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## Effects of sublethal permethrin exposure on larval *Culex pipiens* response to conspecific alarm cues

Margret E.H. Welch

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Effects of sublethal permethrin exposure on larval *Culex pipiens*  
response to conspecific alarm cues

A THESIS

Submitted to the University of Southern Maine  
in partial fulfillment of the requirements for the degree of  
Master of Science in Biology

By

Margret E.H. Welch

2021

**THE UNIVERSITY OF SOUTHERN MAINE  
DEPARTMENT OF BIOLOGICAL SCIENCES**

Date: \_\_\_\_\_

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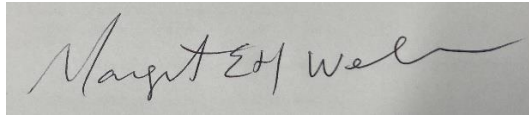
**Effects of sublethal permethrin exposure on larval *Culex pipiens* response to conspecific alarm cues**

Be accepted as partial fulfillment of the requirements for the degree of

**Master of Science in Biology**

**Signatures**

Author:



Date: 31 Aug 2021

Advisory Committee:

Joseph K. Staples  
(Graduate Advisor)



Date: 31 Aug 2021

Christine R. Maher



Date: 28 Aug 2021

Theresa Theodose



Date: 30 Aug 2021

Chair of the Department of Biological Sciences:



Date: 31 Aug 2021

Dean of the College of Science, Technology and Health



Date: 9/13/2021

## Acknowledgments

Over the course of my degree, I've encountered more than my share of supportive individuals. In the academic realm, my thesis advisor, Dr. Staples, tops the list. As an undergraduate, Dr. Staples welcomed me into his lab and encouraged me to actively practice science. After graduation, Dr. Staples agreed to be my primary advisor as I sought a Master's Degree. I equate Dr. Staples to Gandalf because he has challenged me to fight dragons and has assisted me on my journey through Mordor. I don't think there is a higher compliment to be paid.

In addition to Dr. Staples, Dr. Chris Maher and Dr. Terry Theodose form my thesis committee. Both Dr. Maher and Dr. Theodose have demonstrated great patience and support while I completed my course work and thesis. I am a better writer thanks to Dr. Maher's instruction and I have a better understanding of scientific literature thanks to Dr. Theodose. In short, I am a better scientist because of their instruction.

My thesis project would not have been possible without the support of Maine Medical Center Research Institute's Vector-borne Disease Lab. Not only did they provide material support via larvae but they shared expertise and opportunity. Most especially, Chuck Lubelczyk. Chuck taught me how to be a field biologist. His good humor and massive knowledgebase launched me from 'aspiring environmental scientist' into the realm of 'practicing biologist'. Chuck gave me a vocation.

It takes a village to raise a scientist. My village consists of friends and family. I especially need to thank my scout family, who have picked up my slack

this past year so that the kids' experience did not suffer due to my academic ambitions. I am particularly grateful to Sarah Perry (Jeff, Nik, and Cal too) for keeping the thesis conversation going and for giving me the most ingenious progress tracker ever developed. I don't think I'd be this far without that support.

The largest portion of thanks and appreciation goes to Paul. Paul is the solid infrastructure that our family life is built upon. He keeps our family fed, our house clean, and he motivates me to be better. I cannot adequately articulate how vastly grateful I am for Paul. Our two kids, Josie and Kai, need mentioning too. The only reason I attempted college in the first place was to better provide for Josie. She has been my constant field companion since toddlerhood and I adore her help. Kai is an infinite source of love, support, hugs, and kisses...the very stuff motivation is made of.

## Abstract

Organisms living in aquatic environments rely on olfactory, or chemical, information to assess predation risk in environments that are often turbid and difficult to navigate. Utilizing olfactory signals and cues enables aquatic prey to assess predation risk and grade antipredator responses to match the perceived degree of risk, which can improve survivability and fitness. Today, many aquatic habitats can become contaminated with lethal or sublethal concentrations of pollutants and pesticides which, in turn, could influence predator-prey dynamics. The purpose of this study was to examine the behavioral response of larval *Culex pipiens* to a simulated predation event by introducing conspecific alarm cues following exposure to sublethal concentrations of the insecticide permethrin. Larval responses were analyzed for three common antipredator behaviors comprised of distance traveled, mean velocity, and change in mobility. While permethrin exposure resulted in no significant change in behavioral response, significant differences were noted in response to the presence of aqueous extracts from crushed conspecific larvae. Although permethrin exposed larvae did not exhibit statistically significant differences in response to alarm pheromone, a trend showing incrementally smaller intensities in behavioral responses could be seen with increasing concentrations of alarm cue. This research demonstrates that even low concentrations of permethrin exposure can influence larval *Culex pipiens* behavioral response to conspecific alarm cues which could have meaningful implications for larvae existing in predator rich environments contaminated with pesticide.

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## Introduction

**Chemical communication and predator avoidance in aquatic environments.** For prey species living in habitats with high predator diversity, the ability to accurately match potential predation risk with appropriate antipredator behavior can improve survivability and fitness over the course of a prey organism's lifetime (Helfman, 1989). To assess potential risk, prey species may use a combination of visual, auditory, and olfactory (chemical) information (Bronmark & Hansson, 2000). In aquatic systems, where visual and auditory information can be unreliable due to low visibility, turbid conditions or habitat complexity, chemically mediated communication is common (Mortensen & Richardson, 2008; Steiger, Schmitt, & Schaefer, 2011; Xia, Elvidge, & Cooke, 2018). Aquatic prey organisms relying upon chemical communication can gain information regarding the presence of predators and risk of predation (Dahl, Nilsson, & Pettersson, 1998) that would otherwise not be available were prey relying on visual and auditory information alone.

Most major classes of aquatic organisms respond to both heterospecific and conspecific chemical communications (Lima & Dill, 1990; Meuthen, Baldauf, & Thünken, 2012). However, with multiple studies defining similar kinds of communication differently, terminology regarding chemical communications is often ambiguous (Burks & Lodge, 2002; Dicke & Sabelis, 1988; Oldham & Boland, 1996; Wisenden & Millard, 2001). For the purposes of this research, I considered any chemical that relays information (the signal) from one organism

(a sender) to another organism that detects the signal (a receiver) as a semiochemical (Chivers & Smith, 1998; Dicke & Sabelis, 1988; Regnier & Law, 1968; Smith, 1992). Semiochemicals can be further categorized as either pheromones or allelochemicals.

Pheromones are a subset of semiochemicals that enable intraspecific communication (Brown & Eisner, Thomas, Robert, Whittaker H., 1970; Dicke & Grostal, 2001; Dicke & Sabelis, 1988; Oldham & Boland, 1996). Pheromones can be categorized as either releaser pheromones or primer pheromones based on the type of behavior induced by receipt of the pheromone (Regnier & Law, 1968). Releaser pheromones produce a behavioral response in the receiver immediately upon receipt of the signal and are classified into one of three subtypes: sexual attraction pheromones, alarm pheromones, and recruitment pheromones (Regnier & Law, 1968). In contrast to the immediate behavioral response associated with releaser pheromones, primer pheromones induce physiological changes in the receiver, which eventually produce a behavioral response (Regnier & Law, 1968).

Allelochemicals are a broad category of semiochemicals that facilitate interspecific chemical communication (Oldham & Boland, 1996). Allelochemicals are classified by their beneficial or detrimental effects on heterospecific senders and receivers (Dicke & Grostal, 2001; Dicke & Sabelis, 1988; Oldham & Boland, 1996). Allelochemicals are categorized into three subgroups (Nordlund & Lewis, 1976) referred to as allomones, kairomones, and synomones. An allomone results in an adaptively favorable response for the sender but not the receiver

(Nordlund & Lewis, 1976), whereas a kairomone results in an adaptively favorable response for the receiver but not the sender (Nordlund & Lewis, 1976). A synomone results in an adaptively favorable response for both the receiver and sender (Nordlund & Lewis, 1976).

Chemical signaling, including the use of alarm pheromones by prey in the presence of perceived danger (Meuthen *et al.*, 2012) and kairomones emitted by predators and received by prey (Brown & Eisner, Thomas, Robert, Whittaker H., 1970; Ferrari, Wisenden, & Chivers, 2010; Smith, 1992), has been extensively demonstrated in aquatic invertebrates (Ferrari, Messier, & Chivers, 2008; Gall & Brodie Jr., 2009; Laforsch, Beccara, & Tollrian, 2006). Similarly, aquatic prey have demonstrated consistent behavioral responses to chemical cues released by injured conspecifics (Ferrari *et al.*, 2010). However, the terms used to describe both alarm signaling and alarm cues are often misused or used interchangeably in relevant literature (Wisenden, 2019). Additionally, our understanding of the differences between signaling and cues is rapidly expanding (Bairos-Novak, Ferrari, & Chivers, 2019; Wisenden, 2019). As scientific understanding of the distinctions between signals and cues deepens, the definitions of these terms are shifting to more accurately reflect current understanding. For the purposes of this research, the term 'signal' will be used to indicate a voluntary chemical release that benefits the sender and receiver and the term 'cue' will be used to indicate an involuntary chemical release which benefits the receiver but not the sender (Wisenden 2019; Figure 1). Chemicals released from epidermal and midgut tissues of prey during predation are

considered involuntarily released by the sender and are considered alarm cues (Bairos-Novak *et al.*, 2019; Wisenden, 2019). Exposure to alarm cues elicits predictable behavioral responses in receiving conspecifics and can help create associations between predator odor and risk (Ferrari *et al.*, 2010; Smith, 1992; Wisenden, 2000).

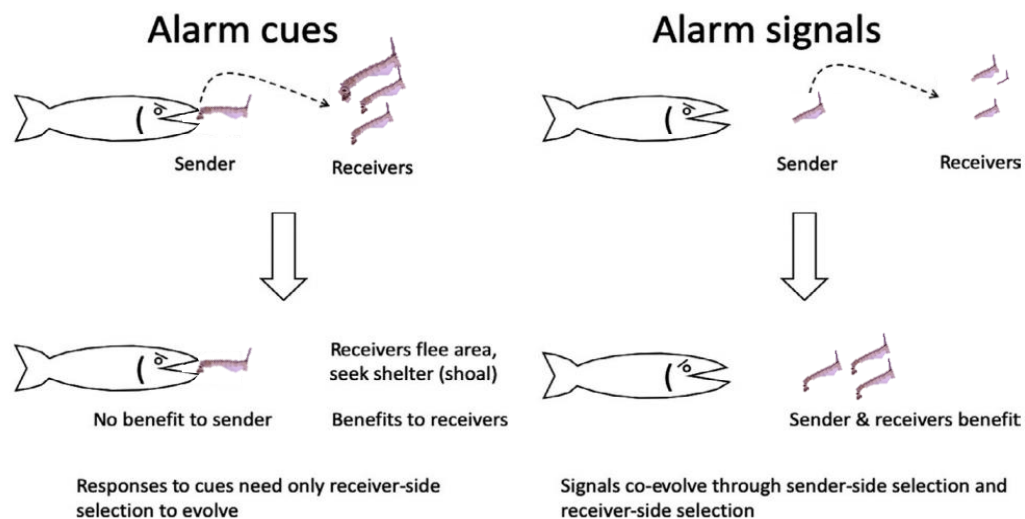


Figure 1. Definitions of alarm cue and alarm signal. Alarm cues are involuntarily released by senders upon injury and only benefit receivers. Prey that receive the alarm signal can respond with antipredator behaviors that can decrease probability of predation. Alarm signals are voluntarily released by senders once predation risk is perceived and prior to an act of predation. Alarm signals benefit both the sender and receiver by enabling both to employ antipredator behaviors before predation begins (adapted from Wisenden, 2019).

In aquatic environments, prey consistently demonstrate specific behaviors in response to alarm cues (Andrade, Albeny-Simoes, Breaux, Juliano, & Lima, 2017; Ferrari, Messier, & Chivers, 2007; Kesavaraju, Damal, & Juliano, 2007; Wisenden, 2003). Behaviors such as decreased movements, slower movement through space, fleeing, and area avoidance (Clark, 1994; Kavaliers & Choleris, 2001; Rodríguez-Prieto, Fernández-Juricic, & Martín, 2006) are considered

antipredator behaviors because they enable prey to avoid predators (Ives & Dobson, 1987; Sih *et al.*, 2010). Thus, successful interpretation of alarm cues enables prey to employ antipredator behaviors and avoid predation.

**Predator-Prey Interactions.** In the 1960's, multiple behavioral studies explored optimal animal foraging and its influence on fitness (Emlen, 1966; MacArthur & Pianka, 1966). Research on optimal foraging focused on distilling complex animal behaviors into broad theories using mathematical models. In these models, assumptions are made so the models can be applied to many measures of costs and benefits, which can then be used to describe animal behavior. In 1966, Robert MacArthur and Eric Pianka developed Optimal Foraging Theory (OFT) to predict foraging behavior based on a series of assumptions rooted in evolutionary theory. As noted by Pike (1984), these assumptions include: 1) individual fitness depends on an organism's behavior during foraging; 2) foraging behavior is heritable; 3) there is a known relationship between foraging behavior and fitness; 4) foraging behavior evolves despite any genetic constraints that may slow the rate of evolution; 5) foraging behavior is limited by functional (or behavioral) constraints and those constraints are known; and 6) foraging behavior evolves more quickly than changes in environmental conditions (Pyke, 1984). In short, OFT predicts that animals evolved to maximize net energy intake per unit of time spent foraging.

Between 1973 and 1981, OFT quickly gained support as a basis for assessing resource allocation in animals (Pyke, 1984). Several reviews tended to accept the assumptions and claims of OFT; however, some found OFT too

dependent on ideal conditions (Pierce & Ollason, 1987). Critics countered that while natural selection tends to maximize fitness, the assumption that any species lives in its optimized state is problematic, because a dynamic environment would likely result in continuously varying optimal states. Thus, species are continuously adapting towards, but never actually reaching, a new optimal state with each environmental shift (Cody, 1974).

Another criticism of OFT centers around its focus on the behavior of the forager (or predator) functioning in an optimum environment, but it does not account for the effect that predation may have on prey response (Pierce & Ollason, 1987). Therefore, in failing to account for potential effects of predators on prey behavior, critiques of OFT concluded that OFT likely misses a fundamental predatory-prey dynamic that could result in deviation between predicted and actual observations in nature (Brown, Laundre, & Gurung, 1999). To address the effects of predation on prey, Charnov (1976) proposed the Marginal Value Theorem (MVT), which describes predator behavior in an environment with patchy resource availability and proposes that when the rate of energy intake in a particular location drops below the mean energy intake for the entire habitat, a predator seeks alternative sources of energy. In other words, when prey are abundant, a predator selectively consumes optimal prey; however, as optimal prey become scarce, the predator begins to select less optimal prey (Charnov, 1976; Robinson & Wilson, 1998) .

Together, OFT and MVT describe adaptive predator resource acquisition in nature. However, neither model fully accounts for the dynamic behavior of prey



in the presence of a predator, and thus both models treat prey as inert participants in the foraging system instead of active participants seeking to avoid predation (Brown *et al.*, 1999). Predators often respond to prey availability by changing location or by seeking other prey species (Persson, 1985). It can therefore be inferred that there is a corresponding adaptive change in prey behavior in the presence of a predator. However, prey species still need to access resources for survival and reproduction. By leaving a resource rich area upon arrival of a predator, prey may reduce their own ability to access food or mates. Therefore, prey may adapt to the threat of predation proportionally, enabling them to gauge the level of threat and thereby minimize energy spent fleeing if such action is not necessary. Helfman (1989) demonstrated this type of threat-sensitive behavior in damselfish (*Stegastes planifrons*) exposed to predatory trumpet fish (*Aulostomus maculatus*). Damselfish reacted to increased amounts of perceived threat by changing avoidance behavior proportionally with increased perception of risk. From this work, Helfman proposed the Threat Sensitivity Hypothesis (TSH).

**Threat Sensitivity Hypothesis.** Prior to publication of the TSH, research primarily focused on qualitative prey response to predator presence or absence, or to the collective threat by groups of prey (Helfman, 1989). However, little research had been carried out on the response of individual prey to different degrees of predation risk. Helfman noted that natural selection should favor prey individuals that can best gauge their antipredator response to perceived predation risk against a perceived threat (Figure 2). Greater threats should

therefore elicit greater antipredator response from prey. Because behavioral traits are a function of natural selection and some traits evolve at the expense of others, Helfman further hypothesized that animals facing conflicting demands on their time and energy balance predator avoidance against other activities that impact fitness in a graded manner (Helfman, 1989). TSH thus was based on three assumptions: 1) conflicting demands on time and energy are present, 2) a tradeoff exists between predator avoidance and other activities that influence fitness, and 3) individuals exchange predator avoidance behavior for other behaviors in a graded way based on perceived risk and benefits (Helfman, 1989).

To test TSH, Helfman (1989) presented damselfish with visual models of trumpetfish and simulated trumpetfish threats of varying degrees by altering the body position of a trumpetfish. Increases in threatening behavior from trumpetfish resulted in stronger antipredator responses from damselfish. Thus, damselfish responded to perceived threats with precise, threat sensitive, antipredator behavior by adjusting antipredator behavior to the magnitude of perceived risk (Helfman, 1989). Subsequent research confirmed threat-sensitive behavior in multiple species in both terrestrial (Monclús, Palomares, Tablado, Martínez-Fonturbel, & Palme, 2009; Papworth, Milner-Gulland, & Slocombe, 2013; Walzer & Schausberger, 2011) and aquatic habitats (Chivers, Mirza, Bryer, & Kiesecker, 2001; Engstrom-Ost & Lehtiniemi, 2004; Foam, Harvey, Mirza, & Brown, 2005; Kesavaraju *et al.*, 2007; Monclús *et al.*, 2009; Papworth *et al.*, 2013; Walzer & Schausberger, 2011).

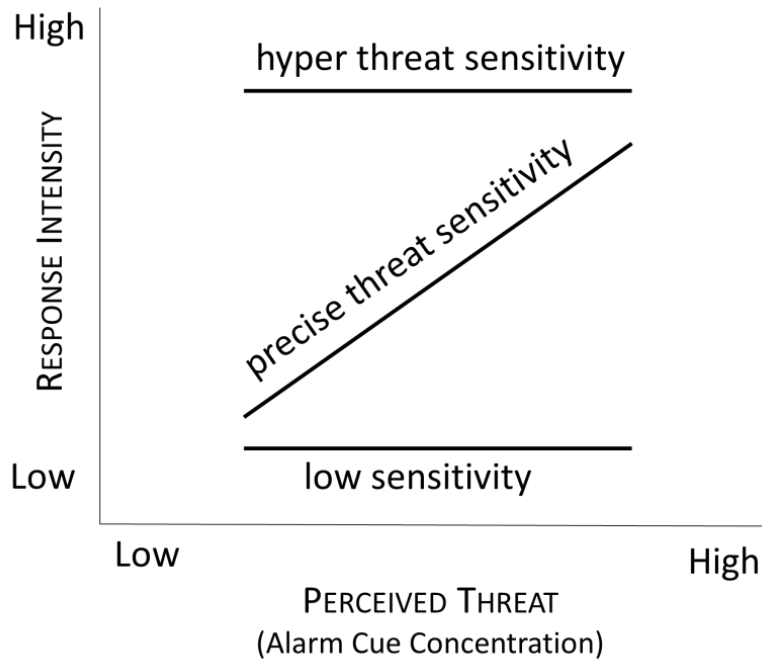


Figure 2. Precise antipredator behavior in response to predation risk. The x-axis represents increasing threat. The y-axis represents intensity of antipredator behavioral response. Threat Sensitivity Hypothesis predicts prey perception of increasing risks will result in increased amounts of antipredator behavior. An antipredator response that is hypersensitive could result in missed foraging and mating opportunities. An antipredator response that is not sensitive enough could result in being predated (Adapted from Helfman 1989).

Whereas threat sensitive behavior has been observed in many different species, the cues that induce precise antipredator behavior vary among species. For example, threat sensitive responses to visual cues have been observed in larval pike (*Esox lucius*) (Engstrom-Ost & Lehtiniemi, 2004). A laboratory experiment exposed larval pike to visual contact with both large and small predatory perch (*Perca fluviatilis*). When larval pike were exposed to large perch, they tended to flee. In contrast, larval pike exposed to small perch tended to freeze or continued to forage. These different behavioral responses suggest flexible antipredator responses based on perceived threat. While there was a

significant difference in behavioral response between control and large perch treatments, the difference between small and large perch treatments was less pronounced, which suggests that antipredator behavior in larval pike has a threshold beyond which increased risk does not result in increased antipredator behavior (Engstrom-Ost & Lehtiniemi, 2004).

Research by Chivers *et al.* (2001) demonstrated threat sensitive behavior in slimy sculpin (*Cottus cognatus*) when exposed to sympatric brook trout (*Salvelinus fontinalis*), a known predator, through both field and laboratory experiments. When presented with a visual model of brook trout, slimy sculpin responded with precise antipredator behavior by avoiding areas containing brook trout large enough to consume them. Slimy sculpin did not avoid areas containing brook trout that were too small to eat them. Interestingly, slimy sculpin responded to chemical cues from brook trout, without visual cues, with antipredator behavior; however, slimy sculpin antipredator response to trout chemical cues was not precise (Chivers *et al.*, 2001). This lack of precise response could suggest that employing antipredator behavior when a brook trout is nearby, regardless of the trout's size, is less costly than not responding to brook trout chemical cues (Chivers *et al.*, 2001).

Whereas multiple species of fish respond to visual predator cues, this modality can be unreliable in turbid and non-linear habitats such as stream and pond beds where many aquatic species thrive (Dahl *et al.*, 1998). When visual cues are unreliable, chemical cues can provide valuable information about predator presence and risk of predation to prey species (Foam *et al.*, 2005). To

determine if juvenile convict cichlids (*Archocentrus nigrofasciatus*) could use ambient chemical cues to form precise responses to perceived risk, Foam *et al.* (2005) performed two experiments exposing convict cichlids to both conspecific alarm cues and predator cues from swordtails (*Xiphophorus hellerii*). In the first experiment, convict cichlids were exposed to conspecific alarm cues and then allowed to forage. In the second experiment, juvenile convict cichlids were exposed to one of four treatments: cues from a conspecific fed a vegetable diet, distilled water, cues from a conspecific fed another conspecific, or cues from a conspecific that had been fed swordtail. Neither experiment produced overt antipredator behavior; however, convict cichlids did adjust feeding posture and foraging patterns when presented with odors of predators fed a prey, which suggests that convict cichlids can use ambient chemical cues to inform antipredator behavior (Foam *et al.*, 2005).

**Aquatic insects and TSH.** Prior to publication of the TSH, research conducted by Sih (1986) documented threat sensitive behavior in two mosquito species: the container dwelling *Aedes aegypti* and the shallow water dwelling *Culex pipiens*. Both mosquito species were exposed to kairomones of the freshwater predator *Notonecta undulata*, and both mosquito species demonstrated antipredator behavior; however, *Cx. pipiens*, which co-occurs with *N. undulata*, spent significantly less time moving and preferred areas at the edge of experimental containers, as opposed to areas in the center of the experimental containers compared to control larvae and to *Ae. Aegypti* larvae (Sih, 1986). Sih (1986) concluded that *Cx. pipiens*' ability to use precise antipredator behavior

after exposure to both *N. undulata* kairomones and alarm cues from ground conspecifics resulted in a lower predation rate. *Ae. aegypti*, which does not co-occur with *N. undulata*, did not demonstrate precise antipredator behavior when exposed to either *N. undulata* kairomones or alarm cues from pierced conspecifics. Sih (1986) concluded that this lack of response resulted in a higher predation rate in the presence of *N. undulata*. Thus, varying degrees of antipredator response in *Ae. aegypti* and *Cx. pipiens* could be due to different methods that each species uses to gauge predation risk and to previous exposure to the predator (Sih, 1986).

As another example, larval eastern tree hole mosquitoes, *Ochlerotatus triseriatus*, exhibit threat sensitive antipredator behavior (Kesavaraju *et al.*, 2007). Researchers catalogued larval behavior after exposure to increasing concentrations of conspecific alarm cues and then to increasing concentrations of cues from the predatory elephant mosquito (*Toxorhynchites rutilus*). *Ochlerotatus triseriatus* larvae reduced filtering activity and increased resting activity as alarm cue concentrations increased, indicating that *O. triseriatus* responds to perceived threat with precise antipredator behavior.

Whereas multiple species show threat sensitive behavior in response to chemical signals and cues, most research to date has focused on identifying the specific chemicals that trigger antipredator behavior or on identifying behavioral responses of prey to predators (Chivers & Smith, 1998; Ferrari *et al.*, 2010; Kats & Dill, 1998). However, the ways in which abiotic environmental influences, such as sublethal levels of pesticide-contaminated runoff (Shumway, 1999), impact

threat sensitive behavior in response to predation risk have been less studied (Kesavaraju *et al.*, 2007; Schwarzenbach *et al.*, 2006; Shumway, 1999).

***Culex pipiens***. *Cx. pipiens* is an important zoonotic bridge vector of West Nile Virus (Fonseca *et al.*, 2004; Hamer *et al.*, 2008; Paul, Harrington, Li Zhang, & Scott, 2005). It is common in temperate climates (Hamer *et al.*, 2008) and thrives in a variety of artificial and natural containers often associated with human activity (Chevillon, Eritja, Pasteur, & Raymond, 1995; Gardner *et al.*, 2013; Loetti, Schweigmann, & Burrioni, 2011). Due to *Cx. pipiens*' close proximity to humans, *Cx. pipiens* larval habitat is often directly and indirectly modified through introduction of pesticides, fertilizers, and xenobiotics (Day, 1989; Gardner *et al.*, 2013; Muturi, Costanzo, Kesavaraju, Lampman, & Alto, 2010; Tilman *et al.*, 2001). Modification of larval mosquito habitat alters larval mosquito development and influences inter- and intra-specific competition among larvae (Gardner *et al.*, 2013; Muturi *et al.*, 2010). Although pesticide exposure via runoff is unlikely to provide a lethal dose to aquatic taxa, sublethal pesticide exposure can induce changes in physiology, metabolism, and behavior (Desneux, Decourtye, & Delpuech, 2007; Fernandes *et al.*, 2016). Additionally, larval *Cx. pipiens*' ability to respond to predator kairomones with threat sensitive antipredator responses has been well documented (Kesavaraju, Khan, & Gaugler, 2011; Sih, 1986).

**Effects of sublethal pesticide exposure on aquatic taxa.** Whereas multiple toxins can contaminate larval mosquito habitat, pesticides, in particular, are highly biologically active and heavily used both globally and in the United States (Stehle, Bub, & Schulz, 2018). Four classes of insecticides:

organophosphates, organochlorines, carbamates, and pyrethroids are commonly used to reduce pest populations (Ramkumar & Shivakumar, 2015) and have been identified in surface waters (Stehle *et al.*, 2018; Stehle *et al.*, 2019).

Pyrethroids, in particular, are heavily relied upon due to their ability to rapidly kill target pests yet have low mammalian toxicity (Nkya, Akhouayri, Kisinza, & David, 2013; Ramkumar & Shivakumar, 2015). For this study, permethrin was used as a representative pyrethroid because it is commonly found in surface waters in sublethal concentrations (Stehle *et al.*, 2018; Stehle *et al.*, 2019). Sublethal effects of pyrethroid, including permethrin, exposure on beneficial arthropods include uncoordinated movements, trembling, tumbling, abdomen tucking, and disruption in kairomone detection (Desneux *et al.*, 2007).

The combined effects of predation and pesticide exposure could be important if the presence of a predator influences the way that aquatic invertebrates metabolize pesticides or if a pesticide alters antipredator behavior of an aquatic invertebrate (Pestana, Loureiro, Baird, & Soares, 2009). The purpose of this study was to examine responses of *Cx. pipiens* larvae to conspecific alarm cues after exposure to sublethal levels of permethrin. I predicted that larval *Cx. pipiens* respond to conspecific alarm cues with less precise antipredator behavior, compared to controls, after sublethal permethrin exposure.



## METHODS

**Experimental Design.** To determine if sublethal permethrin exposure elicits a proportional change in response to increasing concentrations of conspecific alarm cues in larval *Cx. pipiens*, I exposed individual 3rd instar larvae to 1 of 4 concentrations of conspecific alarm cues following 24 h of exposure to a sublethal dose of 0.01 ppm permethrin (3-phenoxyphenyl) methyl 3-(2,2-dichloroethenyl)-2,2-dimethylcyclopropane-1-carboxylate) or an equivalent control dose of technical grade acetone. I used video bioassays to characterize antipredator behavioral response in larval *Cx. pipiens*. Each individual larva was treated as a single experimental replicate. Ten replicates were used per treatment resulting in a total of 80 observations for the behavioral assay (10 larvae x 4 alarm cue concentrations x 1 pesticide treatment = 40 larvae) + (10 larvae x 4 alarm cue concentrations x 1 acetone control treatment = 40 larvae; Table 1). An additional 840 larvae were used for alarm cue treatments ([8 larvae x 10 replicates x 2 control/treatment] + [4 larvae x 10 replicates x 2 control/treatment] + [2 larvae x 10 replicates x 2 control/treatment]).

Table 1. Experimental Design. Individual larvae were exposed to alarm cue treatments after exposure to either a sublethal dose of permethrin or acetone (control). Movement data were quantified prior to and after exposure to an alarm cue treatment. The difference in movement after and before treatments was analyzed for main effects and interaction effects via factorial ANOVA.

Treatment		Response
Pesticide (ppm)	Alarm Cue (quantity of crushed conspecific larvae)	Change in quantitative measure of behavior (after alarm cue exposure – before alarm cue exposure)
Permethrin + Acetone	0	
	2	
	4	
	8	
Acetone (Control)	0	
	2	
	4	
	8	

This experiment was conducted using laboratory reared *Cx. pipiens* (Buckeye strain from Ohio State insectary) housed in the insectary located in Bailey Hall on the Gorham campus of the University of Southern Maine. *Culex pipiens* larvae were reared in an incubator (Percival, model number: DR36VLC8) at 25°C, 75% relative humidity ( $\pm$  10%) with a 15:9 (L:D) photoperiod. Larvae were maintained in Sterilite storage boxes (35.5 cm  $\times$  28 cm  $\times$  8 cm; model number 19638606) containing 250 larvae in 1000 mL of deionized water. They were fed two finely crushed pond pellets (Wardley, PP2118) once daily.

**Permethrin exposure.** To obtain the target sublethal dose of permethrin, I followed the *Guidelines for laboratory and field testing of mosquitos* published by the World Health Organization (World Health Organization, 2005). I began by making a 10,000 ppm stock solution of technical grade permethrin (Chem Service N-12848; CAS 52645-53-1: 80.9% Trans/ 19.1% Cis) by dissolving permethrin in technical grade acetone (Sigma-Aldrich 650501-4L; CAS 67-64-1). I performed multiple serial dilutions to obtain the final concentration of 0.01 ppm permethrin, a concentration demonstrated to fall below LD50 for larval *Culex* spp. mosquitoes (Li & Liu, 2010) and observed in surface waters across the globe including within the United States (Stehle *et al.*, 2019).

Prior to the behavioral assay, larvae were exposed for 24 h to a dose of either 0.01 ppm permethrin diluted in technical grade acetone (treatment) or a control dose of consisting of an equivalent volume of technical grade acetone only. During treatment or control exposure, larvae were maintained in groups of 20 individuals housed in 100 mL of deionized water. After 24 h in either the

treatment or control condition, larvae were rinsed in deionized water and placed in 100 mL of deionized water until they were transferred individually to the recording chamber for analysis.

**Alarm cue collection.** I obtained alarm cues by crushing 0, 2, 4, and 8 (Ferrari et al., 2008) 3rd instar larvae with a plastic, disposable pipette (LabStock, manufacture number: PIP7) in a 3 fl. oz. disposable plastic cup (Great Value, 443468) containing 1 mL of deionized water. Crushed larval bodies were removed from the liquid by slowly pouring the liquid into a 2 mL microcentrifuge tube (USA Scientific, catalog number: 1620-2700), leaving the crushed bodies in the cup. Immediately after preparing the alarm cues, I introduced water containing those cues to larvae via disposable pipette during the behavioral assay.

**Behavioral assay.** To determine if exposure to a sublethal dose of permethrin affects the behavioral response of larval *Cx. pipiens* to conspecific alarm cues, I recorded larval behavior before and after introduction of alarm cue treatments. A petri dish (Greiner bio-one 628161, 60 mm diameter, 15 mm height) containing 19 mL of deionized water and one 3rd instar larvae was placed on a light source (LeeTurn, 50mm diameter, ML010, 3.6W, 90V-265V, white light 6400K) inside a dark box (Figure 3). A 3 oz plastic cup with the bottom cut off was placed in the Petri dish to prevent glare from the edge of the Petri dish from interfering with downstream video analysis.

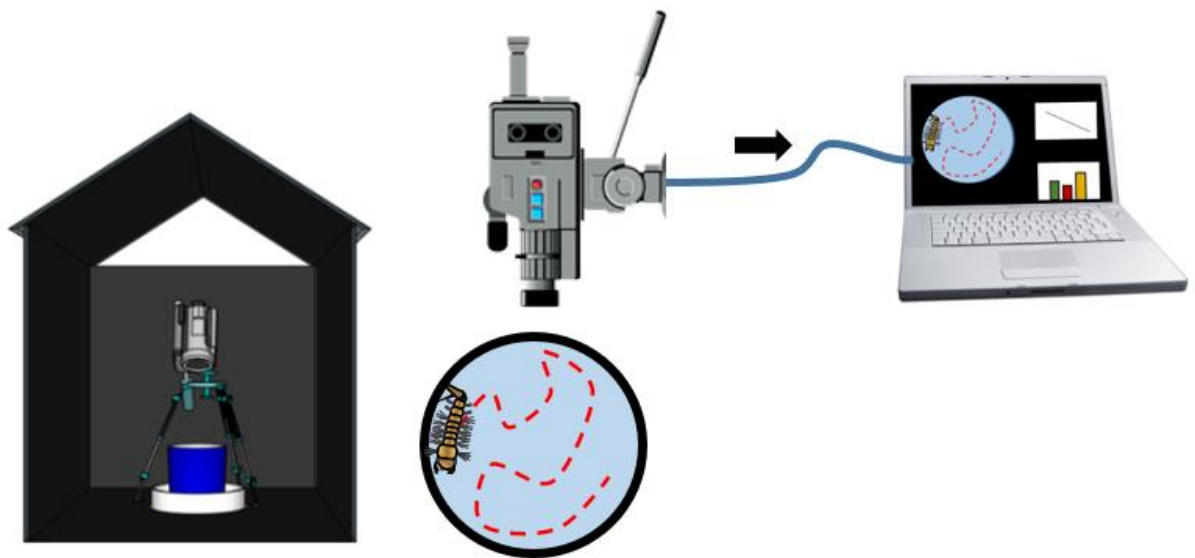


Figure 3. Dark box set-up. Individual larvae were placed in a petri dish on the light pad inside the dark box. The lid to the dark box was placed over the camera. Openings on either end of the dark box allowed access to the camera and an access point for the light pad and camera wires.

A digital video camera (Panasonic HDC-TM700, 30 fps) was mounted on a tripod (Targus TG-5060TR) and positioned so the lens was located 30 cm above the petri dish. After a 5-min acclimation period, larval behavior was recorded for 5 min. After 5 min, and with the camera still running, 1 mL of alarm cue treatment was placed in the middle of the petri dish using a disposable plastic pipette, resulting in a 1:20 ratio of aqueous alarm cue solution to deionized water. The camera continued to record larval behavior for 5 min after the pipette was no longer visible on the screen.

**Video analysis.** To quantify larval behavior during treatment, video footage was converted to a file format compatible with image analysis software and then analyzed by measuring the distance (mm) and velocity (mm/sec) of larval travel between frames or the amount of time (sec) larvae spent immobile between frames (Gulyás, Bencsik, Pusztai, Liliom, & Schlett, 2016). Briefly, FFMPeg software (FFMPeg Developers, 2019) was used with a PC computer to convert videos from their native format (AVCHD) to AVI format, which is compatible with the Fiji video analysis software (Schindelin *et al.*, 2012). Randomized numbers were then assigned to each 5 min video segment for 'before alarm cue' and 'after alarm cue' observations using FFMPeg (FFMPeg Developers, 2019) thus providing blind study for subsequent statistical analysis. Additionally, to accommodate Fiji's file size limitations, each randomized 5 min video was segmented into 1 min clips for analysis.

Table 2. Description of quantified behaviors. Three measures of movement were used to quantify the difference in larval response to alarm cue treatments before and after alarm cue introduction.  $\Delta$  = change.

Metric	Description
Difference in Distance Traveled ( $\Delta$ distance)	total distance traveled (mm) after alarm introduction – total distance traveled (mm) before alarm cue introduction
Difference in Mean Travel Velocity ( $\Delta$ velocity)	mean travel velocity (mm/sec) after alarm introduction – mean travel velocity (mm/sec) before alarm cue introduction
Difference in Total Time Immobile ( $\Delta$ mobility)	total time immobile (sec) after alarm introduction – total time immobile before alarm cue introduction

Animal Tracker plug-in (Gulyás *et al.*, 2016) within the Fiji/ImageJ video analysis software (Schindelin *et al.*, 2012) was used to analyze each 1 min video segment frame by frame so that total distance (mm), velocity (mm/sec) and total time immobile (sec) were quantified for each video. For each metric analyzed, Animal Tracker provided a data file containing distance traveled, velocity, or time spent immobile for each frame in the video (Figure 3). Data files from each 1 min segment were combined and total time immobile (sec), mean velocity (mm/sec), and total distance traveled (mm) were quantified for each 5 min video.

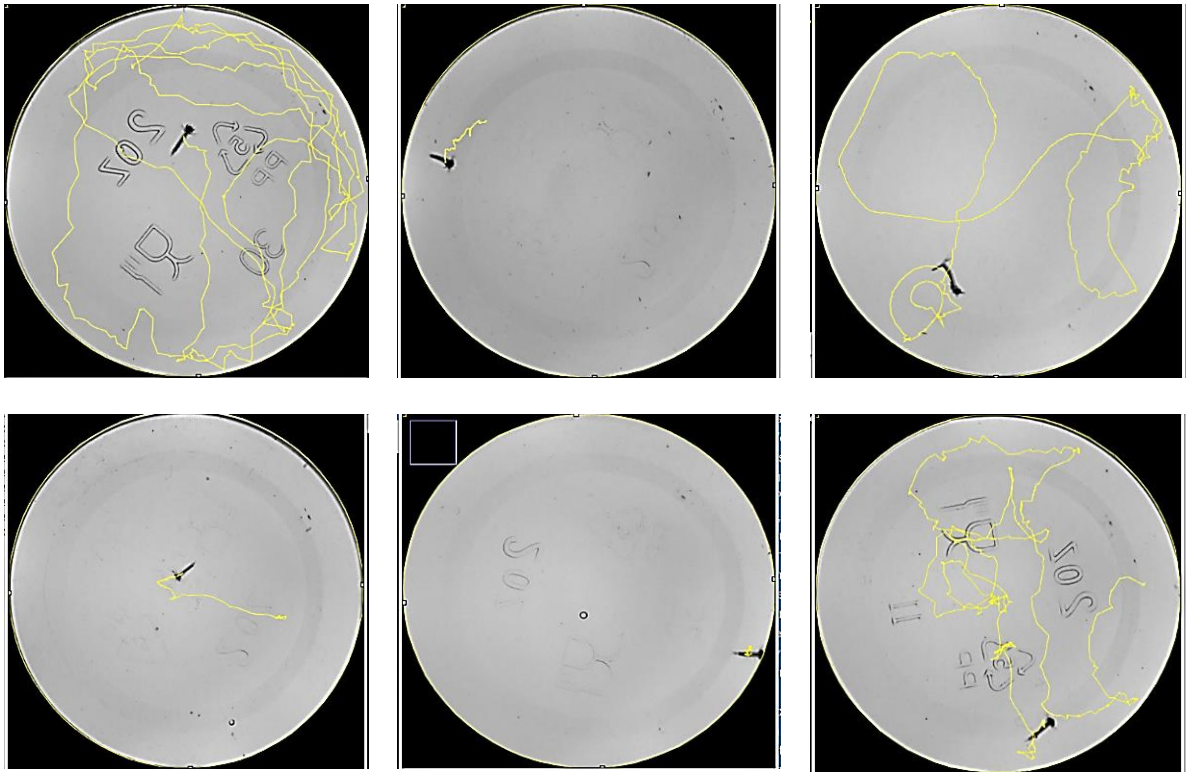


Figure 4. Examples of larval movement tracks. Each video analysis resulted in a data file depicting the path the larva traveled and frame by frame data indicating: distance traveled (mm) between frames, travel velocity (mm/sec) between frames, or duration of mobility (sec). These data were used to quantify the difference in behavior before and after alarm cue exposure.

**Statistical analysis.** To determine if permethrin influences larval *Cx. pipiens*' behavioral response to conspecific alarm cues, data were subjected to a factorial ANOVA using Statistica Software ver.13 (TIBCO Software Inc., 2017). Prior to analysis, data were tested for assumptions of normality via Shapiro-Wilkes test to determine normality of distribution and by Levene's Test for homogeneity of variance. Grubbs test was used to identify outliers (Ahmed *et al.*, 2020) in all response variables; however, overall results of the factorial ANOVA remained unchanged after outlier removal. Because removal of outliers



did not change the results, statistical analysis was performed via factorial ANOVA on data including outliers.

The main effects of permethrin and alarm cue concentration and the interactive effects between permethrin and alarm cue concentration were analyzed via a  $2 \times 4$  factorial ANOVA ( $\alpha = 0.05$ ) consisting of two levels of pesticide treatment (0.01 ppm and 0 ppm)  $\times$  four levels of alarm cue treatment (0 mos. equiv, 2 mos. equiv, 4 mos. equiv, and 8 mos. equiv; Table 1). Effect size is reported as partial eta squared ( $\eta^2_{\text{partial}}$ ) with small, medium, and large effects defined as 0.01, 0.06, and 0.14 respectively (Richardson, 2011). For treatments identified as significant ( $p < 0.05$ ) using factorial ANOVA, post hoc analysis for interactive effects was conducted via Fisher's LSD test (Fisher LSD;  $\alpha = 0.05$ ), and P-values were adjusted for multiple comparisons via the Bonferroni procedure (Cramer *et al.*, 2016). For treatments where no significant interactions effects were observed between treatment levels ( $p > 0.05$ ), a one-way ANOVA was conducted to determine if there were significant differences between treatments.

## Results

**Overview.** Factorial ANOVA identified no significant interactions between permethrin exposure and alarm cue treatment for  $\Delta$  distance traveled ( $F_{(3,72)} = 1.10$ ,  $\eta^2_{\text{partial}} = 0.04$ ,  $p = 0.35$ ),  $\Delta$  time spent immobile ( $F_{(3,72)} = 1.15$ ,  $\eta^2_{\text{partial}} = 0.05$ ,  $p = 0.34$ ), or  $\Delta$  mean travel velocity ( $F_{(3,72)} = 1.90$ ,  $\eta^2_{\text{partial}} = 0.05$ ,  $p = 0.14$ ). Similarly, there was no significant difference in main effects of permethrin

treatment for  $\Delta$  distance traveled ( $F_{(1,72)} = 3.47$ ,  $\eta^2_{\text{partial}} = 0.05$ ,  $p = 0.07$ ),  $\Delta$  time spent immobile ( $F_{(1,72)} = 0.85$ ,  $\eta^2_{\text{partial}} = 0.01$ ,  $p = 0.36$ ), or  $\Delta$  mean velocity traveled ( $F_{(1,72)} = 2.92$ ,  $\eta^2_{\text{partial}} = 0.04$ ,  $p = 0.09$ ). However, significant main effects of alarm cue treatment occurred for  $\Delta$  distance traveled ( $F_{(3,72)} = 4.65$ ,  $\eta^2_{\text{partial}} = 0.16$ ,  $p = 0.005$ ),  $\Delta$  time spent immobile ( $F_{(3,72)} = 5.14$ ,  $\eta^2_{\text{partial}} = 0.18$ ,  $p = 0.002$ ), and  $\Delta$  mean travel velocity ( $F_{(3,72)} = 5.27$ ,  $\eta^2_{\text{partial}} = 0.18$ ,  $p = 0.002$ ). Fisher LSD post hoc tests identified significant differences between groups within the main effects for alarm cue treatments, whereas no significant difference for the main effect of permethrin or interactive effects between permethrin and alarm cue exposure were identified.

**Main effects of permethrin treatment on  $\Delta$  distance travelled.** Larvae exposed to permethrin traveled farther before alarm cue treatment than control larvae however both permethrin exposed and control larvae decreased movement in response to alarm cue introduction. Analysis via factorial ANOVA revealed a non-significant main effect of permethrin on  $\Delta$  distance ( $F_{(1,72)} = 3.47$ ,  $\eta^2_{\text{partial}} = 0.05$ ,  $p = 0.07$ ). One-way ANOVA analysis ( $\alpha = 0.05$ ,  $p > 0.05$ ,  $df = 78$ ) confirmed that there was no significant difference between means of  $\Delta$  distance for permethrin and control treatments (Figure 5).

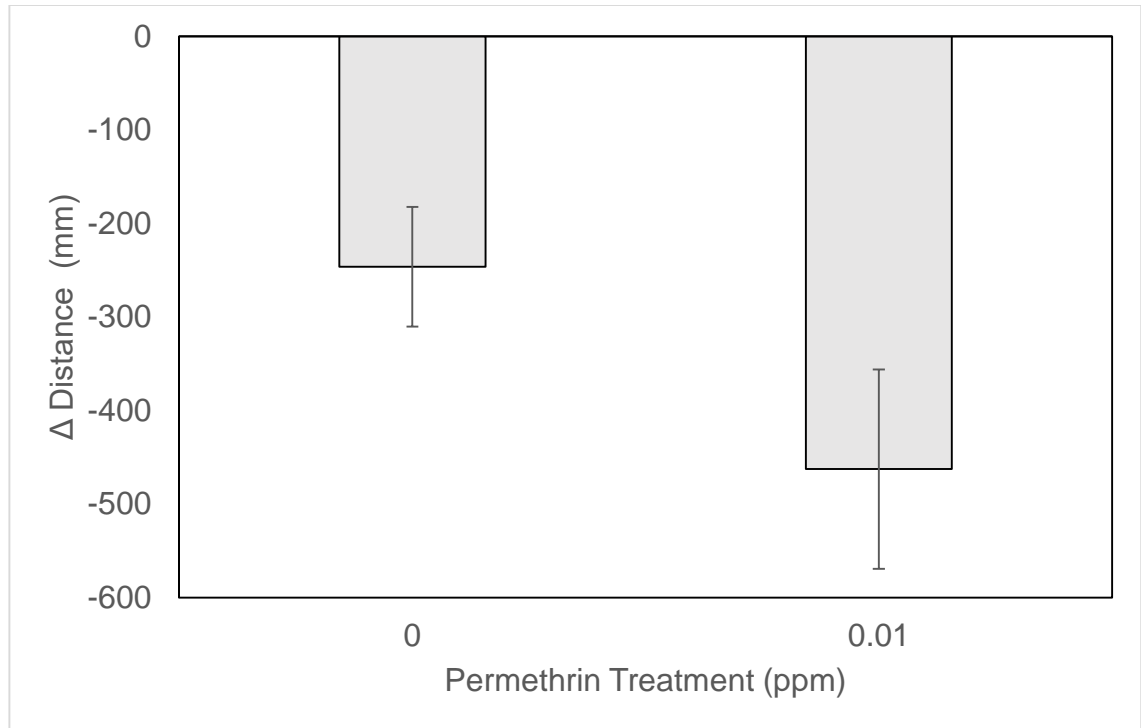


Figure 5. Mean ( $\pm$  SE)  $\Delta$  distance (mm) between permethrin and control treatments. Change in distance traveled between before and after alarm cue treatment was quantified for larvae exposed to an acetone control,  $n = 40$ , and for larvae exposed to 0.01ppm permethrin,  $n = 40$ . Negative numbers indicate larvae traveled farther before alarm cue treatment.

**Main effects of permethrin treatment on  $\Delta$  mobility.** Larvae in both permethrin and acetone treatments responded to alarm cue introduction similarly by decreasing movement. While larvae exposed to permethrin moved less than control larvae, following the introduction of alarm cue, factorial ANOVA revealed no significant effect of permethrin on  $\Delta$  mobility ( $F_{(1,72)} = 0.85$ ,  $\eta^2_{\text{partial}} = 0.01$ ,  $p = 0.36$ ). Similarly, main effects analysis via one-way ANOVA confirmed that there was no significant difference  $\Delta$  mobility between permethrin and control treatments ( $F_{(1,78)} = 0.73$ ,  $\eta^2_{\text{partial}} = 0.009$ ,  $p = 0.39$ ) (Figure 6).

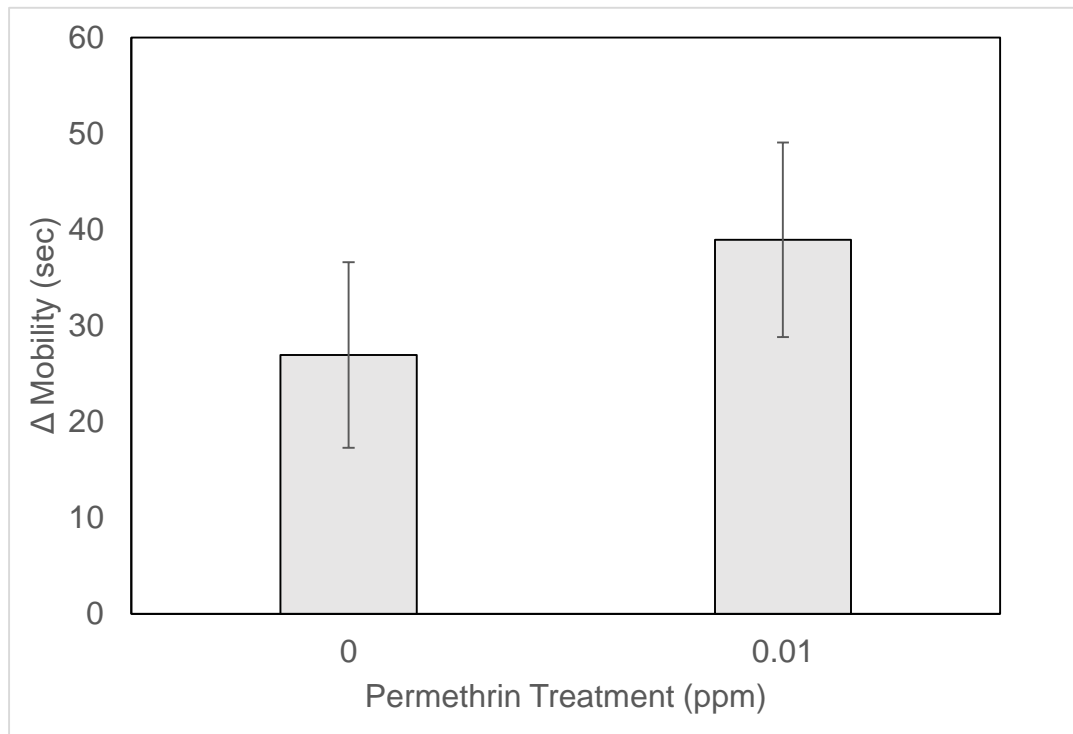


Figure 6. Mean ( $\pm$  SE)  $\Delta$  mobility (sec) between permethrin and control treatments. Change in time spent moving between before and after alarm cue treatment was quantified for larvae exposed to an acetone control,  $n = 40$ , and for larvae exposed to 0.01ppm permethrin,  $n = 40$ . Positive numbers indicate larvae were less mobile after alarm cue treatment.

**Main effects of permethrin on  $\Delta$  velocity.** Larvae exposed to permethrin traveled faster than larvae exposed to acetone before alarm cue introduction. Larvae in both acetone and permethrin treatments moved at similar velocities after alarm cue introduction. Factorial ANOVA revealed no significant main effect for permethrin treatment on  $\Delta$  velocity ( $F_{(1,72)} = 2.92$ ,  $\eta^2_{\text{partial}} = 0.04$ ,  $p = 0.09$ ). Similarly, main effects analysis via one-way ANOVA confirmed that there was no significant difference  $\Delta$  velocity between permethrin and control treatments ( $F_{(1,78)} = 2.43$ ,  $\eta^2_{\text{partial}} = 0.03$ ,  $p = 0.12$ ) (Figure 7).

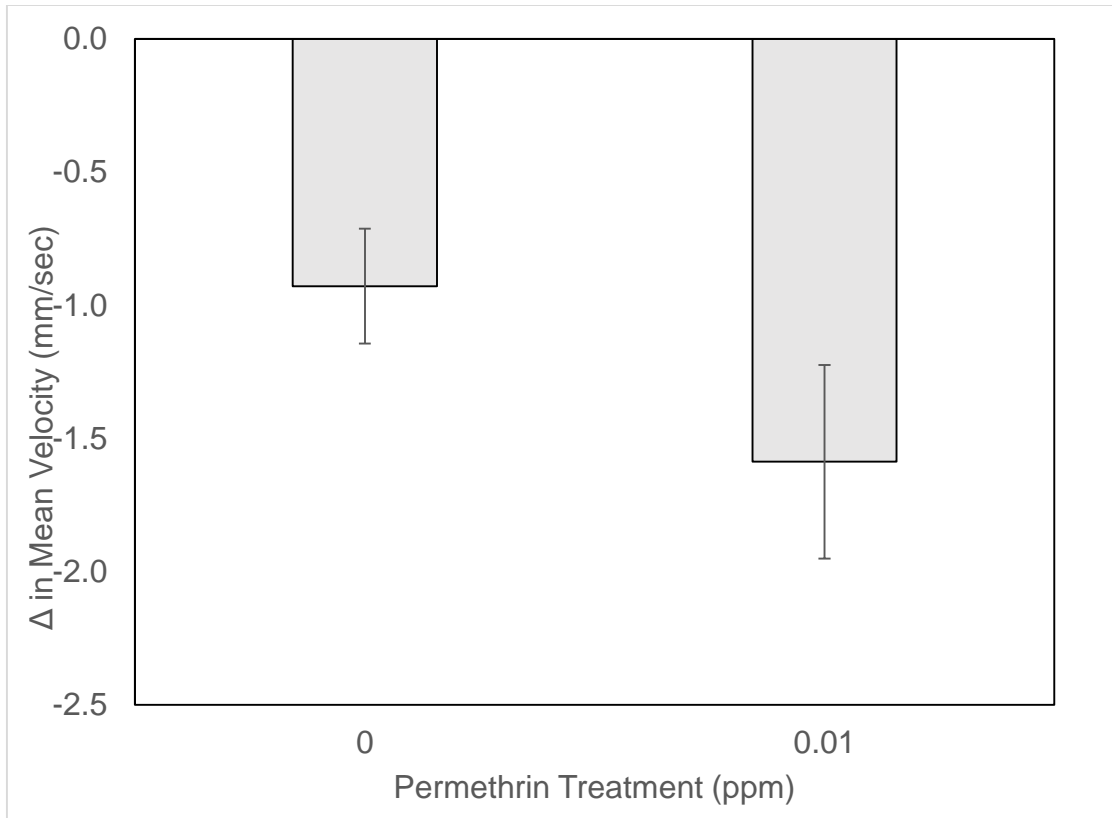


Figure 7. Mean ( $\pm$  SE)  $\Delta$  velocity (mm/sec) between permethrin and control treatments. Change in mean travel velocity between before and after alarm cue treatment was quantified for larvae exposed to an acetone control,  $n = 40$ , and for larvae exposed to 0.01ppm permethrin,  $n = 40$ . Negative numbers indicate larvae traveled faster before alarm cue treatment.

**Main effects of alarm cue on  $\Delta$  distance.** Larvae exposed to alarm cues traveled farther before alarm cue introduction than they did after alarm cue introduction. However, larvae exposed to the 0 alarm cue treatment traveled similar distances both before and after alarm cue introduction. As alarm cue concentration increased, larvae traveled shorter distances; however, the  $\Delta$  distance became smaller, indicating the difference in distance traveled before and after alarm cue introduction lessened as alarm cue concentration increased. Analysis via factorial ANOVA revealed a significant main effect of alarm cue

treatment on  $\Delta$  distance ( $F_{(3,72)} = 4.65$ ,  $\eta^2_{\text{partial}} = 0.16$ ,  $p = 0.005$ ). Fisher's LSD post hoc analysis ( $\alpha = 0.05$ ,  $p < 0.05$ ,  $df = 72$ ) confirmed that there was a significant difference of means between  $\Delta$  distance for alarm cue treatment 0 and alarm cue treatments 2, 4, and 8; yet, there were no significant differences between  $\Delta$  distance of alarm cue treatments 2, 4, and 8 (Figure 8).

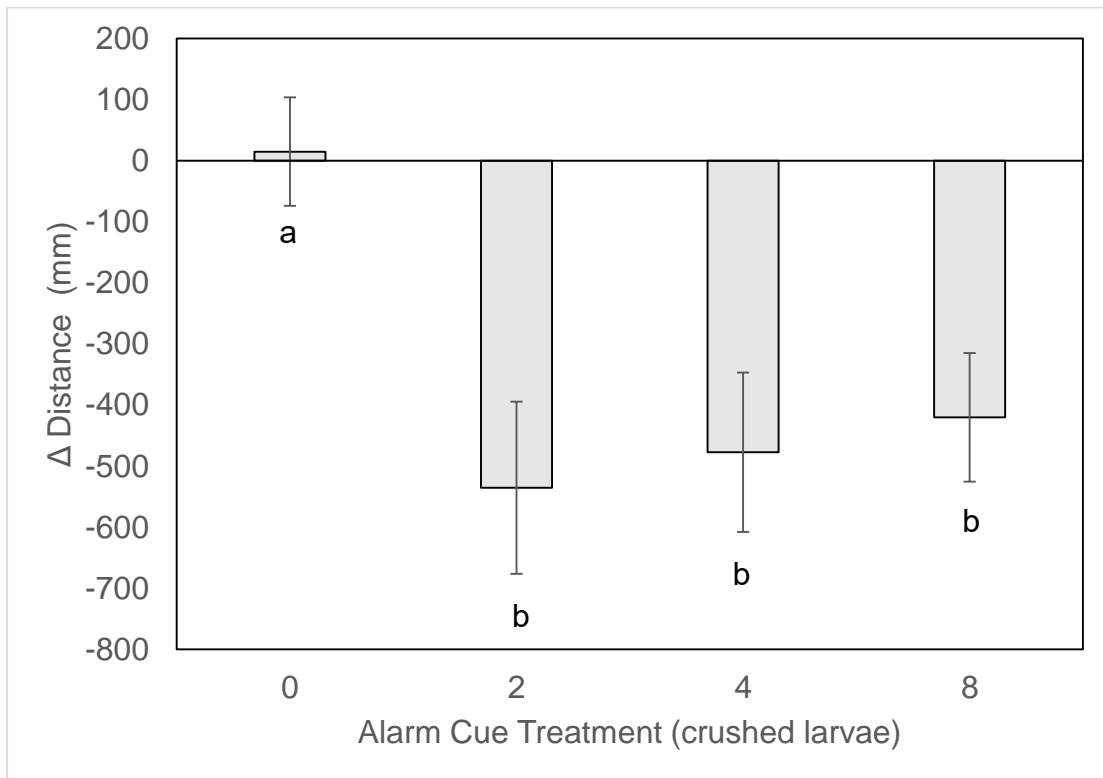


Figure 8. Mean ( $\pm$  SE)  $\Delta$  distance (mm) among alarm cue treatments. Change in distance traveled between before and after alarm cue treatment was quantified for larvae exposed to 4 different alarm cue treatments,  $n = 20$  for each treatment. Negative numbers indicate larvae traveled farther before alarm cue treatment while positive numbers indicate larvae traveled farther after alarm cue treatment. Different letters indicate significant differences ( $p < 0.05$ ) based on Fisher LSD post hoc tests ( $\alpha = 0.05$ ,  $p < 0.05$ ,  $df = 72$ ).

**Main effects of alarm cue treatment on  $\Delta$  mobility.** Larvae exposed to alarm cues were more mobile before alarm cue introduction than they were after

alarm cue introduction. However, larvae exposed to the 0 alarm cue treatment demonstrated similar amounts of mobility both before and after alarm cue introduction. Increasing concentrations of alarm cues did not produce decreasing amounts of mobility; however, as alarm cue concentration increased, the  $\Delta$  mobility became smaller, indicating the difference in mobility before and after alarm cue introduction lessened as alarm cue concentration increased. Analysis via factorial ANOVA revealed a significant main effect of alarm cue treatment on  $\Delta$  mobility ( $F_{(3,72)} = 5.14$ ,  $\eta^2_{\text{partial}} = 0.18$ ,  $p = 0.002$ ). Fisher's LSD post hoc analysis ( $\alpha = 0.05$ ,  $p < 0.05$ ,  $df = 72$ ) confirmed that there was a significant difference between  $\Delta$  mobility for alarm cue treatment 0 and alarm cue treatments 2, 4, and 8; yet, there were no significant differences between  $\Delta$  mobility of alarm cue treatments 2, 4, and 8 (Figure 9).

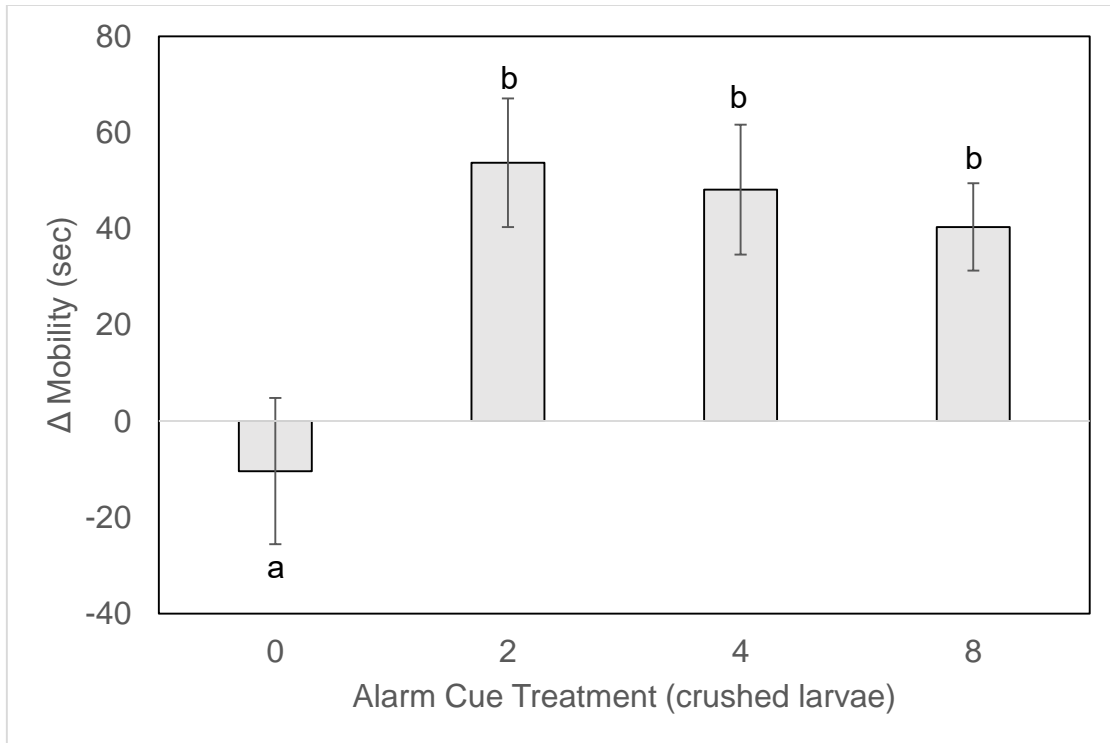


Figure 9. Mean ( $\pm$  SE)  $\Delta$  mobility (sec) among alarm cue treatments. Change in time spent moving between before and after alarm cue treatment was quantified for larvae exposed to 4 different alarm cue treatments,  $n = 20$  for each treatment. Negative numbers indicate larvae were more immobile before alarm cue treatment while positive numbers indicate larvae were more immobile after alarm cue treatment. Different letters indicate significant differences ( $p < 0.05$ ) based on Fisher LSD post hoc tests ( $\alpha = 0.05$ ,  $p < 0.05$ ,  $df = 72$ ).

**Main effects of alarm cue treatment on  $\Delta$  velocity.** Larvae exposed to alarm cues traveled faster before alarm cue introduction than they did after alarm cue introduction. However, larvae exposed to the 0 alarm cue treatment demonstrated similar velocities both before and after alarm cue introduction. The  $\Delta$  velocity decreased as alarm cue concentration increased through treatments 2 and 4; however, the  $\Delta$  velocity between treatments 4 and 8 similar. Analysis via factorial ANOVA revealed a significant main effect of alarm cue treatment on  $\Delta$  velocity ( $F_{(3,72)} = 5.27$ ,  $\eta^2_{\text{partial}} = 0.18$ ,  $p = 0.002$ ). Fisher's LSD post hoc analysis



( $\alpha = 0.05$ ,  $p < 0.05$ ,  $df = 72$ ) confirmed that there was a significant difference between  $\Delta$  velocity for alarm cue treatment 0 and alarm cue treatments 2, 4, and 8; yet, there were no significant differences between  $\Delta$  velocity of alarm cue treatments 2, 4, and 8 (Figure 10).

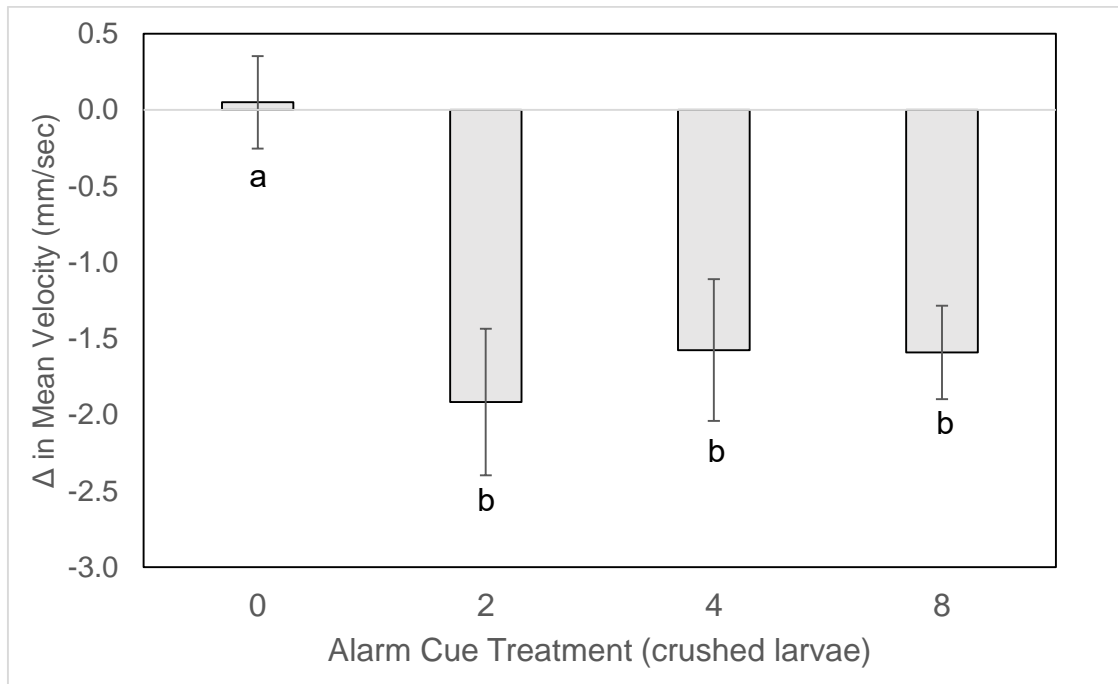


Figure 10. Mean ( $\pm$  SE)  $\Delta$  velocity (mm/sec) among alarm cue treatments. Change in mean velocity before and after alarm cue treatment was quantified for larvae exposed to 4 different alarm cue treatments,  $n = 20$  for each treatment. Negative numbers indicate larvae traveled faster before alarm cue treatment while positive numbers indicate larvae traveled slower after alarm cue treatment. Different letters indicate significant differences ( $p < 0.05$ ) based on Fisher LSD post hoc tests ( $\alpha = 0.05$ ,  $p < 0.05$ ,  $df = 72$ ).

#### **Interactive effects between permethrin and alarm cue treatments on**

**$\Delta$  distance.** Larvae exposed to permethrin and acetone demonstrated similar  $\Delta$  distance when exposed to alarm cue treatment 0. As alarm cue concentrations increased, the  $\Delta$  distance of larvae exposed to permethrin decreased, indicating that the difference in distance traveled before and after alarm cue introduction

lessened as alarm cue concentration increased. Larvae exposed to acetone responded to increasing concentrations of alarm cue with similar measures of  $\Delta$  distance across alarm cue treatments indicating that the difference in distance traveled before and after alarm cue introduction was consistent among alarm cue treatments. Analysis via factorial ANOVA revealed a non-significant interactive effect of permethrin and alarm cue treatment on  $\Delta$  distance ( $F_{(3,72)} = 1.10$ ,  $\eta^2_{\text{partial}} = 0.04$ ,  $p = 0.35$ ). There was no significant difference between larvae exposed to alarm cue treatment 0 between permethrin and acetone treatments. Similarly, the  $\Delta$  distance observed in larvae exposed to acetone was not significantly different across alarm cue treatments. Furthermore, similar  $\Delta$  distance was observed in larvae exposed to acetone across alarm cue treatments. However, larvae exposed to permethrin had a  $\Delta$  distance that decreased slightly as alarm cue concentration increased indicating that the difference in distance traveled before and after alarm cue introduction was slightly different in larvae exposed to both permethrin and alarm cue treatments 2, 4, and 8 when compared to larvae exposed to alarm cue treatment 0 regardless of pesticide treatment (Figure 11).

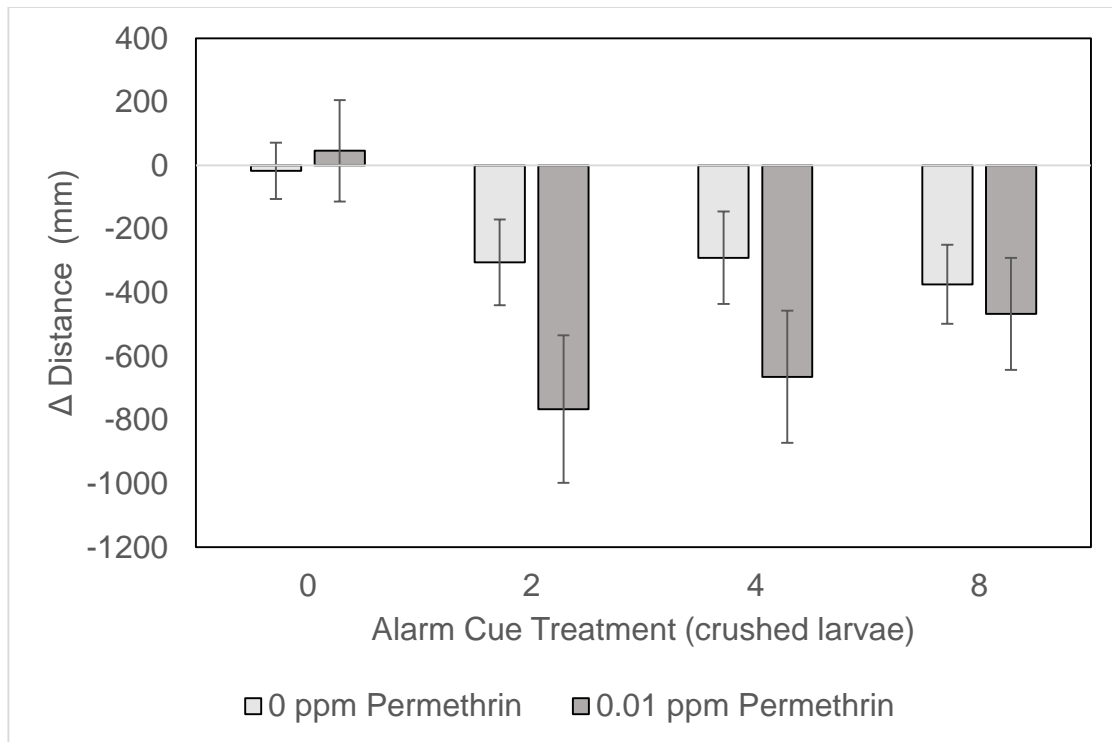


Figure 11. Mean ( $\pm$  SE)  $\Delta$  distance (mm) between permethrin and alarm cue treatments. Change in distance traveled between before and after alarm cue treatments. Larvae were exposed to either an acetone control or permethrin treatment for 24 hours prior to exposure to an alarm cue treatment,  $n = 10$  per treatment group. Negative numbers indicate larvae traveled farther before alarm cue treatment while positive numbers indicate larvae traveled farther after alarm cue treatment.

### Interactive effects between permethrin and alarm cue treatments on

**$\Delta$  mobility.** Larvae exposed to permethrin were more mobile before alarm cue introduction than control larvae for the alarm cue 0 treatment. While larvae exposed to permethrin spent less time mobile than control larvae, larvae exposed to both the control and permethrin treatments responded to increasing concentrations of alarm cue with similar measures of  $\Delta$  mobility across alarm cue treatments, indicating that the difference in time spent mobile before and after alarm cue introduction was similar among alarm cue treatments. Analysis via factorial ANOVA revealed a non-significant interactive effect of permethrin and

alarm cue treatment on  $\Delta$  distance ( $F_{(3,72)} = 1.15$ ,  $\eta^2_{\text{partial}} = 0.05$ ,  $p = 0.34$ ). There was no significant difference in larvae exposed to alarm cue treatment 0 between permethrin and acetone treatments. Similarly, the  $\Delta$  mobility observed in control larvae was not significantly different across alarm cue treatments. Furthermore, similar  $\Delta$  mobility was observed in larvae exposed to permethrin across alarm cue treatments. However, larvae exposed to permethrin and alarm cue treatment 4 had a greater  $\Delta$  mobility than larvae exposed to permethrin and alarm cue treatments 2 and 8 (Figure 12).

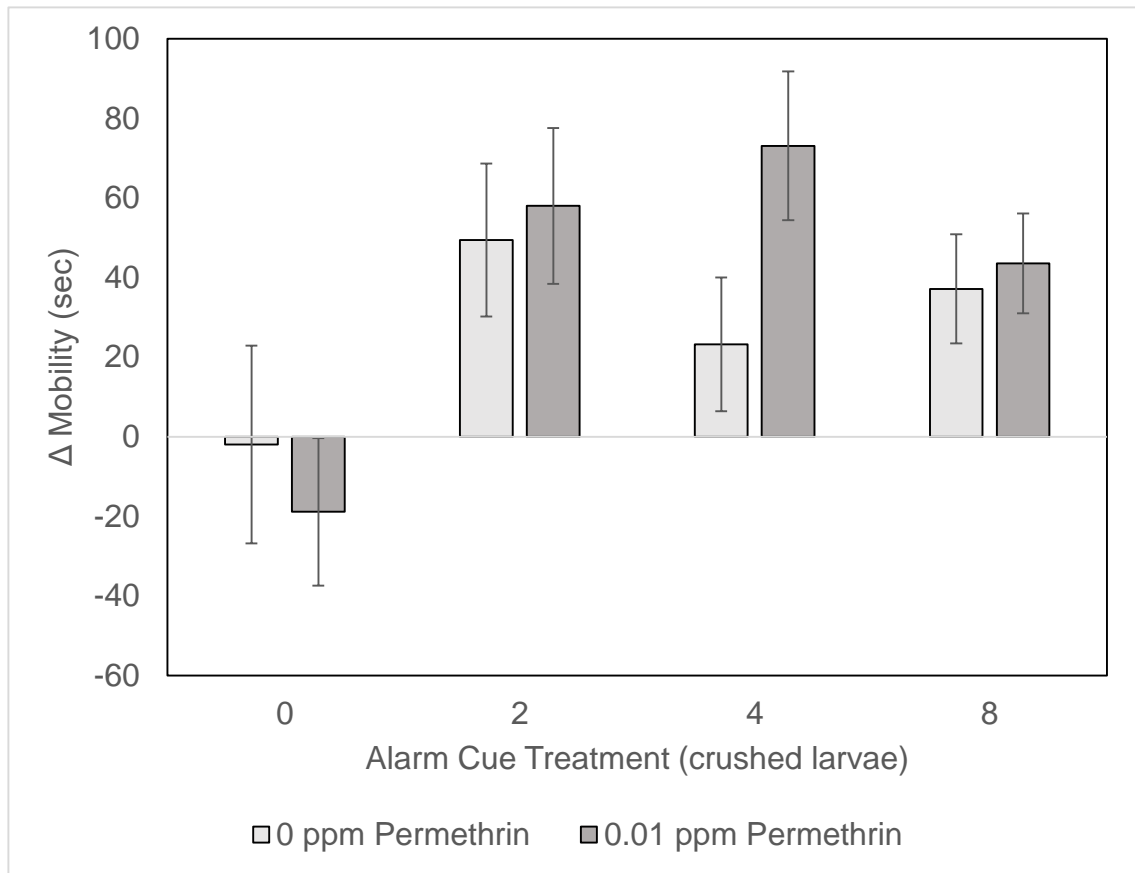


Figure 12. Mean ( $\pm$  SE)  $\Delta$  mobility (sec) between permethrin and alarm cue treatments. Change in time spent moving between before and after alarm cue treatments. Larvae were exposed to either an acetone control or permethrin treatment for 24 hours prior to exposure to an alarm cue treatment,  $n = 10$  per treatment group. Positive numbers indicate larvae were less mobile after alarm cue treatment while negative numbers indicate larvae were less mobile before alarm cue treatment.

**Interactive effects between permethrin and alarm cue treatments on**

**$\Delta$  velocity.** Larvae exposed to permethrin traveled faster after alarm cue introduction than control larvae for the alarm cue 0 treatment, whereas larvae exposed to permethrin traveled faster before alarm cue exposure than control larvae after exposure to alarm cue treatments 2, 4, and 8. Larvae exposed to permethrin treatment responded to increasing concentrations of alarm cue with a

corresponding decrease in  $\Delta$  velocity, indicating that the difference in velocity before and after alarm cue introduction was decreased as alarm cue concentration increased. Analysis via factorial ANOVA revealed a non-significant interactive effect of permethrin and alarm cue treatment on  $\Delta$  velocity ( $F_{(3,72)} = 1.90$ ,  $\eta^2_{\text{partial}} = 0.05$ ,  $p = 0.14$ ). There was no significant difference between larvae exposed to alarm cue treatment 0 between permethrin and control treatments. Similarly, the  $\Delta$  velocity observed in control larvae was not significantly different across alarm cue treatments (Figure 13).

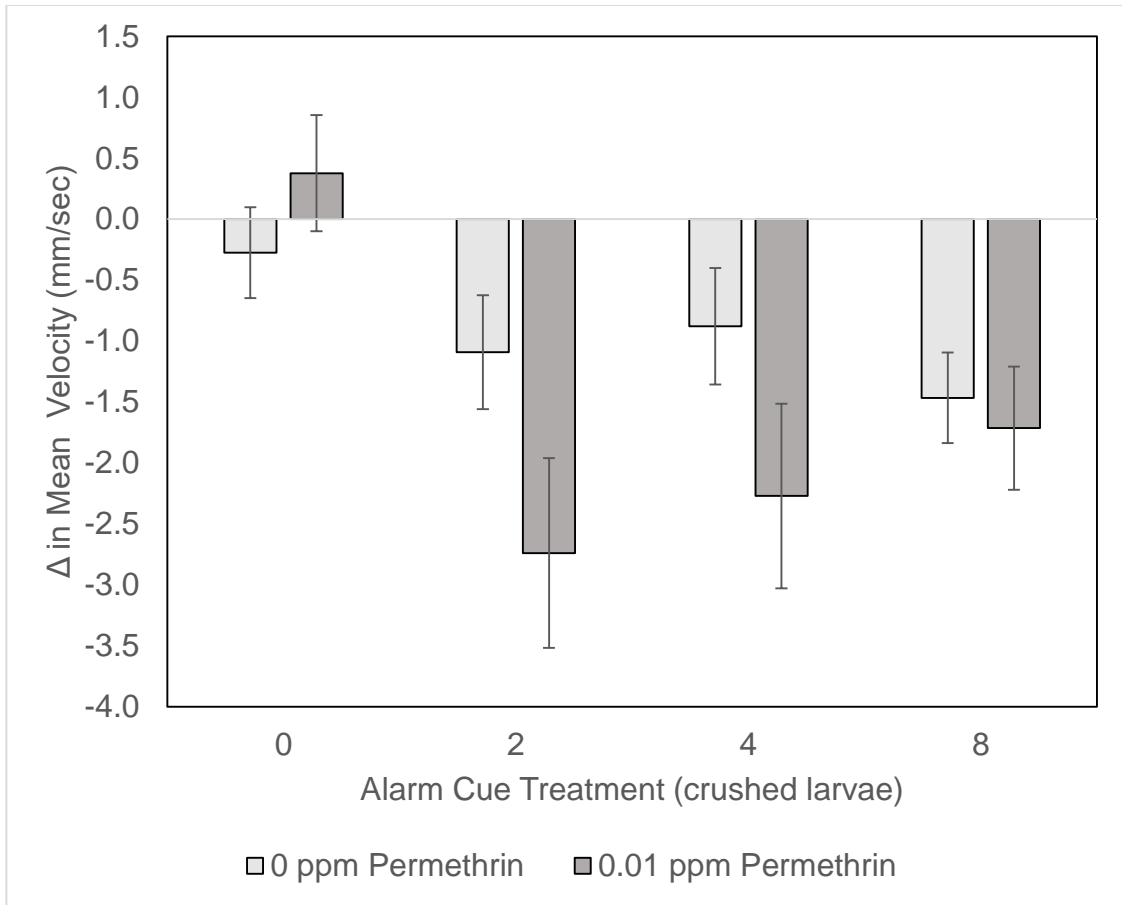


Figure 13. Mean ( $\pm$  SE)  $\Delta$  velocity (mm/sec) between permethrin and alarm cue treatments. Change mean velocity between before and after alarm cue treatments. Larvae were exposed to either an acetone control or permethrin treatment for 24 hours prior to exposure to an alarm cue treatment,  $n = 10$  per treatment group. Negative numbers indicate larvae traveled faster before alarm cue treatment while positive numbers indicate larvae traveled faster after alarm cue treatment.

## Discussion

Failing to avoid a predator can have mortal consequences for prey; however, there are also costs to avoiding predation (Lima & Dill, 1990). When prey reduce activity levels in favor of predator avoidance, they do so at the expense of foraging or other opportunities (Kesavaraju *et al.*, 2007). Prey that

can accurately assess risk and employ antipredator behaviors similar in intensity to predation risk increase fitness and reduce probability of their own predation (Helfman, 1989). Research has demonstrated that larval *Cx. pipiens* respond to crushed conspecifics with antipredator behaviors, suggesting that chemical alarm cues play a role in risk assessment for larval *Cx. pipiens* (Ferrari *et al.*, 2008). Previous research has also demonstrated that exposure to sublethal levels of pesticide can alter larval behavioral responses in *Cx. pipiens* larvae to a range of environmental stimuli (Pestana, Loureiro, Baird, & Soares, 2009).

The purpose of this research was to examine behavioral responses of larval *Cx. pipiens* to alarm cues from crushed conspecific larvae following exposure to sublethal concentrations of permethrin. I predicted that larval *Cx. pipiens* larvae exposed to permethrin would respond to conspecific alarm cues with a less intense behavioral response than to acetone alone. All larvae responded to the introduction of alarm cues with antipredator behavior; however, the behavioral responses observed in this research, measured as  $\Delta$  distance,  $\Delta$  mobility, and  $\Delta$  velocity, did not appear to be threat sensitive. Although results were not statistically significant, observation showed that permethrin exposed larvae tended to react to concentrations of crushed conspecific larvae with slightly less intense behavioral response while larvae exposed to acetone (control) showed no consistent behavioral change to different concentrations of crushed conspecifics. The lack of statistically significantly threat sensitive response from larvae exposed acetone alone in a species known to respond to alarm cues from crushed conspecifics with predictable threat sensitive behavior



(Kesavaraju *et al.*, 2011; Rodríguez-Prieto *et al.*, 2006; Sih, 1986) could indicate that my lowest concentration of crushed conspecifics (alarm cue treatment 2) elicited a maximum antipredator response.

In a similar study, Ferrari *et al.* (2008) demonstrated that larval *Cx. pipiens* respond to crushed conspecific larvae with increasing amounts of antipredator behavior. They observed increased occurrence of antipredator behavior from control to low doses of crushed conspecific larvae and again from low doses of crushed conspecifics to medium doses of crushed conspecifics; however, there was no difference in antipredator behavior between medium and high doses of conspecific larvae. Similarly, my study found increased antipredator behaviors between control (alarm cue treatment 0) and low (alarm cue treatment 2) doses but not between low and medium doses (alarm cue treatment 4) or between medium and high doses (alarm cue treatment 8).

Interestingly, in this study, increasing alarm cue concentrations resulted in decreasing intensity of behavioral responses in permethrin exposed larvae. For example, for  $\Delta$  distance,  $\Delta$  mobility, and  $\Delta$  velocity, larvae exposed to alarm cue treatment 2 responded with a greater behavioral change than larvae exposed to alarm cue treatment 4, and larvae exposed to alarm cue treatment 4 responded with a greater behavioral change than larvae exposed to alarm cue treatment 8. I had hypothesized that increasing concentrations of alarm cue would result in more antipredator behavior, meaning that the change in behavior demonstrated by a larvae exposed to alarm cue treatment 2 would be less than the change in behavior demonstrated by a larvae exposed to alarm cue treatment 4, and the

change in behavior demonstrated by larvae exposed to alarm cue treatment 4 would be less than the change in behavior observed in larvae exposed to alarm cue treatment 8. However, I observed the opposite in this research. One possible explanation could be that after a 'maximum antipredator response' threshold is reached, larvae do not respond to higher alarm cue concentration with more antipredator behavior.

Permethrin is a neurotoxin that is known to inhibit coordinated movement in insects (Desneux *et al.*, 2007). As such, I predicted that larvae exposed to permethrin would have less control over their behavioral response to alarm cue treatments than larvae exposed to an acetone control. I anticipated that an inhibited ability to coordinate movement would result in a less intense behavioral response to alarm cue treatment in permethrin exposed larvae and that acetone exposed larvae would demonstrate a more intense behavioral response to alarm cue treatment. However, after permethrin exposure, larvae responded to increasing concentrations of alarm cues with less change in response as alarm cue concentration increased. Although this proportional change in behavior was not statistically significant ( $p > 0.05$ ) and was in the opposite direction of my original hypothesis, it was still a noticeable pattern. In contrast, larvae exposed to the acetone control treatment did not demonstrate a proportional behavioral change as alarm cue concentration increased, regardless of behavioral response measured. A possible explanation for the proportional behavioral response to increasing alarm cue concentrations observed in permethrin exposed larvae could be that an inhibited ability to detect kairomones (Desneux *et al.*, 2007)

resulted in a higher tolerance to alarm cue treatments, which delayed a potential 'maximum antipredator response' in permethrin exposed larvae.

Similarly, Reynaldi, Meiser, & Liess (2011) demonstrated a decreased alarm response in *Cx. pipiens* after exposure to increasing concentrations of fenvalerate, another common pyrethroid pesticide. Reynaldi *et al.* (2011) measured larval antipredator response 18 h after pesticide exposure and observed more antipredator response compared to larvae measured just 5 h after exposure (Reynaldi, Meiser, & Liess, 2011), which suggests that larvae can recover after exposure to a pyrethroid. Larvae in this study were exposed to permethrin for 24 h and then were removed from the pesticide treatment and allowed to rest in deionized water until used in the bioassay. The time between permethrin exposure and bioassay participation ranged from 5 min to 200 min. If permethrin affected larvae, even minimally, then the time between 'end of exposure' and bioassay participation may have allowed larvae to recover from permethrin exposure.

In summary, I did not find a significant interaction between alarm cue treatment and permethrin treatment. Alarm cue treatment affected larval behavioral response; however, this effect was limited to the lowest concentration of alarm cue treatment. Larvae exposed to permethrin demonstrated greater changes in behavior than larvae exposed to acetone. Larvae exposed to permethrin also showed less intense behavioral responses to alarm cue treatment as alarm cue concentration increased than did larvae exposed to acetone. This work implies that larval *Cx. pipiens* respond to crushed conspecific

larvae with antipredator behaviors, but exposure to high concentrations of alarm cues may elicit a maximum response such that exposure to more crushed conspecifics does not result in more antipredator behavior. Additionally, this work suggests that sublethal permethrin exposure may affect larval *Cx. pipiens* behavioral response to conspecific alarm cues, which could reduce this species' ability to accurately interpret and respond to predation threat in natural surface waters contaminated with pesticide.

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## Appendix A: $\Delta$ Distance Data

### Descriptive Statistics

Table A1.  $\Delta$  distance descriptive statistics data from factorial ANOVA results. STEDV = Standard Deviation. STDEV = Standard Error of the mean.

Effects	Alarm Cue Treatment	Permethrin Treatment	N	$\Delta$ Distance mean	$\Delta$ Distance stdev	$\Delta$ Distance stderr	$\Delta$ Distance -95%	$\Delta$ Distance +95%
Total			80	-354.60	562.89	62.93	-479.87	-229.34
Alarm Cue	8		20	-420.36	471.62	105.46	-641.09	-199.63
Alarm Cue	4		20	-477.36	584.31	130.66	-750.83	-203.90
Alarm Cue	2		20	-535.38	630.16	140.91	-830.31	-240.46
Alarm Cue	0		20	14.70	397.60	88.91	-171.38	200.78
Permethrin		0	40	-246.47	403.77	63.84	-375.60	-117.34
Permethrin		0.01	40	-462.73	674.39	106.63	-678.41	-247.05
Alarm Cue*Permethrin	0	0	10	16.91	278.73	88.14	-216.31	182.48
Alarm Cue*Permethrin	2	0	10	304.79	425.69	134.61	-609.31	-0.27
Alarm Cue*Permethrin	4	0	10	290.22	458.82	145.09	-618.44	38.00
Alarm Cue*Permethrin	8	0	10	373.96	393.12	124.31	-655.18	-92.74
Alarm Cue*Permethrin	0	0.01	10	46.31	503.81	159.32	-314.09	406.71
Alarm Cue*Permethrin	2	0.01	10	765.97	734.14	232.15	-1291.14	-240.80
Alarm Cue*Permethrin	4	0.01	10	664.50	657.59	207.95	-1134.91	-194.09
Alarm Cue*Permethrin	8	0.01	10	466.76	557.00	176.14	-865.21	-68.31

### Factorial ANOVA Results

Table A2.  $\Delta$  distance data factorial ANOVA results. Data was evaluated for effect interactions between permethrin treatments and alarm cue treatments. Main effects were determined for the alarm cue category, independent of permethrin treatment and for permethrin treatment, independent of alarm cue treatment.

Effect	Univariate Tests of Significance, Effect Sizes, and Powers for $\Delta$ Distance Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. Of Freedom	MS	F	p	Partial eta-squared	Non-centrality	Observed power (alpha=0.05)
Intercept	10059353	1	10059353	37.27	0.00	0.34	37.27	1.00
Alarm Cue	3769144	3	1256381	4.65	0.00	0.16	13.96	0.88
Permethrin	935363	1	935363	3.47	0.07	0.05	3.47	0.45
Alarm Cue *Permethrin	891547	3	297182	1.10	0.35	0.04	3.30	0.29
Error	19434622	72	269925					

### Fisher's LSD Results

Table A3.  $\Delta$  distance Fisher's LSD results. Alarm cue treatment had a significant ( $p < 0.05$ ) effect on larval  $\Delta$  distance. To determine the extent to which each treatment affected  $\Delta$  distance, data was evaluated via Fisher's LSD. Alarm cue treatments 2, 4, and 8 were significantly ( $p < 0.05$ ) from treatment 0 but were not significantly ( $p < 0.05$ ) different from each other.

LSD test; variable $\Delta$ Distance, Homogenous Groups, alpha = 0.05, Error: Between MS = 2699E2, df = 72			
Alarm Cue	$\Delta$ Distance	b	a
2	-535.38	****	
4	-477.36	****	
8	-420.36	****	
0	14.70		****

### One-way ANOVA Results

Table A4.  $\Delta$  distance one-way ANOVA results. To verify that permethrin had no significant effect on  $\Delta$  distance, a one-way ANOVA was performed as a post hoc comparison. The one-way ANOVA revealed no significant effect of permethrin on  $\Delta$  distance thereby verifying results from the factorial ANOVA.

Effect	Univariate Tests of Significance, Effect Sizes, and Powers for $\Delta$ Distance							
	Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. Of Freedom	MS	F	p	Partial eta-squared	Non-centrality	Observed power (alpha=0.05)
Intercept	10059353	1	10059353	32.56	0.00	0.29	32.56	1.00
Permethrin	935363	1	935363	3.03	0.09	0.04	3.03	0.40
Error	24095314	78	308914					

## Appendix B: $\Delta$ Mobility Data

### Descriptive Statistics

Table B1.  $\Delta$  mobility descriptive statistics data from factorial ANOVA results. STED = Standard Deviation. SE = Standard Error of the mean.

Effect	Alarm Cue Treatment	Permethrin Treatment	N	$\Delta$ Mobility	$\Delta$ Mobility stdev	$\Delta$ Mobility stderr	$\Delta$ Mobility - 95%	$\Delta$ Mobility +95%
Total			80	32.94	62.49	6.99	19.03	46.85
Alarm Cue	0		20	-10.41	68.02	15.21	-42.24	21.43
Alarm Cue	2		20	53.69	59.88	13.39	25.67	81.72
Alarm Cue	4		20	48.13	60.42	13.51	19.85	76.41
Alarm Cue	8		20	40.35	40.54	9.07	21.37	59.32
Permethrin		0	40	26.95	61.15	9.67	7.40	46.51
Permethrin		0.01	40	38.93	64.02	10.12	18.46	59.41
Alarm Cue*Permethrin	0	0	10	-1.95	78.59	24.85	-58.17	54.27
Alarm Cue*Permethrin	2	0	10	49.41	60.82	19.23	5.90	92.92
Alarm Cue*Permethrin	4	0	10	23.21	53.20	16.82	-14.85	61.26
Alarm Cue*Permethrin	8	0	10	37.14	43.34	13.71	6.13	68.14
Alarm Cue*Permethrin	0	0	10	-18.87	58.58	18.53	-60.78	23.04
Alarm Cue*Permethrin	2	0	10	57.98	61.88	19.57	13.71	102.24
Alarm Cue*Permethrin	4	0	10	73.06	59.13	18.70	30.76	115.36
Alarm Cue*Permethrin	8	0	10	43.56	39.61	12.53	15.23	71.90



**Factorial ANOVA results**

Table B2.  $\Delta$  mobility data factorial ANOVA results. Data was evaluated for effect interactions between permethrin treatments and alarm cue treatments. Main effects were determined for the alarm cue category, independent of permethrin treatment and for permethrin treatment, independent of alarm cue treatment.

Effect	Univariate Tests of Significance, Effect Sizes, and Powers for $\Delta$ Mobility Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. Of Freedom	MS	F	p	Partial eta-squared	Non-centrality	Observed power (alpha=0.05)
Intercept	86815.95	1	86815.95	25.81	0.00	0.26	25.81	1.00
Alarm Cue	2870.79	1	2870.79	0.85	0.36	0.01	0.85	0.15
Permethrin	51910.98	3	17303.66	5.14	0.00	0.18	15.43	0.91
Alarm Cue *Permethrin	11561.62	3	3853.87	1.15	0.34	0.05	3.44	0.30
Error	242197.33	72	3363.85					

### Fisher's LSD Results

Table B3.  $\Delta$  mobility data Fisher's LSD results. Alarm cue treatment had a significant ( $p < 0.05$ ) effect on larval  $\Delta$  mobility. To determine the extent to which each treatment affected  $\Delta$  mobility, data was evaluated via Fisher's LSD. Alarm cue treatments 2, 4, and 8 were significantly ( $p < 0.05$ ) from treatment 0 but were not significantly ( $p < 0.05$ ) different from each other.

LSD test; variable $\Delta$ mobility			
Homogenous Groups, alpha = .05000			
Error: Between MS = 3363.9, df = 72.000			
Alarm Cue	$\Delta$ Mobility	b	a
0	-10.41		****
8	40.35	****	
4	48.13	****	
2	53.69	****	

**One-way ANOVA Results**

Table B4.  $\Delta$  mobility data one-way ANOVA results. To verify that permethrin had no significant effect on  $\Delta$  mobility, a one-way ANOVA was performed as a post hoc comparison. The one-way ANOVA revealed no significant effect of permethrin on  $\Delta$  mobility thereby verifying results from the factorial ANOVA.

Effect	Univariate Tests of Significance, Effect Sizes, and Powers for $\Delta$ mobility							
	Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. Of Freedom	MS	F	p	Partial eta-squared	Non-centrality	Observed power (alpha=0.05)
Intercept	86815.95	1	86815.95	22.15	0.00	0.22	22.15	1.00
Permethrin	2870.79	1	2870.79	0.73	0.39	0.01	0.73	0.14
Error	305669.93	78	3918.85					

## Appendix C: $\Delta$ Mean Velocity Data

### Descriptive statistics: Velocity Data

Table C1.  $\Delta$  velocity data descriptive statistics from factorial ANOVA results. STEDV = Standard Deviation. STDEV = Standard Error of the mean.

Effect	Permethrin Treatment	Alarm Cue Treatment	N	$\Delta$ Velocity mean	$\Delta$ Velocity StDev	$\Delta$ Velocity StdErr	$\Delta$ Velocity -95%	$\Delta$ Velocity +95%
Total			80	-1.26	1.91	0.21	-1.68	-0.83
Permethrin	0		40	-0.93	1.36	0.22	-1.36	-0.49
Permethrin	0.01		40	-1.59	2.30	0.36	-2.32	-0.85
Alarm Cue		0	20	0.05	1.36	0.30	-0.58	0.69
Alarm Cue		2	20	-1.92	2.15	0.48	-2.92	-0.91
Alarm Cue		4	20	-1.58	2.08	0.46	-2.55	-0.60
Alarm Cue		8	20	-1.59	1.37	0.31	-2.23	-0.95
Permethrin *Alarm Cue	0	0	10	-0.28	1.18	0.37	-1.12	0.57
Permethrin *Alarm Cue	0	2	10	-1.09	1.48	0.47	-2.15	-0.04
Permethrin *Alarm Cue	0	4	10	-0.88	1.51	0.48	-1.96	0.20
Permethrin *Alarm Cue	0	8	10	-1.47	1.18	0.37	-2.31	-0.62
Permethrin *Alarm Cue	0.01	0	10	0.38	1.50	0.48	-0.70	1.45
Permethrin *Alarm Cue	0.01	2	10	-2.74	2.46	0.78	-4.50	-0.98
Permethrin *Alarm Cue	0.01	4	10	-2.27	2.40	0.76	-3.98	-0.56
Permethrin *Alarm Cue	0.01	8	10	-1.72	1.59	0.50	-2.86	-0.57

**Factorial ANOVA Results: Velocity Data**

Table C2.  $\Delta$  velocity data factorial ANOVA results. Data was evaluated for effect interactions between permethrin treatments and alarm cue treatments. Main effects were determined for alarm cue category, independent of permethrin treatment and for permethrin treatment, independent of alarm cue treatment.

Effect	Univariate Tests of Significance, Effect Sizes, and Powers for $\Delta$ Velocity Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. Of Freedom	MS	F	p	Partial eta-squared	Non-centrality	Observed power (alpha=0.05)
Intercept	126.58	1	126.58	42.49	0.00	0.37	42.49	1.00
Alarm Cue	8.69	1	8.69	2.92	0.09	0.04	2.92	0.39
Permethrin	47.10	3	15.70	5.27	0.00	0.18	15.81	0.92
Alarm Cue *Permethrin	17.02	3	5.67	1.90	0.14	0.07	5.71	0.47
Error	214.51	72	2.98					

### Fisher's LSD Results

Table C3.  $\Delta$  velocity data Fisher's LSD results. Alarm cue treatment had a significant ( $p < 0.05$ ) effect on larval  $\Delta$  velocity. To determine the extent to which each treatment affected  $\Delta$  velocity, data was evaluated via Fisher's LSD. Alarm cue treatments 2, 4, and 8 were significantly ( $p < 0.05$ ) from treatment 0 but were not significantly ( $p < 0.05$ ) different from each other.

LSD test; variable $\Delta$ Velocity			
Homogenous Groups, alpha = .05000			
Error: Between MS = 2.0963, df = 70.000			
Alarm Cue	$\Delta$ Velocity	b	a
8	-1.59	****	
2	-1.57	****	
4	-1.26	****	
0	0.05		****

### One-way ANOVA Results

Table C4.  $\Delta$  velocity data one-way ANOVA results. To verify that permethrin had no significant effect on  $\Delta$  velocity, a one-way ANOVA was performed as a post hoc comparison. The one-way ANOVA revealed no significant effect of permethrin on  $\Delta$  velocity thereby verifying results from the factorial ANOVA.

Effect	Univariate Tests of Significance, Effect Sizes, and Powers for $\Delta$ Velocity Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degr. Of Freedom	MS	F	p	Partial eta-squared	Non-centrality	Observed power (alpha=0.05)
Intercept	126.58	1	126.58	35.44	0.00	0.31	35.44	1.00
Permethrin	8.69	1	8.69	2.43	0.12	0.03	2.43	0.34
Error	278.63	78	3.57					