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HYALELLA AMPHIPOD SPECIES REDUCE ACTIVITY IN THE PRESENCE OF

FISH KAIROMONES

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HYALELLA AMPHIPOD SPECIES REDUCE ACTIVITY IN THE PRESENCE OF

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Abstract

Behaviors and fitness of freshwater prey species can be shaped by their responses to predator kairomones, but it is often unclear which environmental factors mediate these responses and how the responses vary among species. We hypothesized that the presence of fish kairomones will induce a decrease in activity level in *Hyalella* amphipods because reduction in activity would reduce the threat of mortality from visual predators like fish. We additionally hypothesized that predator diet and the timing of predator food consumption are environmental factors that may modulate antipredator responses of prey. To understand how behavioral antipredator responses vary among species, we measured activity levels of two congeneric freshwater amphipod species, *Hyalella wakulla* and *Hyalella wellborni*, in response to kairomones released by bluegill sunfish (*Lepomis macrochirus*). To evaluate antipredator responses of *Hyalella* to fish kairomones, and behavioral differences between amphipod species, we measured the activity levels of both species in the presence and absence of bluegill kairomones. We varied fish diets to determine how predator diet influences a prey's antipredator response. Fish were fed either commercial freeze-dried bloodworms, live *Hyalella*, or left unfed. We also manipulated time between a fish's last feeding and the exposure of amphipods to their chemicals. We investigated whether this time interval may have an important influence on antipredator response, which would be expected if kairomone release is strongly connected to the act of food consumption and subsequent excretion. We found that both amphipod species dramatically lowered their activity levels in response to the presence of fish kairomones. Activity level of *H. wakulla* dropped by 67% in the presence of fish and *H. wellborni* activity level was reduced by 50%. Additionally, neither the timing of food consumption nor variation in fish diet significantly influenced the antipredator response in either amphipod species. These findings

demonstrate that *Hyalella* amphipods strongly reduce activity in the chemical presence of a common fish, but this behavioral response was not significantly influenced by the fish's diet or the time since the predator last consumed food. This antipredator response is shared by the two *Hyalella* species tested. *Hyalella* amphipods perceive and then alter their behavior in response to one or more chemicals released by fish. This study highlights the substantial effect of predator kairomones on the behavior of freshwater invertebrates like *Hyalella*.

Hyalella Amphipod Species Reduce Activity in the Presence of Fish Kairomones

Introduction

Kairomones are key drivers of species interactions in freshwater communities (Ferrari et al. 2010). Kairomones are allelochemical cues that evoke an adaptively favorable behavioral or physiological response in the receiver of the chemical cue, but not in the emitter (Kost 2008). Kairomones are particularly consequential in predator-prey interactions in which a predator emits chemicals that are received by prey. Aquatic prey species across taxa may perceive and react to predator kairomones from early in development, as soon as their chemosensory apparatus develops during embryogenesis (Naraki et al. 2013; Weiss et al. 2015). Perception of kairomones by prey induces a variety of inducible defenses and antipredator behaviors (Tollrian & Harvell 1999). In some cases, predator kairomones induce life-history changes in prey species, including reproduction at smaller size or younger age (Chakri et al. 2010; Heuschele & Selander 2014; Silberbrush et al. 2019). These life-history changes have been observed across many crustaceans, including amphipods (Abjornnson et al. 2004), copepods (Heuschele & Selander 2014), cladocerans such as *Daphnia* (Weider & Pijanowska 1993; Weber 2003; Chakri et al. 2010), as well as insects (Silberbrush et al. 2019). Predator kairomones can also induce morphological changes, such as deeper tail fins in tadpoles (Releya 2001; Relyea 2004) and development of novel defense structures like neckteeth in *Daphnia* (Barry 2000; Barry 2001; Naraki et al. 2013). Kairomones may also induce reduction in body size in many taxa, such as anurans (Relyea 2004), fishes (Chivers et al. 2008), gastropods (Hoverman & Relyea 2007), insects (Stoks et al. 2005), and zooplankton (Lass & Spaak 2003).

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While predator kairomones commonly induce changes in morphological traits, they can also induce behavioral changes with perhaps an even more pronounced range of plasticity (Relyea 2001). Kairomones induce a variety of behavioral responses in prey that reduce risk of predation mortality, but these responses often involve fitness trade-offs. A common behavioral antipredator response is spatial avoidance of the predator, which can further involve alteration of foraging habits and seeking refuge. These avoidance behaviors have been observed in anurans (Relyea 2001), fishes (Bauman et al. 2019), and amphipods (Camacho & Thacker 2013; Zamzow et al. 2010). Some prey species, such as *Daphnia magna*, have been observed to enter a state of heightened alertness when exposed to predator kairomones. Prey in this state are able to carry out escape behaviors more readily and efficiently than naïve individuals of the same species (De Meester & Cousyn 1997; Pijanowska et al. 2006). Predator kairomones can induce species, such as the amphipod *Gammarus pulex* (Kullmann et al. 2008; Smith & Webster 2015), to undergo conspecific grouping behaviors that reduce the risk of mortality. Among the most common behavioral antipredator response is a reduction in activity. Reduction in activity is a frequently recorded response to predator kairomones, and has been observed in taxa such as anurans (Van Buskirk & Yurewicz 1998; Peacor 2002), gastropods (Sih & McCarthy 2002), and salamanders (Maerz et al. 2001). Reduction in activity by prey reduces detection by visual predators, like fish (Fairbairn 1993; Cothran 2004). Although reduced activity levels and other behavioral responses may reduce risk of predation in prey species, the reduced activity may generate fitness trade-offs that negatively impact prey in other ways. Reductions in activity level mean reductions in other behaviors necessary to increase fitness. For example, reduced activity levels due to kairomone exposure have been linked to reduced foraging behavior in guppies (Gosline & Rodd 2008). It is possible that chronic exposure to kairomones could result in malnutrition in prey populations and

be responsible in part for the body size reductions (Relyea 2005; Chivers et al. 2008; James & McClintock 2017) and reduced brood sizes (Heuschele & Selander 2014) observed in other taxa exposed to predator kairomones. Reduced activity levels also reduce mating opportunities (Khater et al. 2015) and, consequently, prolonged periods of low activity could lead to reductions in overall population size of the effected species. These behavioral changes, coupled with the energetic costs of phenotypically plastic responses to predator kairomones can inhibit fitness and development in many ways at both the individual and population levels (Tollrian & Harvell 1999; Auld et al. 2010).

Much research has focused on identifying the specific chemicals composing fish kairomones, and while many substances have been found that induce kairomone-like responses in prey (Ringelberg & Van Gool 1998; Ferland-Raynond et al. 2010; Weiss et al. 2018; Pijanowska et al. 2020), the relative importance or necessity of these chemicals remains unclear. Kairomones emitted by predatory fish have dramatic and well-documented effects in freshwater ecosystems, and particularly on prey species. However, the chemical identity of kairomones has largely been unknown. Recent studies have characterized a number of fish-emitted substances that induce kairomone-like behaviors and have thus been proposed to be the source of the fish kairomone. Ferland-Raymond et al. (2010) discovered that a negative ion induced antipredator behaviors when fish had consumed prey, but did not induce the behaviors when the ion was absent. Recent studies found that glutamic fatty acids (Weiss et al. 2018), gut bacteria (Ringelberg & Van Gool 1998; Beklioglu et al. 2006), and bile salts (Pijanowska et al. 2020) such as 5α-cyprinol sulfate (Hahn et al. 2019) also induce antipredator responses in prey species. All of these possible kairomone sources are associated with the fish digestive system, suggesting that the fish kairomone is produced by the fish itself as a byproduct of digestion and excretion.

Bile salts, glutamic fatty acids, and negative ions all independently could function as kairomones, with their effects varying among prey and predator species. For example, *Daphnia galeata* express different responses to predatory fish kairomones depending on the fish species (Weber 2003) and *Daphnia pulex* produce neckteeth in response to predatory *Chaoborus* larvae, but respond to fish kairomones through life-history changes when exposed to stickleback fish. If fish kairomones comprise multiple substances, this diversity could explain the predator-specific responses seen in these and other taxa, such as anurans (Relyea 2001) and fish (Smejkal et al. 2017).

Many studies have found that antipredator responses can be induced only when predator kairomones work in conjunction with cues emitted by conspecifics (Ferland-Raymond et al. 2010; Shaffery & Relyea 2016). These conspecific cues consist of disturbance cues released by distressed, but uninjured, conspecifics and alarm cues released by injured or dying conspecifics (Wisenden 2015). In some cases, conspecific cues alone induce some form of antipredator behaviors (Smith & Webster 2015) or they may amplify a response despite not being necessary for the response's induction (Laurila et al. 1997; Wisenden et al. 1999). The multiple possible predator kairomone sources, along with and the potential importance of conspecific chemical cues, suggests the kairomone may be a cocktail of chemical cues that each have independent and sometimes coordinated effects on prey (Weber 2003; Schoeppner & Relyea 2009). Antipredator responses may also be mediated by a host of factors such as the age and sex of individual prey (Wahle 1992; Mathis et al. 2003; Slos et al. 2009) or predators (Eggleston 1990; Cooper & Stankowich 2010). The antipredator response may also differ among prey species even when the species are closely related. Different antipredator responses to the same predator have been

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observed in congeneric species of anurans (Laurila et al. 1995; Relyea 2001) and *Daphnia* (Barry 2000).

Two recently described freshwater amphipod species, *Hyalella wellborni* (Soucek et al. 2015) and *Hyalella wakulla* (Drumm & Knight-Gray 2019), occur broadly in much of the United States (Poynton et al. 2018). Both *H. wakulla* and *H. wellborni* are well-characterized species within the *Hyalella azteca* cryptic species complex. *Hyalella wakulla* reach significantly larger average body sizes than *H. wellborni*, with *H. wakulla* males and females respectively averaging 70% and 18% longer head lengths than male and female *H. wellborni* (Wellborn et al. 2005). This morphological variation is common in the *Hyalella azteca* species complex and a significant driver of this variation may be predatory fish such as bluegill sunfish (*Lepomis macrochirus*) (Wellborn 2002; Cothran 2004; Wellborn et al. 2005). *Lepomis* fish are major predators of *Hyalella* (Wellborn & Cothran 2004) and are visual predators that exhibit size-biased foraging behavior in which they more often consume larger prey (Fairbairn 1993; Wellborn 1994; Cothran 2004). *Hyalella wellborni* mature earlier and have smaller average body lengths at maturation than *H. wakulla*, which both appear to be common adaptations for species in the genus *Hyalella* that live in habitats rich in predatory fish (Wellborn et al. 2005). The smaller size of *H. wellborni* reduces their risk of predation by visual predators such as *Lepomis* who consume larger and more conspicuous individuals (Wellborn 1994; Cothran 2004). However, small species such as *H. wellborni* appear to trade off competitive ability for this greater predator avoidance, as they are outcompeted by larger species like *H. wakulla* when predation pressure is less prevalent (Wellborn 2002). Small-bodied *Hyalella* species such as *H. wellborni* are found in habitats containing *Lepomis* sunfish, while larger-bodied species like *H.*

wakulla are found in fishless habitats or the shallow edges of large bodies of water inaccessible to fish (Wellborn et al. 2005).

We investigated the effects of kairomones on the antipredator responses of *H. wakulla* and *H. wellborni* to assess the influence of these kairomones on antipredator responses in these abundant and geographically widespread North American species. *Hyalella* were exposed to kairomones produced by *Lepomis* sunfish and antipredator response was quantified by recording the amphipods' activity levels. Previous research on the effects of predator kairomones on prey have shown that antipredator responses may be species-specific (Laurila et al. 1997; Relyea 2001; Barry 2002) and be influenced by the presence of conspecific cues (Schoeppner & Relyea 2009; Ferland-Raymond et al. 2010; Shaffery & Relyea 2016). Some conspecific cues are released through the process of a conspecific being consumed by predators (Wisenden 2015), and predator kairomones may also originate in the digestive system (Weiss et al. 2018; Hahn et al. 2019; Pijanowska et al. 2020). The timing of predator feedings may also influence antipredator responses in prey if the process of feeding influences the production of kairomones and conspecific chemical cues, as it would take time for the digestive processes to reach the point at which the kairomone is released. We independently exposed each species of *Hyalella* to any kairomones released from fish that were fed different diets (conspecific amphipods, commercial dehydrated bloodworms, or no food) at different times (2 hours before trial or 24 hours before trial) to determine the effects of conspecific cues and timing of food consumption on the antipredator behaviors of *Hyalella* (Figure 1). The activity levels of *H. wakulla* and *H. wellborni* were assessed to determine the influence of fish kairomones on antipredator responses of the two species.

Study System and Methods

Antipredator Response in Hyalella

Hyalella respond to the risk of predation in a number of ways, including developmental changes in response to presence of fish kairomones that result in changes in adult body size (James & McClintock 2017). Amphipods also perform predator-avoidance behaviors, such as altering habitat use (Camacho & Thacker 2013). In this experiment, the antipredator response of *Hyalella* was determined by observing changes in amphipod activity level. Reduction in activity is a common antipredator response across many taxa in environments that contain visual predators (van Buskirk & Yurewicz 1998; Peacor 2002; Sih & McCarthy 2002). This defense strategy reduces predation by predators like *Lepomis* that rely on visual detection of prey (Fairbairn 1993; Cothran 2004).

Animal Collection and Housing

Non-hybridized bluegill sunfish (*Lepomis macrochirus*) were obtained from Nabatak Outdoors in Claremore, Oklahoma. *Hyalella wellborni*, a small-bodied amphipod species, was collected at Brier Creek, Marshall County, OK (33° 59' N, 96° 49' W) where bluegill are common predators. *Hyalella wakulla* were collected at Cowan Creek, Marshall County, OK (33° 52' N, 96° 50' W) which does not support a population of predatory bluegill or other centrarchid fish species. While *H. wellborni* and *H. wakulla* co-occur regionally, they do not typically coexist in the same water bodies and are not found together in either Brier Creek or Cowan Creek. Both amphipod species have historically been regarded as *Hyalella azteca,* along with other North American members of the *Hyalella* genus. These species have a broad distribution across much of North America (Poynton et al. 2018).

After collection, the two *Hyalella* species were housed in separate aerated 25 L aquariums where they were fed a diet of periphyton grown from each species' source stream. Bluegill sunfish were housed individually in 20-gallon aquariums when not being actively used in a trial. Fish were acclimated for one week before initiating experimental trials. Depending on their experimental treatment, bluegill were fed a diet of either commercial dehydrated bloodworms (Tetra®) or live *Hyalella*. Amphipods and fish were kept under a natural day-night cycle, which was approximately 12:12h. All trials were conducted at midday. All aquariums were kept in a greenhouse environment with an evaporative cooling system. The temperature of each tank was measured twice daily. Water temperatures in the holding aquariums and experimental units ranged from 22.2-31.9°C over the course of the experiment.

Experimental Design

Our experiment was designed to determine how the antipredator behavior of *Hyalella* amphipods is influenced by the chemical presence of predatory fish, the fish's diet, and the time between a fish's last consumption of food and the amphipods' exposure to the fish's kairomones (Figure 1). Experiments were performed in 4L plastic containers (28 cm long x 18 cm wide x 9 cm deep) divided by a Nitex® mesh barrier (0.75 mm) such that fish kairomones could diffuse through the mesh, but fish and amphipods could not cross the barrier (Figure 2). Each experimental unit was filled with tap water that was aged and dechlorinated for 24 hours. The underside of each container was marked with a line along the middle of the *Hyalella* side of the barrier (Figure 2). Activity level was determined by recording the number of times amphipods crossed the activity level marker, with a lower number of crossings indicating a lower activity level. Therefore, activity level in this experiment equates to total line crosses per 2 minutes. This

activity level was used to gauge the amphipods' level of antipredator response for each trial. The water temperature of each experimental unit was measured immediately before each trial.

Ten bluegill sunfish were used throughout the experiment. All fish measured between 8-10 cm in standard length (length from tip of the snout to beginning of the caudal fin) to control for variability in kairomone production due to body size. To control for potential kairomone production variability due to sex, only male fish were used. Every trial was also conducted from 3:30-4:30pm. Before each trial, each fish was randomly assigned to one of the 10 possible treatment groups. This randomization process was conducted by randomly assigning each fish one of the three possible dietary treatments and one of the two possible timing treatments before each trial. The *Hyalella* species that would be exposed to each fish's kairomones was also randomly assigned. For both *H. wakulla* and *H. wellborni*, fish were fed either 10g of live *Hyalella* 2 hours before the trial, 10g of live *Hyalella* 24 hours before the trial, 10g of commercial freeze-dried bloodworms (Tetra®, larval *Chironomidae* sp.) 2 hours before the trial, 10g of bloodworms 24 hours before the trial, or left unfed (Figure 1). Each trial also consisted of no-fish control treatments for each *Hyalella* species in which amphipods were placed in fishless experimental units to measure their activity level when no fish were present (Figure 1). Fish were left unfed for 48 hours before being given their dietary treatment to ensure that their stomach contents were emptied prior to each trial (Ha et al. 2009). After the designated time interval, fish were placed on one side of the mesh barrier of one of 10 randomly assigned experimental units and given 5 minutes to acclimate. Trials for each species of *Hyalella* were conducted simultaneously and such that there was one replicate of each treatment for each species in every trial. There were a total of 10 replicates per treatment per species in this experiment.

Once fish were placed in the experimental units, 11 *Hyalella* amphipods were placed on their respective side of the barrier in each container (both those with fish and those without). The *Hyalella* were given 2 minutes to settle before their activity level was measured over a 2-minute period. *Hyalella wellborni* and *H. wakulla* were tested independently. In each trial, fish assigned to the *Hyalella* dietary treatment were fed the conspecific species, such that *H. wakulla* were only exposed to fish that consumed *H. wakulla* and *H. wellborni* were only exposed to fish that consumed *H. wellborni*. Each experimental unit held 3 male and 8 female adult amphipods to approximate the naturally occurring 1:3 male-to-female ratio (Wellborn & Bartholf 2005). Only gravid female amphipods were used. Amphipods were considered adults if they measured either equal or greater in size than the smallest gravid female of a sample of amphipods taken at the beginning of the experiment. This minimum size was determined to be a body length of 5 mm in *H. wakulla* and 3 mm in *H. wellborni*. No individual *Hyalella* were used in more than one trial. At the conclusion of each trial, each fish was returned its own 20-gallon aquarium until the next trial. Each tub was thoroughly rinsed with aged, dechlorinated tap water and non-toxic aquarium cleaner, then left to dry for 48 hours between trials.

Statistical Analysis

Data were collected independently and in the same manner for both *Hyalella* species. Data for both species were collected simultaneously, with both species being observed in every trial. The assumptions of normality and homogeneity of variance were tested for all treatments using the Shapiro-Wilks test and Levene's test, respectively. While Levene's test demonstrated that the assumption of homogeneity was met for all treatments, the Shapiro-Wilks test showed that data were not normal for the diet, feeding time, or fish presence treatments of either *H.*

wakulla or *H. wellborni*. As a result, a Kruskal-Wallis test was used to analyze the results of the diet treatments and Mann-Whitney U tests were used to analyze the results of the feeding time and fish presence treatments. Mann-Whitney U tests were also used to determine whether activity level differed between *H. wakulla* and *H. wellborni*. Data from *H. wakulla* and *H. wellborni* were analyzed independently. An alpha value of 0.05 was used for all tests. A Dunn test was used for post-hoc analysis of the Kruskal-Wallis results. Some studies have shown that temperature has an influence on activity level (Qin et al. 2021), so a further correlation test was run to determine whether temperature variation between trials had a significant influence on results. Experimental unit temperatures at the time of each trial ranged from 22.2-31.9°C over the course of the experiment.

Results

The presence of bluegill in trials resulted in a large decrease in activity level in both *H. wakulla* and *H. wellborni*. The average activity level of *H. wakulla* was 67% lower when fish were present when compared to the treatments in which fish were absent (Figure 3a). This difference in activity level between fish presence and absence treatments was significant ($z=-2.24$, $p=0.03$). In *H. wellborni* treatments average activity levels were 50% lower when fish were present (Figure 3a). The difference in activity level for the presence or absence of bluegill was also significant for *H. wellborni* ($z=-2.02$, $p=0.04$). The activity level of the two *Hyalella* species did not significantly differ from one another when fish were present ($z=-1.11$, $p=0.27$) or when they were absent ($z=0.49$, $p=0.62$).

Bluegill diet did not influence activity level of either amphipod species, with fish diets consisting of conspecific *Hyalella*, bloodworms, or unfed fish controls exhibiting similar activity

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levels (Figure 3b). There was no significant difference in activity level among the three diet treatments in either *H. wakulla* (H=1.2, df=2, p=0.37) or *H. wellborni* (H=2.0, df=2, p=0.36). The time interval between a fish's consumption of prey and exposure of predators to the amphipods also had no influence on activity level in either *H. wakulla* (Z=-0.04, p=0.97) or *H. wellborni* (z=-0.24, p=0.81) (Figure 3c). The correlation test also found that relationship between activity level and temperature was not significant in either the *H. wakulla* (r=-0.53, p=0.12) or *H. wellborni* (r=-0.27, p=0.47) trials.

Discussion

Both *H. wakulla* and *H. wellborni* exhibited a strong behavioral response in the chemical presence of *Lepomis*. Both amphipod species significantly reduced their activity levels in the chemical presence of bluegill, regardless of the fish's diet or feeding schedule. This reduction in activity was pronounced in both species, with *H. wakulla* showing a 67% reduction in activity in the presence of fish kairomones compared to controls where fish were absent, while *H. wellborni* experienced a 50% reduction in activity level when fish were present (Figure 3a). The two *Hyalella* species share a strong response to fish kairomones. Differences in diets of the fish (*Hyalella*, bloodworms, or unfed) did not significantly affect the activity level of either amphipod species. Unfed fish and those fed a diet of amphipods or bloodworms all produced similar magnitudes of antipredator response, suggesting that consumption of conspecifics is not necessary for induction of antipredator responses in *Hyalella*. This result indicates the fish kairomone that *Hyalella* respond to originates from the fish itself rather than the prey item.

Reduced activity levels, fitness, and morphological responses

The dramatically reduced activity levels we observed in our experiment may be a factor in the morphological antipredator responses that other studies have observed in *Hyalella*. In a similar study, small groups of *H. wakulla* were exposed to *Lepomis* kairomones, and chronic exposure to these kairomones resulted in significantly smaller adult body lengths in the amphipods (James & McClintock 2017). The reduced activity levels induced by predator kairomones in our study may contribute to this reduction in body length. Reduction in activity level can negatively impact the fitness of prey populations by reducing individuals' ability to forage for food or seek mates (Auld et al. 2010; Khater et al. 2015). Reduction in nutrient uptake caused by reduced activity may lead to slower growth and the smaller adult body sizes observed by James & McClintock (2017). Longer term, a persistent reduction in foraging and mating opportunities could cause lower rates of reproduction and reduced population size, as poorly nourished adults reduce the viability of the prey population as a whole (Langerhans et al. 2005; Auld et al. 2010; Khater et al. 2015).

Reduced activity levels are a common response to predator kairomones in a variety of other taxa. Many taxa, such as anurans (van Buskirk & Yurewicz 1998; Peacor 2002), gastropods (Sih & McCarthy 2002), and salamanders (Maersz et al. 2001) reduce their activity levels to avoid detection by predators when exposed to predator kairomones. Development of smaller body size is also a common antipredator response across taxa, being documented in anurans (Relyea 2004), fishes (Chivers et al. 2008), gastropods (Hoverman & Relyea 2007), insects (Stoks et al. 2005), and zooplankton (Lass & Spaak 2003), which suggests that the relationship between reduced activity levels and reduced body sizes may be common across aquatic taxa. Reduced activity levels may also be connected with the state of heightened alertness observed in other taxa. Prey individuals experiencing this heightened alertness remain

more sensitive to disturbances as a means of increasing the effectiveness of their escape response (De Meester & Cousyn 1997; Pijanowska et al. 2006). This behavioral state could require increased energy expenditure (Weider & Pijanowsa 1993). Amphipods also seek refuge in response to predator cues (Camacho & Thacker 2013; Zamzow et al. 2010) which may further disrupt foraging and mate seeking activities, and perhaps restrict them to environments less optimal for foraging and selecting mates. These behavioral responses may also work in concert with the life-history changes that can result from exposure to predator cues. Groups of *Hyalella* that were chronically exposed to predator cues were younger and smaller at first reproduction (James & McClintock 2017), which can subsequently cause a reduction in offspring size (Gosline & Rodd 2008) and the number of eggs in the first clutch (Heuschle & Selander 2014). Similar life-history changes have been observed in other aquatic crustaceans such as copepods (Heuschele & Selander 2014) and *Daphnia* (Weider & Pijanowska 1993; Chakri et al. 2010).

Influence of predator diet

In our experiment, the diet of *Lepomis* fish (*Hyalella*, bloodworms, unfed) did not have a significant influence on the activity level of either *H. wakulla* or *H. wellborni*. This result suggests that neither consumption nor digestion of prey are necessary to induce the observed antipredator response. It is possible, however, that the 48-hour fasting period used in other studies (Ha et al. 2009) was not long enough to allow evacuation of all food content from the digestive system of the *Lepomis* in our study before amphipods were exposed to their presumed kairomones. The relative importance of predator diet and conspecific cues to antipredator responses is unclear from previous studies and may vary by prey or predator species, experimental design, and environmental factors. While the importance of predator diet has not

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been studied extensively in *Hyalella*, it has been documented in other amphipods. For example, *Gammarus minus* reduce activity levels and seek refuge when exposed to injured conspecifics, but increase activity level and move upward in the water column when exposed to cues from injured heterospecifics (Wisenden et al. 1999). Some studies have found that larval anurans require predators to feed on conspecifics for antipredator responses to be induced. For example, kairomones can induce altered behavior and morphology in tadpoles when they are exposed to kairomones from conspecific-fed predators, but not when the tadpoles are exposed to starved or heterospecific-fed fish (Shaffery & Relyea 2016). Furthermore, reduced activity levels have been observed in tadpoles in response to a negative ion produced only by fish that were fed other tadpoles (Ferland-Raymond et al. 2010).

In contrast to studies that suggest antipredator responses can only be induced by the consumption of conspecifics, there is also evidence that neither conspecific cues or prey consumption are always necessary to evoke a response in prey. For example, when examining the community-level response to a predator's presence when no prey cues where involved, Marino et al. (2015) found that the mere presence of a predator significantly reduced the abundance of macroinvertebrates in a bromeliad community. The disparity between these studies may be related to prey responding not to a single chemical, but rather a collection of different chemicals produced by both predator and prey. For example, tadpoles have been documented to seek refuge in response to crushed conspecifics, but reduce activity level and undergo morphological changes in response to chewed or digested conspecifics (Schoeppner & Relyea 2009). Similarly, some tadpoles still exhibit a weak antipredator response when exposed to predators that were fed heterospecifics, but have a stronger response when the predators consume conspecifics (Laurila et al. 1997). The results of these studies suggest that prey species'

antipredator responses are dictated by a variety of chemicals and while each may induce a particular response, all chemicals are needed to induce the full suite of responses for any one species. While cues from consumed conspecifics did not influence *Hyalella* activity levels in our experiment, these cues from injured or deceased conspecifics may still be required to induce other responses such as the morphological changes (James & McClintock 2017) and refuge-seeking behaviors (Camacho & Thacker 2013) observed in other studies. Furthermore, *Hyalella* in our experiment may have responded to fish kairomones strongly because they were tested in small groups rather than individually. Conspecific amphipods release a stress-induced cue (Hettyey et al. 2015; Wisenden 2015) that cause more pronounced antipredator responses than is seen in solitary amphipods (James & McClintock 2017).

Variation in antipredator response between Hyalella species

Both species of *Hyalella* used in this experiment reacted strongly to the presence of *Lepomis* and the magnitude of their behavioral response did not significantly differ. This similarity in response suggests that activity level reduction is a conserved trait in *Hyalella*. However, it has been observed that closely-related species may have different responses to the same predator in other studies using anurans (Laurila et al. 1997; Relyea 2001), *Daphnia* (Barry 2000), and fishes (Smejkal et al. 2017). The similarity in antipredator behavior seen in this study is interesting given the two *Hyalella* species' habitat preferences and associated adaptations to different predator regimes. *Hyalella wakulla* is significantly larger than *H. wellborni* in adult body size (Wellborn et al. 2005). The larger size of *H. wakulla* makes individuals of the species more vulnerable to predation by *Lepomis*, which preferentially consume larger prey (Wellborn 2002; Wellborn & Cothran 2004). *Hyalella wellborni* are common in fish-rich habitats where

their comparatively smaller bodies make them less vulnerable to fish predators. *Hyalella wellborni* commonly co-occur with *Lepomis*, while *H. wakulla* are rarely found in habitats with predatory fish (Wellborn et al. 2005). However, the trade-off for the antipredator adaptation of *H. wellborni*'s small size is that larger species like *H. wakulla* outcompete them in environments where predatory fish are scarce (Wellborn 2002). These predator-dependent size differences were reflected in our own study, as *H. wakulla* were not found in the fish-rich Briar Creek alongside *H. wellborni*. Similarly, *H. wellborni* could not be found in the fishless Cowan Creek where *H. wakulla* resided.

We expected these ecological differences between *H. wakulla* and *H. wellborni* to shape the species' behavioral responses to fish kairomones. Further, we predicted that *H. wellborni*, who are regularly exposed to *Lepomis* kairomones in their natural environment, would exhibit a larger behavioral response to *Lepomis* than *H. wakulla* who would be naïve to predator centrarchid fish like bluegill. In other taxa, such as the cladoceran *Daphnia magna*, prey populations that have been exposed to predator kairomones react to predators more quickly or strongly than naïve populations (De Meester & Cousyn 1997; Pijanowska et al. 2006). However, we observed that the *H. wakulla* that had never been exposed to *Lepomis* responded to fish kairomones to the same degree as the *H. wellborni* that had been collected from a *Lepomis*-rich environment. That we found *H. wakulla* to respond strongly to *Lepomis* kairomones despite not naturally coexisting with *Lepomis* may indicate that activity level reduction is a conserved behavioral response in *Hyalella*. As in most crustaceans, *Hyalella*'s ability to receive and respond to chemical cues derives from a system of chemoreceptors (Hallberg & Skog 2011). Similarities in their chemoreceptors would result in the two species picking up on same chemical cues, providing a possible explanation for *H. wellborni* and *H. wakulla* responding similarly to

Lepomis in this study. While the two species experienced similar reductions in activity level, it is possible that they may have differences in their other antipredator responses. For example, the species may differ in how their body size changes in response to predator kairomones as that aspect of antipredator response has only been studied in one *Hyalella* species (James & McClintock 2017).

Conclusions

We found that neither predator diet nor the timing of predator food consumption had a significant influence on antipredator behavioral responses in *Hyalella*. These results suggest that cues from digested conspecifics were not necessary for the induction of antipredator responses in *Hyalella*, in contrast to similar studies with other taxa (Wisenden 1999; Ferland-Raymond et al. 2010; Shaffery & Relyea 2016). Further, our findings also suggest that the fish kairomone(s) that induce(s) *Hyalella* activity reduction derives from the fish itself, rather than the prey species that is being consumed. We also found that the *H. wakulla* and *H. wellborni* both experienced a significant reduction in activity level in the presence of fish and the responses of both species were similarly unaffected by predator diet or the time interval between predator feedings. This result suggests that antipredator responses are similar between congeneric species, even if species are adapted to different predator regimes. Further research should be conducted to quantify the repercussions of reduced activity levels on the fitness of *Hyalella* to determine whether a connection exists between reduced activity levels and decreased average body size (James & McClintock 2017). Finally, it would be instructive to investigate if the small-bodied *H. wellborni* also has reduced body size in response to predators as has been observed in *H. wakulla*

(James & McClintock 2017) to determine whether a decrease in body size is a common

antipredator response among *Hyalella* of all ecological backgrounds.

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Hyalella species		Fish Presence vs. Absence		Fish Diet		Timing of Food Consumption
H. wakulla	X	Fish Present Fish Absent	X	Fish fed bloodworms Fish fed *H. wakulla* Fish not fed	X	Fish fed 2 hours before trial Fish fed 24 hours before trial
H. wellborni	X	Fish Present Fish Absent	X	Fish fed bloodworms Fish fed *H. wellborni* Fish not fed	X	Fish fed 2 hours before trial Fish fed 24 hours before trial

Figure 1. Overview of experimental design. The antipredator response of each *Hyalella* species (*H. wakulla* and *H. wellborni*) were measured independently. Antipredator response was measured by observing the amphipods' activity level. The activity level of each amphipod species was measured in response to all combinations of the presence or absence of a *Lepomis* sunfish, the diet of the *Lepomis* (commercial dehydrated bloodworms, conspecific *Hyalella*, or no food), and the time since the fish was last fed (either 2 or 24 hours before *Hyalella* were exposed to fish kairomones). 10 replicates per treatment were conducted for each species.
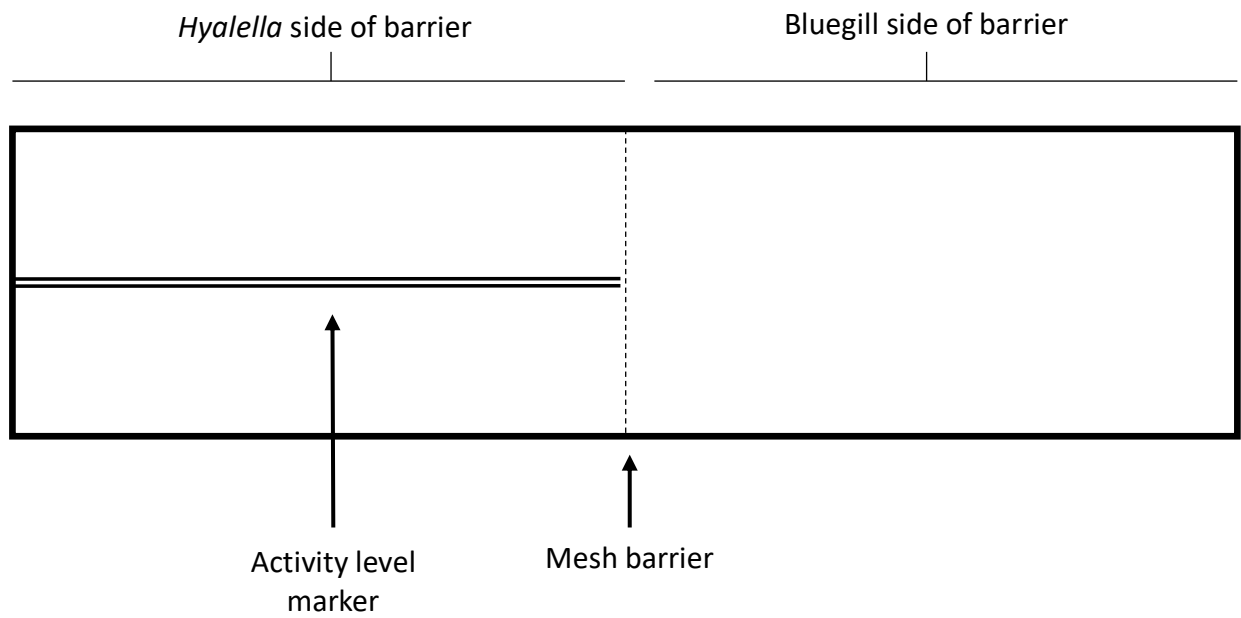
Figure 2. Design of each 4.5 L (28 cm x 18 cm x 9 cm) experimental unit. *Lepomis* (Bluegill) and *Hyalella* were placed on their respective sides of the barrier before collecting observational data. Activity level was measured by recording the number of times an amphipod crossed the activity level marker (a line drawn across the exterior of the unit). See text for further details.
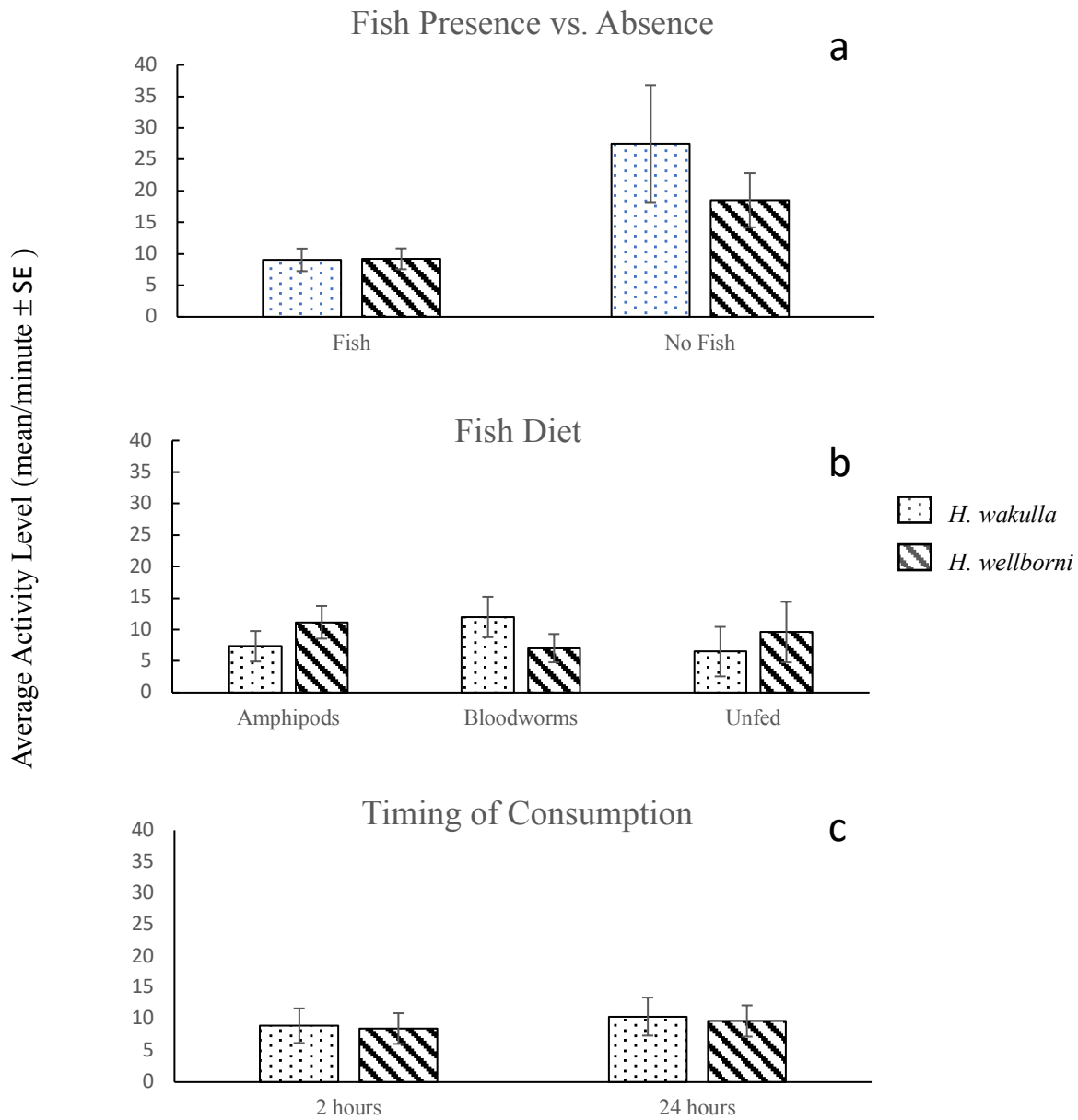
Figure 3. Average activity level of *Hyalella wakulla* and *Hyalella wellborni* based on: (a) presence or absence of fish; (b) the diet of the fish; and (c) the time interval between consumption by fish and beginning of experimental trial. Activity level was measured by recording the number of times the activity level marker was crossed (see Fig. 1). Data are means ± 1 SE.
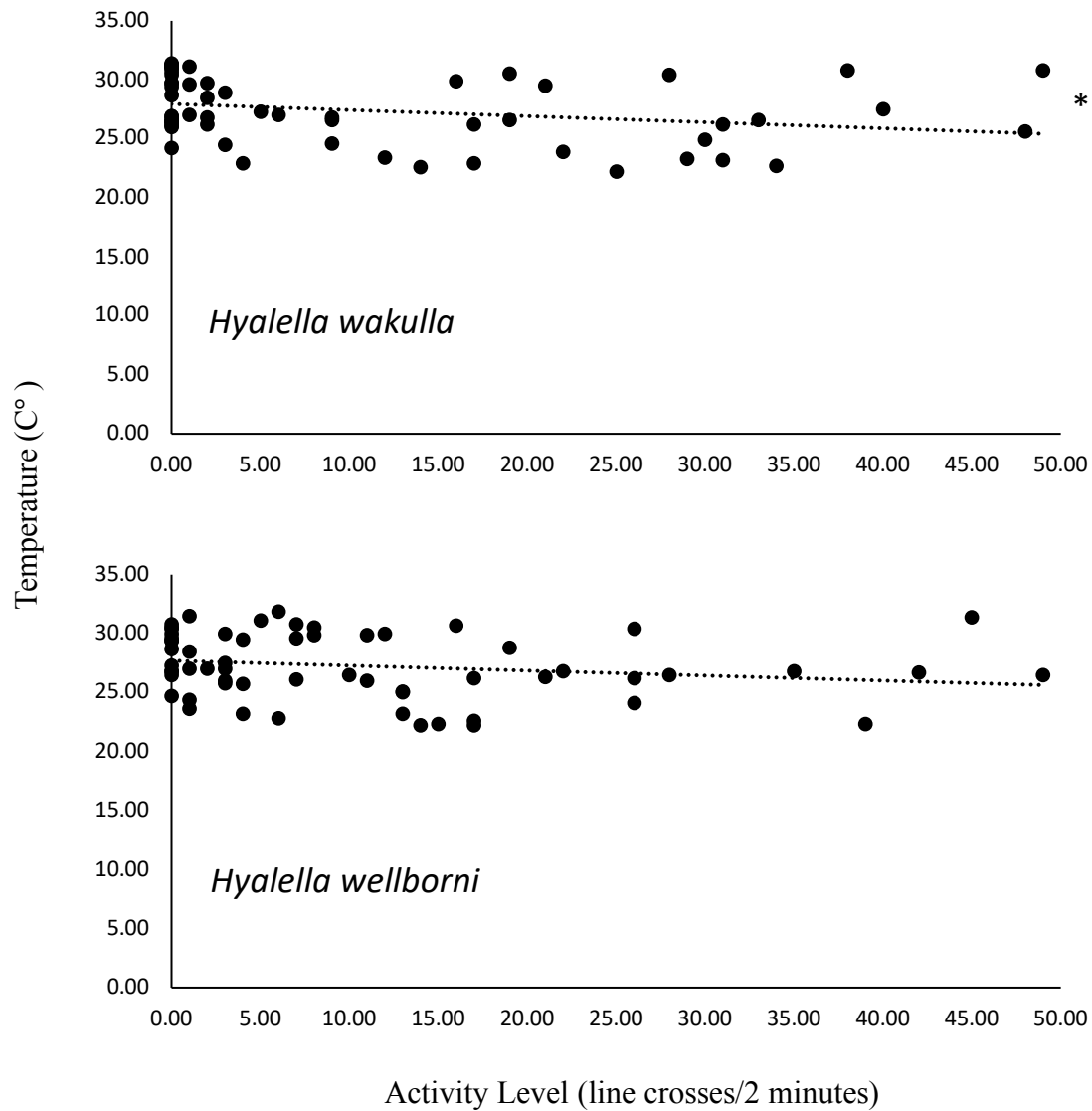
Figure 4. Correlation between water temperature and activity level in *Hyalella wakulla* (top) and *Hyalella wellborni* (bottom). Activity level was measured by recording the number of times the activity level marker was crossed over a 2-minute time period (see Fig. 1). No significant linear correlation was found for either *H. wakulla* (r=-0.53, p=0.12) or *H. wellborni* (r=-0.27, p=0.45). The asterisk denotes one trial with an activity level of 95.

Trial Number	*Hyalella wakulla*						*Hyalella wellborni*						Temperature (°C)
	Amphipod (2 hours)	Amphipod (24 hours)	Bloodworm (2 hours)	Bloodworm (24 hours)	Unfed	No Fish	Amphipod (2 hours)	Amphipod (24 hours)	Bloodworm (2 hours)	Bloodworm (24 hours)	Unfed	No Fish	
Trial 1	30	0	22	12	29	9	4	26	1	1	0	13	24.00 ± 0.18
Trial 2	6	2	2	33	31	17	21	0	22	42	49	28	26.60 ± 0.08
Trial 3	9	0	1	5	0	40	1	0	3	3	0	0	27.00 ± 0.11
Trial 4	2	1	2	0	0	3	0	12	1	3	0	19	29.20 ± 0.16
Trial 5	0	0	0	0	0	0	7	26	3	2	10	17	26.40 ± 0.11
Trial 6	0	0	0	0	0	28	8	0	0	4	7	0	30.50 ± 0.18
Trial 7	0	0	0	19	0	0	45	16	1	7	0	8	30.70 ± 0.17
Trial 8	3	9	19	48	0	95	4	11	3	13	13	35	25.90 ± 0.21
Trial 9	38	16	0	21	1	49	6	5	11	0	0	26	30.40 ± 0.79
Trial 10	14	17	31	25	4	34	14	17	15	6	17	39	22.60 ± 0.10

Table 1. Activity level for all replicates of each treatment for both *H. wakulla* and *H. wellborni*. Activity level was measured by recording the number of times the activity level marker was crossed (see Fig. 1). This table also provides average recorded water temperature $\pm$ 1 SE at the time of each trial.