Behavioral avoidance of injured conspecific and predatory chemical stimuli by larvae of the aquatic caddisfly *Hesperophylax occidentalis*

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Abstract: Prey animals often encounter situations that hinder their ability to conduct normal fitness-enhancing behaviors. Mating and foraging are frequently interrupted by predator vigilance and avoidance, and antipredator behavior. Many caddisfly larvae build protective cases that are carried with them throughout the aquatic life cycle. However, they are still vulnerable to predation, yet it is unknown the extent caddisflies use chemical cues for predator recognition and avoidance. We exposed larval caddisfly *Hesperophylax occidentalis* (Banks, 1908) to predatory, conspecific, and heterospecific chemical cues to determine if caddisfly larvae can use chemical stimuli alone for predator recognition and avoidance. Exposure to predator and injured conspecific chemicals elicited significant decreases in activity, while exposure to injured and uninjured heterospecific chemicals yielded no significant change in activity. The extended latency to move following exposure to predator kairomones indicates larval caddisflies utilize chemical cues for predator recognition and avoidance, and a similar decrease in movement associated with exposure to injured conspecifics suggests the presence of a chemical alarm cue.

Résumé : Les proies animales font souvent face à des situations qui les empêchent d'avoir les comportements qui normalement augmentent leur fitness. L'accouplement et la recherche de nourriture sont souvent interrompus par la vigilance et l'évitement des prédateurs et par les comportements antiprédateurs. Plusieurs larves de trichoptères construisent des fourreaux protecteurs qu'elles transportent avec elles pendant toute la partie aquatique de leur cycle biologique. Elles demeurent, néanmoins, vulnérables à la prédation; cependant, on ne sait pas dans quelle mesure elles utilisent des signaux chimiques pour reconnaître et éviter leurs prédateurs. Nous avons exposé des larves de trichoptères *Hesperophylax occidentalis* (Banks, 1908) à des signaux chimiques de prédateurs, d'insectes de même espèce et d'organismes d'espèces différentes pour déterminer si les larves de trichoptères peuvent à partir des seuls signaux chimiques reconnaître et éviter leurs prédateurs. Une exposition à des produits chimiques provenant de prédateurs ou de larves blessées de leur propre espèce provoque une diminution significative de l'activité, alors qu'une exposition à des produits chimiques d'organismes hétérospécifiques blessés ou sains n'entraîne aucun changement significatif de l'activité. La période prolongée de latence avant la reprise du mouvement après une exposition aux kairomones de prédateurs indique que les larves de trichoptères utilisent les signaux chimiques pour reconnaître et éviter leurs prédateurs; une diminution similaire du mouvement associée à une exposition à des larves blessées de leur propre espèce laisse croire à l'existence d'un signal chimique de détresse.

[Traduit par la Rédaction]

Introduction

Prey animals must often balance activities such as foraging and reproduction with predator avoidance (Lima and Dill 1990; Lima 1998). Predator avoidance and corresponding antipredator defenses are inherently costly because prey must forgo activities that would otherwise enhance fitness (Lima and Dill 1990; Lima 1998). Therefore, prey should be able to accurately measure the current level of predation risk and react accordingly to minimize the costs of this trade-off (see reviews in Lima and Dill 1990; Lima 1998).

Prey use a variety of behaviors to measure and respond to the threat of predation. In aquatic ecosystems where predators are often cryptic and sedimentation or dense vegetation frequently reduces visual acuity, chemical cues can provide reliable information in the absence of all other stimuli (see review in Kats and Dill 1998). Specifically, aquatic prey often use chemical cues such as kairomones and alarm cues to assess predation risk and decrease their probability of predation. Kairomones are chemical stimuli that elicit beneficial behavioral changes in heterospecific receivers (Brown et al. 1970). When exposed to predator kairomones, aquatic organisms often reduce activity, increase drift, increase shelter use, or a host of other predator avoidance behaviors (see reviews in Wooster and Sih 1995; Kats and Dill 1998). Alternatively, alarm cues are chemical stimuli released by injured individuals that elicit similar responses as predator kairomones when received by nearby conspecifics (Smith 1992; Chivers and Smith 1998). Alarm cues function to warn conspecifics of immediate danger (Smith 1977; Smith 1992; Mathis and Smith 1993*a*; Chivers and Smith 1998) or attract additional predators that disrupt the predation event (Mathis et al. 1995; Chivers et al. 1996*a*; Chivers and Smith 1998). More recently, Chivers et al. (2007) demonstrated

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that alarm cues may also provide protection against pathogens, parasites, and ultraviolet-B radiation (at least in fishes). Some species have evolved to respond to alarm cues from sympatric heterospecifics that occupy the same prey guild (Mathis and Smith 1993*b*; Brown et al. 2001; Mirza and Chivers 2001*a*). This cross-species response is most likely to occur in species occupying the same prey guild and microhabitat, and having the same predators. Under these conditions, responses to heterospecific alarm cues should provide similar survival benefits as a response to conspecific alarm cues. In fact, several studies have documented the increased survival benefits experienced by organisms responding to chemical alarm cues. For example, Wisenden et al. (1999) demonstrated that the amphipod *Gammarus minus* Say, 1818 survives encounters with green sunfish (*Lepomis cyanellus* Rafinesque, 1819) longer when they are simultaneously exposed to conspecific alarm cues. Similar fitness benefits experienced by prey responding to alarm cues have also been found in amphibians and fishes (Hews 1988; Mathis and Smith 1993*a*; Chivers et al. 2002).

Invertebrates use chemical cues extensively, yet little is known about the use of chemical cues in one of the largest orders of aquatic insects. Caddisflies (Trichoptera) are one of the most widely distributed aquatic insect orders with 1400 recognized species in the United States and Canada alone (Merritt et al. 2008). Except for a few species, caddisfly larvae (henceforth caddisflies) are entirely aquatic and occupy a great diversity of freshwater habitats (Merritt et al. 2008). Many species of caddisflies construct portable cases that function as defense against some predators (Otto and Svensson 1980; Johansson 1991; Nislow and Molles 1993; Wissinger et al. 2006) and may increase the efficiency of respiration (Williams et al. 1987).

Even with defensive cases, caddisflies are still vulnerable to a variety of vertebrate and invertebrate predators, and previous work on caddisfly–predator interactions has primarily focused on the direct influence of predation (e.g., Otto and Svensson 1980; Kohler and McPeek 1989; Johansson 1991; Johansson and Nilsson 1992; Nislow and Molles 1993; Johansson and Englund 1995; Otto 2000; Wissinger et al. 2006). Nevertheless, some empirical evidence exists to suggest caddisflies use chemical cues to detect predators. Kuhara et al. (2001) found that caddisflies exposed to sculpin *Cottus nozawae* Snyder, 1911 stimuli reduced activity and food intake during the most risky time of day. However, the experimental design in that study permitted both chemical and hydrodynamic cues. Malmqvist (1992) examined caddisfly activity in response to chemical cues from predators, but got conflicting results between the two caddisfly species tested. In addition, the study was weakly replicated and statistical analyses were not performed on the caddisfly data. Pestana et al. (2009) exposed caddisflies to predatory chemical cues and a pesticide, and found respiration rates decreased with pesticide exposure but increased when exposed to predators. Experiments by Boyero et al. (2006) eliminated all but chemical cues and showed that larvae adjusted their selection of case type according to the specific predatory threat. Although these studies suggest caddisflies utilize chemical cues, the extent to which chemical cues are used for immediate predator recognition and predator avoidance behavior is unknown. In addition, it is unknown if caddisflies possess chemical alarm cues that would further enhance their ability to detect and avoid predators.

To determine if caddisflies utilize chemical cues in immediate predator avoidance behavior and to determine if caddisflies possess a chemical alarm cue, we examined the behavioral response of the caddisfly *Hesperophylax occidentalis* (Banks, 1908) to chemical stimuli from (1) a potential predator (rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792)), (2) injured conspecifics, (3) injured heterospecifics (the amphipod *Gammarus lacustris* G.O. Sars, 1863; henceforth amphipod) known to possess chemical alarm cues (Wudkevich et al. 1997), (4) uninjured heterospecifics (*G. lacustris*), and (5) a blank control.

Materials and methods

Animal collection and maintenance

Caddisflies used in this study were collected on 29 October and 19 November 2008 from a single pond (41.5709°N, 111.8485°W) in Paradise, Utah. Caddisflies were collected by dip net and placed in plastic buckets for transport to Utah State University. Larvae were then transferred to a 37 L glass aquarium containing 15 L of tap water filtered by reverse osmosis (henceforth tap water). Larvae were transferred to the holding aquarium by draining most of the water in the buckets and dumping all larvae into the holding aquarium. This method was used to transfer test larvae to prevent acclimation to the simulated predation event (see below). The aquarium was kept in an environmental chamber at 16.5 °C and a 12 h light : 12 h dark cycle. Larvae were fed ad libitum on Timothy hay pellets (Bunny Basics/T, Oxbow Pet Products, Murdock, Nebraska, USA).

While collecting caddisflies, we sampled (hook and line) and visually identified the presence of rainbow trout in the collection pond. We therefore assume that the caddisflies used in this study were not naïve, but were experienced with trout predators.

Amphipods were collected from the same pond as the caddisflies on 19 November 2008. The amphipods and a small amount of detritus (food) were transferred to a 37 L aquarium with 15 L of tap water and maintained in the same environmental chamber as caddisflies.

Stimulus preparation

Hesperophylax occidentalis larvae were tested with five different stimuli: (1) blank tap-water control ($N = 20$), (2) uninjured amphipods ($N = 20$), (3) injured amphipods ($N = 20$), (4) injured caddisfly larvae ($N = 20$), and (5) rainbow trout ($N = 20$; Table 1). All stimuli (except rainbow trout stimulus, which was frozen following preparation) were prepared with tap water maintained at 16.5 °C with an aerator and used immediately following preparation. The rainbow trout treatment was prepared by catching four rainbow trout (fork length range 24.0–25.5 cm) by hook and line and placing two each into 39 L containers with 20 L of tap water. The trout were removed from the container 1 h after introduction and immediately returned to the pond of capture after stimulus collection. The stimuli from the two containers were homogenized and frozen at –80 °C in 2 L aliquots after collection. Although rainbow trout were sampled from a separate pond as the caddisflies, caddisflies

Table 1. Treatments tested in experiments 1 and 2, including sample size, mean mass (g) of larval caddisfly *Hesperophylax occidentalis* in each treatment, mean latency to emerge (s), and mean latency to move (s).

			Latency (s)	
Treatment	Sample size	Mass (g)	Emerge	Move
Experiment 1				
Blank control	20	0.157±0.02	23.00±5.34	55.10±11.41
Rainbow trout	20	0.173±0.02	102.50±14.16	117.40±15.60
Experiment 2				
Blank control	20	0.163±0.06	42.70±8.97	49.15±8.36
Uninjured amphipod	20	0.169±0.02	32.20±6.73	45.00±7.70
Injured amphipod	20	0.174±0.02	56.10±10.55	62.40±11.35
Injured caddisfly	20	0.177±0.07	95.35±17.00	101.10±16.73

Note: Values are mean ± SE.

were present in the pond with stimulus trout. We thus cannot rule out the presence of dietary cues in the trout stimulus.

Immediately prior to testing, the trout stimulus was thawed and maintained at 16.5 °C with an aerator. The injured caddisfly treatment was prepared by crushing 20 caddisflies in 800 mL of tap water with a mortar and pestle. The caddisflies were starved for 7 days prior to stimulus collection to remove any food cues. Stimulus from injured amphipods was prepared by grinding 30 individual amphipods, starved for 72 h, in 800 mL of tap water using a mortar and pestle. The uninjured amphipod stimulus was prepared by placing 30 individuals, without food, in a 1 L plastic container with 800 mL of tap water for 72 h prior to experimentation. All solutions were filtered through 100% polyester (Poly-Fil, Fairfield Processing Corp., Danbury, Conneticut, USA) to remove large solid particles. Stimuli were coded prior to experimentation so that the observer was blind to treatment selection.

Behavioral bioassays

Two experiments were performed between 1300 and 2000 on 5 and 26 November 2008. In experiment 1, caddisflies were exposed to rainbow trout stimuli and a blank control to determine whether larvae utilize predatory chemical cues in predator recognition and avoidance. These trials were conducted in a plastic container (9 cm × 9 cm, 6 cm height) with 140 mL of stimulus. In experiment 2, caddisflies were exposed to a blank control, as well as stimulus water from uninjured amphipods, injured amphipods, and injured conspecifics. Experiment 2 was conducted to determine the presence of chemical alarm cues in caddisflies, as well as whether cross-species responses to heterospecific alarm cues occur in this system. To ensure a strong concentration of stimuli and to minimize the unnecessary sacrifice of caddisflies and amphipods, these trials were conducted in 35 mm × 10 mm round plastic dishes with 10 mL of stimulus. All trials were conducted inside an environmental chamber at 16.5 °C. Prior to each trial, the test container was rinsed with tap water and a randomly chosen treatment was poured into the test container. A caddisfly was selected from the holding aquarium, grasped between the thumb and forefinger, and held for 3 s. The larva was then dropped into the center of the test container from 1 cm above the stimulus water and the trial began. This method simulates a predatory attack (Johansson 1991; Johansson and Englund 1995) and is similar to that used in Lefcort et al. (2000); larvae retreated into their protective case when grasped. After dropping the larva into the test container, the latency to emerge from the case (time between start of trial and emergence of the head, but not legs) and latency to begin moving (time between start of trial and emergence and movement of the legs) were recorded. Upon completion of testing, larvae were weighed to the nearest 0.01 g and maintained in a holding aquarium. There was no difference in the mass of larvae in the six treatments (Kruskal–Wallis test, $H = 7.80$, $P = 0.10$). Individual larvae were tested only once.

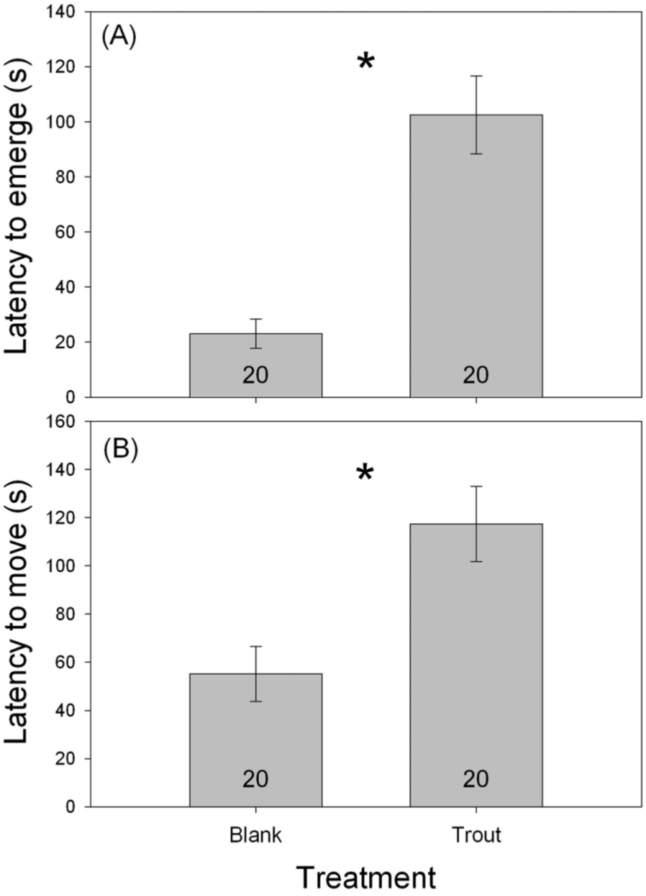
Statistical analysis

Data from experiment 1 were analyzed with a Mann–Whitney rank-sum test (SigmaPlot version 15; Systat Software, Inc., San Jose, California, USA). Data from experiment 2 were analyzed by Kruskal–Wallis tests (Minitab version 15; Minitab Inc., State College, Pennsylvania, USA) followed by nonparametric multiple comparisons (WINKS SDA version 6.0; Texasoft, Cedar Hill, Texas, USA). We performed linear regressions (SigmaPlot version 11; Systat Software, Inc., San Jose, California, USA) to test for differences in response time between different-sized caddisflies.

Results

Caddisflies significantly increased their time to emerge from cases (Mann–Whitney U test, $U = 53$, $P < 0.001$; Fig. 1A) and begin moving (Mann–Whitney U test, $U = 96.5$, $P = 0.005$; Fig. 1B) when exposed to chemical stimuli from rainbow trout compared with the blank control (experiment 1, Table 1). In experiment 2, there were significant differences among larval responses to the four stimuli for both response variables (latency to emerge: Kruskal–Wallis test, $H = 13.37$, $P = 0.004$; Fig. 2A; latency to move: Kruskal–Wallis test, $H = 10.04$, $P = 0.018$; Fig. 2B). When exposed to chemicals released by injured conspecifics, caddisflies also showed a significant increase in latency to emerge (nonparametric multiple comparisons: $Q = 2.73$, $P < 0.02$) and a significant increase in latency to begin moving (nonparametric multiple comparisons: $Q = 2.55$, $P < 0.05$) compared with the blank control (Figs. 2A, 2B; ex-

Fig. 1. Responses of larval caddisfly *Hesperophylax occidentalis* exposed to chemical stimuli from a predator (rainbow trout, *Oncorhynchus mykiss*) and a blank control. (A) The latency (mean ± 1 SE) for the heads of caddisflies to emerge from the cases. Mann–Whitney *U* test: *, $P < 0.001$, (B) The latency (mean ± 1 SE) for caddisflies to emerge from cases and begin moving. Mann–Whitney *U* test: *, $P = 0.005$. Sample sizes are given within the bars.

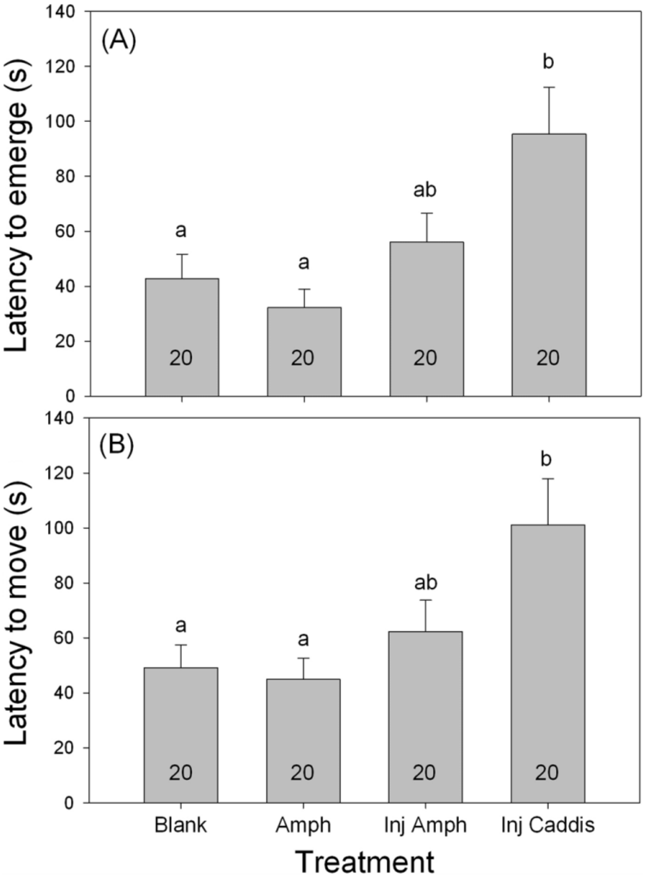


periment 2, Table 1). When larvae were exposed to chemical stimuli from injured amphipods, they exhibited responses intermediate of the blank and trout or injured caddisfly treatments; however, this response was not significantly different from the blank control for either response variable (all *P* values >0.50; Figs. 2A, 2B; experiment 2, Table 1). Finally, larvae exposed to water containing uninjured amphipods did not alter activity compared with the blank control (all *P* values >0.50; Figs. 2A, 2B; experiment 2, Table 1).

Larvae that emerge from cases but did not immediately begin moving may be attempting to acquire more information from the chemical stimuli or the surrounding environment. Therefore, the difference between latency to move and latency to emerge may indicate additional chemoreception or predator inspection behavior. The latency to emerge was subtracted from the latency to move for each test larva, and there was no significant difference between the treatments for this behavior (Kruskal–Wallis test: $H = 6.12$, $P = 0.106$).

Because the larvae varied in size within a treatment and because ontogenetic changes in antipredator response occur in other prey species (Puttlitz et al. 1999; Mathis and Vincent 2000; Brown et al. 2001; Marcus and Brown 2003), we used linear regression to look for differing responses between larvae of different sizes in each treatment; where necessary, data were transformed with a square-root function to meet assumptions of normality. A significant correlation between mass and either latency behavior was detected in only one treatment. Within the injured caddisfly treatment, larger larvae took longer to emerge from cases and begin moving (emerge: $F_{[1,18]} = 6.19$, $P = 0.02$; move: $F_{[1,18]} = 4.28$, $P = 0.05$; Figs. 3A, 3B).

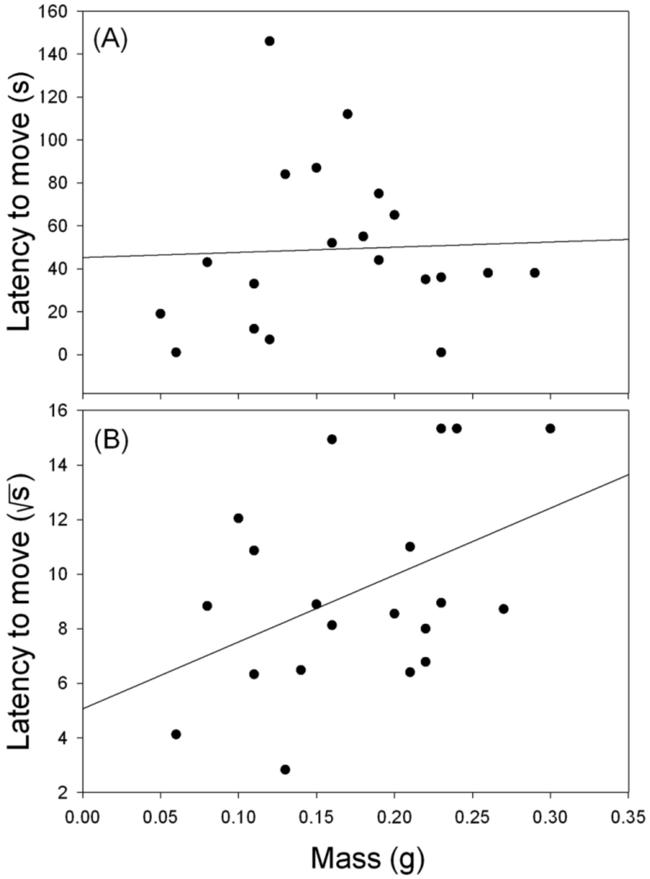
Fig. 2. Behavioral responses of larval caddisfly *Hesperophylax occidentalis* exposed to chemical stimuli from injured conspecifics (Inj Caddis), injured heterospecifics (amphipod *Gammarus lacustris*; Inj Amph), uninjured amphipods (Amph), or a blank control (Blank). (A) The latency (mean ± 1 SE) for the heads of caddisflies to emerge from cases. Kruskal–Wallis ANOVA: $P = 0.004$. (B) The latency (mean ± 1 SE) for caddisflies to emerge from cases and begin moving. Kruskal–Wallis ANOVA: $P = 0.018$. Different letters above bars indicate significant differences between treatments ($P < 0.05$). Sample sizes are given within the bars.



Discussion

Caddisflies showed a significant increase in time to egress from their protective cases when exposed to chemical cues from predatory rainbow trout. The threat of predation is not constant, and the level of risk prey experience can fluctuate extensively (Lima and Dill 1990). Caddisflies that are able to adjust their behavior to the immediate level of predation

Fig. 3. Mass (g) of larval caddisfly *Hesperophylax occidentalis* in the (A) blank treatment in relation to the latency to move ($R^2 < 0.002$, $P = 0.86$) and the (B) injured caddisfly treatment compared with latency to move ($R^2 = 0.19$, $P = 0.05$). Data for injured caddisfly treatment were transformed with a square-root function.



risk should have a higher probability of survival. Larvae remaining inside their protective cases after a predator has been chemically identified should reduce the likelihood of being detected and attacked. Rainbow trout are active predators that extensively utilize visual and hydrodynamic (i.e., lateral line) cues during foraging in clear and turbid waters (Montgomery et al. 2002; Rowe et al. 2003). The prey-capture efficiency of rainbow trout decreases with reductions in prey movement (Ware 1973), and caddisflies that remain motionless and feign death after a predatory attack do indeed increase their probability of survival (Johansson 1991; Johansson and Englund 1995). The combination of chemical information clearly enhances this antipredator response and should result in an even greater reduction of predation risk.

The use of predator kairomones by caddisflies is not limited to immediate changes in activity. When exposed to chemical cues from three different predators (dragonfly niads, larval fire salamanders (genus *Salamandra* Laurenti, 1768), and brown trout (*Salmo trutta* L., 1758)), caddisflies adjusted their choice of case type according to the level of predation risk (Boyero et al. 2006). When larvae were removed from their cases and given a choice between mineral and organic cases (mineral cases provide greater protection from many predators; Otto and Svensson 1980), those exposed to chemical cues from potential predators entered cases faster and consistently chose mineral cases over organic cases (Boyero et al. 2006). The combination of immediate responses to perceived predatory threat and adaptive long-term behavioral responses should minimize the risk of predation for caddisflies.

The decrease in activity exhibited by caddisflies in response to injured conspecifics was similar to the response to trout kairomones, indicating the presence of a chemical alarm cue. Alarm cues have previously been documented in a variety of aquatic organisms (reviewed by Chivers and Smith 1998), but not in Trichoptera. The presence of a chemical alarm cue increases the probability of prey detecting a predator that has recently attacked a conspecific. Alternatively, as demonstrated by Mathis et al. (1995), if the alarm cue also attracts additional predators, then the predation event might be disrupted and the sender may survive the encounter. This model seems problematic in a prey organism that is swallowed whole; however, fishes often swallow caddisflies and subsequently spit them out in an effort to dislodge them from their protective cases (Johansson 1991; Johansson and Englund 1995). This process can be repeated multiple times, and may provide ample opportunity for the release of the alarm cue and attraction of other predators. Nevertheless, how the alarm cue functions in wild caddisfly populations remains to be studied.

It is likely the caddisflies used in this study were experienced with trout, and larger caddisflies reduced activity to a greater extent than small larvae when exposed to the alarm cue (Fig. 3B). Large caddisflies are at a higher instar than smaller individuals and should therefore be older and presumably more experienced with local predation threat. The increased antipredator response with increasing mass (i.e., age) suggests that some component of learning may be involved in the response of larvae to the alarm cue. Other freshwater insects have been shown to use learning in predator recognition and avoidance. By pairing the alarm cue with novel predatory chemical stimuli, damselfly and mosquito larvae can learn to avoid novel predators (Wisenden et al. 1997; Ferrari et al. 2008). With learning being an important component of predator recognition and avoidance, further research should focus on its role, as well as predator diet, on caddisfly antipredator behavior.

Caddisflies did not significantly increase the latency to emerge from their cases in response to amphipod alarm cue. However, a trend toward reduced activity is present (Figs. 2A, 2B). If caddisflies were to reduce activity toward amphipod alarm cues, then it would likely confer survival benefits because receivers would increase the chance of detecting nearby predators. On the other hand, if a predator is consuming heterospecifics, the presence of heterospecific alarm cues may indicate some degree of safety from predation and result in a weaker antipredator response. Predator recognition is often influenced by the diet of the predator (Gelowitz et al. 1993; Wilson and Lefcort 1993; Chivers et al. 1996*b*; Chivers and Mirza 2001; Mirza and Chivers 2001*a*), and some organisms do not respond to predator cues when the predator has only been eating heterospecifics (Gelowitz et al. 1993; Stabell and Lwin 1997; Belden et al. 2000). Nevertheless, the role heterospecific alarm cues have in caddisfly antipredator behavior needs further investigation.

Prey fitness is increased from responding to conspecific and heterospecific alarm cues by decreasing the probability of encountering the predator or increasing the chance of escape after detection (Mirza and Chivers 2001*b*). Chemical alarm cues are abundant in aquatic ecosystems and many species of gastropods, insects, crustaceans, amphibians, and fishes possess analogous systems where antipredator behavior is elicited from injured conspecific chemical stimuli (reviewed by Chivers and Smith 1998). Among freshwater invertebrates, the presence of such alarm systems has been well documented and includes amphipods (genus *Gammarus* Fabricius, 1775; Williams and Moore 1985; Wisenden et al. 1999), daphnia (*Daphnia magna* Straus, 1820; Pijanowska 1997), crayfish (genus *Orconectes* Cope, 1872; Hazlett 1994), mosquito larvae (*Culex pipiens* L., 1758; Sih 1986), mayfly larvae (order Ephemeroptera; Scrimgeour et al. 1994; Huryn and Chivers 1999), and damselfly larvae (genus *Enallagma* Charpentier, 1840; Chivers et al. 1996*b*; Wisenden et al. 1997).

The survival of prey organisms is dependent upon accurate and reliable information about predation risk. In aquatic environments, chemical cues can provide the information necessary for prey to respond to the threat of predation and increase their probability of survival. Our study indicates that caddisflies utilize chemical cues for immediate predator recognition and antipredator behavior. Moreover, the caddisfly *H. occidentalis* appears to possess and utilize chemical alarm cues, expanding our knowledge of chemical alarm cues in aquatic taxa.

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