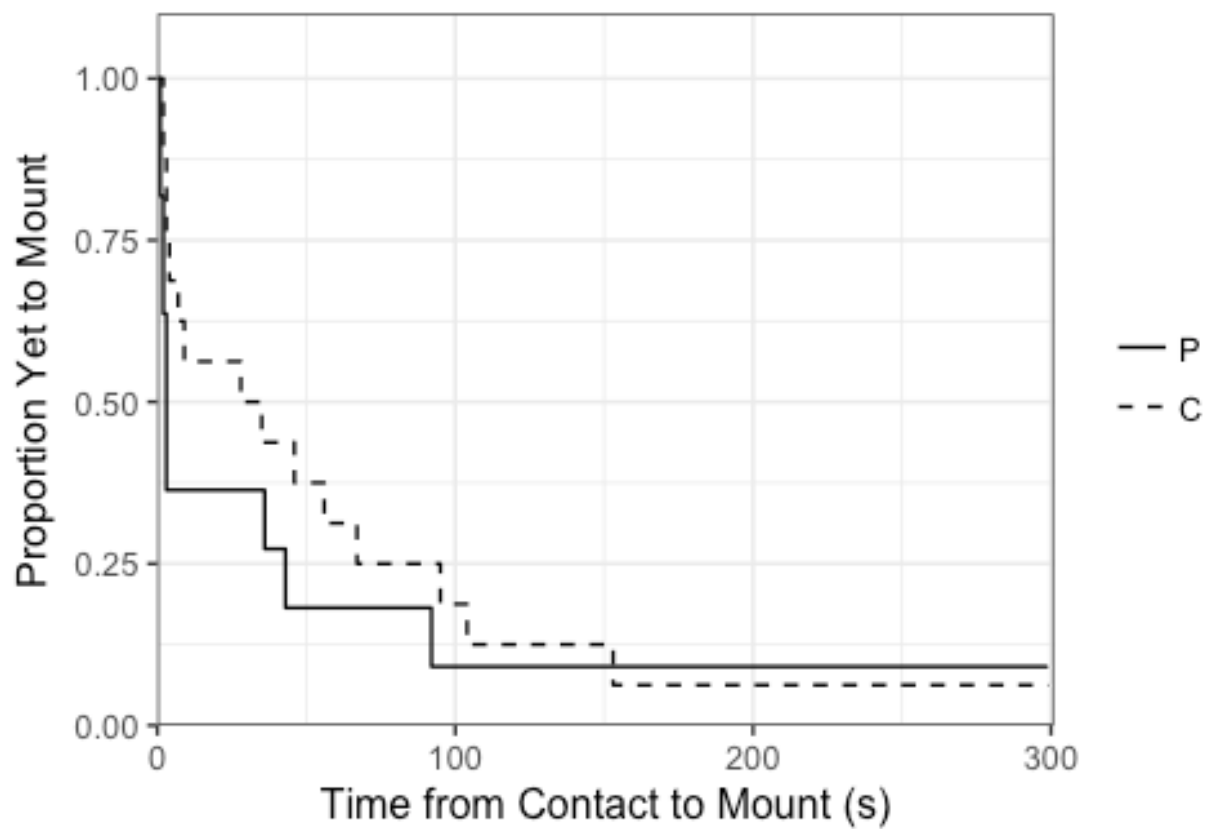


Fig 4. Male choice: time from contact to mount 1. The proportion of males (all unexposed) that had not yet mounted a female at different times, for males whose first mount was with the pesticide female (P) and for males whose first mount was with the control female (C), regardless of which female he first contacted.

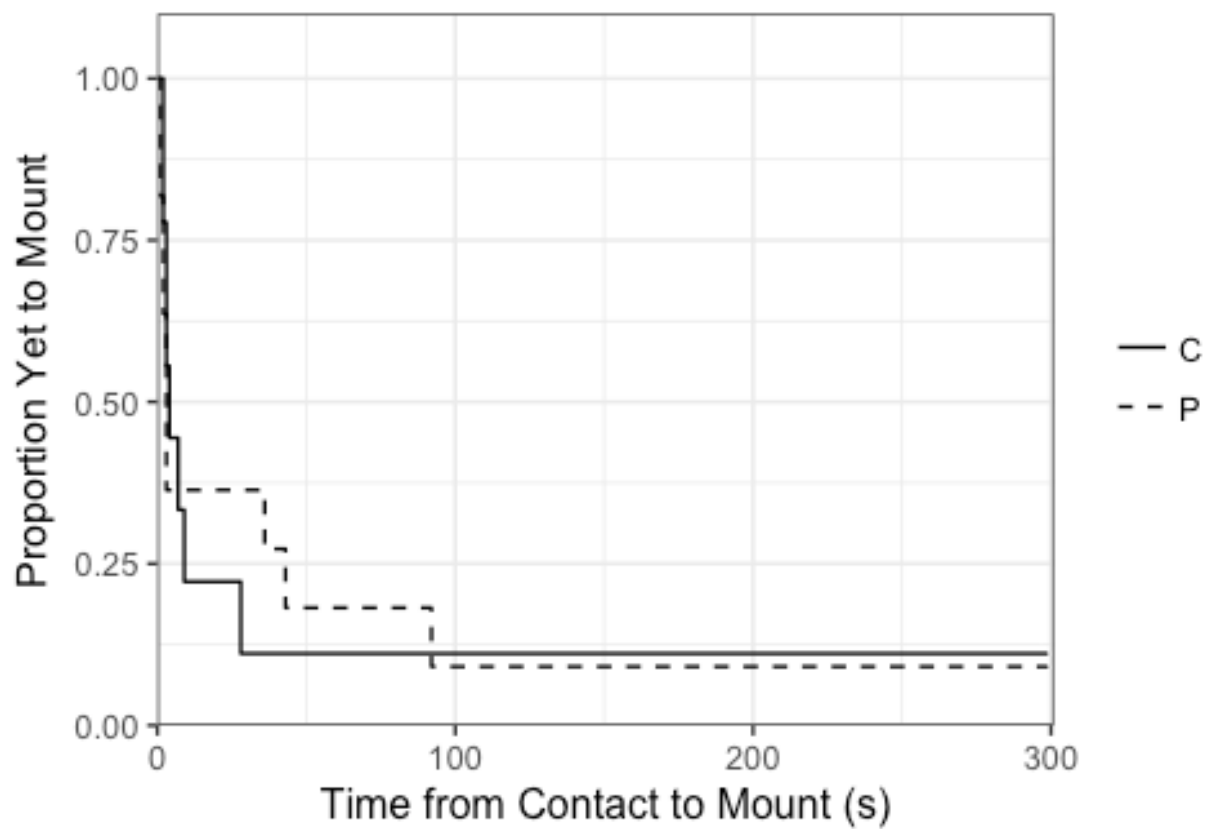


Fig 5. Male choice: time from contact to mount 2. The proportion of males (all unexposed) that had not yet mounted a female at different times, for males that first mounted the same female that he first contacted, when that female was the pesticide female (P) versus when she was the control female (C).

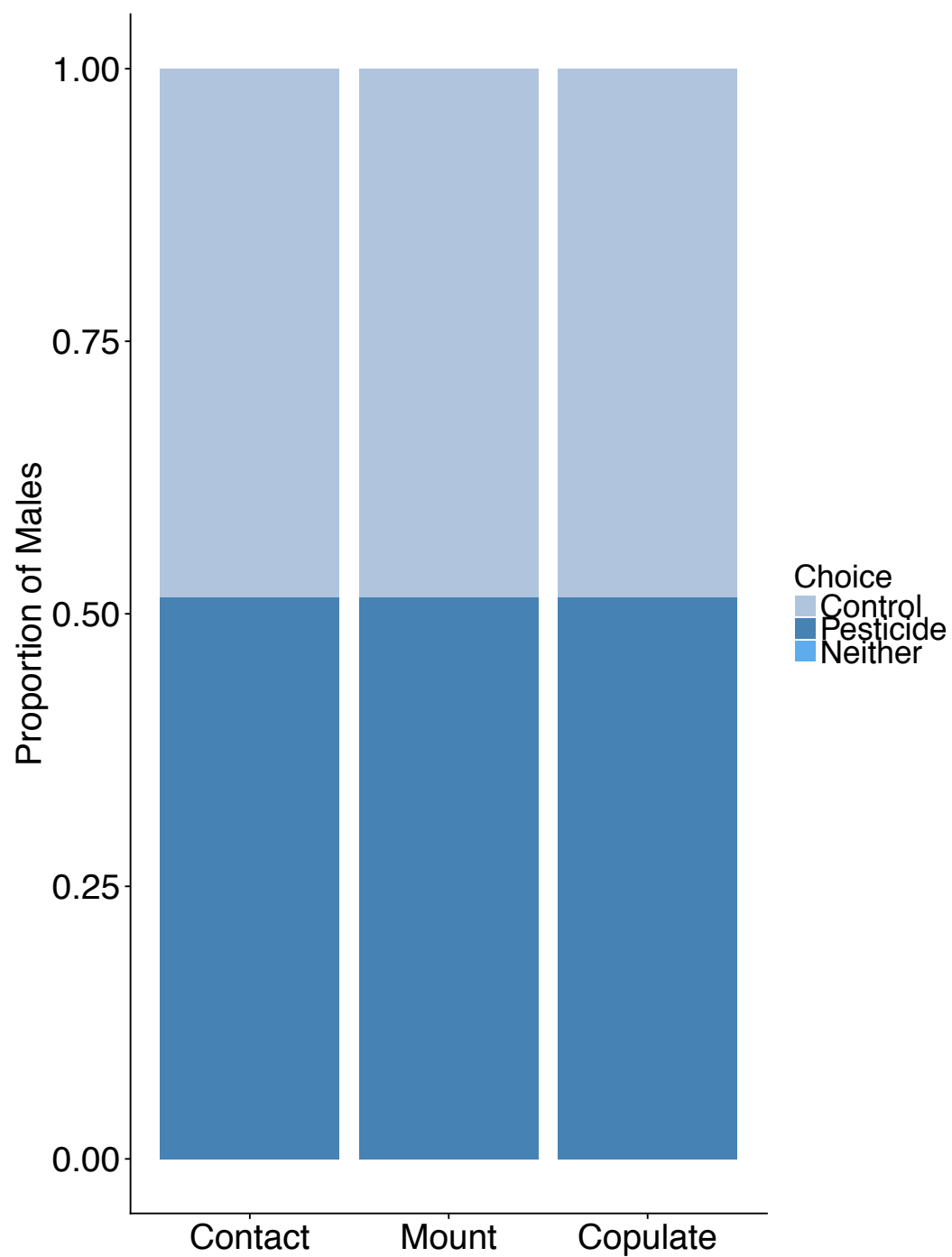


Fig 6. Male choice: pesticide vs. control live-female. The proportion of unexposed males that first contacted, first mounted, and first attempted copulation with each type of female (pesticide, control) or neither, within 5 min.

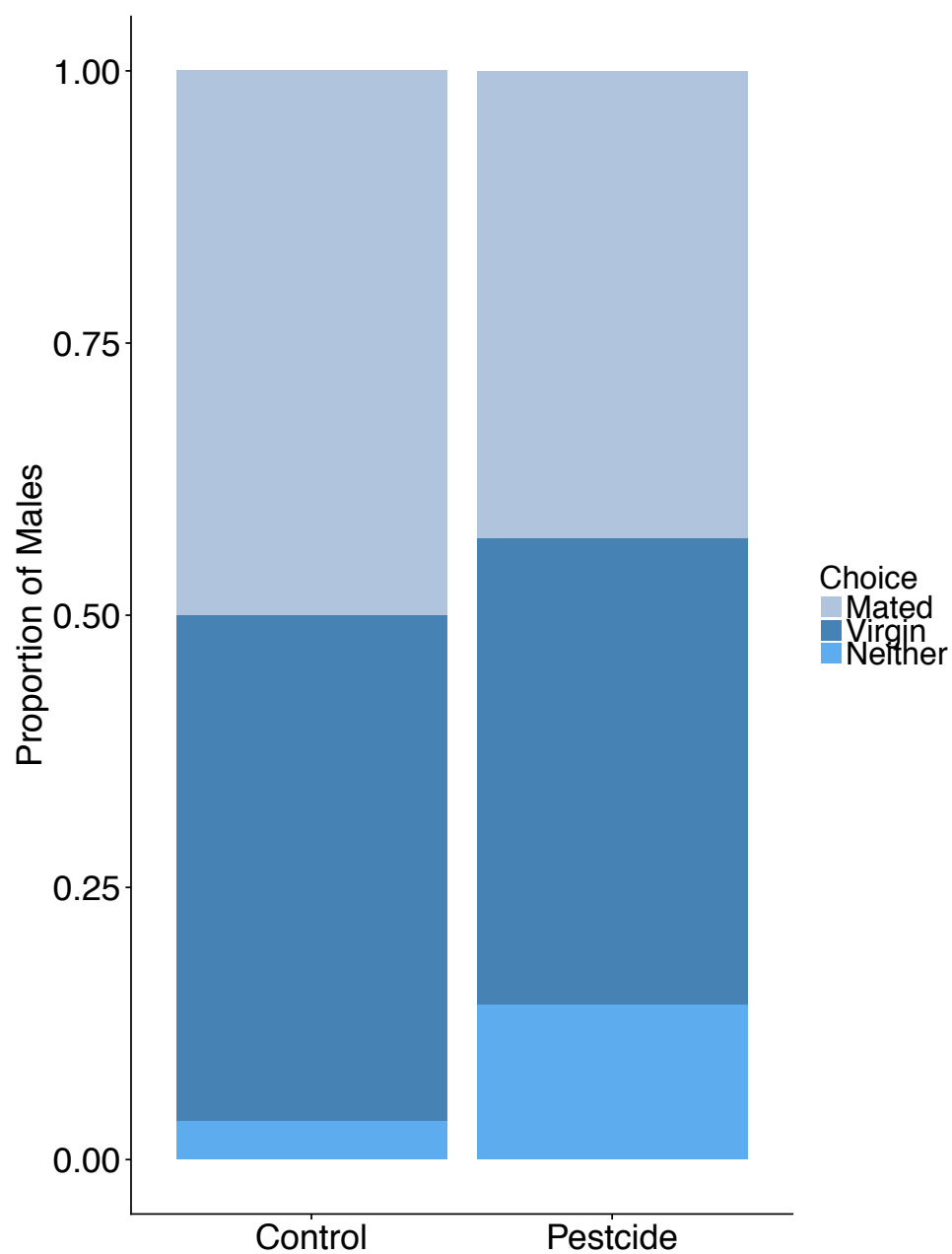


Fig 7. Male choice: mated vs virgin female, contact. The proportion of males that had not been exposed to pesticide (Control) and had been exposed to pesticide (Pesticide) that first contacted the female that was virgin versus with the female that had mated, or neither, within 5 min.

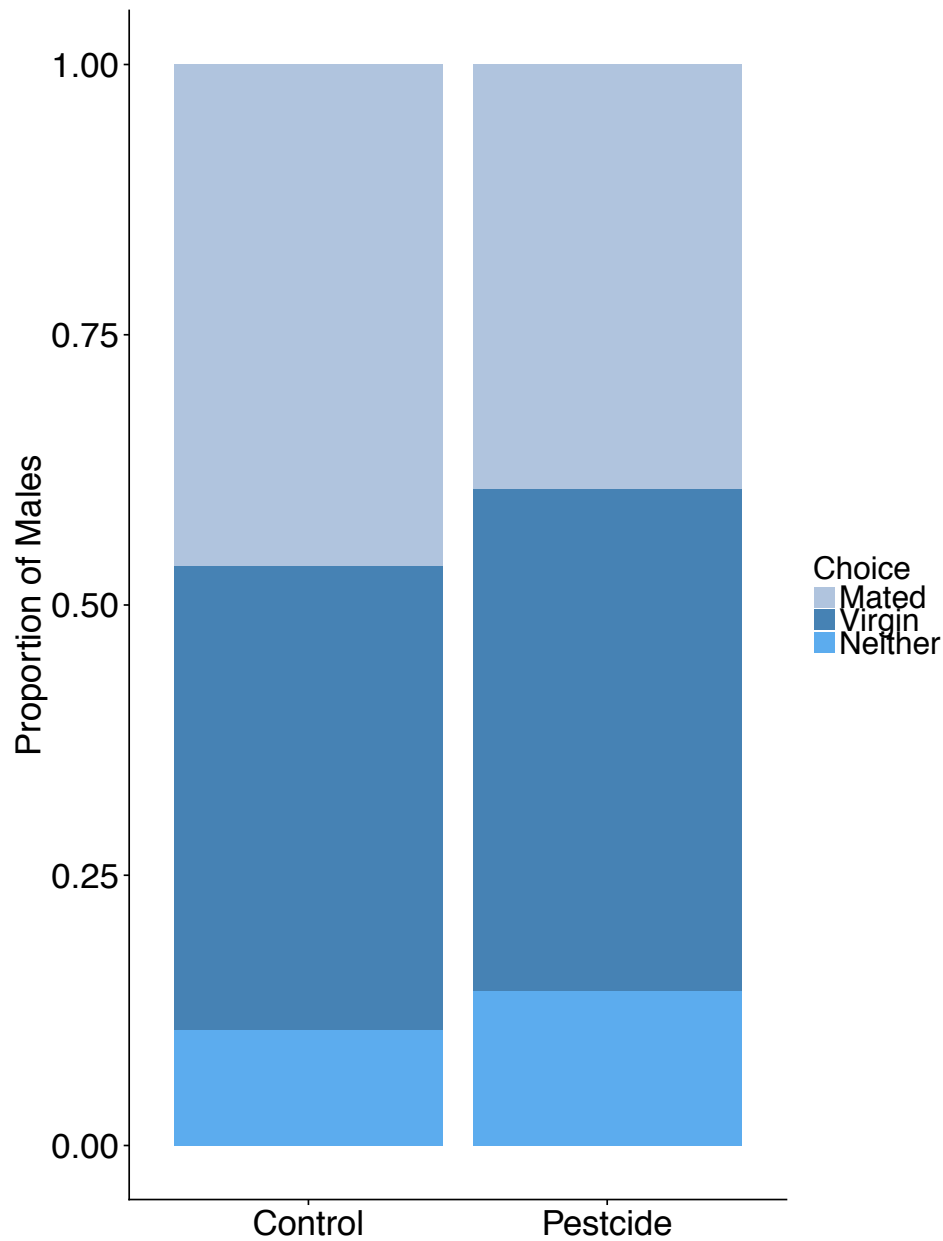


Fig 8. Male choice: mated vs virgin female, mount. The proportion of males that had not been exposed to pesticide (Control) and had been exposed to pesticide (Pesticide) that first mounted the female that was virgin versus with the female that had mated, or neither, within 5 min.

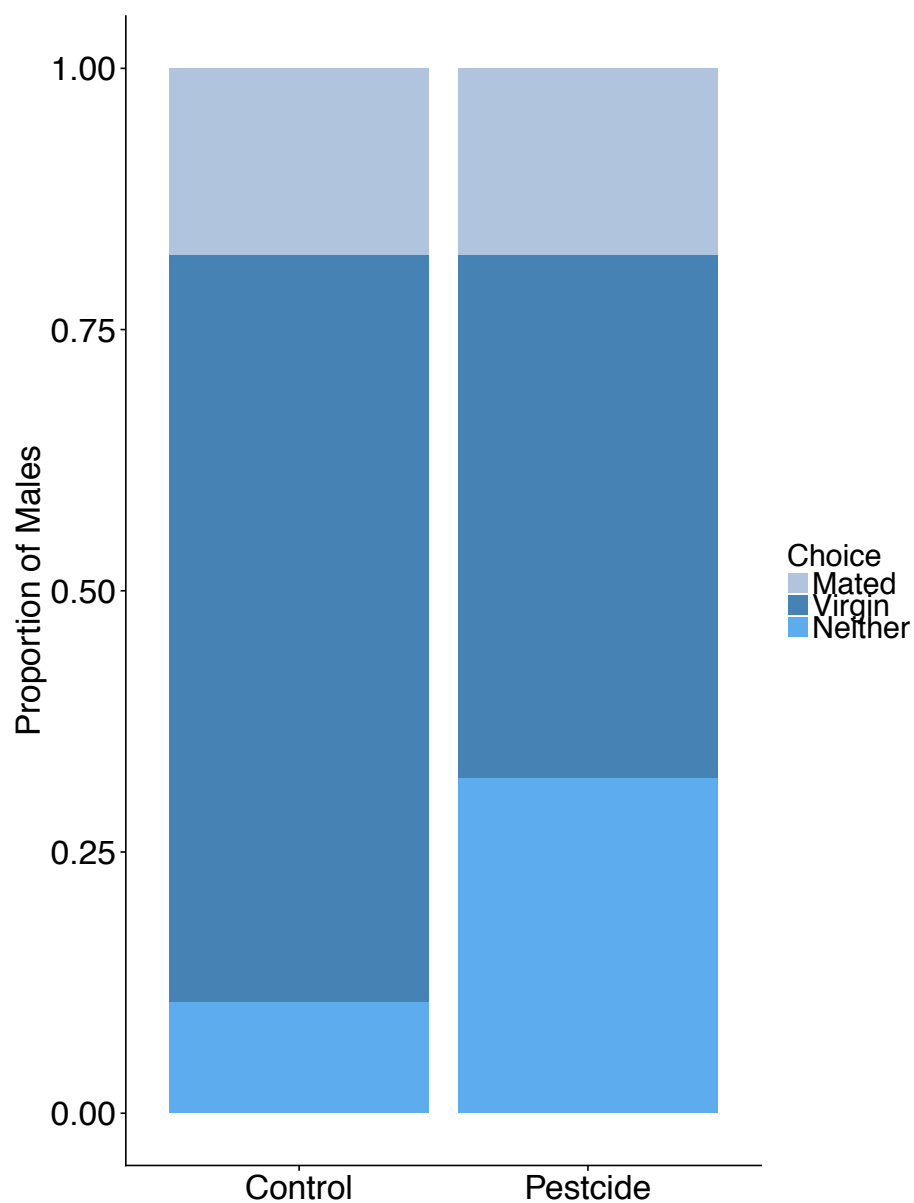


Fig 9. Male choice: mated vs virgin female, copulate. The proportion of males that had not been exposed to pesticide (Control) and had been exposed to pesticide (Pesticide) that first attempted copulation with the female that was virgin versus with the female that had mated, or neither, within 5 min.

Table 4. The number of males, control or pesticide, that first contacted, mounted, and attempted copulation with the virgin versus the mated female.

Male		Mated	Virgin	χ^2	p
Control	Contact	14	13	$\chi^2 = 0.04$	0.85
	Mount	10	13	$\chi^2 = 0.04$	0.84
	Copulate Attempt	6	20	$\chi^2 = 7.54$	0.006
Pesticide	Contact	12	12	$\chi^2 = 0.00$	1.00
	Mount	12	13	$\chi^2 = 0.39$	0.53
	Copulate Attempt	5	13	$\chi^2 = 4.26$	0.04

Female Choice of Pesticide versus Control Males. Significantly more control males contacted, mounted, and copulated with the female when she was presented with a pesticide and control male (Fig 10; $\chi^2 = 19.70$, $df = 1$, $p < 0.0001$; $\chi^2 = 33.11$, $df = 1$, $p < 0.0001$; $\chi^2 = 27.13$, $df = 1$, $p < 0.0001$).

Indirect Effects

There was no significant treatment effect on mean number of offspring of offspring over seven days (Table 5).

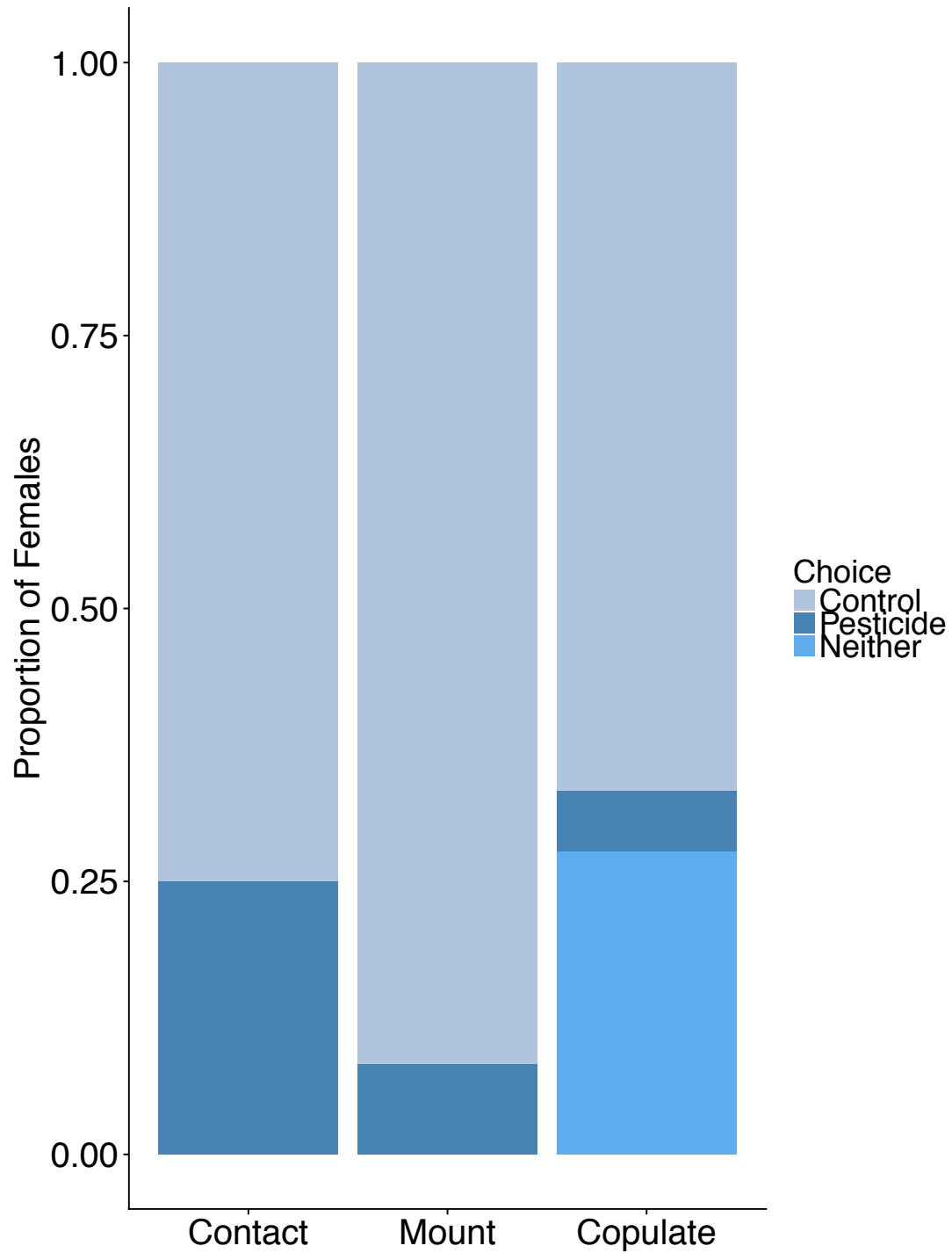


Fig 10. In the *Female Choice of Pesticide versus Control Males* experiment, the proportion of females (all unexposed) for which the first contact, first mount, and first copulation were with each type of male (pesticide, control) or neither, within 5 min.

Table 5. The mean number of offspring per mother and offspring sex ratio when a mother mated with either a control or a pesticide male

Male	Mean Offspring per Mother	% Daughters	n (number of mothers)
Control	55.30 ± 3.01 (30-87)	81%±	24
Pesticide	51.94 ± 4.55 (26-86)	81%±	18
	t = 0.63, df = 30, p = 0.54	t = 0.07, df = 39, p = 0.95	

Discussion

In the present study, female and male exposure to imidacloprid affected multiple aspects of mating behavior in the parasitoid wasp *Spalangia endius*.

Male Mate Choice: Females Exposed to Imidacloprid Exhibited Less Receptivity

Female exposure to imidacloprid had no detectable effect on mating until the female receptivity stage. When females were alive, clean males did not preferentially contact or mount pesticide or control females, but males copulated significantly more often with the control female. This increased copulation may have resulted from almost all control females being receptive and almost no pesticide females being receptive. When females were dead, males did not preferentially contact, mount, or attempt to copulate with either female. These results with both the live and the dead females indicate that males do not detect pesticide residues on the female or do not avoid females upon detecting the pesticide. *S. endius* females are attracted to imidacloprid on a glass cover slip at a quantity equivalent to that found in a single granule of fly bait containing imidacloprid, 11.07 µg (Burgess & King 2016). Male response has not been

examined, and the quantity likely to be on a female in this study is less than 0.00179 μg , but unknown, so response may have been different with greater pesticide residues on females. (The 0.00179 μg was calculated by dividing the amount added to each vial by the number of females in a vial.)

Imidacloprid may be hindering female receptivity by acting on the nerves and muscles involved in her opening her genital cavity or by inhibiting her ability to detect male mating cues, e.g., by affecting her sensory cells. Both possibilities are plausible because imidacloprid acts on the nervous system and inhibits effective nerve cell communication (Tomizawa & Casida 2005). Sublethal pesticide exposure has also been shown to cause abnormalities of the ovaries in other insect species (George & 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).

The plausibility of imidacloprid exposure preventing females from detecting male mating cues is supported by data on the confamilial species *N. vitripennis*. In *N. vitripennis*, female perception of male sex pheromone decreases when females have previously been exposed to sublethal doses of imidacloprid; response to host odors is also reduced (Tappert et al. 2017). Decreased pheromone perception after insecticide exposure has also been demonstrated in other insect species (Wei & Du 2004; Delpuech et al. 2012; Delpuech et al. 1998a; Delpuech et al. 1998b; Delpuech et al. 1999). Although female-specific pheromones have been found in *S. endius*, male-specific ones have not (Nichols et al. 2010). Regardless, males give off behavioral cues such as vibration after mounting and backing up while on the dorsal abdomen, and pesticide exposure may hinder a female's ability to detect these cues.

Female Mate Choice: Males Exposed to Imidacloprid Rarely Mated

Control males contacted, mounted and copulated significantly more often than pesticide males, even though there was little visible difference 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. Body washes and extraction of volatiles show that *S. endius* females have two compounds not found in males (Nichols et al. 2010). One of these compounds has been identified as methyl 6-methylsalicylate. In *S. endius*, low concentrations of this female-emitted pheromone component are associated with male courtship (wing fanning), while higher concentrations are associated with male behavioral arrestment and are usually emitted in these concentrations towards the end of copulation and by previously mated females, which causes males to retreat (Nichols et al. 2010; Mowles et al. 2013). Tappert et al. (2017) tested imidacloprid effects on the confamilial parasitoid wasp *N. vitripennis*, by having males absorb imidacloprid solution through the anal orifice. This exposure delays subsequent copulation and the overall mating rate and decreases his pheromone detection ability. Alterations in chemical communication after pesticide exposure have been exhibited in other insect species as well (Wei & Du 2004; Delpuech et al. 2012; Delpuech et al. 1998a; Delpuech et al. 1998b; Delpuech et al. 1999). Future studies should examine the response of pesticide males to isolated methyl 6-methylsalicylate (female pheromone).

Males Exposed to Imidacloprid Still Prefer to Copulate with Virgins

Males typically avoid and retreat from females that have already mated (King et al. 2005). This is thought to be evolutionarily beneficial to both the male and the female because females rarely become receptive again after mating once. In the choice of virgin versus mated

females experiment, the proportion of males that preferentially responded first to virgin females was not dependent on whether or not the male had been exposed to imidacloprid previously. Surprisingly, neither control nor pesticide males preferentially contacted or mounted either female but they both attempted to mate with the virgin female more often. This contrasts with data on the Florida *S. endius* strain where males preferentially mounted and copulated with the virgin female. This may be due to strain differences, and future studies should examine other strains and species to determine if this is specific to the Florida strain. The difference in males:females may explain why a greater proportion of pesticide males responded to the female in this experiment than in the female choice experiment. Males:females was 1:2 in this experiment as opposed to 2:1 in the female choice experiment. The typical sex ratio of *S. endius* is approximately 80% female in the lab and 61%-75% in the field (Donaldson & Walter 1984).

Economic Concerns

When males mounted the pesticide females, they were often persistent with attempting copulation for the remainder of the trial, rather than moving to the control female. This resulted in a significant reduction in mating because males also did not discriminate in which female they first contacted and first mounted. Even if a male were to move on from an unreceptive female, it is unlikely that he would quickly encounter a female that was not experiencing the same sublethal effects. *S. endius* males stay proximate to their emergence site and begin mating almost immediately after eclosion (King 2006), so it is likely that when one potential mate has been exposed to insecticide, then other accessible mates will also often have been exposed. If males spend time attempting to mate with multiple unreceptive females, they will have less time to find and mate with receptive females, assuming time is a limiting factor, which it may be because of

how long it likely takes to fly around in search of females after mating with those near the emergence site. Thus, male mating response to pesticide females could result in an increase of virgin females because either the females have been exposed to pesticide and are unreceptive or they do not have males with which to mate. An increase in virgin females would result in a male-biased population and curtail population growth.

Males' lack of mating response to females after the male's exposure is also concerning. Even if an exposed male does attempt to mount and copulate with a female, if she is also exposed to pesticides she may not be receptive to his attempt. These negative effects of female exposure and of male exposure are of significant concern for the biocontrol potential and economic value of beneficial parasitoids. If both males and females are displaying very little mating activity at pesticide concentrations that kill few to no parasitoids, populations will need continual augmentation rather than allowing populations to build on their own.

Indirect Effects

Total number of offspring, total number of emerged flies, and offspring sex ratios did not depend on whether a mother's mate had been exposed to pesticide or not. This suggests that male exposure did not affect ability to copulate and transfer sperm successfully or damage sperm. In the Florida strain of *S. endius*, sex ratios were not detectably affected by whether a mated female had been exposed to imidacloprid or not prior to encountering hosts (Burgess et al. 2016). In contrast to *S. endius*, sex ratios of other species are often affected by pesticide exposure (see Table 3), but those studies usually focus on maternal or host exposure. Sublethal pesticide exposure causes abnormalities on the testes of the reduviid *Rhynocoris kumarii* (George & Ambrose 2004). This species is not arrhenotokous, but rather traditionally sexual, so only

offspring production, probably not sex ratio, would be affected. However, if testes are also affected in arrhenotokous wasps, then sperm transfer ability may be harmed, resulting in a male-biased sex ratio. Further studies should examine how paternal exposure affects sex ratio in species where sex ratio is affected after maternal exposure.

Conclusions

Biological control of agricultural pest species is estimated at a \$4.5 billion value in the United States alone (Losey & Vaughan 2006). Unfortunately, in an integrated pest management approach, biological control agents are likely to encounter a range of pesticides and pesticide concentrations while searching for pest species (Burgess et al. in prep). In addition to causing mortality at field-relevant concentrations, there may also be sublethal effects, i.e., individuals that survive may experience altered behavior and physiology, hindering these insects' ability to act as effective biological control agents, such as by interfering with host location (Stapel et al. 2000), parasitization rate (Burgess et al. 2016), and offspring sex ratios (see Table 3). Very few studies on how sublethal pesticide exposure affects mating behaviors have been done (see Table 2), but they are crucial to determine the effectiveness of biological control agents, especially when considering long-term control of the pest and population stability of the control agent.

Imidacloprid effects on insects can be much more complex than simple debilitation interfering with all behaviors. For example, when red imported fire ants are exposed to 0.25 $\mu\text{g/ml}$ or more of imidacloprid, the ants reduce their sugar-water consumption, digging, and foraging; but ants exposed to 0.01 $\mu\text{g/ml}$ increase their sugar-water consumption and digging (Wang et al. 2015). In the present study, I demonstrate that when either male or female parasitoid

wasps were exposed to a concentration of the neonicotinoid imidacloprid it induced little to no mortality, significantly altered some mating behaviors, and in some cases resulted in an almost a complete lack of successful mating. If mating is inhibited in hymenopterans, populations will become more male-biased. This is of economic concern because only females parasitize hosts, so insufficient mating may result in the need for continual release of mated females to augment natural male biased populations. Examination of field-realistic conditions where both parasitoids are exposed and examination of different exposure methods and doses are crucial to further understand how the ability of these parasitoids to provide biological control is being affected in the field.

Chapter 3

PESTICIDE EXPOSURE IN FIELD-REALISTIC CONDITIONS

Introduction

Imidacloprid is highly soluble in water, so it is plausible that environmental factors like the wetness of manure and used animal bedding may dissolve some of the pesticide after it has been applied. Burgess et al. (2016) provided evidence for this in the Florida strain of *S. endius* using the pesticide imidacloprid. In an experiment where females were presented with hosts not in used fly-rearing media (mixture of fly larva medium [Lab Diet, St. Louis, MO, USA], pine shavings, fish meal, and water), females that had previously contacted the pesticide showed decreased rates of host parasitization relative to control (nonpesticide) females. In contrast, in an experiment where hosts were presented in media, pesticide and control females did not differ. An ELISA (enzyme-linked immunosorbent assay) demonstrated that being with media resulted in less pesticide residue remaining on the parasitoids. The media may have washed off some of the pesticide before it was absorbed through nonsclerotized parts of the parasitoids cuticle, or burrowing through and walking on the media may physically remove imidacloprid. Here I examine whether the presence of media mitigates any deleterious sublethal effects on mating as it did with parasitization rate.

Methods

See “Laboratory Colonies” and “Parasitoid Exposure to Imidacloprid” in Chapter 2 for general colony and exposure methods.

Media

Male Media Exposure. The male media experiment had three treatments, pesticide males (PNM = pesticide, no media; n = 28), pesticide males that had then been allowed to burrow through media (PM = pesticide, media; n = 28), and control males that had been allowed to burrow through media (CM = control, media; n = 28). After pesticide exposure or lack of exposure (controls), the next 24 h males were either put in a clean polystyrene petri dish (85 mm diameter) or put in a petri dish containing a strip of moistened used fly media (approximately 5 cm wide, 8 mm high), which went across the center of the dish contacting both sides and the top and bottom of the dish. The media was composed of a mixture of fly larva medium (Lab Diet, St. Louis, MO, USA), pine shavings, fish meal, and water. On the side of the media strip that was opposite to where the male was placed, 10 *M. domestica* pupae were placed to encourage the male to contact the media (which observations indicated he did). Ten pupae were added to one side of the clean dishes as well. Preliminary experiments with dry florescent paint on *S. endius* demonstrated that males and females walk on and through media (pers. obs.). After the 24 h in a dish, the male was removed and immediately placed in a test tube with a virgin unexposed female. Duration to first contact, first mount, and first copulation were recorded for up to 5 min. New petri dishes, test tubes, and media were used for each trial.

Female Media Exposure. Methods were basically as described in the Male Media Exposure experiment. After exposure to pesticide, females were allowed to burrow through media or not (n = 24 PNM: pesticide, no media; n = 35 PM: pesticide, media). Control females were not exposed to pesticide but were exposed to media (n = 34 CM: control, media). Then, for each female, mating interactions with a virgin unexposed male was recorded.

Statistical Analysis

Alpha was set at 0.05 for each response variable (contact, mount and copulation) within each experiment because patterns can differ among response variables (King et al. 2005). Duration until first contact, duration from contact to mount, and duration from mount to copulation were compared using log-rank tests on Kaplan-Meier survival curves generated with the “survival” package (Therneau 2015) in R version 3.1.2 (R Core Team 2015). Survival analysis accounts for the possibility that nonresponders might have responded if the duration of observation had been longer. Whether a parasitoid mounted after contact and copulated after mounting differed by treatment (pesticide versus control; pesticide with media versus pesticide without media) was compared to 50% using chi-square tests of independence with Yates correction for continuity. A separate chi-square test was used for each response variable within each experiment.

Results

Male Media Exposure. Pesticide-media males did not differ from either pesticide no-media males or control-media males in duration until contact, but differed from both in duration

until copulation, with pesticide-media males less quick to copulate. Males that had been allowed to burrow through media after exposure to pesticide did not differ from males that had not been allowed to burrow through media in their duration to first contact of a female; however, duration to mount and duration to copulation did depend on treatment method (Table 6; Fig. 11, 12, 13). Pesticide-exposed and control males that were both allowed to burrow through media without pesticides for 24 h were then compared. Duration to contact and duration to mount did not depend on treatment; however, duration to copulation did depend on treatment method (Table 6; Fig. 11, 12, 13).

Table 6. Survival analysis comparing male time to contact when he was exposed to pesticide or not and then allowed to burrow through untreated media (PM vs CM) and comparing time to contact when he was exposed to pesticide and allowed to burrow through media or not (PM vs PNM).

Behavior	Treatment	χ^2 (p-value)	Fig
Contact	PM vs CM	1.90 (0.16)	11
	PM vs PNM	1.80 (0.18)	11
Mount	PM vs CM	3.80 (0.052)	12
	PM vs PNM	5.40 (0.02)	12
Copulate	PM vs CM	5.10 (0.02)	13
	PM vs PNM	4.70 (0.03)	13

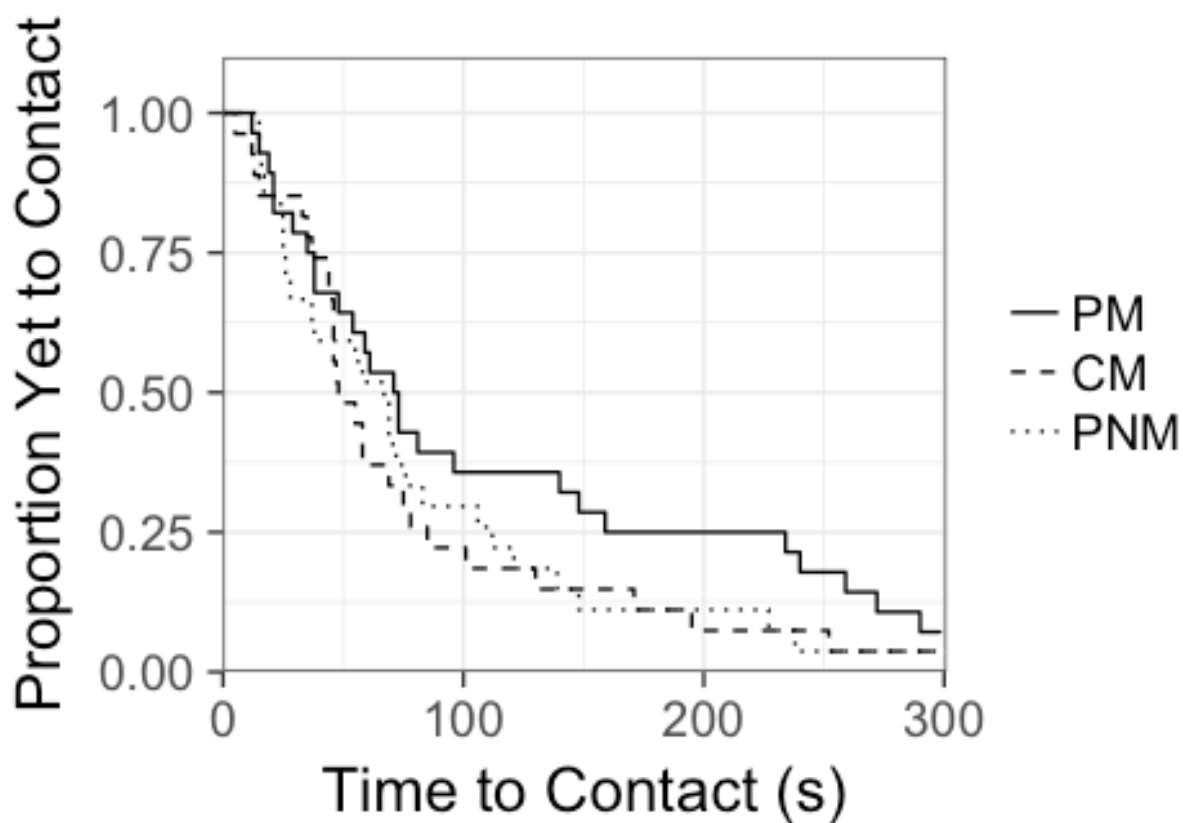


Fig 11. Male exposure: time to contact. The proportion of males that were exposed to pesticide and allowed to burrow through media (PM), exposed to pesticide and not allowed to burrow through media (PNM), or exposed to an acetone control and allowed to burrow through media (CM) that had not yet contacted a female at different times. Survival curves were compared for PM-PNM and CM-PM.

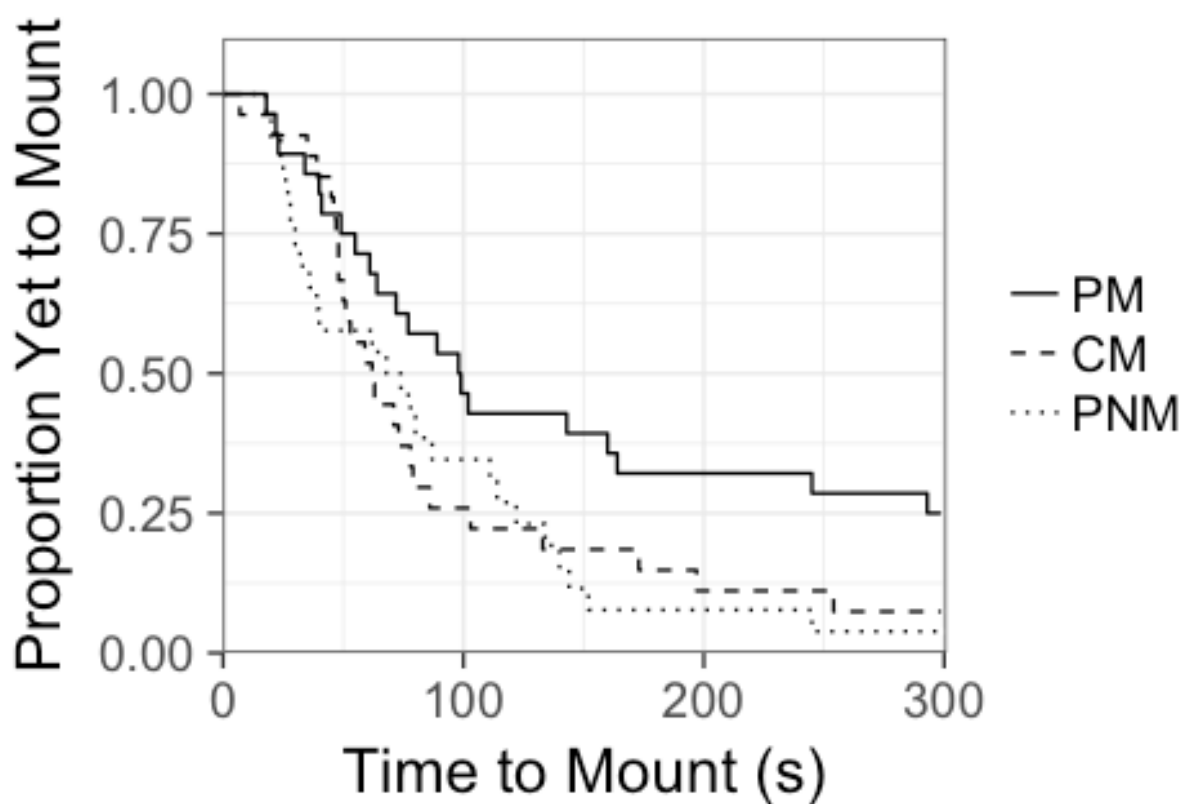


Fig 12. Male exposure: time to mount. The proportion of males that were exposed to pesticide and allowed to burrow through media (PM), exposed to pesticide and not allowed to burrow through media (PNM), or exposed to an acetone control and allowed to burrow through media (CM) that had not yet mounted a female at different times. Survival curves were compared for PM-PNM and CM-PM.

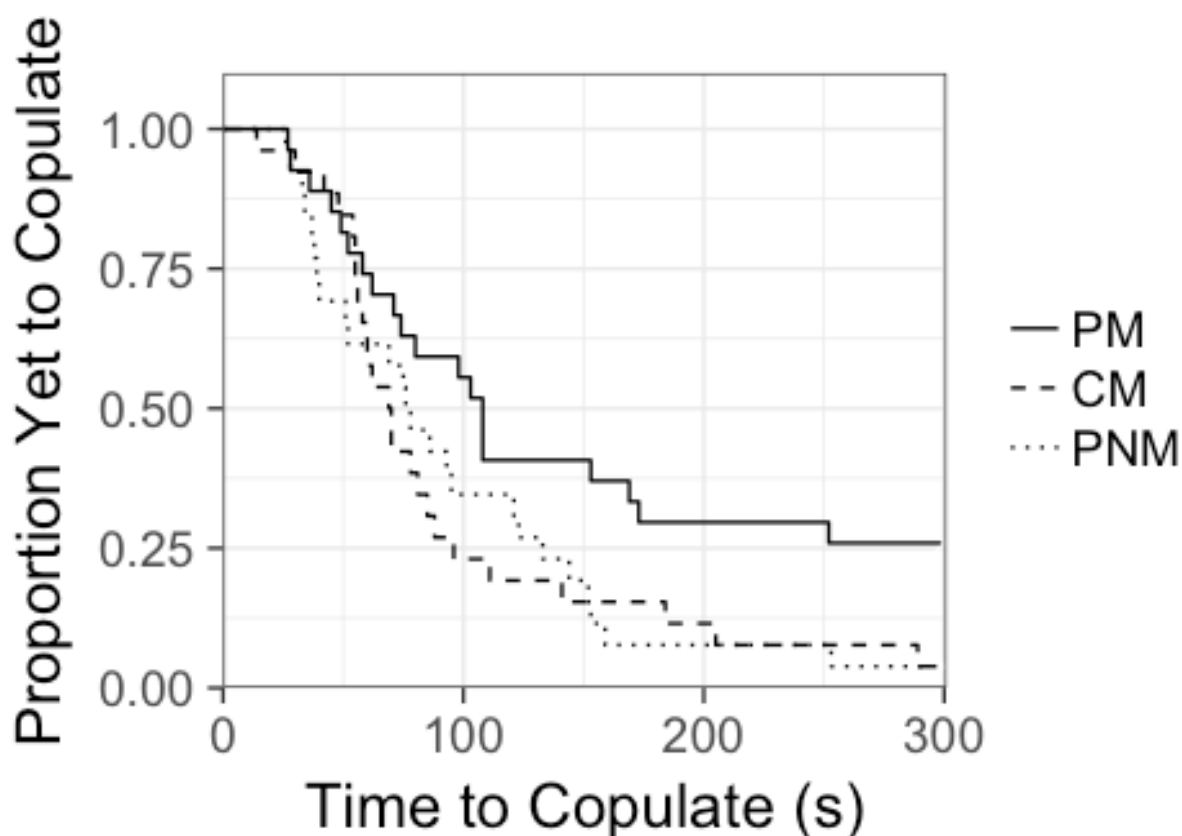


Fig 13. Male exposure: time to copulate. The proportion of males that were exposed to pesticide and allowed to burrow through media (PM), exposed to pesticide and not allowed to burrow through media (PNM), or exposed to an acetone control and allowed to burrow through media (CM) that had not yet copulated with female at different times. Survival curves were compared for PM-PNM and CM-PM.

Female Media Exposure. Pesticide females did not differ from either pesticide no-media females or control-media females in duration until contact and duration until mount. Duration to copulation of pesticide females was not different from pesticide no-media females but was slower than for control media females. Pesticide females that had been allowed to burrow through media and pesticide females that had not been allowed to burrow through media did not differ significantly in their duration to contact, duration to mount, and duration to copulation (Table 7; Fig. 14, 15, 16). Pesticide-exposed and control females that were both allowed to

burrow through media without pesticides for 24 h was compared. Duration to initial contact and duration to mount did not depend on treatment; however, duration to copulation did depend on treatment method (Table 7; Fig. 14, 15, 16).

Table 7. Survival analysis comparing female time to contact when she was exposed to pesticide or not and then allowed to burrow through untreated media (PM vs CM) and comparing time to contact when she was exposed to pesticide and allowed to burrow through media or not (PM vs PNM).

Behavior	Treatment	χ^2 (p-value)	Fig
Contact	PM vs CM	3.20 (0.08)	14
	PM vs PNM	0.30 (0.58)	14
Mount	PM vs CM	3.40 (0.07)	15
	PM vs PNM	0.80 (0.38)	15
Copulate	PM vs CM	5.30 (0.02)	16
	PM vs PNM	0.40 (0.51)	16

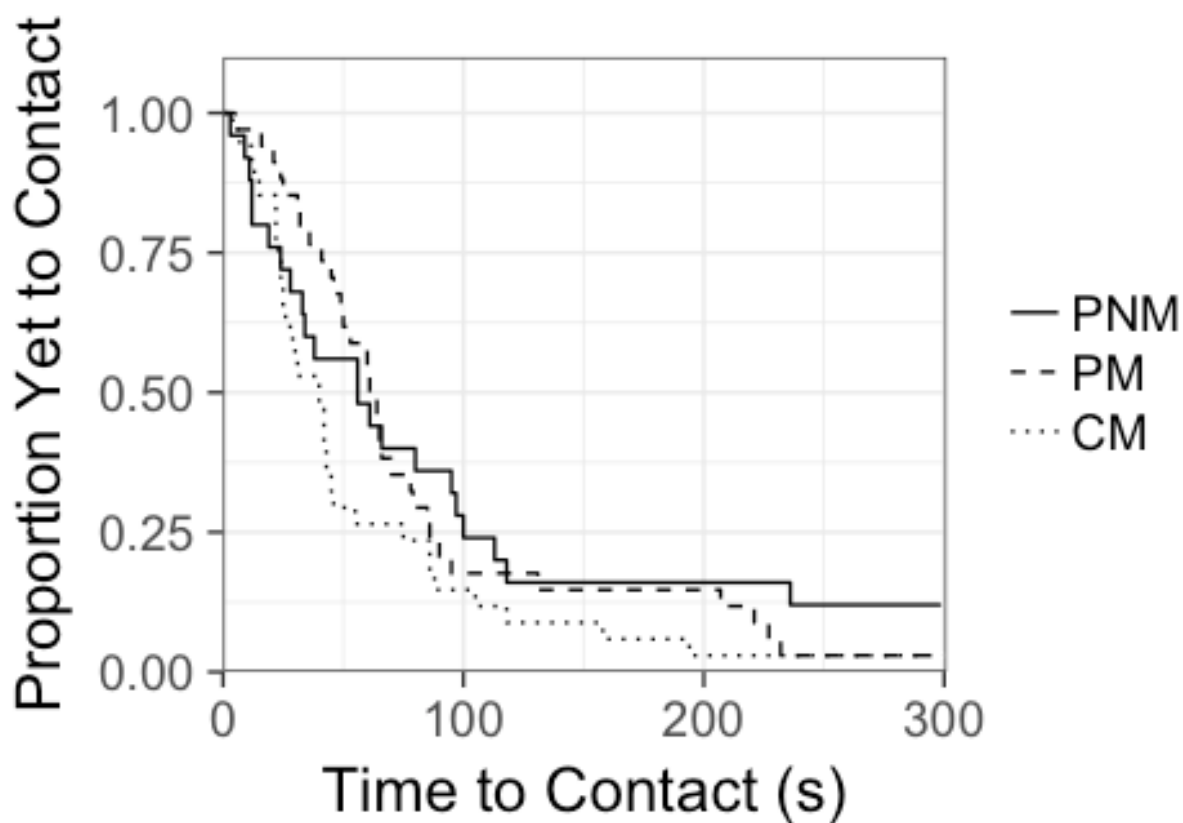


Fig 14. Female exposure: time to contact. The proportion of females that were exposed to pesticide and allowed to burrow through media (PM), exposed to pesticide and not allowed to burrow through media (PNM), or exposed to an acetone control and allowed to burrow through media (CM) that had not yet been contacted by a male at different times. Survival curves were compared for PM-PNM and CM-PM.

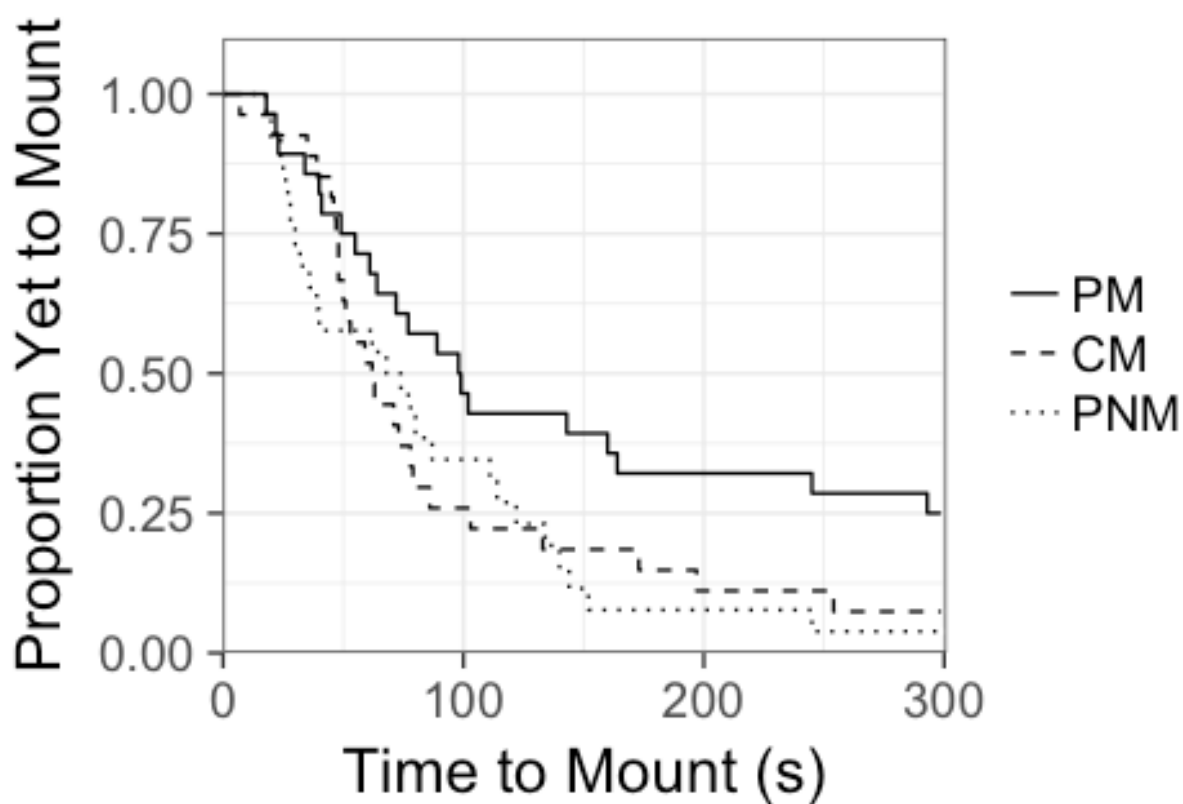


Fig 15. Female exposure: time to mount. The proportion of females that were exposed to pesticide and allowed to burrow through media (PM), exposed to pesticide and not allowed to burrow through media (PNM), or exposed to an acetone control and allowed to burrow through media (CM) that had not yet been mounted by a male at different times. Survival curves were compared for PM-PNM and CM-PM.

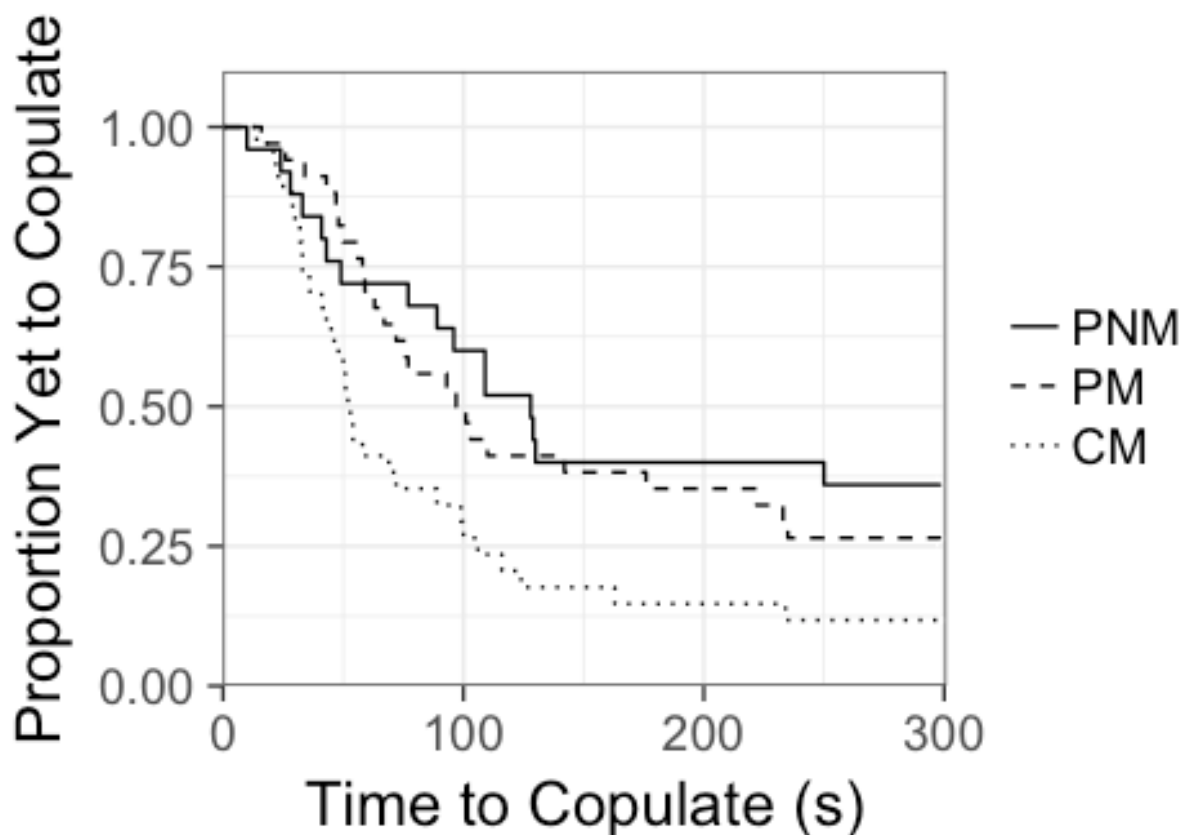


Fig 16. Female exposure: time to copulate. The proportion of females that had not yet copulated at different times, among females that were either exposed to pesticide and allowed to burrow through media (PM), exposed to pesticide and not allowed to burrow through media (PNM), or exposed to an acetone control and allowed to burrow through media (CM) that had not yet been mounted by a male at different times.. Survival curves were compared for PM-PNM and CM-PM.

Discussion

Media

Media does not mitigate pesticide effects on females or males.

Whether or not females burrowed through untreated media after exposure did not affect the time it took them to contact, mount and copulate with a male. Results of the male media exposure experiment were similar to those of the female media exposure experiment. Whether or

not males burrowed through untreated media after exposure did not affect the time it took them to contact, mount and copulate with a female.

These results contrast with results of the previously described study of parasitization rates in the Florida strain of *S. endius*, where hosts being in media eliminated the effect of imidacloprid on parasitization (Burgess et al. 2016). In the present study, if the media removed any imidacloprid, then the amount removed was not enough to mitigate the negative effects of imidacloprid on subsequent mating. The mass per area of imidacloprid that wasps were exposed to in that study and the present study were the same, but as noted previously, the lethality of that exposure is less for the present Illinois strain than for the Florida strain. Thus, explanations for the effect of imidacloprid being mitigated in the parasitization study (Burgess et al. 2016) but not in my mating study include: the Illinois strain being less sensitive to imidacloprid and thus less affected by some removal of it by media, removal by media being sufficient to mitigate imidacloprid's effects on parasitization but not on mating, and burrowing through a larger quantity of media (Burgess et al. 2016) removing more imidacloprid than walking on and maybe through a smaller quantity (the present study).

Some sublethal effects last at least 24 h after exposure ends.

The mate choice experiments (Chapter 2) showed that some aspects of mating were negatively affected when either the female or the male *S. endius* had been exposed to imidacloprid in the previous 24 h. The media experiments show that pesticide effects can be even more persistent than that; some aspects of mating were still negatively affected when both pesticide and control parasitoids had an intervening 24 h period with media after exposure to

pesticide or no pesticide and before introduction to the opposite sex. Specifically, for pesticide females, duration to copulation was affected. For pesticide males, duration to mount and from mount to copulation was affected. These results suggest that the parasitoids are not completely recovering from a low dose of pesticide after 24 h without exposure. Other studies on recovery rates show a range of recuperation periods. Recovery in foraging ability can take 2 -18 d after exposure to various insecticides, although not including imidacloprid, in the parasitoid *Microplitis croceipes* Cresson (Hymenoptera: Braconidae; Stapel et al. 2000). In the bumble bee *Bombus terrestris* Linnaeus (Hymenoptera: Apidae), elimination of bodily pesticide residues takes approximately 48 h after ingestion of sublethal doses of imidacloprid (Cresswell et al. 2014).

The difference in mating responses by pesticide parasitoids relative to control parasitoids in the media experiments was greater than in the mate choice experiments (Chapter 2). Specifically, In the female mate choice experiment, males that had been exposed to imidacloprid showed little to no response to the female, whereas in the male media experiment, approximately 75% of pesticide males copulated within trials of the same duration. In the male mate choice experiment, almost no pesticide females exhibited receptivity, whereas in the female media experiment, approximately 60% of pesticide females copulated within 5 min. This difference between media and choice experiments may be explained by the media experiments' longer duration since pesticide exposure providing time for recovery or by the experimental design being no choice in the media experiments versus choice in the mate choice experiments. Preference for a high-quality mate is generally lower in no-choice experiments than in choice experiments (Dougherty & Shuker 2015). At least some insects are able to recover from pesticide exposure rather rapidly. For example, an aphid species that was moved from

imidacloprid-treated leaves to clean leaves after 24 h began eating the clean leaves and recovered from the deleterious exposure effects (Nauen 1995). Ability to recover may well be less when doses are greater.

Economic Concerns

Whether laboratory results are relevant to field conditions is a concern with any laboratory study. Field studies are often more logistically difficult and expensive than laboratory studies. A compromise can be attempting to recreate field-realistic conditions in the laboratory. Occasionally, hosts may pupate in locations where wasps would not need to be exposed to decaying organic matter to reach them, similar to the lack of media in the mate choice experiments, for example, in a poultry house location where extreme numbers of hosts pupated together on the surface of manure (King pers. comm.), To mimic fly pupation in decaying organic matter, e.g., soiled bedding, in the present experiments, pesticide-exposed parasitoids were allowed to burrow through used fly-rearing media. Presence of media did not mitigate deleterious effects of imidacloprid. Time without exposure may mitigate deleterious effects of imidacloprid, but time without exposure was confounded with choice versus no choice. Thus, future research on *S. endius* might directly test for recovery. Unfortunately, parasitoids may stay in areas where they encounter pesticides for extended periods because females preferentially contact imidacloprid (Burgess & King 2016), and parasitoids, especially males, do not move far from their emergence site in their natural environment (King 2006). Future studies should also examine different exposure methods that match field conditions, such as treating media with pesticide and then allowing parasitoids to mate on it or burrow in it for hosts.

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