DIET AND FAMILIARITY INFLUENCE ON PREDATOR RECOGNITION BY CHEMICAL
CUES IN CRAYFISH

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ABSTRACT

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Prey often alter their morphology, physiology, and/or behavior when presented with predatory cues. Alteration in behaviors (i.e. habitat use, food consumption) are consequences of non-consumptive effects that can alter the dynamics of prey resources and cause changes in food web structures. One key factor in determining predation threat level by predators is the composition of the diet of the predator. We wanted to test the ability of prey to determine threat level based on cues produced by different predators on various diets. Odors from two different species of fish, bass (*Micropterus salmoides)*, a natural predator of crayfish, and cichlid (*Oreochromis aureus x Oreochromis niloticus)*, a non-natural predator of crayfish that were fed a vegetarian pellet, a protein diet, a heterospecific crayfish, and a conspecific crayfish were collected. Anti-predator behavior was tested by placing the prey, crayfish (*Orconectes virilis),* in a y-maze and analyzing the side of choice arena the crayfish spent time in, shelter usage of the crayfish, walking speed, walking forward and backward, climbing, and posture when presented with predator odors. Our results show that crayfish spent less time in odors containing conspecific diets, but when in this odor, crayfish spent most of the time hiding in the shelter when odors were emitted from a natural bass predator. However, these results were not present when exposed to non-predatory cichlid odors. Therefore, crayfish can determine different threat levels based off of chemical signals emitted from a potential or real threat, when paired with diet, eliciting predator avoidance behaviors.

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INTRODUCTION

Within the interaction of predators and their prey, a loss by prey means death whereas a loss by the predator simply means a lost lunch. As a result of this imbalance in risk, there is a stronger selective pressure on prey to avoid predation, or at least, reduce the risk of predation (Dawkins and Krebs 1979). Prey have evolved a number of different methods for reducing the chance of predation events including morphological, physiological, and behavioral changes (Sih 1992; Munoz and Blumstein 2012; Weissburg et al. 2014). Crucian carp develop deeper bodies in response to predatory pike presence which, due to pike's gape limited attack, reduces the predation risk from pike (Brönmark and Miner 1992). By sensing chemical cues from predatory jumping spiders, spitting spiders decreased egg incubation time because carrying eggs can make them more vulnerable to predators (Li and Jackson 2005). Also, crayfish have been noted to reduce feeding time and increase time in shelters or spend more time in a lowered posture position when presented with a predatory alarm cue (Tomba et al. 2001; Gherardi et al. 2011). For any of these anti-predator responses to occur, prey need to detect the presence of a predator from sensory information and extract the degree of risk of a predation event from that information (Weissburg et al. 2014).

The detection of predation risk and the subsequent response of prey has been captured under the general concept of non-consumptive effects (NCE) and has been specifically termed the landscape of fear (Laundré et al. 2001). The original concept of the landscape of fear was developed after the reintroduction of wolves to Yellowstone altered foraging decisions. Female elk and bison increased vigilance behaviors in areas that contained wolves, especially females with calves (Laundré et al. 2001). Within the concept of the landscape of fear, predator cues (e.g., visual, auditory, chemical) dispersed spatially and temporally within an environment create a varied and diverse sensory landscape of aversive signals (Abrahams et al. 2007; Large et al. 2011; Wilson

and Weissburg 2013; Jurcak and Moore 2014). These aversive signals, when sensed by prey, produce the alterations in morphology and behavior that can even have trophic effects (Schmitz et al. 1997). Interactions between a plant-grasshopper-spider food chain have been analyzed (Laws and Joern 2015). Predatory spiders directly affected grasshopper foraging by reducing activity, indirectly causing an increase in plant abundance (Beckerman et al. 1997; Schmitz 1998). The presence of spiders had no impact on grasshopper populations directly, concluding that the grass abundance was a result of a non-consumptive effect (Wineland et al. 2015). The authors concluded that the grasshoppers were spending more time avoiding predators rather than utilizing that energy to forage. These results have led to the additional concept of sphere of influence (Turner and Montgomery 2003) where there is a sphere of sensory signals surrounding a predator which potentially alters a prey's response. While prey responses can alter trophic level dynamics, the response to predatory cues is not universally constant across a population or across a particular set of stimuli.

Differences in an organism's behavioral patterns and sensory ecology can alter non-consumptive responses. For example, free living great tits that were considered fast explorers, had a reduction in body mass in response to predators, compared to those deemed slow explorers. These results suggest that fast explorers may be at a higher risk, therefore reducing their body size (Abbey-Lee et al. 2016). Similar phenotypic changes in prey, as a result of predator detection, has occurred in several other taxa as well (Lima 1986; Witter and Cuthill 1993; Pérez-Tris et al. 2004). A decrease in body size can allow the prey to become more agile (Gosler et al. 1995), and oftentimes less nutritious (Smith et al. 2014), deterring predation. In terms of prey responses to predator detection in aquatic environments, soft-shelled clams buried themselves more often and at greater depths in the presence of predatory green crabs. Further research was done to conclude

that clams that dug deeper holes in the sediment had higher survival rates (Flynn and Smee 2010). Given this variability in responses to predatory cues, several researchers have called for an increased effort in understanding the sensory ecology of prey (Ferrari et al. 2009; Weissburg et al. 2014).

Understanding how prey detect and respond to predatory cues necessitates an understanding of both the generation of cues by the predators and the sensory capabilities of the prey (Dall et al. 2005; McCoy et al. 2012). Within aquatic habitats, detection often occurs at a large distance via chemical cues produced by predators (Brönmark and Hansson 2012). In many aquatic environments, chemical signals are a primary modality for acquiring information about the environment (Weissburg et al. 2002). Chemoreception plays a vital role in detecting the presence of chemical cues, signals, and alarm odors (Hazlett and Schoolmaster 1998; Wisenden 2000; Chivers and Mirza 2001). Many organisms emit alarm odors when injured which serves to warn others that a predation event has occurred. Alarm odors can be both intentional and non-intentional (Gil et al. 2017) and have been demonstrated in a plethora of taxa including gastropods, crayfish, amphibians, and several freshwater species (See review: Ferrari et al. 2010). However, these signals are restricted to conspecific organisms and are not directly related to the predator themselves. Chemically-based cues emanating from predators has also been shown to alter behaviors and work has shown that the intensity of a prey's response is often based upon the diet of the predator.

Diet cues are metabolic products that arise from post-digestion of prey and that are released into the surrounding environment (Ferrari et al. 2010). The diet provides the metabolic building blocks for chemical cues and studies have shown that these predatory cues are important in aquatic systems (Murray and Jenkins 1999; Hazlett and Schoolmaster 1988; Turner 2008;

Scherer and Smee 2016). Information deduced by prey from injured conspecifics appears to be less informative than cues simply from digested conspecifics (Grason 2017). For example, although tadpole activity was inhibited in response to crushed conspecifics, increased effects on behavior decisions and morphological defenses were measured with cues from predator digestion (Schoeppner and Relyea 2009). The shape and size of the prey consumed may also play a role in predator detection. Remarkably, shallow bodied goldfish displayed greater predator avoidance when presented with odor from pike fed shallow bodied goldfish compared to deep bodied goldfish, and vice versa (Chivers et al. 2007). Thus, diet is an indicator of predation threat and changes in the diet might alter a prey's calculation of potential predation threats (Scherer and Smee 2016).

Prey behavior can be very sensitive to small changes in predator diet (Turner 2008). In general, there should be a higher vulnerability to predators that consume conspecifics, whereas predators consuming heterospecifics should pose as a lesser threat (Ferland-Raymond and Murray 2008). For example, freshwater snails (*Physella virgate)* exhibited a more fearful response (crawling out) when faced with crayfish *(Procambarus simulans)* that were fed conspecific *Physella virgate* compared to those fed subtropical gastropods (Alexander and Covich 1991). Additionally, Ferland-Raymond and Murray (2008) observed bull frogs growing larger, while mink frogs remained similar sizes when exposed to dragonflies being fed mink frogs. Although there have been several studies conducted (Ferrari et al. 2010) demonstrating the effects that predator diet has on prey as genetic familiarity becomes increasingly similar between an animal and an alarm cue, the information contained within that cue also becomes more relevant and useful to that animal (Gil 2017).

By altering diet composition of a natural predator and a non-natural predator, we wanted to identify the role of digested conspecifics versus heterospecifics in crayfish behavior and foraging activities to determine what diet poses the greatest threat. This was accomplished by testing different diet compositions of predators (vegetarian and carnivorous) to determine which elicits the most responses and inhibits the most behavior on prey species. The focus of this study was to eliminate all other cues to determine how predator diet directly affects prey behavior. Because evidence from several other studies has shown increased fear responses in prey from predators consuming conspecifics compared to heterospecifics, we hypothesized that crayfish would be most fearful (in descending order), of odor containing conspecific crayfish, heterospecific crayfish, mealworms, and finally the vegetarian diet alone. We also predicted that these responses would be heightened when paired with a natural predator compared to a non-natural predator due to the recognition of natural predator odors.

METHODS

Organisms

Largemouth Bass (*Micropterus salmoides*)

Male and female largemouth bass (*Micropterus salmoides*) were purchased from Harrietta Hills Trout Farm in Harrietta, MI. The total length (TL) of each fish was measured from the tip of the snout to the end of the longest lobe of the caudal fin. All bass (average TL ± SEM: 15.8 ± 0.03 cm) were collectively housed in two metal troughs (237.5 cm x 86.4 cm x 60.1 cm: L x W x H) and were provided two PVC pipes (10.2 cm in diameter and 30.5 cm long) for shelter. Each trough was fed by unfiltered river water from the nearby east branch of the Maple River and was covered with window screening to prevent predation and fish escapes. Fish were fed commercial pellets (Purina AquaMax® Sportfish/Grower 600) before and after being transferred to feeding troughs to be fed treatment diets (explained below).

Cichlid (*Oreochromis aureus* x *Oreochromis niloticus*)

All male cichlids (*Oreochromis aureus* x *Oreochromis niloticus*) were purchased and shipped from White Brook Tilapia Farm (Edgerton, MO) to the University of Michigan Biological Station (UMBS) in Pellston, MI. All cichlids (average TL ± SEM: 4.14 ± 0.01 cm) were communally housed in a large aquarium (60 L) containing two Aqua-tech power filters recirculating water. The bottom of the aquarium was covered with a gravel substrate (~2 cm) and around 30 terra cotta pots to provide shelter and prevent injuries. The aquarium was covered with Plexiglas to prevent escapes as well as potential predators. Fish were fed commercial tilapia food (Allied Aqua® Tilapia Fingerling Pellet) before and after being transferred to feeding troughs to be fed treatment diets (explained below).

Crayfish (*Orconectes virilis* and *Orconectes rusticus*)

Female form II (non-reproductive) crayfish (*O. virilis)* (study organism) and male and female form II crayfish (*O. virilis* and *O. rusticus)* (feeders) were collected from Maple Bay of Burt Lake, MI (45°28'N, 84°40'W) during the summer of 2017 with the use of hand nets. Only *O. virilis* with all sensory appendages and walking legs intact were used in the current study as test subjects. All crayfish had an average intraorbital carapace length of 3.29 ± 0.03 cm (average ± SEM). The chelae were measured from the tip of the largest cheliped to the carpal joint, and all crayfish had an average chelae length of 2.93 ± 0.04 cm (average ± SEM).

Crayfish species were housed at the (UMBS) Stream Research Facility in artificial streams made of cinder blocks lined with 4 mil plastic sheeting (150 cm x 50 cm x 41 cm: L x W x H). The streams were fed by river water from the Maple River and water exited the streams through a cinderblock covered with window screening downstream. This unfiltered water provided fresh oxygen and detritus for food. The crayfish were housed in individual plastic containers (36.6 cm x 36.6 cm x 10.1 cm: L x W x H) which physically isolated them from each other to reduce social and agonistic interactions (Karavanich and Atema 1998). Crayfish were used once throughout the experiment to avoid habituation. After each trial, the crayfish were placed in a community tank.

Monitoring Health of Fish

All procedures were approved under protocols PRO00007576 (University of Michigan) and 1016353-1 (Bowling Green State University). Any fish that showed signs of distress or disease were immediately removed from the experiment and placed into a quarantine tank equipped with an aerator. Antibiotics (Melafix) were administered in the quarantine tank. Fish in quarantine were closely monitored by personnel. Isolation reduced the spread of disease and prevented further

injuries from other animals. Once the fish recovered, they were placed back into the community tank of similar species.

Experimental Design

To examine the interaction of diet and predator species on the anti-predator response of crayfish, a 2 x 4 fully factorial experimental design was used. The first factor was predator species which consisted of two conditions: (1) largemouth bass, a natural predator of crayfish and (2) a non-natural potential predator, cichlid (Gherardi et al. 2011). The second factor was predator diet which consisted of four conditions: (1) control vegetarian diet (Tetra Spring & Fall Diet), (2) protein control diet (*Eisenia hortensis,* Big Red Worms brand), (3) heterospecific diet (crushed *O. rusticus* frozen pellets)*,* and (4) conspecific diet (crushed *O. virilis* frozen pellets). Each treatment had 15 trials (except one non-natural cichlid x protein control diet treatment in which crayfish escaped) for a total of 119 trials.

Natural predator (Bass):

- Control vegetarian diet N = 15

- Control protein diet N = 15

- Heterospecific diet N = 15

- Conspecific diet N = 15

Non-natural predator (Cichlid):

- Control vegetarian diet N = 15

- Control protein diet N = 14

- Heterospecific diet N = 15

- Conspecific diet N = 15

Protocol

All trials were run between 7:30 AM and 12:00 PM during the summer (June and July) of 2017. Each crayfish was placed in the middle of the choice arena (Figure 1) within a y-maze at the start of each trial. Each trial consisted of a 15-minute acclimation period and an 11.5-minute trial period. During the trial period, water (and fish odor) flowed through the y-maze. Each trial was divided into four time-periods based on previous dye trials. The first time period, from 0:00 to 2:30 minutes represented pre-odor time (river water was flowing, but the river water + fish odor had not reached the choice arena section of the y-maze). Minutes 2:30 to 5:30, river water + fish odor was present on one half (right side) of the y-maze while only river water was present on the other side (left side). At 5:00, the valves controlling water and odor delivery were switched. The time period from 5:30 to 8:30, the river water + fish odor was present on both sides of the y-maze. From 8:30 to 11:30, the river water + fish odor was present on the left side of the y-maze, while only river water was present on the right side. A breakdown of the temporal sequence of odor presentations can be found in Table 1. After each trial, the crayfish were returned to their housing units. Between trials, the arena was rinsed with river water and drained to remove any chemical cues from the previous trials.

Experimental Arena

A two-current choice laminar flume (y-maze) was constructed out of 1.27 cm thick white Plexiglas modeled from Jutfelt et al. (2016) (Figure 1). The entire bottom of the y-maze was covered with a thin (~ 1mm) layer of sand that was glued to the flooring using spray adhesive (Loctite: Professional Performance Heavyweight Bonding). The y-maze consisted of three separate sections: the flow conditioning section (96.5 cm x 66.0 cm x 19.1: L x W x H) the choice arena section (63.5 cm x 66.0 cm x 19.1 cm: L x W x H) and the water outflow section (26.7 cm

x 66.0 cm x 19.1 cm: L x W x H). The flow conditioning section consisted of two inflow ports (3.8 cm) located 8.9 cm above the floor of the y-maze and a center wall that separated the flow (96.5 cm long). Each side of the flow conditioning section contained two rows of black window screening (33.0 cm) (one with baffle plates made of silicone and one section without a baffle), one row of white egg crating (1.7 cm² holes) and three separate collimators, each 5.1 cm wide, made from plastic honeycomb material (33.0 cm x 32.3 cm: L x H; Plascore). The end of the last honeycomb was aligned with the end of the wall.

The choice arena section was free of any structures except two PVC half pipes that were potential shelters for the crayfish (11.4 cm x 9.1 cm x 4.6 cm: L x W x H) and placed 39.1 cm from the honeycomb. The water outflow section consisted of a center wall (26.7 cm long) splitting the outflow section in half to separate the two flows. On either side of the center wall was a single row of egg crating (1.7 cm² holes) with black window screening (66.0 cm) attached to the front wall with silicone. Along the back wall of this section were 8 outflow holes (1.3 cm^2) located 8.9 cm above the floor of the y-maze.

Two identical constant pressure head tanks delivered water to each inflow connector (Figure 2). A constant pressure head tank consistent of a 208.2 L plastic barrel that circulated water to a 20 L bucket that sat on top of the barrel. A submersible water pump (Little Giant Pump; Model # 2E-38 N) delivered water via a 119 cm plastic tube to the bucket. To create a constant head, an overflow hole (0.79 cm$^{2)}$ was cut into the side of the bucket 26.7 cm above the bottom of the bucket. The outflow of this bucket returned to the barrel. A larger hole (6.28 cm^2) was located at the base of the bucket in order to allow water to flow into the y-maze. Connected to each head tank was a switchable y-valve (Bosworth; Model # Y-10501) that split the flow into one of two separate hoses. One hose of each head tank was connected to another y-valve (Camco

plastic Garden hose y-valve) which fed water into the y-maze. Thus, each head tank could deliver water to either side of the y-maze depending on the position of the initial y-valves. One constant head tank always supplied river water and the other constant head tank supplied river water and fish odor. The water circulated from the barrels, through the buckets, and into the y-maze. Both valves were switched simultaneously in order to change the delivery of river water and river water + fish odor to the other side of the y-maze. Opening both valves at the same time did not disrupt the flow. The flow rates were tested several times with different colored dyes before the initial trial to ensure that there was minor mixing of the odors in the choice arena.

A camera (Sony; Model # HDR-CX405) was attached to a wooden frame 129.5 cm directly above the choice arena section of the y-maze to video record crayfish movements. In order to have consistent lighting, a tarp was draped over the frame holding a light, and secured using zip ties.

Feeding Troughs and Procedures

All fish (donors) were transferred from their previous housing troughs to four separate feeding troughs (182.9 cm x 61.0 cm x 61.0 cm: L x W x H). Donors were fed the assigned diet in individual feeding tanks for at least 48 hours to flush out any metabolites from their previous diets (McLennan and Ryan 1997). Based on prior measurements, the fish were placed in feeding troughs depending on size and species to produce a similar ratio of fish length per volume of water. The troughs were labeled A through D and each trough was further divided in half to create two equal sections and labeled either 1 or 2 to uniquely identify each holding tank (Figure 3). Each trough was supplied by unfiltered river water which exited the holding tanks through an outflow tube at the bottom of the trough. PVC pipes were provided for shelter for fish. Each trough was covered with window screening to prevent potential predators and objects from entering. Due to the number

of fish and number of trials, both species of fish were reused in random groups throughout the entire experiment. A minimum of 48 hours elapsed before re-use of any fish in this experiment. If a fish was injured or a death occurred during feeding, the fish was replaced with another fish of a similar size that was fed the same diet.

Bass Feeding

Thirty-two uninjured bass (average TL ± SEM: 15.76 ± 0.03 cm) were housed in four separate metal troughs with each trough housing eight fish (182.9 cm x 61.0 cm x 61.0 cm: L x W x H). Within each section, four bass were housed. The total length of the four fish in each trough averaged 63.0 ± 0.13 cm (average TL ± SEM). After all bass trials were run, the bass were transferred back into their original housing troughs.

Cichlid Feeding

Sixty-four uninjured cichlids (average TL ± SEM: 4.16 ± 0.02 cm) were housed in four separate metal troughs (same as bass). Due to their aggressive behavior, each cichlid was housed individually in plastic containers (20.32 cm x 21.59 cm x 8.26 cm: L x W x H) with window screening covered holes (7.62 cm x 7.62 cm: L x W) on each of the four sides to allow flow. Tupperware® containers also helped with handling and reducing stress for the fish. The same tank separation mechanism that was used for the bass was also used for the cichlids. However, eight fish were housed in one section of the metal trough at a time instead of four to size match the fish as close as possible. The total length of the eight fish in each section averaged 33.18 ± 0.12 cm (average TL ± SEM) to match the bass as closely as possible. After all cichlid trials were run, the cichlids were transferred back into their original housing troughs.

Odor Collection Housing and Procedures

After 48 hours on the specified diet, fish were transferred from feeding troughs into odor collection housing. This eliminated the possibility of alarm cues from the animal based diets or other food cues to influence the odors in the y-maze. The fish (4 bass or 8 cichlids), based on their diet, were placed in a 189.3 L container (Sterilite; Model # 554024470) containing 173 L of water and an aerator. The container was covered with window screening and a tarp to prevent contamination from outside sources and any fish escapes. Fish were kept in the odor collection housing for 24 hours without feeding. After the 24 hour collection period, the fish were returned to their feeding troughs. While individual fish were reused, the combination of 4 bass or 8 cichlids was unique for each of the 119 trials to prevent the same stimulus from being repeated.

Diet Production and Feeding

Four unique diets were provided for the fish. To control for dietary amount (Turner 2008), all food was cut and divided into 2 gram portions for the odor donor fish. The control vegetarian diet was chosen because of the lack of protein within the pellets. The vegetarian/control diet consisted of 2 gram portions of Tetra Pond Spring & Fall Diet (Tetra Pond). The control protein diet was chosen as a non-crayfish protein diet and consisted of worms from Big Red Worms brand (DMF Bait Company). The containers of worms were stored in a refrigerator to ensure the worms would not perish. All worms were cut up into 2 gram portions and fed to the fish live. Heterospecific diets were made using frozen male and female crayfish (*O. rusticus*) taken from a community tank that were crushed by using a coffee grinder (Hamilton Beach; Model # 80333) (van Oosterhout et al. 2014). The crushed crayfish were placed on a sheet of Parafilm® and put in the freezer for anywhere between 24 to 72 hours to solidify to make into pellets. Conspecific diets

were made by using the same methods; however, male and female *O. virilis* crayfish were used. The choice of 2 grams was based on preliminary trials with bass consumption rates.

Data Collection

Each trial was recorded on a handheld video camera and behavioral parameters were quantified either by a researcher blind to treatments or by a motion capture program (ProAnalyst® Xcitex Inc.) (Lite Edition). Behavioral parameters quantified included time spent on each side of the choice arena, time spent in shelter, walking in a forward or backward direction, walking speeds, climbing behaviors, and body posture. Motion capture using Xcitex ProAnalyst generated x, y coordinates of the crayfish for every second. A researcher blind to treatment determined the time spent on sides of the arena, time in the shelter, and whether the crayfish was climbing the walls of the choice arena. Shelter use was defined as the point where the joint connecting the abdomen and cephalothorax was completely covered by the shelter and no longer in view. Arena side was determined when more than half of the body was on a side. If the body was evenly split between the two sides, the side in which the head was facing was used to determine the side. Climbing was defined as when all appendages were not touching the substrate, while the crayfish was maintaining a vertical position. The crayfish was noted to be in a lowered posture position whenever the underside of the tail was visible to when the tail started to curl and fully tuck under the body. When a crayfish was moving in a direction it was facing, this was considered to be walking forward, while a crayfish moving in a direction opposite to which it was facing was considered to be walking backward.

Data Analysis

A 2 x 4 MANOVA (multivariate analysis of variance) was performed to test for any effects of predator species (two conditions) and diet (four treatments) on crayfish behaviors in

the y-maze. Percent of time spent in shelter was calculated by dividing the time spent in a shelter by the total time on that side of the y-maze. This produced a proportion that was transformed using an arcsine square root transformation used in the statistical analysis. If statistical differences were detected, a Fisher least significant difference (Fisher-LSD) post hoc analysis was used to determine specific differences between the treatment groups. All statistics were performed in STATISTICA (Statsoft Inc., Tulsa, OK; version 13.2).

RESULTS

Overall MANOVA Interaction

A MANOVA found a significant relationship with an interaction effect between diet and donor (Wilks' Lambda = 0.602, $F_{(42, 291.5, 0.05)}$ = 1.55, p = 0.021). In addition to this interaction effect, there was a main effect due to diet (Wilks' Lambda = 0.556, $F_{(42, 291.5, 0.05)}$ = 1.52, p = 0.026) (Table 2).

Time Spent in Odor Side of Y-maze

Overall, crayfish spent significantly less time in the fish odor side of the y-maze when being presented with odor from bass being fed conspecifics compared to odor from bass being fed heterospecifics (Fisher-LSD, $p < 0.05$). The time crayfish spent in bass vegetarian diet odor (165 s) steadily increased for bass fed worms and bass fed heterospecifics and then dropped significantly for conspecific bass odor (140 s). Although not significant, the opposite effect was seen in cichlids. None of the other comparisons showed any significant difference (Figure 4).

Percent Shelter Use

Crayfish exposed to bass odor with a conspecific diet spent significantly more time in the shelter on that side of the y-maze than every other odor treatment (Figure 5, Fisher-LSD $p < 0.05$). Crayfish spent more than half of the time in the shelter when presented with conspecific bass odor, which was not seen for any other combinations of diet and donor. There were no differences between the percentage of time the crayfish spent in the shelter for any of the cichlid odors as well as any of the bass conditions other than the conspecific diet treatment. None of the odor or predator treatments caused a change in percent shelter use on the river water side of the y-maze (Figure 6).

Walking Speed

When the crayfish were in the fish odor only, the crayfish walked significantly faster when presented with diet odor containing worms digested from cichlids compared to heterospecific diets fed to bass (Fisher-LSD $p < 0.005$). Crayfish walked near 0.6 cm/s when presented with vegetarian, heterospecific, and conspecific cichlid odor, and dramatically increased to 0.9 cm/s when exposed to cichlid worm odor. Also, there was a notable decrease in walking speed demonstrated by crayfish presented with heterospecific bass odor (0.3 cm/s) compared to all other treatments ($\sim$ 0.6. cm/s). Conversely, there were no significant differences for walking speeds when the crayfish was in river water only (no odor) across all diets ($p > 0.05$).

Non-Significant Behaviors

Regardless of donor or dietary components, there were no significant differences across all interactions for the crayfish time spent walking forward, walking backward, in lowered a posture position, or climbing when in fish odor or river water ($p > 0.05$).

DISCUSSION

The results from this study indicate two significant findings. First, crayfish behavior is influenced differentially by native (or a real threat) and non-native (or a potential threat) fish predators. For the time spent in each of the different fish odors, the results are almost a mirror image of each other. Crayfish spent around the same time in both fish odors when fed vegetarian diets. Then, for bass, the time spent increases as potential threat increases, but interestingly, the opposite is seen in cichlids. Finally, crayfish exposed to conspecific bass odor spent significantly less time in that area of the choice arena than crayfish exposed to conspecific cichlid odor (Figure 4). Although the components of the food the fish consumed were identical, crayfish are able to depict the differences between predators. Also, crayfish spent more time actively avoiding predation by hiding in the shelter for a longer period of time when exposed to conspecific bass odor compared to all other treatments (Figure 5). This trend was not seen when crayfish were in river water with no fish odor (control) (Figure 6).

Second, the degree to which those behaviors are altered is based on the dietary consumption of potential predators. Crayfish spent less time on the side of the choice arena containing heterospecific bass odor compared to conspecific bass odor (Figure 4) and when located on that side of the y-maze, spent significantly more of their time in hiding (Figure 5). Crayfish also walked slower when presented with bass heterospecific odor compared to odor containing worms digested from cichlids. Given the differences in behavioral reaction due to the interaction of fish species and diet, crayfish appear to have the ability to not only determine the differences between an actual predator compared to a potential predator, but can also determine threat level based on the previous diet of the predators. The lack of other sensory cues, including

alarm cues from feeding, indicate that crayfish can determine predatory threat levels based off of chemical signals.

Dietary effects on prey behavior have been demonstrated in other systems (See review: Scherer and Smee 2016). Prey perceive predators that eat organisms more genetically related to themselves as more threatening. Snails exhibited higher refuge behaviors after being presented with odors from crayfish that were fed closely related heterospecifics as opposed to more distantly related heterospecifics (Turner 2008). Also, damselflies decreased movement and foraging activity when exposed to odor from pike fed damselflies compared to odor from pike fed mealworms (Chivers et al. 1996). Decreased foraging activity by mud crabs occurs when exposed to blue crab urine produced from a conspecific mud crab diet as opposed to heterospecific oysters as a diet (Weissburg et al. 2016). Even the amount of prey consumed by predators may also have an impact on prey behavior. Within a tri-trophic cascade, predators that consumed greater amounts of food evoked responses in prey from further distances than those that were fed less (Weissburg and Beauvais 2015). Like studies on other animals (Nolte et al. 1994; Berton et al. 1998), crayfish appear to be able to calculate threats based on dietary cues and that calculation is influenced by the relatedness of dietary consumption. While most studies have used quite phylogenetically dissimilar prey items, our results show that even very closely related species can be discriminated by prey.

Though predator dietary effects are commonly demonstrated across many different prey (See review: Chivers and Mirza 2001), these effects, in crayfish, appear to be limited to native predators and are not generalized to potential predators, or at least to cichlids. Our findings indicate that crayfish do not generalize predatory threats beyond native predators even when non-native predators are fed conspecific diets, which is contrary to other studies (Mathis and

Smith 1993; Dixon et al. 2012; Rosell et al. 2013). Previous work has shown that prey can detect threats from non-native species if dietary cues are based on conspecifics (Marquis et al. 2004; Gonzalo et al. 2007; Nunes et al. 2012). Non-native (introduced or invasive) predators can have large effects on native prey populations through direct predation, but within the context of this experiment, crayfish would exhibit very little non-consumptive effects due to the inability to recognize this unique combination of diet and non-native predators. Thus, any NCEs exhibited by crayfish to non-native predators may either be weak or non-existent until the populations of crayfish gain experience with the non-native predator.

The lack of response from the crayfish to non-native predators suggests that the cues produced by these two predators are dissimilar enough that the crayfish does not recognize the predation threat by chemical signals alone. Given that the cues generated by predators are based on the building blocks of dietary cues, the production of cues appears to differ despite similar metabolic building blocks. Contrary to our findings, Schoeppner and Relyea (2009) found when dragonflies (predator) consumed prey more phylogenetically related to tree frogs, then tree frogs would decrease activity. Similar results were found in a study showing that the genetic variation between different salamander species fed to garter snakes played a role in salamander predator avoidance (Sullivan et al. 2005). However, our results indicate that crayfish do not respond differently when presented with a non-conspecific diet based odor cue.

The introduction of invasive species has become more widespread; therefore, predator detection is becoming more important. Organisms must be able to detect these predatory threats within the landscape of fear. Prey react to cues based on the type and magnitude of the chemical stimulus, thus creating a sphere of influence (Turner and Montgomery 2003; Hay 2009; Weissburg and Beauvais 2015). Our study indicates the size of the sphere of influence will be

impacted by predator diet and predator species. The direction of flow carrying these odors within the sphere may also affect the size and shape of an area where non-consumptive effects will occur (Atema 1996; Wilson et al. 2013). However, prey need to identify cues emitted from predators and recognize that a cue is perceived as threatening in order to respond appropriately (Werner and Anholt 1993; Acquistapace et al. 2003). For instance, the introduction of invasive Nile perch into Lake Victoria caused a major decline in cichlid populations, most likely because cichlids were unable to identify the perch as a predator (Witte et al. 1992). Correspondingly, crayfish that coexisted with several fish species (predators and non-predators) were able to distinguish between predator odors, whereas non-experienced crayfish generalized all fish as predators (Gherardi et al. 2011). The sphere of influence may depend on known cues from a natural predator compared to a non-native predator, as well as odors from a digested conspecific compared to a heterospecific organism (Mathis and Smith 1993; Grostal and Dicke 2000; Cai et al. 2011). The sphere should appear larger for prey that consider a predator as threatening, whether based on visual cues (size or shape) or chemical cues (i.e., odor, diet, alarm cues) therefore altering behaviors over a larger area.

Since diet produces metabolic by-products, some of which are inadvertently released by all organisms, these cues can be especially informative in how prey make decisions about potential predation threats. Generally, higher concentrations of prey within a diet and decreased distances to the predator cause cue intensities to increase (Ferrari et al. 2008; Johannesen et al. 2017). Larger animals, because of their physical size and higher rates of consumption, are expected to produce higher concentrations of cues. Evidence of size effects has been shown for crucian carp activity patterns based on northern pike diet. Larger bodied pike produced stronger avoidance behaviors for crucian carp (Pettersson et al. 2000). Similar size effects have been

reported for alarm cues emitted from large conspecifics, which inhibited juvenile roach's feeding activity to a greater degree than cues emitted from small and medium sized animals. However, the NCEs varied based on the experience level of the roach. Naïve roach were unable to recognize predatory cues, seeing them as unthreatening, therefore spending more time foraging compared to experienced roaches (Jachner 2001). Taken together, these results demonstrate that diet and predator naivety influence the size of a non-consumptive effects. Thus, NCEs due to predatory dietary cues may cause an alteration in habitat use, changes in grazing choices, and fluctuating food consumption in prey that can cause changes in food web structures (Turner and Peacor 2012).

By detecting chemical cues within a sensory landscape, prey often adjust their behavior, morphology, or physiology as a result of predatory diet-based cues (Weissburg et al. 2014). Decreasing movement or foraging is a common behavior exhibited by prey presumably to reduce their susceptibility to predation (Lima and Dill 1990; Gherardi et al. 2011; Kenison et al. 2017). Evaluation of cues, and the inherent predation threat the cues signal, are contingent upon the recognition of predator. Information extracted from cues is reliant on the diet of the predator, but also on the familiarity of the predator and the interaction of these two factors. These factors can contextualize the threat of predation. The misidentification of predators could be deadly, but constantly reacting to any predatory cue is costly in terms of reduced foraging effort and other behaviors necessary for survival. Overall, this study concludes that prey can determine different threat levels very precisely based on chemical signals emitted from a potential threat or a real threat. Subtle differences in diet, such as that between two species in the same genera, can reduce this perceived threat significantly.

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APPENDIX A – TABLES

Table 1 – Temporal sequence of odor presentations in y-maze.

Time	Left Side	Right Side
0:00-2:30	River Water	River Water
2:30-5:30	River Water	River Water + Fish Odor
5:30-8:30	River Water + Fish Odor	River Water + Fish Odor
8:30-11:30	River Water + Fish Odor	River Water

Table 2 – Overall MANOVA table for effects of predator species and diet. An asterisk indicates significance ($p < 0.05$).

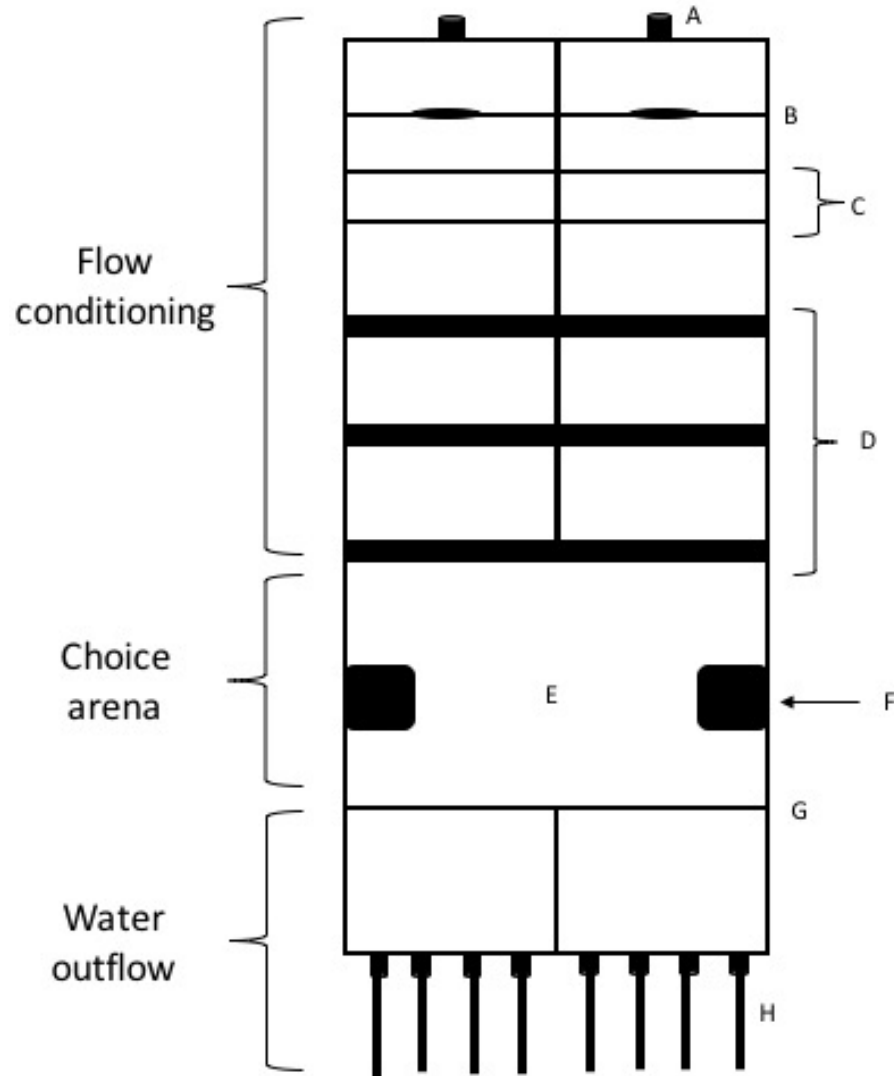| Effect	Test	Value	F	Effect df	Error df	p
Predator Species	Wilks	0.86	1.10	14	98.00	0.36
Diet	Wilks	0.56	1.52	42	291.48	0.026*
Predator Species x Diet	Wilks	0.56	1.49	42	291.48	0.033*

APPENDIX B – FIGURES

Figure 1. Schematic of two-current choice flume arena (y-maze). The letters represent the following: (A) inflow tube; (B) baffle plate to decrease turbulence; (C) window screening; (D) honeycomb to ensure constant flow and prevent crayfish from escaping; (E) choice arena for the crayfish; (F) shelter; (G) window screening; (H) flume drains. Water flowed in the direction of A → H.
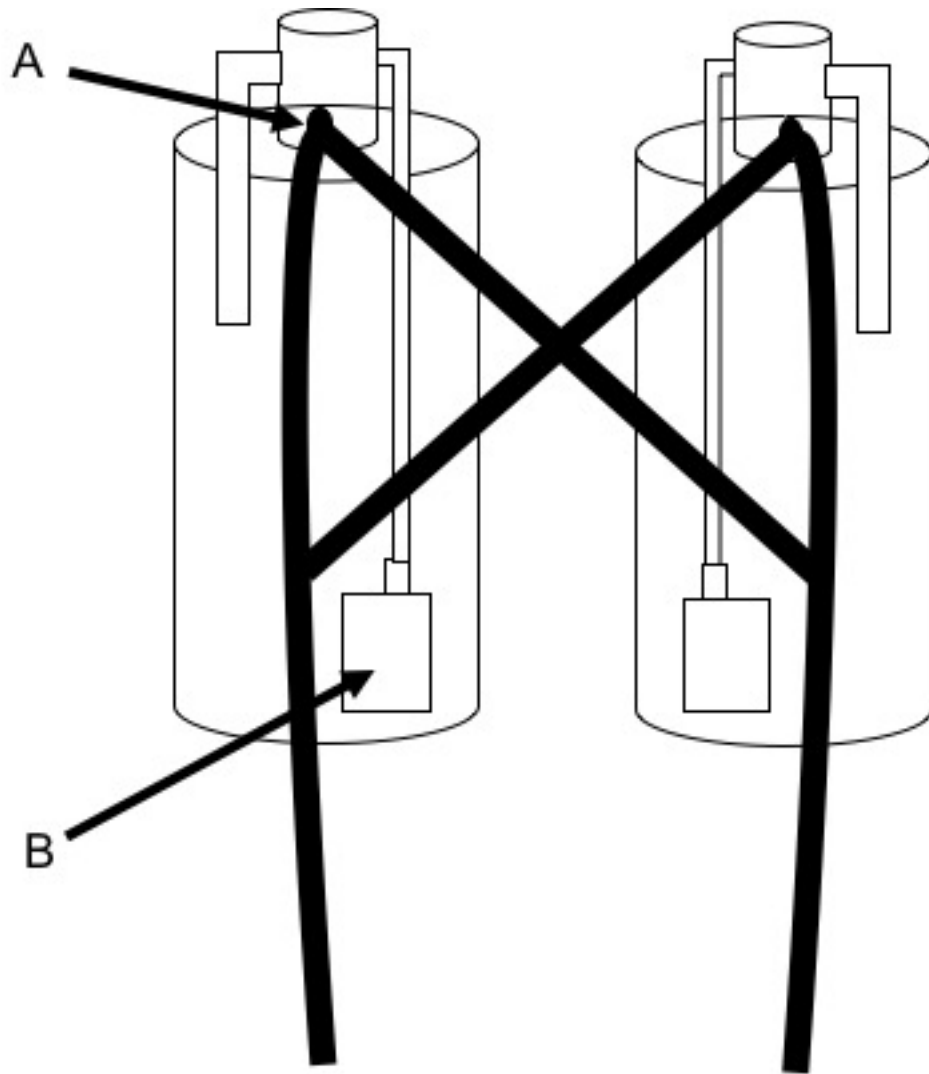
Figure 2. Schematic of constant head tank which consisted of buckets and barrels to distribute the water to the y-maze. The letters represent the following: (A) y-valve to switch the side of the odor halfway through each trial; (B) submersible water pump to circulate the water. This is not drawn to scale for clarity purposes.
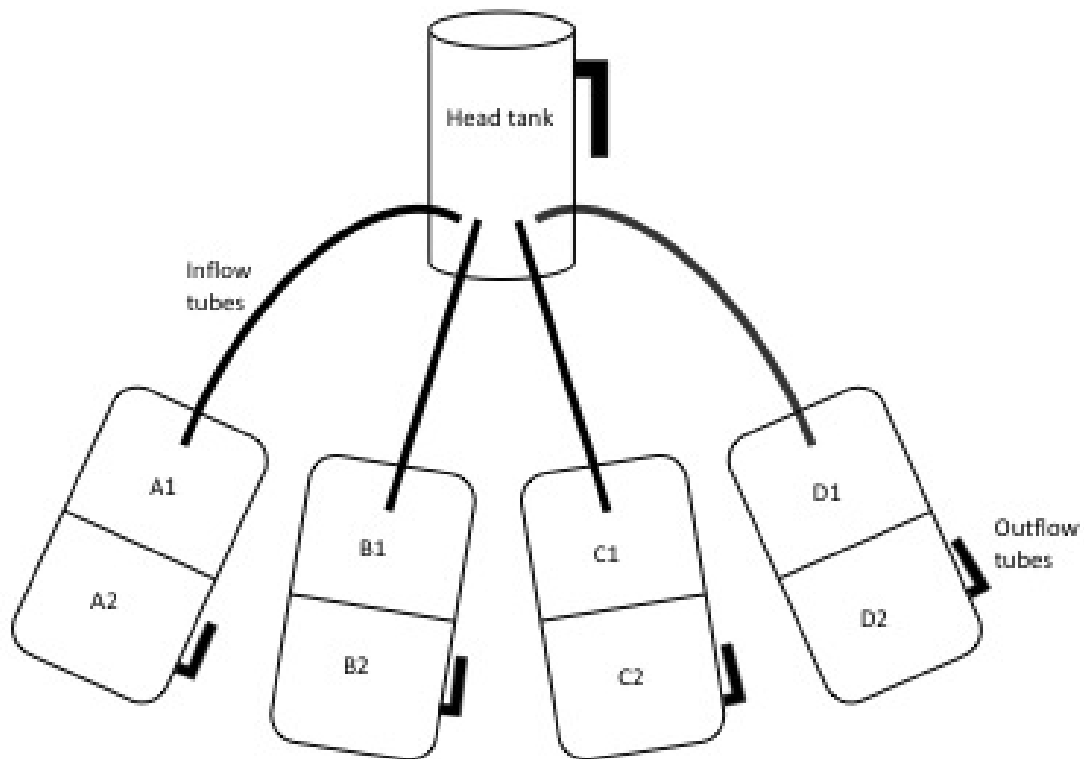
Figure 3. Schematic of housing and feeding area for the fish while their odor was not being collected. Water that came from a nearby stream was pumped into the head tank, which then flowed into the troughs through the inflow tubes. Once the head tank troughs were filled to maximum capacity, water was released through the outflow tubes. The numbers and letters were used to identify each individual feeding section.
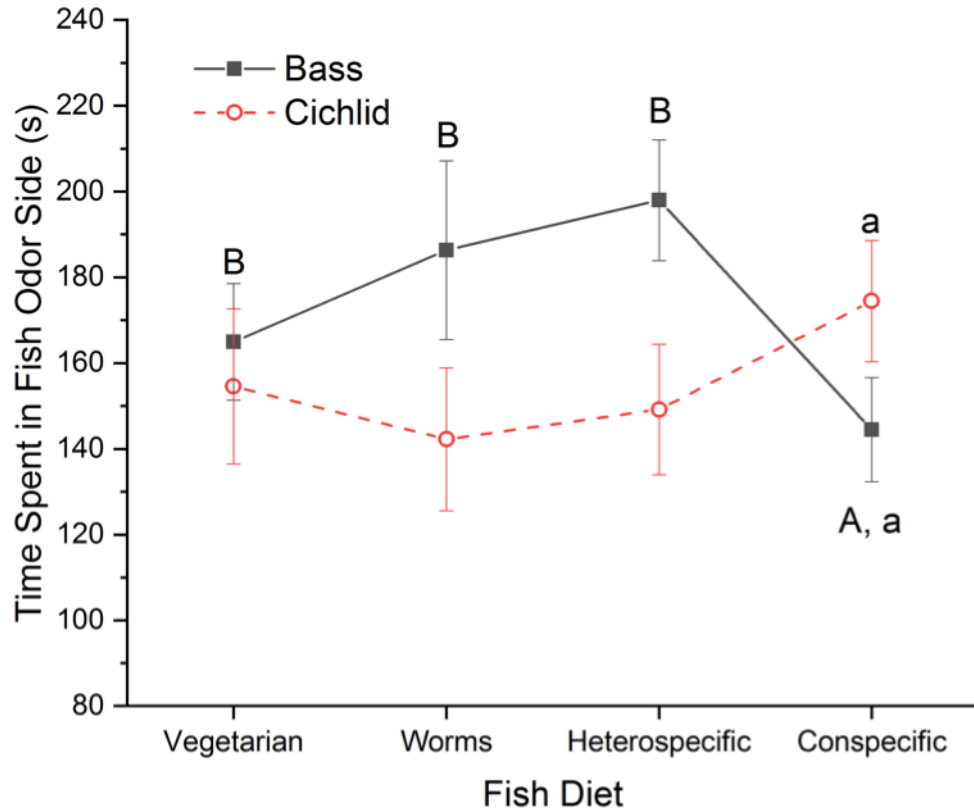
Figure 4. Mean (± SEM) of amount of time (s) crayfish spent on the side of the y-maze containing fish odor as a function of species and diet. Red open circles and dotted lines are for the cichlid odor donors and the black closed squares with solid lines are for the bass odor donors. Capital letters represent significant differences for comparison within a species. Data points with different letters are significantly different from each other ($p < 0.05$). Lower case letters represent significant differences between species compared at the same diet ($p < 0.05$).
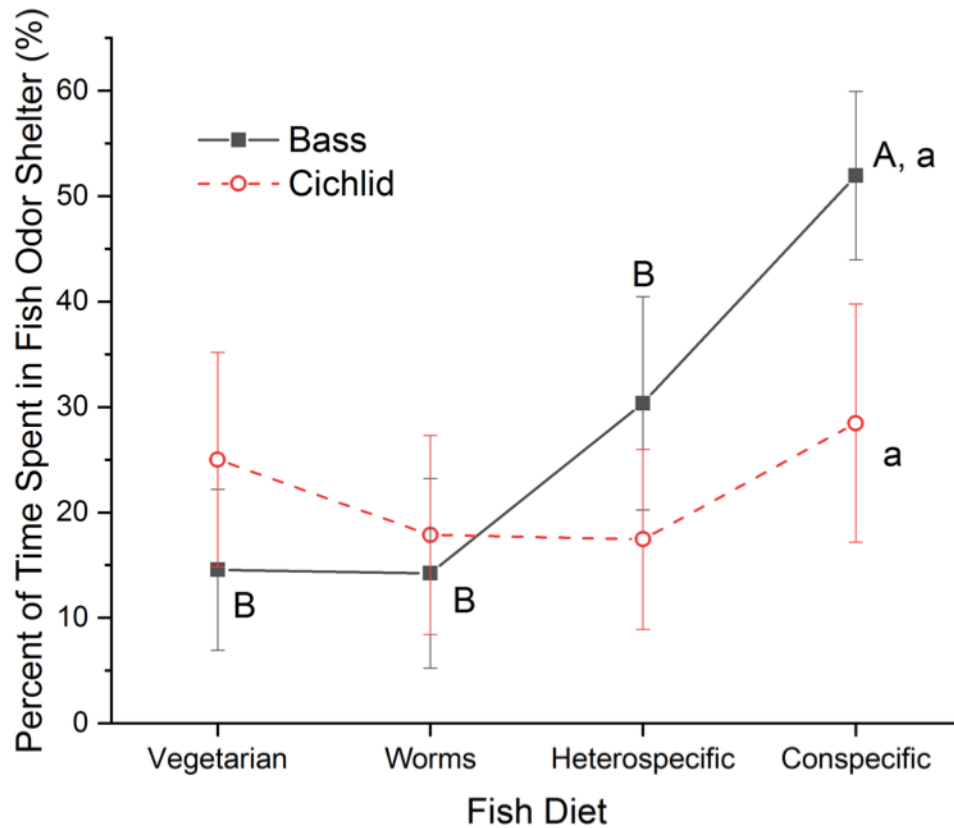
Figure 5. Mean (± SEM) of percent time crayfish spent in the shelter on the side with the fish odor as a function of species and diet. Red open circles and dotted lines are for the cichlid odor donors and the black closed squares with solid lines are for the bass odor donors. Capital letters represent significant differences for comparison within a species. Data points with different letters are significantly different from each other (p < 0.05). Lower case letters represent significant differences between species compared at the same diet (p < 0.05).
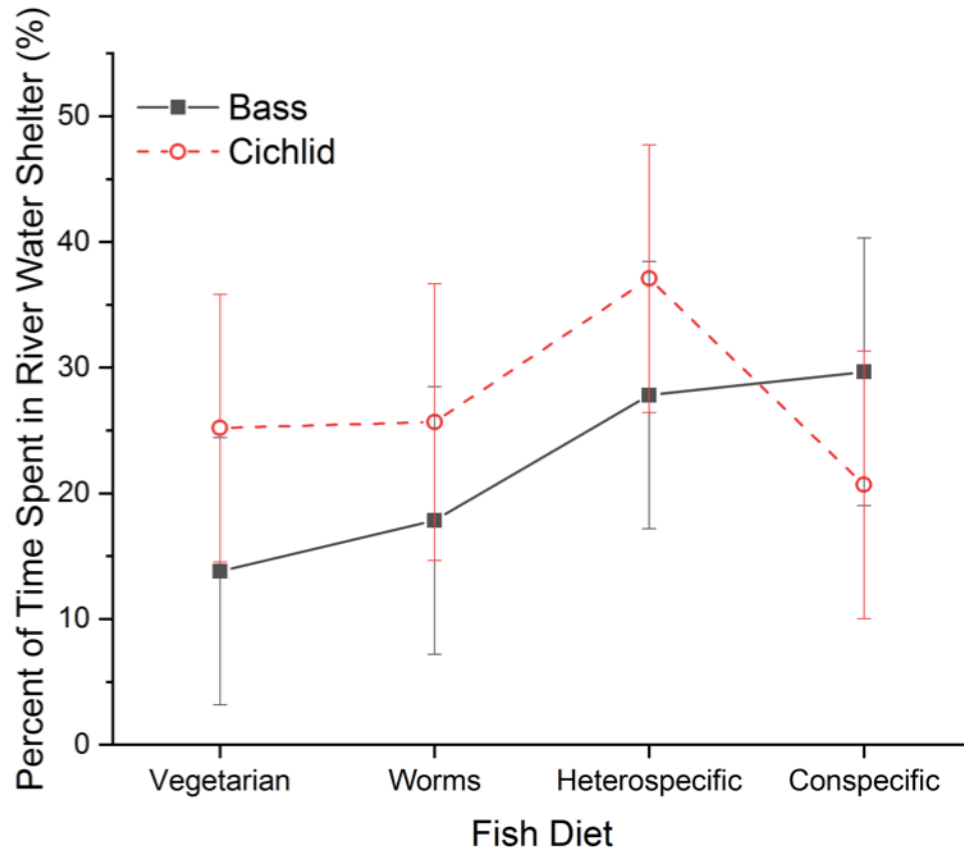
Figure 6. Mean (± SEM) of percent time crayfish spent in the shelter on the side with the river water as a function of species and diet. Red open circles with dotted lines are for the cichlid odor donors and the black closed squares with solid lines are for the bass odor donors. There were no significant differences for donor species or donor diet (p > 0.05).