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PRIMARY RESEARCH PAPER

Mosquito larvicides disrupt behavior and survival rates of aquatic insect predators

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Abstract Understanding the environmental effects of chemicals used in pest control on aquatic systems is crucial to ensure the conservation of beneficial non-target organisms. Mosquito larvicides are commonly applied to aquatic habitats; however, their non-target effects are not well understood. Our goal was to determine lethal and behavioral effects of insect growth regulators (IGRs) and surface films (SFs) on predaceous diving beetle adults and larvae, and damselfly and dragonfly nymphs in roadside ditch habitats. Behavioral effects were determined via bioassays quantifying predator movement, location, and larval mosquito predation. Using Principal Components Analyses, correlated behaviors were determined for each taxa, and compared within SF and IGR treatment levels. Surface films were lethal to beetle adults in the genus *Laccophilus* (Dytiscidae) at recommended and high concentrations. *Laccophilus* adult behavior changed in response to IGRs, those exposed to recommended and high concentrations were more active than controls. Larval *Laccophilus* mosquito consumption varied between SFs and IGRs. We conclude that SFs can harm aquatic organisms that require atmospheric oxygen, and both larvicides may have sublethal effects on hunting behaviors of aquatic insects. Future studies should focus on different larvicides, and a wider variety of aquatic taxa that interact with mosquito larvae.

Keywords Culicidae · Predation · Non-target effects · Larvicides · Predator behavior

Introduction

Predaceous insects are often at the top of the food chain in semi-permanent bodies of water lacking fish (Batzer & Wissinger, 1996), which makes them important top-down regulators of the aquatic invertebrates that they prey upon. Aquatic dipteran larvae like mosquitoes are one of such prey groups that inhabit these semi-permanent habitats, which makes these habitats targets of pesticide application as a means of vector suppression. Since the middle of the twentieth century, there has been growing interest in studying the environmental impacts of pesticide use. The publication of stories like *Silent*

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Spring (Carson, 1962) alerted the public to the consequences of imprudent DDT use and, importantly, clearly explained how target pests may not be the only organism that can be harmed. Recent studies have shown how insect communities are indirectly affected by pesticide use through changes in behavior (Cox & Wilson, 1984; De Jiu & Waage, 1990; Wiles & Jepson, 1994; Kunkel et al., 2001; Claver et al., 2003; Martinou et al., 2014; Sánchez-Bayo, 2021), predatory release (Dennett et al., 2003; Douglas et al., 2015), or reduction of prey items (Hershey et al., 1998). However, most of this focus has been on beneficial terrestrial arthropods in an agricultural setting or aquatic invertebrates exposed to agricultural runoff. The few studies that have considered the effects of chemical exposure on the behavior and hunting ability of aquatic mosquito predators include a study on the effects of non-pesticide pollutants on damselfly nymph behavior (with *Daphnia* prey) (Van Gossum et al., 2009), dragonfly mosquito consumption after exposure to silver nanoparticles (Murugan et al., 2015), and the effects of two broadly toxic mosquito larvicides—cypermethrin (a synthetic pyrethroid) and the insect growth regulator (IGR) diflubenzuron, on damselfly hatching, survival, and growth (Subrero et al., 2019).

Current mosquito control practice uses an integrated approach to lower adult populations and the pathogens they transmit. Surveillance, source reduction, and public education are all essential components of integrated mosquito management (Mazzacano & Black, 2013). Although source reduction is the most effective method for controlling container-dwelling mosquitoes (Connelly & Carlson, 2009; Mazzacano & Black, 2013) (i.e., *Aedes* spp.), the use of larvicides may be necessary to prevent adults from emerging from habitats that cannot be drained. For example, other medically important mosquito genera like *Culex* and *Anopheles* lay their eggs on the water's surface, generally in larger bodies of water like ponds, roadside ditches, wetlands, and tire ruts (Clements, 1999). Additionally, floodwater mosquitoes like *Psorophora* and some species of *Aedes* lay their eggs on soil in anticipation of a flood event and emerge in large numbers in large pools created by heavy rain, or from newly flooded irrigation ditches (Gouge et al., 2016). Thus, widespread use of larvicides is used to control these species.

Larvicides are chemicals or modified pathogens developed specifically to kill larvae of a target pest species (Lawler, 2017). Common types of larvicides include dipteran-killing bacteria, growth regulators (IGRs), surface films (SFs), and organophosphates (Connelly & Carlson, 2009; Mazzacano & Black, 2013). These chemicals come in liquid and solid forms; liquids are sprayed on the surface of a target habitat, and solids are simply tossed into the water. All forms of larvicides have recommended dosages that can be calculated by approximating the surface area or volume of the target body of water. Naturally occurring mosquito pathogens like *Bacillus thuringiensis* Berliner 1915 var. *israelensis* (Bti) and *Saccharopolyspora spinosa* Mertz & Yao 1990 (spinosad) create toxins that disrupt the targets' digestive system (Lawler, 2017). Organophosphates (e.g., Temephos or Abate®) are pesticides that target the insect's nervous system (Mazzacano & Black, 2013). Organophosphates are used less commonly due to higher toxicity to a wide variety of non-target vertebrate and invertebrate taxa (Mazzacano & Black, 2013). Insect Growth Regulators (e.g., methoprene or Altosid®) mimic juvenile fly hormones that regulate molting in larvae (Miura & Takahashi, 1973, 1974). Surface films (e.g., Agnique Monomolecular Film®) create a physical barrier between the water and atmosphere, preventing larvae and pupae from accessing atmospheric oxygen (Miles et al., 2002) and may also be lethal via flooding of the insect's siphon (Corbet et al., 2000).

Surface films and IGRs are especially useful when many pupae are found in a body of water, as mosquitoes in this stage do not eat and other Diptera-specific larvicides (e.g., Bti and spinosad) need to be ingested to be lethal (Connelly & Carlson, 2009; Mazzacano & Black, 2013). Additionally, past studies have reported declines in the abundance of various predaceous insects in aquatic sites after the application of IGRs (Steelman & Schilling, 1972; Norland & Mulla, 1979) and SFs (Takahashi, 1984; Miles et al., 2002), making these chemicals good candidates for observing potential behavioral effects. This study aims to answer questions relating to the non-target toxicity of IGRs and SFs, both of which are routinely used today and understudied in terms of their non-target effects on mosquito predators.

Understanding how natural predators of mosquitoes respond after exposure to larvicidal chemicals will better equip us for future challenges in both ecosystem health and mosquito control research. Negative effects of larvicides have been observed on non-target aquatic insects that are known to prey on mosquito larvae (e.g., aquatic beetles, hemipterans, odonates) (Steelman & Schilling, 1972; Miura & Takahashi, 1973 & 1974; Norland & Mulla, 1975; Breaud et al., 1977; Takahashi et al., 1984; Miles et al., 2002; Antwi & Reddy, 2015; Lawler, 2017). Harming non-target predators that exist in habitats were larvicides have been applied might be beneficial for future mosquito populations, as these taxa have longer generation times compared to small aquatic Diptera (Chase & Knight, 2003; Merritt et al., 2008). Even if these chemicals are not directly lethal to non-target predators, there may be sublethal behavioral effects that negatively influence their locomotion and hunting behavior. These sublethal effects may in turn reduce the effectiveness of these predators at regulating larval mosquito numbers.

We hypothesized that when exposed to larvicides, non-target aquatic mosquito predators will exhibit behaviors different to those in treatments containing no larvicides. We predicted that predaceous diving beetle adults and larvae would be more negatively affected by SFs than the odonates as they need to access the atmosphere for oxygen (Merritt et al., 2008), and that SFs would harm aquatic beetles in the same way they are intended to kill mosquito larvae and pupae. Odonates obtain their oxygen using gills (Merritt et al., 2008). Thus, we predicted that dragonfly and damselfly nymphs exposed to any concentration of SF would not differ from their control-treatment counterparts in behavior. We also predicted that IGRs would most negatively affect predaceous diving beetle larvae behavior, as IGRs are designed to halt molting in holometabolous insects (e.g., Diptera, Lepidoptera, Coleoptera).

Furthermore, we hypothesized that survival rates and prey consumption would vary among these predator groups exposed to different treatments. Our predictions for survival and prey consumption align with the above predictions for behavior, with SFs being more harmful (i.e., higher mortality and lower prey consumption) to atmosphere-breathing predators, and predaceous beetle larvae potentially being the most negatively affected by IGRs. In terms of larvicide concentration for all hypotheses, we predicted that the higher concentrations would show more negative effects (if any negative effects were observed) on predators than lower concentrations. Higher amounts of a hydrophobic substance (like SFs) will cover a wider surface area of water, increasing its effectiveness as a suffocant. Likewise, a higher concentration of a toxin (like IGRs) in a body of water increases the chance of exposure to harmful levels of a dissolved chemical. Although past studies have examined lethal concentrations and changes in abundance of non-target insects after larvicide exposure (Steelman & Schilling, 1972; Miura & Takahashi, 1973 & 1974; Norland & Mulla, 1975; Breaud et al., 1977; Takahashi et al., 1984; Antwi & Reddy, 2015; Lawler, 2017), this is the first to directly examine changes in hunting and locomotion behavior of non-targets after being exposed to SFs and IGRs.

Materials and methods

Collection and treatments

We conducted laboratory observations examining larvicidal effects on common predatory taxa known to prey on mosquito larvae (Kumar & Hwang, 2006; Floore et al., 2007; Culler & Lamp, 2009; Shaalan & Canyon, 2009). We tested for behavioral changes in predatory taxa after exposing them to larvicides. We also examined the number of mosquito larvae eaten among different larvicide types and concentrations within each predator group during behavioral trials. Predators in all experiments were collected from larvicide-free sites around Hattiesburg, MS (Lake Sehoy: 31.352768°N, − 89.362825°W, Lake Thoreau Environmental Center (LTEC): 31.368154°N, − 89.432707°W, and Petal River Park: 31.342412°N, − 89.275838°W) one week prior to experiment start. Individuals were kept alive at the University of Southern Mississippi (USM) campus in biological incubators on a 12:12 light:dark cycle at 27 °C and fed two 4th instar *Culex* larvae daily. The predator groups included *Pachydiplax longipennis* Burmeister 1839 nymphs (Odonata: Libellulidae), *Ischnura* spp. nymphs (Odonata: Coenagrionidae), and *Laccophilus fasciatus rufus* Melsheimer 1884 adults and larvae (Coleoptera: Dytiscidae), which were identified to lowest taxonomic unit using keys by Wright & Peterson (1944), Epler (1996), and Merritt et al. (2008). There are no reliable species keys for *Laccophilus* larvae in the southern US (Epler, 1996), but our specimens were most likely *Laccophilus fasciatus rufus*, the most commonly collected adult of this genus in southern Mississippi (Pitcher & Yee, 2018). Damselfly nymphs were identified to genus and based on historical data of commonly collected damselfly adult species by county in MS (Krotzer et al., 2008), they were likely one of three species: *Ischnura hastata* Say 1839, *Ischnura posita* Hagen 1861, or *Ischnura ramburii* Selys 1850.

For Experiment 1, four plastic tubs (91.5×61.0×20.0 cm) contained one of the following larvicide concentrations: 0% (control, no chemicals), 10% of the recommended application concentration (low), recommended concentration (medium), and double the recommended concentration (high). Recommended concentrations for larvicidal chemicals are given on the product label as a set amount added per estimated surface area (Agnique Monomolecular Surface Film®) or total volume of water (Altosid® Insect Growth Regulator). Although double recommended concentrations will likely not be seen in a real-world setting, this treatment was added to determine if any potential negative effects could be seen in these organisms if none were present in recommended and below. For SFs, we used volumes based off recommended concentrations and surface area of our treatment tubs: 10% = 0.025 ml, recommended = 0.25 ml, and double = 0.5 ml. For solid Altosid® IGR briquettes, we based weights off the average weight of five briquettes (6.474 g) and recommended usage by volume of water being dosed in our tubs: 10% = 0.0842 g, recommended = 0.8416 g, and double = 1.6832 g. Plastic tubs were filled with 37.9 l of well water, and chemicals were added and homogenized via stirring.

Treatment (larvicide type x concentration) exposures were conducted outdoors at the USM LTEC. To prevent escape and any interaction among individuals during exposure, each insect was placed in a 14×6 cm mesh pouch made of Phifer© no-see-um fiberglass screening. Multiple mesh pouches were fastened to the sides of the treatment tubs using thumbtacks and suspended halfway into the water to provide access to the surface for respiration (necessary for *Laccophilus* adults). We took this approach as it exposed all predators to the same concentration of pesticides, which were difficult to replicate in smaller volumes. We included four replicates for every predator (4) by chemical treatment (8) combination. Individuals were subjected to chemical solutions for 24 h in the absence of food. Then, predators were removed from their pouches, rinsed, and placed into fresh water, and feeding behavior plus overall activity were observed on the same day post-exposure.

Behavioral observations

Following chemical exposure, predators were placed into laboratory containers for predatory trials. Clear acrylic containers (18×5×10 cm) were filled with 500 ml of reverse osmosis (RO) water. Two stalks of a common local aquatic plant (*Ludwigia palustris* (L.) Elliott) found in roadside ditches and wetlands were added to each observation container (Pitcher & Yee, 2014). Plant stalks were triple rinsed with tap water to remove debris and potential live invertebrates. Plant stalks, long enough to reach the bottom of the container, were suspended from a rectangular piece of cardboard fully covering each containers' opening (Fig. 1). A single predator was placed in each container, allowed to acclimate for 15 min, and then live prey (10, 4th instar *Culex quinquefasciatus* Say 1823 larvae) were introduced. Prey mosquitoes were collected as egg rafts in the wild, and reared following protocol outlined in Gerberg et al. (1994).

Behavior and predation observations were recorded over 30 min starting after the addition of prey. Observations, belonging to four categories, were recorded once per minute for every container during this time by a single observer (J. Nelsen): (1) Activity: swimming (freely moving in water column), walking (using legs for locomotion and touching a surface), and resting (attached to a surface and not walking, or attached to the water's surface in the case of beetles obtaining oxygen), (2) the surface they were touching: plant, container wall, container floor, or open space (freely swimming), (3) the predator's depth in the water column: at the water's surface, top 1/2, middle 1/2, or bottom 1/2, and (4) predatory activity: striking at prey (actively attempting to catch prey), eating prey (processing prey with mandibles), or neither. Predator behaviors were selected based on dytiscid behavioral observations outlined in Yee (2010). Prior to these behavioral trials, we chose to standardize the recorded behaviors to these four subcategories across all four predator groups. Although dytiscid adults are active hunters, their larvae can be sit-and-wait predators (Yee, 2010). Dragonfly and damselfly nymphs are also ambush predators, making

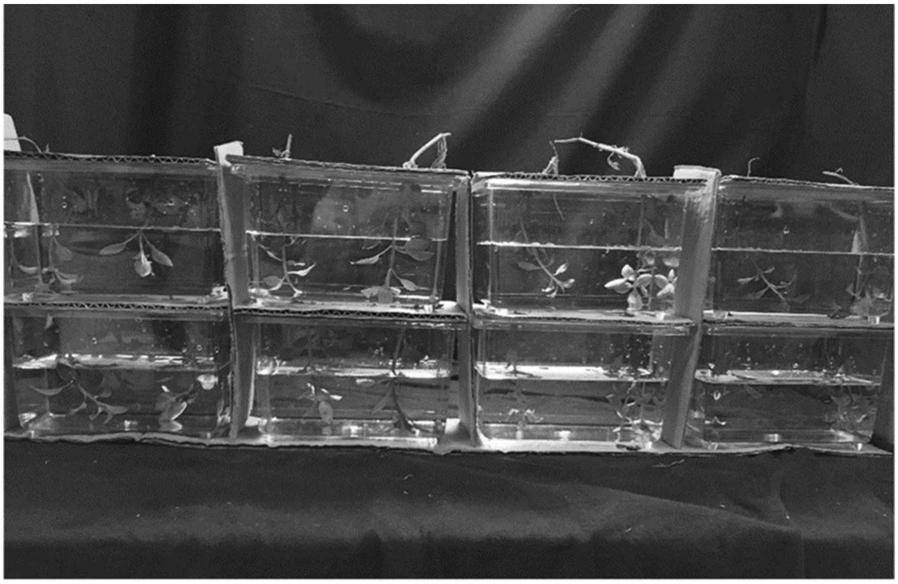

Fig. 1 Arrangement of viewing chambers in behavioral experiment

observation methods similar to Yee (2010) (i.e., aquaria with plant structure) appropriate for odonates found in these roadside habitats. Behaviors unique to the beetle adults and larvae (atmospheric siphoning) were denoted above.

Observations were recorded in a darkened room with a black curtain background and single light source positioned to illuminate all containers as evenly as possible. Eight containers were observed at a time, and cardboard was placed in between observation chambers to eliminate any potential visual stimulus from neighboring chambers. Number of prey eaten was also recorded at the end of the observation period. Predators in all treatments were of same size/instar within orders to control for different feeding behaviors and energy requirements (Merritt et al., 2008). All four replicates of each predator and treatment combination were observed on the same day (e.g., reps 1–4 of damselflies in IGRs). As only eight containers were able to be observed at one time and there were a total of 16 individuals being observed on a given day, we observed replicates 1–2 of each concentration first, and placed replicates 3–4 in an incubator to account for any behavioral differences that might be caused by time spent sitting in fresh water.

Data for trials were the proportion of times predators were observed performing each action over the 30 min period. After observational trials, the same individuals were reared over a week-long period to assess post-exposure survival. Individuals were placed in cups containing RO water and a wooden tongue depressor to provide structure, and stored in incubators set at 27 °C, 12:12 light:dark cycle. Individuals were fed two mosquito larvae once per day.

Behavioral analysis

To meet assumptions of normality, proportions of the behaviors within each category (activity, surface, depth, predation) were arc-sine square root transformed. A Principal Components Analysis (PCA) was then conducted on this transformed data, to reduce the number of correlated behaviors. A PCA was generated from each predator group and larvicide type combination (e.g., damselfly x SF) to account for natural behavioral differences among predators (Merritt et al., 2008), as well as behavioral changes brought on by the different modes of action between IGRs and SFs. Principal components (PCs) with eigenvalues ≥ 1 were retained for further analysis. Rotated factor loading scores (the degree to which each behavior is associated with a principal component axis) were analyzed using one factor multivariate analyses of variance (MANOVA) with concentration (high, medium, low, and control) as the factor. The results of the MANOVAs were interpreted by examining Standard Canonical Coefficients (SCCs) generated by SAS, which help identify the PCs that are most responsible for any multivariate effects. Behaviors were considered important for a specific

PC if loadings were ≥ ±40 (Yee, 2010). To determine statistical differences among important independent variables (e.g., concentration = high, medium, low, or control), a Tukey's post hoc analysis with a significance threshold of $P < 0.05$ was conducted after each MANOVA. Effects of treatments on specific behaviors were then determined based on these PC loading scores. All behavior analyses were performed in SAS (SAS Institute, Inc., 2004). Statistical power for all analyses was calculated using G*Power software (Faul, et al., 2009), and ranged from 0.06 to 0.995.

Survival and predation analyses

Assumptions of normal distribution were not met for one-week survival in damselflies, *Laccophilus* adults, and *Laccophilus* larvae after conducting a Shapiro–Wilk's goodness of fit test. However, variance assumptions (homoscedasticity) were met for damselfly and beetle adult and larvae survival data based on visual inspection. As analysis of variance (ANOVA) is robust against departures from normality, we did not transform the data and instead conducted a two-factor factorial ANOVA to compare survival within predator groups (Blanca Mena et al., 2017). Dragonfly nymph survival data were not analyzed because all individuals survived one week. Larval mosquito consumption data did not meet assumptions of normality (Fig. S1) but met the assumptions of homogeneity (Fig. S2). Thus, a two-factor factorial ANOVA was conducted with larvicide type and concentration as factors and was used to compare prey consumption separately within each of the four predator groups. In all analyses, a Tukey's post hoc analysis with a significance threshold of $P < 0.05$ was conducted to determine significant differences between treatment levels, unless noted otherwise. Statistical analyses for survival and predation were performed in R and JMP (R Core Team, 2019; SAS Institute, Inc., 2019).

Results

Behavior

Based on the PCAs, the first four PCs explained 85.71% of the variation in Laccophilus adult behaviors in IGRs. There was a significant effect of concentration on *Laccophilus* adult behavior in IGR trials, with PC1 contributing most to that effect (Table 1). For dragonfly nymphs, damselfly nymphs, and *Laccophilus* larvae, there were no significant effects of concentration on behavior for IGRs (Table 1) or SFs (Table 2). For *Laccophilus* adults, PC1 separated individuals that were observed resting and in contact with plant structure (negative values) from those that spent more time swimming in open space and being in contact the aquarium floor (positive values) (Table 3). Within PC1, mean values for control individuals were significantly lower than mean values for both high and medium (recommended) concentration treatments (Fig. 2).

Survival and predation

All dragonfly nymphs survived regardless of larvicide type and concentration. For damselflies, there were

Table 1 Results of four separate one-factor MANOVAs comparing PCA axes correlated with each predator group's swimming and hunting behavior concentrations within IGR treatments. Standardized Canonical Coefficients (SCCs) show amount of contribution of the dependent variable (concentration) in significant MANOVA effects

	Source	df	Error df	Pillai's Trace	P-value	Standardized Canonical Coefficients			
						PC1	PC2	PC3	PC4
Damselfly nymphs	Concentration	12	33	1.229	0.070	–	–	–	–
Laccophilus adults	Concentration	12	33	1.316	**0.041**	**1.410**	0.610	0.419	0.855
Dragonfly nymphs	Concentration	12	33	1.036	0.193	–	–	–	–
Laccophilus larvae	Concentration	18	27	1.530	0.144	–	–	–	–

P-values that are less than 0.05 have been bolded

Table 2 Results of four separate one-factor MANOVAs comparing PCA axes correlated with each predator group's swimming and hunting behavior concentrations within SF treatments

	Source	Df	Error df	Pillai's Trace	P-value
Damselfly nymphs	Concentration	12	33	1.054	0.178
Laccophilus adults	Concentration	3	3	0.129	0.925
Dragonfly nymphs	Concentration	12	33	1.214	0.077
Laccophilus larvae	Concentration	9	36	0.582	0.486

Table 3 Rotated factor pattern of first four Principal Components (PCs) generated by PCA on square root proportions calculated from behavior data of *Laccophilus* adults exposed to IGRs. Absolute values deemed important (≥ 40) are in bold

Category	Behavior	PC1	PC2	PC3	PC4
Activity	Rest	**− 89**	− 10	11	− 32
	Walk	5	− 20	− 21	**72**
	Swim	**96**	16	− 9	2
Contact	Plant	**− 86**	− 35	− 25	5
	Wall	0	0	0	0
	Floor	**44**	**82**	− 6	− 1
	Space	**89**	18	28	− 3
Depth	Surface	− 11	− 28	23	**− 74**
	Top	− 9	**− 88**	2	− 9
	Mid	0	28	12	**88**
	Bottom	26	**85**	− 20	24
Predation	Strike	0	0	0	0
	Eat	9	− 10	**97**	− 13
	Neither	− 9	10	**− 97**	13

P-values that are less than 0.05 have been bolded

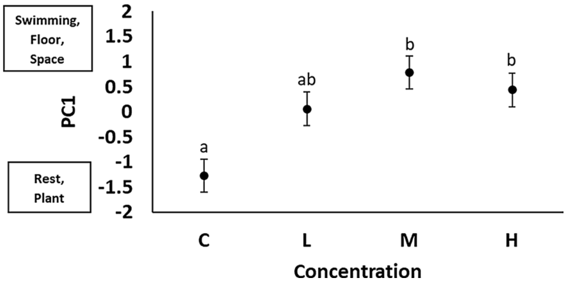

Fig. 2 Behavioral differences for *Laccophilus* adults among concentrations of growth regulator (IGR) concentrations (Control = C, Low = L, Medium = M, and High = H). Data are mean behaviors for PC1 (± 1 SE). Behaviors strongly associated with PC1 are listed along the edges of the figure (e.g., "Rest" and "Plant" are negatively associated with PC1). Groups that do not share letters are significantly different based on Tukey's post hoc adjustment

no significant effects of larvicide type ($F_{1,3}=0.077$, $P=0.784$), concentration ($F_{1,3}=1.923$, $P=0.153$), or interaction of type and concentration on the survival ($F_{1,3}=0.077$, $P=0.972$). *Laccophilus* larvae showed significant differences in one-week survival when grouped by larvicide type only ($F_{1,3}=11.524$, $P=0.0024$), with growth regulator-exposed individuals (mean = 6.93 d) living longer than SF individuals (mean = 4.19 d); no significant differences were seen in concentration ($F_{1,3}=0.706$, $P=0.558$), or the interaction of type and concentration ($F_{1,3}=0.016$, $P=0.9972$). *Laccophilus* adults showed significant differences in survival by type ($F_{1,3}=81$, $P<0.0001$) concentration ($F_{1,3}=17$, $P<0.0001$), and the interaction of type and concentration ($F_{1,3}= 17$, $P<0.0001$). Specifically, adults in all IGR concentrations and SF low and control concentrations had significantly higher survival than *Laccophilus* adults in medium and high concentrations of SFs (Fig. 3). All dead adult *Laccophilus* had died during 24 h exposures prior to behavioral trials, all individuals that survived larvicide exposure remained alive past one week.

Prey consumption with larvicide type and concentration as factors was analyzed within predator groups. For damselflies, significant differences were seen when comparing individuals grouped by concentration ($F_{1,3}=3.189$, $P=0.0418$) but not larvicide type ($F_{1,3}=0.649$, $P=0.429$) or the interaction of type and concentration ($F_{1,3}=0.647$, $P=0.592$). Individuals in the low concentration treatments ate fewer mosquito prey than those in the recommended concentration treatments, with control and high as intermediates. No significant effects on prey consumption were found for dragonflies by type ($F_{1,3}=1.724$, $P=0.202$), concentration ($F_{1,3}=0.322$, $P=0.810$), and the interaction of type and concentration ($F_{1,3}=0.667$, $P=0.581$).

Laccophilus larvae exhibited differences in prey consumption when grouped by larvicide type only ($F_{1,3}=9$, $P=0.006$), with significantly more mosquito prey being eaten by IGR-exposed individuals

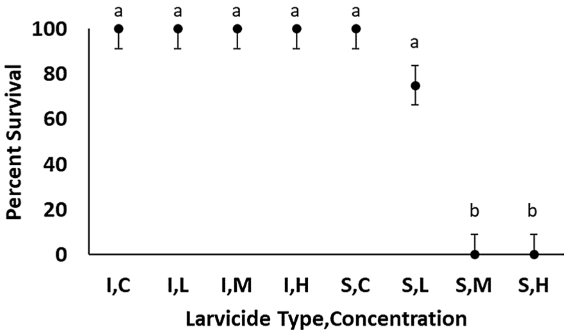

Fig. 3 Effects of larvicide type (surface film = S and insect growth regulator = I) and concentration (Control = C, Low = L, Medium = M, and High = H) on *Laccophilus* adult survival after larvicide exposure (means ± 1 SE). For survival data, all *Laccophilus* adults that were dead had died on the day of exposure. Treatments that do not share letters are significantly different based on Tukey's post hoc adjustment

than SF-exposed individuals after conducting a Tukey's post hoc analysis. For *Laccophilus* larvae, there were no significant differences in prey eaten by concentration ($F_{1,3} = 0.111$, $P = 0.953$) or the interaction of type and concentration ($F_{1,3} = 0.704$, $P = 0.560$). *Laccophilus* adults died after exposure to SFs, but we conducted this same analysis as the above predator groups, and instead using zeroes for the number of mosquitoes consumed by the dead adults, which showed significant differences in type ($F_{1,3} = 23.05$, $P < 0.0001$) and concentration alone ($F_{1,3} = 4.273$, $P = 0.015$), but not with the interaction of type and concentration ($F_{1,3} = 1.86$, $P = 0.163$). After conducting a Tukey's post hoc analysis for both larvicide type and concentration, *Laccophilus* adults exposed to IGRs ate more mosquito larvae than adults exposed to SFs. By concentration only, adults in control treatments ate more mosquitoes than high and recommended concentrations, with low as an intermediate.

Discussion

We examined behaviors of four different predators after being exposed to different concentrations of two common mosquito larvicides. We hypothesized that effects of these chemicals on predator behavior will vary by larvicide type and concentration depending on the physiology and life history traits of each group (e.g., mode of obtaining oxygen, metamorphosis type) (Merritt et al., 2008), and that there would also be differences in behavior when comparing larvicide-exposed individuals to control individuals. From our experiments, we witnessed direct lethal effects of SFs, and sublethal behavioral effects of IGRs in *Laccophilus* adults. Specifically, *Laccophilus* adults were more active in recommended and high concentration treatments of IGRs than chemical-free controls, with surface preference also varying among these treatments. *Laccophilus* adults exposed to recommended and high levels of IGRs swam in open water and were in contact with the floor of the aquarium more often and rested on plants less often than control individuals. Because increased movement of these beetle adults is associated with IGR exposure, these results may suggest an irritative effect of methoprene on adult diving beetles. Effects of irritation via insecticides have been previously documented in agricultural settings. For instance, Wiles & Jepson (1984) found increased locomotion and changes in location preference in ladybeetle adults after pyrethroid exposure (albeit pyrethroids have different modes of action than IGRs).

There were no significant behavioral effects of either larvicide type and their concentration on dragonfly nymphs, damselfly nymphs, or *Laccophilus* larvae. However, there was a lethal effect of SFs on *Laccophilus* adults. All *Laccophilus* adults exposed to high and medium concentrations of SFs died (including one in low concentration) before we could analyze their behavior, which also resulted in low statistical power (0.06) for this groups' MANOVA. The lack of a full data set and low power prevented a full comparison of behavioral effects between larvicide types in *Laccophilus* adults. However, given that SFs were effective at killing adult beetles does make examinations of their behavioral differences somewhat moot. The analysis for *Laccophilus* larvae in SFs also had a low power (0.438), and more replicates would have helped determine behavioral differences. One explanation that may clarify why there were no behavioral effects seen in damselfly and dragonfly nymphs is that odonates have biological gills (Merritt et al., 2008) and thus do not need to interact with the surface as often as adult beetles. Although dytiscid larvae use siphons to obtain atmospheric oxygen, they also have gills (Kehl, 2014), which is likely why immediate SF mortality was only seen in adult beetles.

For insect survival after exposure to larvicides, we hypothesized that survival would differ based on the larvicide type and concentration. Miura & Takahashi (1973) observed an LC_{50} of methoprene (IGR) at 2.0 ppm in *Laccophilus* adults. However, this study used a sustained release of liquid methoprene in a laboratory setting for 48–72 h. For our experiments, we exposed all predators to crushed solid methoprene briquettes, mixed in large plastic tubs for 24 h. Therefore, even though our concentrations were higher than the LC_{50} described in Miura & Takahashi (Low = 2.23 ppm, Medium = 22.23 ppm, High = 44.47 ppm), the *Laccophilus* adults in our study may have survived due to these differences. Additionally, Miura & Takahashi (1984) also found no significant changes in *Laccophilus* mortality after collecting individuals from sites that were recently dosed with IGRs. In medium and high concentrations, SFs prevented these beetles from utilizing the water's surface tension and accessing the atmosphere, which likely starved them of oxygen (Merritt et al., 2008). In contrast, survival was higher in the low concentration because the oil layer was likely thin enough for the beetles to still gain access to atmospheric oxygen, or there was not enough oil to cover the entire surface area in the tub. Although this showed that SFs are lethal to *Laccophilus* adults in an artificial setting, we were not able to determine how this larvicide affected beetle adults in the wild based on previous roadside habitat surveys (unpublished data).

Besides effects on predators directly, we also recorded the proportion of 4th instar *Culex* mosquito larvae eaten by each predator after the behavioral trials. For this, we hypothesized that there would be differences in mosquito consumption within predator and larvicide type groups based on the concentration they were exposed to. We did find significant differences in prey consumption across three of the predator groups but not dragonflies. Although there was a significant effect of concentration only (combining both SF and IGR data) for damselflies, with individuals in low concentrations eating less than the recommended concentration, concentration as a factor alone does not provide much information. We also note that the power for the above analysis was nearing 0.8 (0.796), and a larger sample size may be necessary to find significant effects on damselfly predation caused by larvicides. We were not able to identify damselfly nymphs to species, as keys for juvenile damselflies require mature nymphs, which we did not use in our behavioral studies as we did not want them to molt into adults mid-experiment/chemical treatment. Although damselflies could have been one of three species, we used specimens of equal body size in behavioral trials. Overall, these results suggest that in this laboratory assay, unless directly lethal (i.e., *Laccophilus* adults in SFs), larvicides did not affect prey consumption in dragonflies, damselflies, and *Laccophilus* adults over a 30-min period.

Laccophilus larvae exposed to IGRs ate more prey than those exposed to SFs, but this includes all concentrations grouped together, including controls. Surface films may affect the ability of *Laccophilus* larvae to hunt and process prey, but the lack of significant effects within the interaction of concentration and larvicide type do not allow us to determine if SF-exposed individuals were significantly different than control individuals. Similar results were seen when removing controls and rerunning this analysis, with the only significant effect being larvicide type ($F_{1,2} = 7.0$, $P = 0.016$), again with more mosquitoes being eaten by IGR-exposed individuals versus SFs. This suggests that more replicate trials would have needed to be conducted to determine the true effects of these larvicides on the predation ability of *Laccophilus* larvae.

It is important to understand how a chemical may change the way an organism moves or finds its food, but the few studies that have investigated behavioral responses of beneficial insects to pesticides often occur in an agricultural context (Cox & Wilson, 1984; De Jiu & Waage, 1990; Wiles & Jepson, 1994; Kunkel et al., 2001; Martinou et al., 2014; Claver et al., 2003; Sánchez-Bayo, 2021). For example, Kunkel et al. (2001) studied predatory ground beetles (Family: Carabidae) in turfgrass systems and observed weakened mobility and increased grooming activity when exposed to imidacloprid, a neonicotinoid. A study by Ahmad et al. (2003) reported lower aphid consumption, longer development times, and higher mortality in *Coccinella septempunctata* Linnaeus 1758 larvae after eating aphids exposed to neem oil. These examples exhibit how pesticides may negatively affect the mobility and prey-capturing effectiveness of beneficial arthropods. However, no such study to date has examined behavioral responses of aquatic mosquito predators to pesticide exposure. Conducting studies to learn more about the sublethal effects of mosquito larvicides on beneficial predaceous aquatic insects will provide future researchers with useful information on the capability of these organisms to be utilized in biological control.

Similar to agricultural pests, using a single chemical too frequently will cause a buildup of resistance in the local mosquito population (Connelly & Carlson, 2009). Biocontrol using natural enemies of mosquito larvae is a relatively new approach in public health entomology (breeding organisms for biocontrol is a far more prevalent practice in agricultural pest control (Leppla & LeBeck, 2021)). Mosquitofish (*Gambusia affinis* Baird & Gerard 1853) are one of the more commonly used organisms implemented in mosquito IPM practices (Kumar & Hwang, 2006). However, fish are only effective as long as the habitat is inundated with water and introducing non-native larvivorous fish to control mosquitoes has been shown to have negative impacts on endemic fish and amphibian populations (Rupp, 1996; Kats & Ferrer, 2003; Kumar & Hwang 2006; Benelli et al., 2016). In contrast, insects have evolved to take advantage of temporarily inundated habitats and avoid large vertebrate predators like fish (e.g., fully aquatic adults can fly and disperse, some taxa lay desiccation-resistant eggs, aquatic larvae mature quickly or burrow and aestivate) (Williams, 1996; Merritt et al., 2008; Strachan et al., 2015). Thus, a more effective organism to use in biological control is one that occupies the same ecological niche as mosquito larvae and can withstand the same environmental pressures. Aquatic insects like adult and juvenile beetles, hemipterans, predatory fly larvae, and odonates inhabit semi-permanent aquatic habitats and prey on aquatic Diptera like mosquitoes. Without communities of these predaceous invertebrates that regularly consume mosquito larvae, mosquito populations would likely be higher in these pools (Kumar & Hwang, 2006; Mogi, 2007; Connelly & Carlson, 2009; Shaalan & Canyon, 2009).

This study showed that recommended amounts of SFs are directly lethal to *Laccophilus* adults, likely in the same manner they are intended for killing mosquito larvae (blocking atmospheric access). Individuals in this genus of predaceous diving beetles are relatively small in body size (~ 4.5 mm length), whereas adults in the entire family of Dytiscidae range from 1 to 45 mm in length (Yee, 2014). Surface films may have different effects depending on the size of the animal. For instance, a dytiscid from a larger-sized genus may be able to break through the oil barrier and siphon air. In addition to predaceous diving beetles, there are other aquatic insects that siphon air, which include predatory Hemiptera like corixids, notonectids, *Toxorhynchites* spp. larvae, belostomatids, and other beetles whose larvae are predatory (e.g., Hydrophilidae), all of which have been shown to prey on mosquito larvae (Shaalan & Canyon, 2009). Experiments examining the direct lethality and sublethal behavioral effects should be conducted on individuals belonging to aquatic insects like these. Moreover, surfactants like SFs likely affect a much wider variety of organisms. There are many other invertebrates that rely on surface tension to move around and gather food (e.g., water striders, semi-aquatic spiders, whirligig beetles), or complete stages of their life cycle (e.g., oviposition and subsequent larval emergence). Do SFs affect how these invertebrates perform these tasks? This should be addressed to gain a better understanding of the effects that SFs have on aquatic invertebrate communities.

If sublethal effects on non-targets do occur in the wild, larvicides may significantly alter food webs because any impairment or developmental effects on an individual that reduces its ability to hunt and acquire food may also reduce that individual's effectiveness to control pests in that system (Desneux et al., 2007; Douglas et al., 2015). The preservation of known mosquito-eating non-target organisms is important for both maintaining the structure of the trophic web of the habitats they live in and the regulation of pathogen vectoring mosquito species (Kumar & Hwang, 2006; Merritt et al., 2008; Connelly & Carlson, 2009; Culler & Lamp, 2009).

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Data availability The data that support our findings are available upon request.

Declarations

Conflict of interest The authors have no conflict of interest.

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