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Drift and activity responses of black flies (*Simulium vittatum*) in the field: influences of tactile and injury-released stimuli from simulated predation

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Abstract

Prey must manage threat from many types of predators; therefore, selection should favor sensory mechanisms that allow the refinement of defensive behavior. To assess responses to tactile and chemical stimuli related to predation, we observed drift and activity of larval black flies (*Simulium vittatum*) to simulated predator contact intended to imitate benthic and drift predators as well as a combination of tactile and injury-released stimuli. In the field, larvae responded to tactile stimuli applied to the head with a higher frequency of curling and posterior abdominal segments with a higher frequency of drifting. Additionally, chemical cues from injured conspecifics followed by tactile stimuli applied to the head resulted in a higher frequency of drifting than to either cue independently and this effect was more pronounced at night. The results of our study indicate that larval black flies may utilize multiple cues to determine their antipredator and predator avoidance strategies.

Keywords

antipredator, *Simulium*, black flies, drift, chemical cues, tactile stimuli.

1. Introduction

In natural settings prey species must manage threat from a variety of predators (Cohen & Briand, 1984; Sih et al., 1998; Siddon & Witman, 2004). The general strategies employed by prey to cope with ubiquitous threat include behavioral responses for evading encounters with predators or escaping a predator after an encounter occurs (Sih, 1987; Lima & Dill, 1990; Brodie et

al., 1991). However, in many cases responses that reduce the threat from one predator may make prey more susceptible to another. Understanding the ecological and evolutionary circumstances shaping the appropriate strategy by prey in scenarios with multiple predators has been the focus of both empirical and theoretical studies (e.g., Wooster & Sih, 1995; McIntosh & Peckarsky, 1999; Ruetz & Stephens, 2003). These studies suggest that the availability of resources, type of predators, and time of day exert strong influences on antipredator decision-making.

The complex nature of predator–prey interactions and the sometimes conflicting demands that defensive behavior may place on prey may lead to context-dependent and threat-sensitive predator avoidance and antipredator strategies (Helfman, 1989; Kotler et al., 1992; Matsuda et al., 1993; Lima, 1998; Nevai et al., 2007). Predator–prey interactions among several species of aquatic insect reveal a range of defensive behavior depending on the source of the predatory threat (reviewed by Kats & Dill, 1998; Dicke & Grostal, 2001; Ferrari et al., 2010). Typically these responses to predation are manifest as altered per capita drift or changes in activity (Wooster & Sih, 1995). This pattern of behavior has been especially well documented within the Ephemeroptera and Trichoptera, which tend to respond to cues from predators and injured conspecifics by adjusting the timing of drift (Kohler & McPeck, 1989; McIntosh & Peckarsky, 1996; McIntosh et al., 2002) or decreasing overall levels of activity (Crowl & Covich, 1994; Scrimgeour et al., 1994; Huryñ & Chivers, 1999). When multiple predators are present, prey tend to favor a form or combination of behaviors that reduces the risk to the most dangerous predator perceived at a given time (McIntosh & Peckarsky, 1999).

Black flies (Diptera: Simuliidae) provide an additional opportunity to examine how the source and nature of predator threat influence the type of behavioral response exhibited by prey. Members of the Simuliidae are widely distributed, often found at high densities, and preyed upon by a variety of vertebrate and invertebrate predators (Adler et al., 2004; Malmqvist et al., 2004; Currie & Adler, 2008). Aquatic larvae tend to anchor themselves to substrates within the stream with silken strands produced from salivary glands but can move within their habitat by looping or drift (Adler et al., 1983; Reidelbach & Kiel, 1990). Once anchored, larvae use retractable labral fans to filter particulate matter from the water column and mandibles to scrape

organic material from the substrate (Hershey et al., 1996). While larval feeding rates do not vary with diel periodicity (Mulla & Lacey, 1976), drifting rates are higher at night (Adler et al., 1983). Furthermore, individuals will drift in response to predators (Simmons, 1982; Ciborowski & Craig, 1991; Malmqvist & Sackmann, 1996; Meissner et al., 2009). In addition, larval *Simulium tuberosum* and *S. vittatum* may retract their labral fans, curl the anterior portion of their body over their posterior to form the shape of a 'C', and flatten themselves against the substrate when exposed to tactile stimuli, injury-released cues and invertebrate predators (Simmons, 1982; Sullivan et al., 2011, 2013).

Previous work with *S. vittatum* larvae at our field site suggests that larval exposure to kairomones from an invertebrate predator or injury-released cue from conspecifics ends with one of three results: lack of a detectable behavioral response, curling the anterior portion of the body and flattening against the substrate, or drifting from the area (Sullivan et al., 2011, 2013). The behavioral responses appear to differ based on levels of perceived threat related to the type of predator and time of day. For instance, drifting in response to benthic predators may provide a substantial benefit because it quickly removes an individual from a risky habitat, but carries with it risks of being preyed upon by drift predators foraging in the water column (especially during daylight hours), reduced pupation and decreased foraging (Wooster & Sih, 1995; Kiel et al., 1998). Curling and flattening against the substrate would likely make larvae less conspicuous or accessible to drift predators, such as fish, but would presumably have less defensive value against a benthic predator foraging along the substrate. The ability to balance the potential benefits and costs of antipredator decision-making in the presence of multiple predator types requires an accurate evaluation of predation risk but it is uncertain which stimuli associated with predation elicit the different responses in *S. vittatum* and if those responses vary according to the diel cycle.

Our goal with this study was to examine the influence of some ecological mechanisms, namely the type of stimulus and time of day, on changes in drift and activity by larval *S. vittatum*. To that end, we conducted two experiments to determine the type of behavior performed by larval black flies under field conditions. First, we evaluated how individual larvae responded to simulated predator contact directed toward different regions of their body in an effort to mimic attack from both benthic and drift predators. Second, we observed

the effects on behavior of a combined tactile and chemical stimulus to assess the plasticity of the behavioral response to tactile cues when presented with a combination of predator stimuli. In each case, experiments were performed during the day and night to determine if prey responses exhibited diel variability.

2. Materials and methods

2.1. Description of field site

We conducted the two sets of trials to evaluate the behavioral responses by larval *S. vittatum* to tactile and chemical cues during 17–18 June 2010. All trials were conducted in Houghton Creek, NY, USA (42°26'N, 78°10'W). Houghton Creek is a first-order tributary of the Genesee River with a bedrock substrate mostly free of gravel and cobble at our field site, with depths ranging from approximately 8.0–10.0 cm at the time of our study. Larval flies were found in dense aggregates attached to the substrate with silken threads.

2.2. Drift and activity responses of larvae to tactile stimuli

We examined the behavioral responses of individual *S. vittatum* to tactile stimuli that were designed to mimic predation during day (15.00) and night (23.00) trials. We used the following three procedural treatments to evaluate behavioral responses by individual larvae: a sham treatment, the application of a tactile stimulus to the head, or the application of the stimulus to the posterior abdomen. The application of the tactile stimulus to different body regions was intended to indicate drift predators foraging from above in the water column above (e.g., fish) or benthic predators on the surface of the substrate (e.g., macroinvertebrates). The sham procedure was performed by submerging the tip of a teasing needle 30–50 mm upstream from a focal individual and slowly bringing the needle to within 2–3 mm of the head without making physical contact. This allowed us to evaluate the effects of current perturbations, investigator movement, and visual stimuli on larval behavior. The application of the tactile stimuli involved submerging the tip of a teasing needle 30–50 mm upstream from a focal individual and slowly bringing the needle into contact with the individual so that moderate pressure (using the sub-terminal portion of the needle tip to prevent damaging larval tissue) was applied to the head or the most posterior 1–2 abdominal segments. We recorded if individual larvae (1) failed to respond, (2) curled their anterior

end, or (3) drifted in response to the procedural treatments. Curling larvae withdraw the labral fans and fold the anterior portion of the body against the posterior portion of the abdomen (Simmons, 1982; Adler et al., 2004; Sullivan et al., 2011, 2013). Drifting larvae detach from the substrate and flow to a downstream region. In addition, we recorded the duration of any curling responses by larvae exposed to the different treatments. We tested 75 individual larvae to each of the procedural treatments during each of our trials (15.00 and 23.00), for a total of 450 larvae tested during this study.

During this behavioral assay, we processed larvae slowly in an upstream direction through the creek and maintained a minimum distance of at least 5 cm between test subjects to minimize the disruption of nearby individuals. In addition, our observations during the night trial were made using headlamps fitted with red filters because Simmons (1982) showed that congeneric larvae did not relocate or drift in response to red light.

2.3. Drift and activity responses of larvae to tactile and chemical stimuli

In this experiment, we sought to determine if the behavioral responses of larvae to a tactile stimulus could be modified by prior exposure to a chemical stimulus from an injured conspecific. To achieve this we exposed individual larvae to one of four different procedural treatments: a distilled water control, a tactile stimulus applied to the head as described previously, an injury-released stimulus from conspecifics, and the injury-released cue followed immediately by the application of the tactile stimulus to the head. The distilled water control would allow us to assess how local changes in current velocity or some other procedural disturbance influenced the response of focal larvae. To prepare the injury-released cue, we collected larvae to serve as stimulus donors and crushed those individuals for 1 min using a mortar and pestle. We then added enough distilled water to the pulverized tissue to achieve a concentration of 1 ml water per organism (100 ml total volume from 100 stimulus donors) and poured the solution through glass wool to remove fragments of tissue. The injury-released stimulus was used within one hour of preparation. We used 10 ml syringes to dispense the chemical treatments approximately 2.5 cm upstream from the focal individual and 1 cm above the substrate of the stream. We dispensed 1 ml of the stimulus over a period of 3–5 s in an effort to minimize disturbances associated with hydrodynamic effects. We conducted our behavioral assay in an upstream direction to avoid repeatedly exposing individual larvae to elevated levels of

the stimuli before testing and we moved slowly through the creek to minimize the disruption of larvae associated with investigator movement. We again recorded if individual larvae (1) failed to respond, (2) curled their anterior end, or (3) drifted. In addition, we recorded the duration of any curling responses by larvae exposed to the different treatments. During the course of our experiment, we exposed 75 individual larvae to each of the treatments during each of our trials (at 15.00 and 23.00) for a total of 600 larvae tested over the course of this study.

During this behavioral trial, we processed larvae slowly in an upstream direction through different portions of the creek to avoid repeatedly exposing individual larvae to the chemical stimuli and reduce the likelihood of retesting individuals from the previous night. We maintained a minimum distance of at least 5 cm between test subjects to minimize the disruption of nearby individuals not associated with the exposure to the stimulus. We again relied on headlamps fitted with red filters to perform our observations during the night trial.

2.4. Statistical analyses

For each trial, we used contingency tables to determine if there were differences in the frequency of larvae that failed to respond, curled, or drifted when exposed to the procedural treatments during day (15.00) and night (23.00) trials. For our first experiment, we utilized 3×3 tables to evaluate the responses of flies to each of three tactile stimuli (sham, stimulus directed to the head, or stimulus directed to the posterior abdomen), and for our second experiment, we used 3×4 tables to assess the responses of larvae to each of four tactile and chemical stimuli (water, tactile stimulus to the head, injury-released stimulus, or the combination of injury-released and tactile stimuli). We then calculated adjusted residual values to determine which cell(s) within the table were major contributors to the omnibus chi-square value (Sheskin, 2007). The adjusted residuals are reported as z scores and, as a result, those with an absolute value equal to, or greater than, the critical two-tailed value ($z_{0.05} = 1.96$), is significant at $\alpha = 0.05$ level. A cell with a significant residual allows for the rejection of the null hypothesis that there is no difference between the observed and expected cell frequencies had there been no association between larval behavior and procedure. The sign of the residual value indicates whether the observed frequency of that cell is above or below the expected frequency. Lastly, we used a series of 2×3 contingency tables to

determine if there were diel differences in the frequency of larva that failed to respond, curled, or drifted in response to each of the treatments.

In addition to analyzing the frequency data obtained from our trials, we compared the duration of the curling responses (of those individuals that curled) among the different treatments using Kruskal–Wallis one-way analyses of variance (ANOVA) by ranks for each set of trials. We conducted post-hoc multiple comparisons when the main effect was significant and adjusted the experimentwise error rate using sequential Bonferroni tests using the Dunn–Šidák method (Sokal & Rohlf, 1995). We used the nonparametric statistical analysis because our data failed to meet the assumptions of normality and homoscedasticity.

3. Results

3.1. *Drift and activity responses of larvae to tactile stimuli*

The results of the daytime trial (15.00) indicate that the type of behavior exhibited by larval black flies is not homogenous with respect to the location of the application of the tactile stimulus (chi-square test: $\chi^2 = 224.31$, $p < 0.001$, Table 1). In general, the frequency of larvae that responded (either with curling or drifting) to the sham procedure was significantly less than the expected value, while those that received tactile pressure to the head were significantly more likely to curl, and those that received the stimulus to the posterior abdomen were significantly more likely to drift. In addition, the amount of time that larval black flies remained curled differed significantly among the treatments during daytime trials (Kruskal–Wallis ANOVA: $H_{2,83} = 18.97$, $p < 0.001$, Figure 1a). Post-hoc comparisons indicate that larvae curled for a significantly longer period when receiving the tactile stimulus to the abdomen versus the head ($p = 0.019$) or sham treatment ($p < 0.001$). Furthermore, larvae curled for a significantly longer period when receiving the tactile stimulus to the head versus the sham ($p < 0.001$).

The data from the nighttime trials (23.00) also indicate that the type of behavior exhibited by larvae is not homogenous with respect to the type of tactile stimulus to which they are exposed (chi-square test: $\chi^2 = 235.92$, $p < 0.001$, Table 1). Responses by larvae followed the same general pattern as seen during the earlier trials with larvae exposed to the sham procedure least likely to respond behaviorally, those receiving tactile stimulus to the

Table 1.

Frequencies of larval black flies curling, drifting and not responding to a sham treatment or a tactile stimulus directed to the head or abdomen.

Houghton Creek	No. curling	No. drifting	No. not responding
Day (15.00)			
Sham	6 (-4.16)	0 (-4.12)	69 (7.12)
Head	53 (4.82)	0 (-4.12)	22 (-1.51)
Abdomen	24 (-0.76)	51 (8.25)	0 (-5.48)
Night (23.00)			
Sham	1 (-3.92)	0 (-4.58)	74 (6.17)
Head	38 (4.96)	1 (-4.36)	36 (-0.11)
Abdomen	13 (-1.03)	62 (8.95)	0 (-6.06)

Adjusted residual values are in parentheses. Residual values with absolute values equal to, or greater than, the critical two-tailed value $z_{0.05} = 1.96$ indicates a significant departure from expected. The sign of the residual value indicates whether the observed frequency is above or below the expected frequency.

head most likely to curl, and those receiving pressure to the posterior abdomen most likely to drift. In addition, the amount of time that larval black flies remained curled differed significantly among the treatments during nighttime trials (Kruskal–Wallis ANOVA: $H_{2,52} = 6.87$, $p = 0.032$, Figure 1b). Post-hoc comparisons indicate that larvae curled for a significantly longer period when receiving the tactile stimulus to the abdomen versus the head ($p = 0.002$) or sham treatment ($p < 0.001$). Furthermore, larvae curled for a significantly longer period when receiving the tactile stimulus to the head versus the sham ($p < 0.001$).

Lastly, we found that the frequencies of larvae that failed to respond, curled, or drifted for each stimulus did not differ between day and night trials for the sham procedure ($\chi^2 = 3.75$, $p = 0.153$) or tactile stimulus to the abdomen ($\chi^2 = 4.34$, $p = 0.057$), but did show a significant difference for the tactile stimulus directed to the head ($\chi^2 = 6.85$, $p = 0.033$).

3.2. Drift and activity responses of larvae to tactile and chemical stimuli

The results of the daytime trial (15.00) show that the type of behavior exhibited by larval black flies is not homogenous with respect to the chemical and tactile stimuli to which they are exposed (chi-square test: $\chi^2 = 67.71$, $p < 0.001$, Table 2). In summary, the frequency of animals that did not respond (either with curling or drifting) was significantly higher than expected

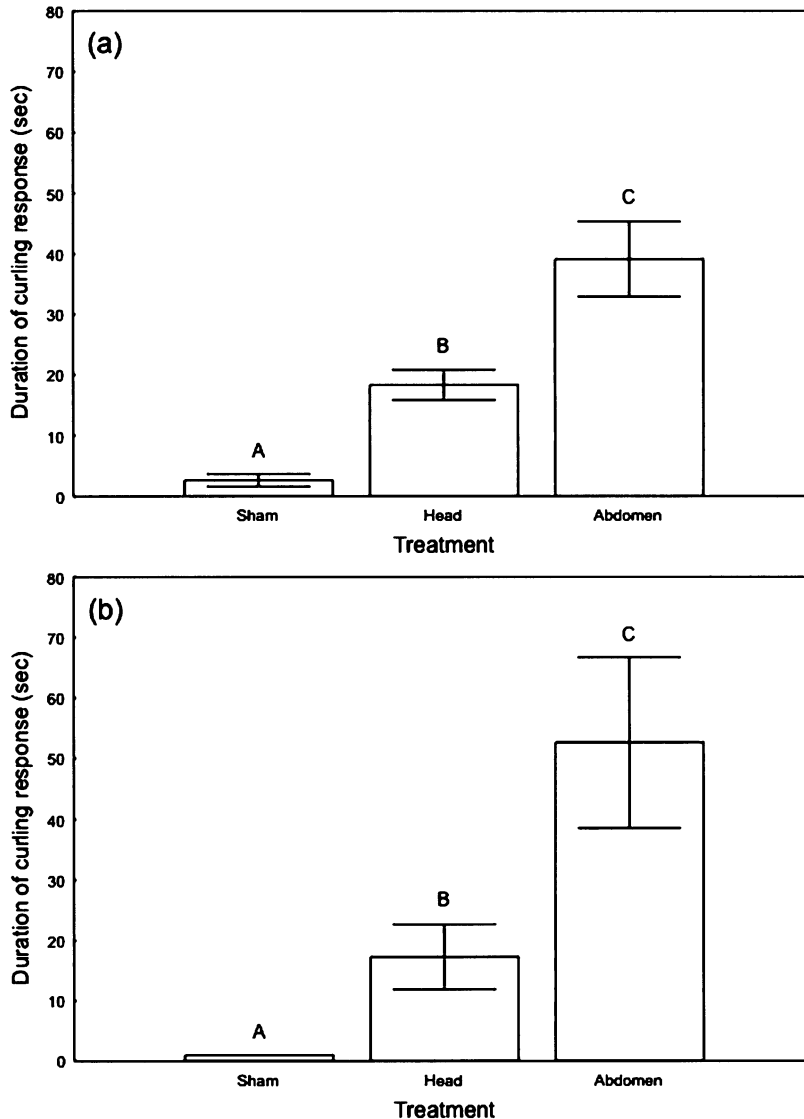


Figure 1. Mean duration \pm SEM of the curling behavior of larval black flies during (a) day trials (15.00) and (b) night trials (23.00). Larvae curled in response to a sham treatment (day: $N = 6$; night: $N = 1$) or a tactile stimulus directed to the head (day: $N = 53$; night: $N = 38$) or abdomen (day: $N = 24$; night: $N = 13$). Bars capped with different letters indicate significant differences among treatments at the $\alpha = 0.017$ level obtained via the Dunn-Šidák correction.

Table 2.

Frequencies of larval black flies curling, drifting and not responding to water, a tactile stimulus directed to the head, injury-released stimulus or a combination of the tactile stimulus directed to the head and injury-released stimulus.

Houghton Creek	No. curling	No. drifting	No. not responding
Day (15.00)			
Water	8 (-4.49)	0 (-1.32)	67 (4.48)
Head	51 (2.86)	1 (-0.57)	23 (-2.56)
Injured	43 (1.50)	1 (-0.57)	31 (-1.28)
Head + Injured	35 (0.13)	5 (2.46)	35 (-0.64)
Night (23.00)			
Water	12 (-3.02)	2 (-3.25)	61 (4.94)
Head	43 (2.83)	7 (-1.92)	25 (-1.35)
Injured	36 (1.51)	7 (-1.92)	32 (-0.13)
Head + Injured	20 (-1.51)	41 (7.09)	14 (-3.28)

Adjusted residual values are in parentheses. Residual values with absolute values equal to, or greater than, the critical two-tailed value $z_{0.05} = 1.96$ indicates a significant departure from expected. The sign of the residual value indicates whether the observed frequency is above or below the expected frequency.

when exposed to the water control, the frequency of larvae that curled was significantly higher than expected when they experienced the tactile stimulus to the head, and the frequency of animals that drifted was significantly higher than expected when they experienced the combined stimulus (injury-released cue followed by the tactile stimulus applied to the head). In addition, the amount of time that larval black flies remained curled differed significantly among the treatments during daytime trials (Kruskal–Wallis ANOVA: $H_{3,137} = 13.66$, $p < 0.003$, Figure 2a). Post-hoc comparisons indicate that larvae spent significantly less time curled when exposed to the water control versus the application of the stimulus to the head ($p < 0.001$), the injury-released cue ($p < 0.001$) and the combined chemical and tactile treatment ($p < 0.001$). The time spent curled did not differ among the non-control treatments ($p = 0.999$ in each comparison).

The data from the nighttime trials (23.00) also indicate that the type of behavior exhibited by larvae is not homogenous with respect to the type of chemical and tactile stimulus to which they are exposed (chi-square test: $\chi^2 = 126.83$, $p < 0.001$, Table 2). Responses by larvae followed the same general pattern as the earlier trial as larvae that did not respond was significantly higher than expected when exposed to the water control, the frequency

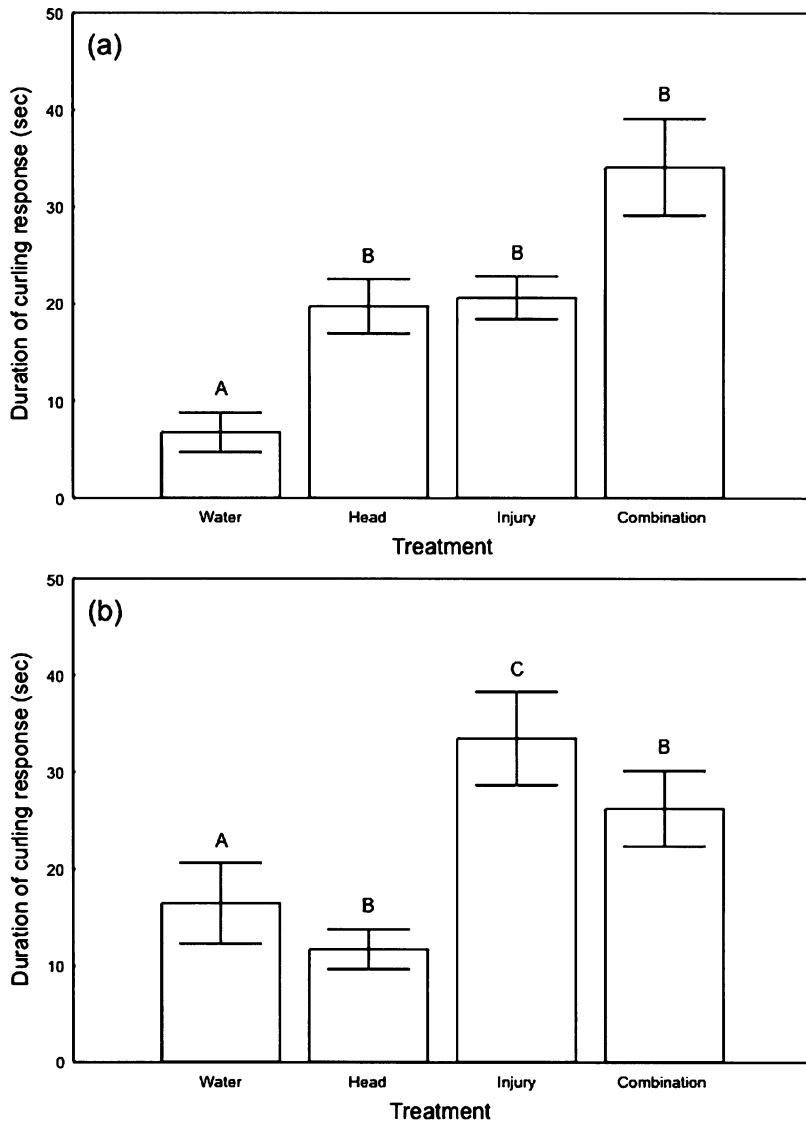


Figure 2. Mean duration \pm SEM of the curling behavior of larval black flies during (a) day trials (15.00) and (b) night trials (23.00). Larvae curled in response to water (day: $N = 8$; night: $N = 12$), a tactile stimulus directed to the head (day: $N = 51$; night: $N = 43$), injury-released stimulus (day: $N = 43$; night: $N = 36$) or a combination of the tactile stimulus directed to the head followed by injury-released stimulus (day: $N = 35$; night: $N = 20$). Bars capped with different letters indicate significant differences among treatments at the $\alpha = 0.009$ level obtained via the Dunn-Šidák correction.

of larvae that curled was significantly higher than expected when they experienced the tactile stimulus to the head, and the frequency of animals that drifted was significantly higher than expected when they experienced the combination of chemical and tactile stimulus. The amount of time that larval black flies remained curled also differed significantly among the treatments (Kruskal–Wallis ANOVA: $H_{3,111} = 21.76$, $p < 0.001$, Figure 2b). Post-hoc comparisons indicate that larvae spent significantly less time curled when exposed to the control versus the injury-released cue ($p < 0.001$) and the combined chemical and tactile treatment ($p < 0.001$), but significantly more time curled compared to the application of the tactile stimulus to the head ($p < 0.001$). In addition, larvae exposed to the combination of chemical and tactile stimuli spent significantly less time curled versus injury-released cue ($p < 0.001$) but did not significantly differ from the application of the stimulus to the head ($p = 0.999$). Lastly, individuals that were exposed to the injury-released cue curled for longer than those receiving the stimulus to the head ($p = 0.023$).

Lastly, we found that the proportions of larvae that failed to respond, curled, or drifted for each stimulus did not differ between day and night trials for the water control ($\chi^2 = 3.08$, $p = 0.214$), tactile stimulus to the head ($\chi^2 = 5.26$, $p = 0.072$), or injury-released stimulus ($\chi^2 = 5.14$, $p = 0.077$) but did show a significant difference for the combination of chemical and tactile stimuli ($\chi^2 = 41.26$, $p < 0.001$).

4. Discussion

In their review of studies investigating the impact of predators on population densities of stream-dwelling prey Wooster & Sih (1995) note that predatory invertebrates and vertebrates often exert different effects on individuals. Predatory invertebrates tend to alter the rates of prey drift whereas predatory vertebrates elicit changes in levels of prey activity. These responses may be adaptive given that invertebrates often act as benthic predators whose attacks are near the substrate (Wiley & Kohler, 1981; Peckarsky & Wilcox, 1989). Additional work with benthic feeding fishes shows that the impact of these predators on prey may be similar (Culp et al., 1991; Huhta et al., 2000). Conversely, other vertebrates tend to be drift predators that generally rely on visual cues to pluck prey from the water column (Allan, 1978; McIntosh et al., 2002). In the former instance, drifting would quickly remove an

individual from a risky habitat; in the latter, flattening against the substrate or reducing activity would likely make larvae less conspicuous or vulnerable. The results of our first experiment are consistent with these hypotheses, albeit with stimuli associated with simulated predation rather than actual predators. Our application of the tactile stimulus to the posterior abdominal segments was intended to simulate a predator attack from the substrate (e.g., a macroinvertebrate foraging along the streambed) whereas the tactile stimulus to the head was intended to simulate a predator attack from above (e.g., a fish foraging in the water column). Although this procedure was, in some ways, a crude attempt to simulate different predators based largely on the direction of the predator stimulus, the pattern of responses is consistent with the above predictions: the tactile stimulus to the head region resulted in a higher frequency of curling among larval *Simulium*, while the stimulus directed to the posterior abdominal segments resulted in a higher frequency of drifting. In addition to the consistencies with the predictions of Wooster & Sih (1995), the specialized behavior in our simulation is consistent with observations of drift in response to predators (e.g., Ciborowski & Craig, 1991; Meissner et al., 2009), rather than movement within their habitat by looping (Reidelbach & Kiel, 1990).

The responses observed by larval *Simulium* to predators (Wiley & Kohler, 1981; Simmons, 1982; Sullivan et al., 2013) may be problematic in habitats with both benthic and drift predators. Our data suggest that larvae are sensitive to different stimuli associated with predation and may modify defensive responses accordingly, presumably according to the level of risk perceived. Here we found that the exposure to injury-released cue from conspecifics combined with the application of the tactile stimulus to the head significantly increased the frequency of larval drift relative to other treatments during both daytime and nighttime trials. We should note that the current study lacks an additional control that combines a water stimulus prior to the application of the tactile stimulus to the head so we cannot say unequivocally that the response to the combined stimulus is not related to a general disturbance from the injection and not a response to the detection of the injury-released stimulus. However, our data as well as those of previous studies (Sullivan et al., 2011, 2013), show a minimal impact of the water control on either duration of curling or drift. In addition, these results are consistent with studies of mayflies that show more pronounced responses to the combined contact and chemical cues from stoneflies (Williams, 1987; Ode & Wissinger, 1993).

How the chemical stimulus alters the decision-making in larval black flies is uncertain but could serve as a type of warning signal that sensitizes prey to additional or impending stimuli (e.g., Ode & Wissinger, 1993). Interestingly, stimuli from damaged conspecifics could indicate the presence of either benthic or drift predator foraging in the immediate vicinity as either could inflict tissue damage on focal or nearby individuals. Thus, chemical cues could provide information regarding predator proximity that could inform decisions related to curl or drift.

Our data also suggest a degree of diel variability related to the frequency of larval drift. In our first experiment we found that a higher than expected frequency of larvae drifted when the tactile stimulus was applied to the abdomen during both day and night trials. The difference in the frequencies that did not respond, drifted or curled was marginally significant between day and night trials in is treatment group ($p = 0.057$) due to an increased number of drifting larvae at night. The relatively high rate of drift by larvae 'attacked' at the posterior abdomen during the day when drift predators are presumably active could be related to an imminent threat from the substrate versus a potential threat in the water column. We also found that the responses of larvae receiving the tactile stimulus to the head to differ between day and night, in this case driven by a higher frequency of larvae not responding. There is no obvious explanation for this particular result, although it could represent a response by flies that incorporated information related to the cost of leaving a suitable foraging area with the likelihood that a drift predator was actually foraging at that time of night. In our second experiment we found that a higher than expected frequency of larvae drifted when exposed to the combination stimulus during both day and night trials as well as a significant difference in the frequencies that did not respond, drifted or curled in this treatment group between the day and night. The increased rate of drift in response to the combined treatment at night was expected as previous work shows that nocturnal drift reduces the risk of predation by drift predators (Elliot, 1969; Malmqvist & Sjöström, 1987; Douglas et al., 1994; McIntosh et al., 2002) and influences larval *Simulium* responses to chemical stimuli (Sullivan et al., 2013). This supports our hypothesis that these two stimuli administered concurrently is indicative of elevated predation threat.

The analysis of activity changes in response to simulated predation and injury-released cues, as measured by duration of curling, suggests that larval

responses are not quite as straightforward as the drift data alone would indicate and highlights the importance of using multiple response variables when examining antipredator behavior. In our first experiment, we found that larvae curled for a significantly greater length of time when they were attacked on the lower abdomen than head. This ranking certainly matches our assumptions about the level of risk associated with the different tactile stimuli but begs the question: why do any larvae curl and not drift when exposed to that level of threat? The pattern of response exhibited by larvae 'attacked' in the posterior abdomen could be an attempt to reduce the costs associated with drifting (e.g., leaving a suitable foraging patch, encountering drift predators) and may be linked to recent foraging success or experience with predators (e.g., Whitham & Mathis, 2000; Verdolin, 2006; Roberts & Liebgold, 2008). The curling data from our second experiment that observed responses of larvae to chemical and tactile cues shed additional light on our questions about threat assessment and antipredator decision-making. In the early trials (15.00) the duration of curling is significantly higher for all treatments (versus the control), but a different pattern emerges at night. One interpretation of the duration of curling from the night portion of the trial (23.00) is that the injury-released cue is the most threatening stimulus to which larvae were exposed. However, this takes into account only one type of response. When both forms of behavior are taken into consideration, we suggest that combination of tactile and chemical stimuli represents the greatest threat as most larvae drift (approx. 55%), whereas when exposed to the injury-released cue alone only approx. 9% drift. In this case, it seems that the best strategy for exposure to the injury-released cue is to reduce activity (i.e., curl) and evaluate subsequent stimuli. It may also be interesting to note that curling in response to the tactile stimulus directed at the head is relatively short at night, perhaps because drift predators are less active at night at this field site.

Tactile and chemical stimuli relay important information to aquatic prey about the type, proximity and motivation of predators and may lead to the manifestation of predator-specific responses which may increase the probability of survival. Furthermore, the most effective response to a predator may exhibit some degree of diel variability, especially among aquatic insects. The results of our study demonstrate that black fly larvae utilize multiple cues to determine their antipredator and predator avoidance responses, and defensive behavior in this species hinges on a number of decisions about drifting, curling, and even the duration of curling that is likely linked to the internal

state of the individual. Subsequent studies of antipredator decision-making by larval *S. vittatum* should include how factors such as predator type, experience with various predators, and resource availability influence the strategy utilized by larvae to balance the costs and benefits associated with predator-specific responses (Lima & Dill, 1990).

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