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On the Rocks: Changes in Substrate Alter Antipredator Behavior in Benthic Stream Fish

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**ON THE ROCKS: CHANGES IN SUBSTRATE ALTER ANTIPREDATOR BEHAVIOR
IN BENTHIC STREAM FISH**

A Master's Thesis

Presented to

The Graduate College of
Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Sarah White

May 2021

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ON THE ROCKS: CHANGES IN SUBSTRATE ALTER ANTIPREDATOR RESPONSES IN BENTHIC STREAM FISH

Biology

Missouri State University, May 2021

Master of Science

Sarah White

ABSTRACT

Natural selection should strongly favor characteristics that make prey difficult for predators to distinguish from the background, including both morphological and behavioral crypsis. The Rainbow Darter (*Etheostoma caeruleum*) is a small, cryptic, benthic fish that inhabits gravel-bottomed streams and is preyed upon by predators such as the benthic Knobfin Sculpin (*Cottus immaculatus*) and the pelagic Longear Sunfish (*Lepomis megalotis*). In three experiments, I tested whether the behavior of darters was influenced by the opportunity for crypticity offered by their substrate and by the presence of cues from benthic and pelagic predators. First, darters in the non-breeding season chose substrates that were most similar in reflectance values to their bodies. This choice was expressed as preferential occupation of a darker mixture of dark and light rocks in comparison to a homogeneous mixture of light rocks only. This preference was present regardless of the level of predation risk by the benthic (sculpin) predator. Second, during the breeding season, the behavior of darters was counter to what I hypothesized, with darters showing higher levels of activity on the darker background when predation risk was high. This result could be due to changes in reflectance values from bright breeding coloration leading to the light rocks offering a more cryptic background. Third, in the non-breeding season, darters showed the predicted response of decreasing activity on the darker substrate, particularly when predators were present. Effects appeared stronger to a benthic predator (sculpins) than to a pelagic predator (sunfish). In addition, darters increased vigilance behavior in response to presence of cues from both predators regardless of substrate type and showed less swimming behavior in the predator treatments compared to the control treatment. Therefore, behavior of darters is influenced by the levels of both predation risk and habitat crypticity, which may vary between breeding and non-breeding seasons.

KEYWORDS: antipredator behavior, benthic, chemical cues, evolution, behavior

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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I thank my lab supervisor, Ben Dalton, whose expertise and reminders of “stop being so stressed” got me through. He is truly a one-of-a-kind mentor, and to this day one of the most influential persons I have encountered. I thank two of my closest friends and alums of the Mathis Lab, Katy Gardner and Stephanie Morrison, for all of their kindness and support throughout the years. I would not have believed in myself enough to apply to grad school without them, and I look up to them in many areas of life.

I dedicate this thesis to Cheryl Ann White.

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INTRODUCTION

A common result of high predation risk is that prey individuals evolve to blend in with their backgrounds because these backgrounds provide better opportunities for remaining concealed and reduce the potential of capture by predators (Kjernsmo & Merilaita, 2012). Background matching is primarily due to similarities in coloration or patterning between the prey and their background surroundings (Merilaita & Stevens, 2011). However, crypsis can be enhanced by behaviors such as freezing or reduction in movement (behavioral crypsis), particularly for motion-sensitive visual predators (Main, 1987; Stevens et al., 2011; Kenison et al., 2017).

Maintaining crypticity can be challenging because backgrounds are often heterogeneous in color and geometry (e.g., Boyero, 2003). The heterogeneous composition of habitat backgrounds can compromise or enhance cryptic opportunities, with changes in pattern and color affecting a predator's ability to detect a prey individual (Merilaita et al., 2001). Prey can increase their probability of remaining undetected by choosing to occupy patches that offer the highest level of crypticity (Marshall et al., 2016). Relatively few studies have examined background color preferences in prey (review by Stevens & Ruxton, 2019). For example, young Coho Salmon, *Oncorhynchus kisutch*, preferred substrates that reduced contrast between fish body surfaces and the background (Donnelly & Dill, 1984), and moths, *Hypomecis roboraria*, moved to backgrounds that offered better crypticity (Kang et al., 2013).

In addition to habitat choice, prey can further reduce detection by a predator by altering their behavior depending on the level of crypticity offered by the background (Stevens & Ruxton, 2019). For example, freezing or decreased activity might be an effective antipredator

strategy when prey are difficult to detect against a cryptic background, but this behavior might be less effective once prey have been detected (Martín et al., 2009). Moreover, some backgrounds offer crypticity to the predators as well as the prey. When predators are easy to detect, such as on a non-cryptic background, prey can afford to employ detection-avoidance strategies and to flee only if these strategies fail (Cooper, 2008). However, when it is difficult to spot a predator from a distance, such as in a cryptic habitat, an optimal strategy for prey is to initiate fleeing immediately (Broom & Ruxton, 2005). For example, Longfin squid, *Loligo paeleii*, employed camouflage and freezing in the presence of an active predator and flight in the presence of an ambush predator (Staudinger et al., 2011). Another complicating factor is that both color and pattern of prey and habitat can vary seasonally. Thus, decisions about which patches to occupy and how to behave within those patches can also vary depending on season (Bergen & Beldade, 2019).

Stream fishes offer a good opportunity to study the influence of background matching on prey behavior because background substrates are often patchy. For example, within a short distance, prey can be exposed to substrate patches of different types of rocks, soil, moss, and woody debris (Gooderham et al., 2007), with different background types offering different levels of crypticity for prey. In the Ozarks region of the USA, darters (Percidae) are common prey fishes that are susceptible to a wide range of predators, including both benthic (e.g., sculpin, *Cottus* sp.: Phillips & Kilambi, 1996) and pelagic (e.g., sunfish, *Lepomis* sp.: Becker & Gabor, 2012) predatory fishes. Darters are typically found on the rocky bottoms of the streams due to the absence of a swim bladder, the air sac that provides buoyancy in most fishes. The antipredator behavior of darters has been fairly well studied, with darters exhibiting both freezing/reduced activity (Crane et al., 2009) and fleeing (Johnson & Mathis, 2021) in response

to predators. Increased predation risk is detected by darters via detection of alarm cues released from the damaged tissues of conspecifics (Anderson & Mathis, 2016), as well as detection of the scent of predatory fish scents or “kairomones” (Gibson & Mathis, 2006). Darters also have color vision with single, middle-wavelength-sensitive cones as well as double, long-wavelength-sensitive cones, yet appear to lack sensitivity to ultraviolet light or short-wavelength-sensitive cones (Gumm et al., 2012).

Sculpins in the genus *Cottus* are common benthic predators used in studies of darter antipredator behavior (Gibson & Mathis, 2006). Sculpins are ambush predators, capitalizing on their general crypticity and ability to hide under rocks (Pflieger, 1997). Although visual detection of prey is important, sculpins have also been observed as using lateral-line sensitivity as well as chemosensory recognition as a means of prey detection (Kanter & Coombs, 2002). Color vision has not been studied in most *Cottus* species, but Elkhorn Sculpin (Cottidae), a benthic marine species, has been reported to have color vision with peak sensitivities and highest visual perception at wavelengths at 554 nm (Matsuda et al., 2005). Many species of sunfishes (Centrarchidae) are piscivorous fishes that are common in the same habitats as darters and sculpins. Although they tend to be active primarily in the pelagic zone, they can consume benthic as well as nonbenthic prey (Thorp et al., 1989). Sunfishes also have color vision and can use visual cues to make predation decisions (Mitchem et al., 2018) with peak sensitivities at 536 nm for single cones and 620 nm for double cones (Hawryshyn, 1988).

Rainbow Darters (*Etheostoma caeruleum*), the subject of this study, are sexually color monomorphic during the non-breeding season, exhibiting a range of mottled brown and grey colors (Pflieger, 1997). Females retain this coloration throughout the year, but during the breeding season (early spring through late summer), males develop striking blue and red bars

across the posterior end toward the caudal fin and have blue and red on the spiny and soft dorsal fins (Pflieger, 1997). The seasonal color change in male Rainbow Darters (as well as other darter species) can vary dependent on populations, with expression of blue and red/orange relating to body size (Zhou et al., 2014). During the breeding season, Rainbow Darters undergo a seasonal shift, with brighter coloration of red and blue on the fins, making them appear conspicuous and serving as a sexual signal for male and female interactions (Martin & Mendelson, 2016). Thus, substrate usage might differ for Rainbow Darters between seasons. This seasonal change in conspicuous color pattern has been observed to alter antipredator behaviors of darters, with a change in response to threat of predators from decreased movement (freezing) during the non-breeding season when males appear more cryptic and increased movement (fleeing) when males appear conspicuous (Moran et al., 2014).

For this study, I tested whether different color/patterns of rock substrates would influence behavior of Rainbow Darters (which will hereafter be simply referred to as “darters”). I used naturally occurring variation in rock cobble collected from the study site (Figure 1) to form two substrate types: light-colored (white, off-white, light gray) rocks versus a darker mixture composed of rocks collected from the same stream as the darters that contained an unmanipulated range of colors (from light to dark browns, dark grays, and reds; Figure 2). In the first experiment, I tested whether darters preferred to occupy light versus darker substrates during the non-breeding season and whether this choice was influenced by the presence of chemical cues from a benthic predator, the Knobfin Sculpin (*Cottus immaculatus*). Qualitatively, non-breeding Rainbow Darters appeared to be more cryptic against the darker substrate, and this observation was verified by comparing spectral reflectance values of the two types of substrate with the reflectance values for the darters. In a second experiment, conducted in the breeding

season, I tested whether darters would behave more cryptically following exposure to predatory cues of Knobfin Sculpins (which will hereafter simply be referred to as “sculpins”) when on the darker substrate in comparison to the light-colored substrate. The third experiment was similar to Experiment 2 except that a second predator, the pelagic Longear Sunfish (*L. megalotis*) was added and that the study was conducted during the non-breeding season.



Figure 1. Composition of substrate in the James River at the collection site.



Figure 2. Hand-selected white, off-white, tan, and grey rocks made up the light substrate (left) while a natural mixture of all colors made up the composition of the darker substrate (right).

METHODS

Experiment 1: Effect of Risk from a Benthic Predator on Darter Substrate Preference in the Non-breeding Season

Collection and Maintenance. Compliance for working with fish was approved through the CITI Program (Appendix A). This study was approved by the Institutional Animal Care and Use Committee (IACUC) on 04/30/2019 (IACUC, ID #19-007.0; Appendix B.) and was completed using collection permits granted by the Missouri Department of Conservation (MDC ID #18594, #18079). Rainbow Darters and Knobfin Sculpins were collected by seine from the James River (Greene CO., MO) during September 2020. Darters were placed in a 75-L communal tank filled with well water. Clay pots along the bottom served as refugia and an arbitrary selection of rocks scooped from a mixture collected from the river serving as substrate. Darters (n = 40; 46-67 mm) were allowed to acclimate to the housing tank for 2 wk prior to testing, and were kept on a 12:12 light:dark cycle at 20° C. Knobfin Sculpins (n = 4; 5.2-10.83 g) were placed in a communal 151-L tank at the same temperature and light:dark cycle as the darters. Darters were fed frozen brine shrimp (*Artemia* sp.) and sculpins were fed bloodworms (*Chironomidae* sp.) every other day.

Substrate Types. Cobble collected from arbitrary sites in the same stream as the darters was placed in buckets for use as both light and darker substrates. For the darker substrate treatment, I scooped rocks from buckets containing unmanipulated mixtures of stones in color proportions as they occurred most frequently in the river (personal observation). To generate a light-colored substrate, I scooped rocks from the buckets and then separated the lightest-colored

stones (white, light gray, tan) by hand. Substrate mixtures consisted of pebbles between 0.3-50mm.

Reflectance Spectra Measurements. To verify that the darker mixture of rocks offered a more cryptic substrate than light-colored rocks, I compared the reflectance spectra of both substrate types to that of the darters. Substrate and darter light reflectance values were obtained using a UV-VIS spectrometer (Ocean Optics USB2000) equipped with a tungsten-deuterium source of light (Ocean Optics DH2000-BAL) and a white fluoropolymer standard (WS-1-SL Spectralon; NH, USA). I held the RPH reflectance probe (Ocean Optics) at a 45° angle over samples while the probe's case shielded interfering light. Reflectance measurements were recorded using a 100 ms integration time. Darters ($n = 10$) collected during the non-breeding season were arbitrarily chosen from the communal tank to collect reflectance data. The RPH probe was placed on each side of the body directly behind the operculum, and two measurements were taken per side for each fish and averaged. The same method was used to measure spectral reflectance of individual rocks in each substrate type ($n = 40$ per substrate type). I arbitrarily selected each rock without replacement by reaching into a container for each substrate type and selecting a rock without looking. Reflectance values of darters as well as the light and darker substrates were then calculated at single cone mid-wavelengths (508 nm), double cone long-wavelengths (604 nm), and rod (523 nm) sensitivities of Rainbow Darters (Gumm et al., 2012). A generalized linear model was used to determine differences between reflectance values for each background type and values for the bodies of darters. Reflectance data residuals were square-root transformed to correct for nonnormality and a Tukey's post-hoc test was used to determine differences between groups.

Predator and Control Cue Collection. During substrate choice tests, darters were exposed to chemical cues from a benthic predator (sculpins) to provide high predation risk and a control of “blank” water to provide low/no risk. Rainbow Darters exhibit antipredator behavior in response to sculpin chemical cues and can distinguish between sculpin and nonpredatory fish (Gibson & Mathis, 2006), so inclusion of additional non-predator control scents was not necessary. To generate the sculpin cues, sculpins were placed in individual containers of deionized water that was proportional to their mass (100 mL/g). Containers were oxygenated but not filtered so that active chemicals would not be removed. After 24 h, each sculpin was removed, and the remaining water was strained through glass wool to remove particulate matter. The water was then partitioned into 20-mL tubes and frozen at -20°C for later use. The control (blank) cue was prepared using the same methods, except that no sculpin was added to the water.

Food Cue Collection. Darters in the lab can sometimes show low levels of activity (personal observations). Because a typical antipredator response for darters is decreased activity (e.g., Anderson & Mathis, 2016), I provided darters in all treatments with food cues to stimulate initial activity. For the food cue, frozen brine shrimp (*Artemia* sp.) cubes were placed in well water with a 1:1 proportion of mass:water (100mL/g) for 15 min. The mixture was then filtered with glass wool to remove any pieces of shrimp, and the remaining water was then placed into 20-mL tubes for immediate use.

Testing Protocol. Individual darters were removed from the communal tank 1 h after being fed and placed in an aerated 9.5-L testing tank. For this substrate choice experiment, the tanks were divided down the middle length-wise, and the two substrate types (light or darker) were placed on randomly-selected opposite sides. Darters (n = 16 per treatment) were placed in testing tanks 48 h prior to testing to allow for acclimation. Fluorescent lights were placed directly

over the individual tanks, and a 12:12 light:dark cycle was maintained. I then used a syringe to add the 20-mL of food cue to the water over the air stone to ensure rapid dispersal. Randomly selected stimulus treatments of either predator cue (sculpin) or control cue (blank) were then immediately added in the same way and location (administered at air stone for rapid dispersal) as the food cue. The observer was blind to the identity of stimulus treatments. During trials, time spent on each background type (light vs. darker) was recorded for 10 min.

Statistical analyses were completed using Program R (version 3.6.2) with Base R, car (Fox & Weisberg, 2019), and ARTool (Kay et al., 2021) packages. To determine the effect of predation risk on background choice, I compared the time spent on the darker substrate in the blank versus the sculpin treatments using a one-way ANOVA following a square root transformation, which normalized the residuals.

Experiment 2: Effect of Substrate Type and Risk from a Benthic Predator on Darter Behavior in the Breeding Season

Collection and Maintenance. New darters and sculpins were collected for this experiment. Rainbow Darters ($n = 60$; 36-57mm) were collected from the James River (Greene Co., MO) in August 2019. Knobfin Sculpins ($n = 20$; 6.0-11.46 g) were collected from Pierson Creek (Greene Co., MO) in November 2019. Housing and maintenance were as in Experiment 1. Sex was determined by expression of bright color of the males. It is possible some males were not expressing nuptial coloration, so “sex” was used as a proxy to differentiate darters as either colorful (“male”) or not colorful (“female”).

Cue Collection and Background Types. Sculpin ($n = 5$) were arbitrarily selected from the pool of 20 that were collected, and control cues were collected as in Experiment 1.

Composition of substrate types were the same as in Experiment 1.

Testing Protocol. For this experiment, each testing tank (the same size tanks used in Experiment 1) received only one of the two substrate types. Darters ($n = 11$ per treatment) were placed individually in randomly chosen testing tanks (light vs. darker). Focal darters were fed 1 hr prior to being placed in testing tanks and were allowed to acclimate for 48 h. At the beginning of each trial, darters were given a food scent cue as described in Experiment 1 to encourage movement. Immediately following addition of the food cue, either the sculpin predator cue or control water cue was added to the tanks in the same manner as in Experiment 1. The observer was blind to the identity of stimulus treatments.

Darter behaviors that were recorded were number of darts (any change in location of the darter: Anderson & Mathis, 2016), time spent in the upper water column (denoted by a dotted line at 5cm above the substrate: Brown et al., 2006), and time spent in vigilance posture (anterior end of body lifted off the substrate by the pectoral fins: Wisenden, 1995). Trials lasted 10 min, and each darters' length and "sex" (whether or not it was expressing nuptial coloration) was recorded after all trials on a given day were completed. Fixed factors were cue type (sculpin predator vs. control) and background type (light vs. darker substrate); sex was included as a random factor. The three response variables (number of darts, time spent in vigilance, and time spent in the upper water column) were analyzed separately. Data were normalized via aligned rank transformed (Higgins & Tashtoush, 1994) before using a two-way ANOVA with Type III sum of squares to compare means.

Experiment 3: Effect of Substrate Type and Risk from Benthic and Pelagic Predators in the Non-breeding Season

Collection and Maintenance. New Rainbow Darters ($n = 60$; 35-66 mm) and Longear Sunfish (*Lepomis megalotis*) ($n = 3$, 124 mm-96 mm, 35.9-15.5 g) were collected in November 2020 from the James River (Greene Co., MO). Knobfin Sculpin ($n = 5$) were different individuals than in Experiment 2 but were arbitrarily selected from the same pool of sculpins. Sunfish were housed in a 37.9-L communal tank and fed bloodworms every other day. Darters and sculpins were maintained as in Experiments 1 and 2. Cue collection for both Longear Sunfish and Knobfin Sculpin in this experiment was the same as in Experiments 1 and 2.

Testing Protocol. Testing protocol was the same as in Experiment 2 with individual tanks containing either the light substrate or the darker substrate. Observation of darters ($n = 9$ per treatment) took place during a 10 min time period as in Experiment 2. I made the following changes in behavioral response variables following preliminary observations of darters in the presence of cues from the pelagic predator: I added an index number of swims (every observed 2 s interval spent swimming and not touching the substrate = 1 “swim”). I excluded time spent in the upper water column as a response variable. Mean responses were compared using two-way ANOVAs with Type III sum of squares to determine effect of substrate and predator type (benthic vs. pelagic) on behavior (number of darts, number of swims, and time spent in vigilance). Data was aligned rank transformed to correct for departures from normality (Higgins & Tashtoush, 1994). Post-hoc comparisons for significant differences of factors were completed using Tukey’s multiple comparison tests.

RESULTS

Experiment 1: Effect of Risk from a Benthic Predator on Darter Background Preference in the Non-breeding Season

Spectral Reflectance of Substrates and Darters. The light-colored rocks had uniformly higher reflectance relative to the darker mixture, which had uniformly intermediate reflectance, relative to the darters, which uniformly had the lowest reflectance (Figure 3). Reflectance was significantly different between groups (light substrate vs darker substrate vs darter) at each of the peak wavelength sensitivities for cone types and rods of darters with groups different from each other following a Tukey's post-hoc tests ($F_4 = 2.764$, $p < 0.001$; Figure 4). Reflectance values for darters had less contrast to that of the darker mixture of rocks.

Substrate Preferences. Predation risk (blank versus sculpin) did not significantly affect choice of substrate ($F = 0.001$, $p = 0.970$) using a one-way ANOVA, therefore we combined the data for the two treatments to determine whether there was an overall preference for the darker substrate. Darters spent significantly more time overall on the darker substrate (that the reflectance data concluded was more similar to the darters' bodies) than the random expectation (one-sample Wilcoxon test; $V = 364$, $p = 0.030$, one-tailed; Figure 5).

Experiment 2: Effect of Background Type and Risk from a Benthic Predator on Darter Behavior in the Breeding Season

With one exception, there were no effects of sex on any response variables (all p 's > 0.38). For time spent in the upper water column, there was a sex \times substrate interaction ($F_{1,44} = 4.149$, $p = 0.048$; Figure 6); males spent more time in the upper water column on the darker

substrate and females spent little time in the upper water column on either substrate ($F_{1,44} = 7.989, p = 0.007$; Figure 6). Due to there being no other significant effects, sex will not be considered in any further analyses.

For number of darts, there was a significant interaction between cue and substrate ($F_{1,44} = 4.98, p = 0.031$; Figure 7), with darters being more active in the predator treatment on the darker substrate and more active on the light substrate in the control treatment. There was no significant effect of cue ($F_{1,44} = 0.582, p = 0.459$) or substrate ($F_{1,44} = 0.897, p = 0.348$) on the number of darts observed. There was no significant difference in vigilance behavior influenced by the cue ($F_{1,44} = 0.738, p = 0.398$), substrate ($F_{1,44} = 0.216, p = 0.644$), or an interaction between cue and substrate affecting time spent in vigilance ($F_{1,44} = 2.369, p = 0.130$). For time spent in the upper water column, there was no significant influence from the interaction of cue treatment and substrate type ($F_{1,44} = 3.343, p = 0.070$). No effect of cue type ($F_{1,44} = 0.012, p = 0.915$) or substrate ($F_{1,44} = 0.474, p = 0.495$) were present that influenced time spent in the upper water column.

Experiment 3: Effect of Background Type and Risk from Benthic and Pelagic Predators in the Non-breeding Season

There was a significant interaction between cue and substrate on number of darts ($F_{2,48} = 3.291, p = 0.046$, Figure 8), with darters on the darker substrate darting the least in response to the sculpin cue, an intermediate level of darts to the sunfish cues, and the most darts in response to the control cue. There also was a significant main effect of cue on number of darts ($F_{2,48} = 3.521, p = 0.037$), with darters moving less often in the predator treatments. There was no main effect of substrate ($F_{1,48} = 0.496, p = 0.484$). Cue significantly affected time spent in vigilance

posture ($F_{2,48} = 3.806, p = 0.029$; Figure 9), with darters spending most time in vigilant behavior when sculpin cue was introduced and the least amount of time vigilant in the control. The effect of substrate was not significant ($F_{1,48} = 0.807, p = 0.373$), nor was the interaction between cue and substrate ($F_{2,48} = 1.322, p = 0.276$). Cue significantly affected number of swims above the substrate ($F_{2,48} = 5.329, p = 0.008$; Figure 10) as darters in the control treatment swam the most, and darters in the sculpin treatment were observed swimming the least. The effects of substrate ($F_{1,48} = 1.335, p = 0.253$) and the interaction between cue and substrate was not present ($F_{1,48} = 0.264, p = 0.769$).

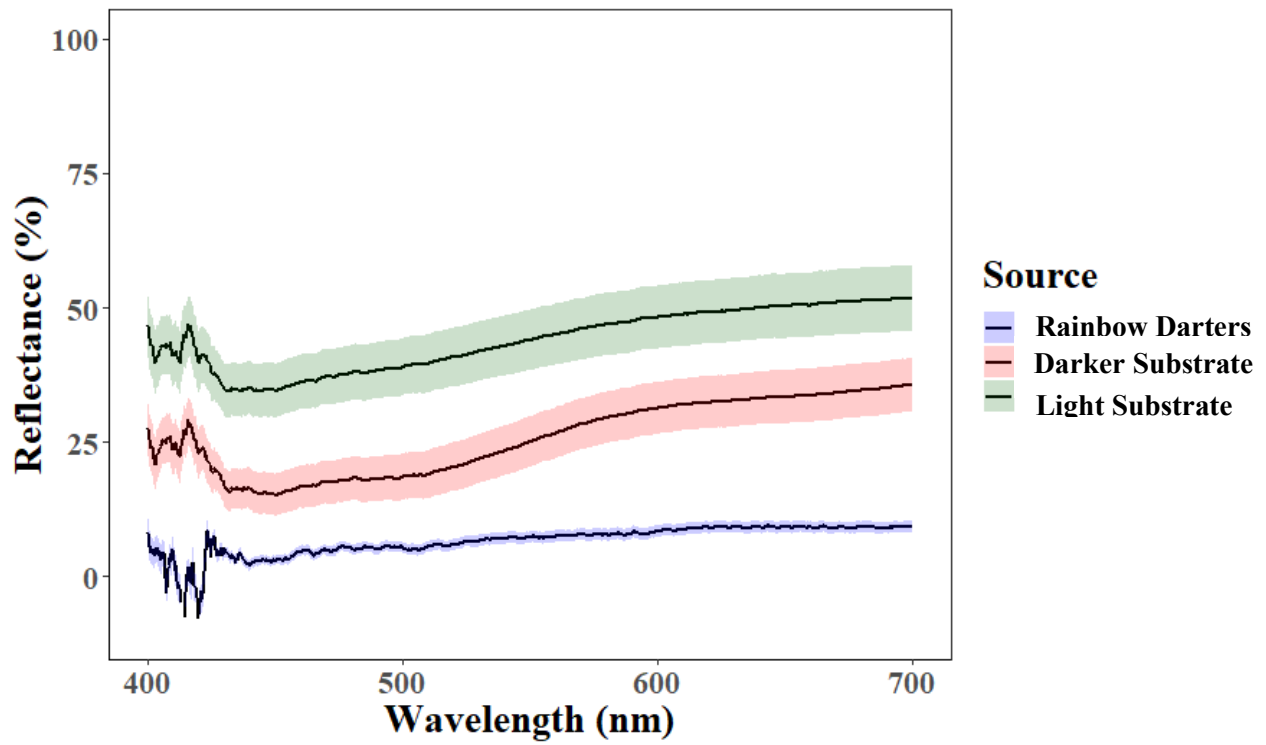


Figure 3. Mean and 95% C.I. reflectance values for darter bodies, darker substrate, and light substrate. The light rock substrate had the highest average reflectance values compared to the darker substrate, making the light substrate higher contrast to darter bodies.

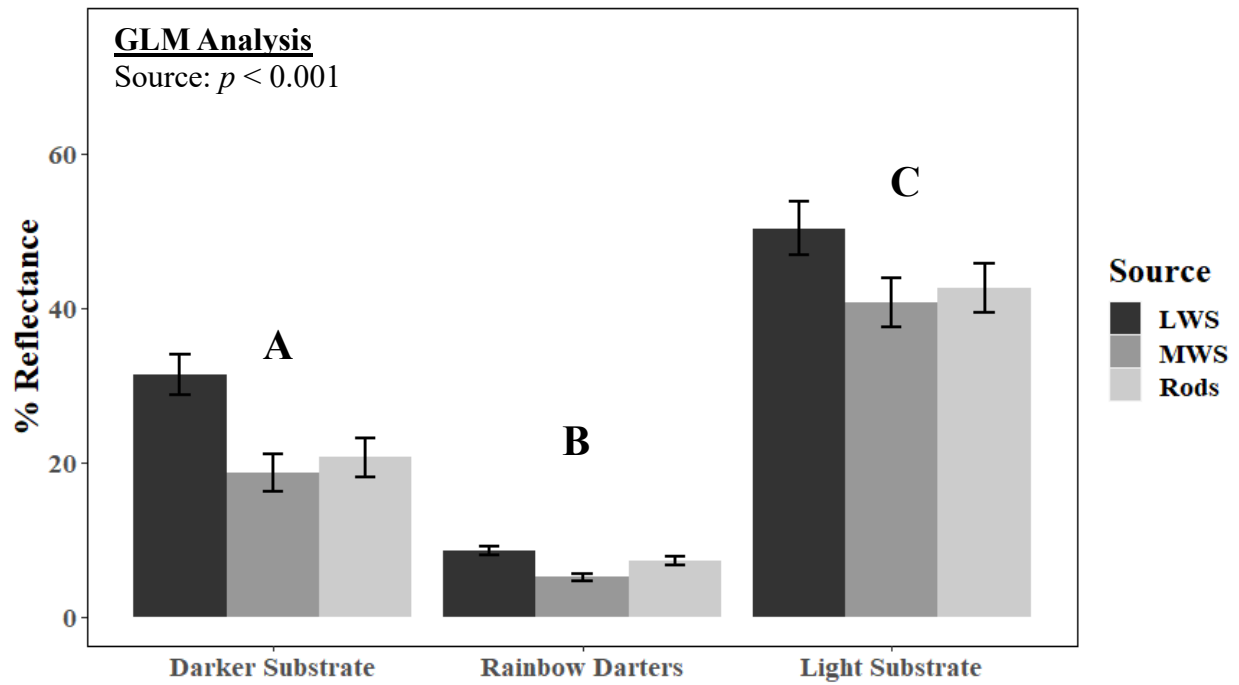


Figure 4. Mean (\pm SE) reflectance values show differences between the different backgrounds compared to the darters at Rainbow Darter mean peak sensitivities for long wavelength sensitivity (LWS), middle wavelength sensitivity (MWS), and rod sensitivity. Mean values of the groups were square root transformed and compared using a generalized linear model. Means that do not share a letter are significantly different from one another.

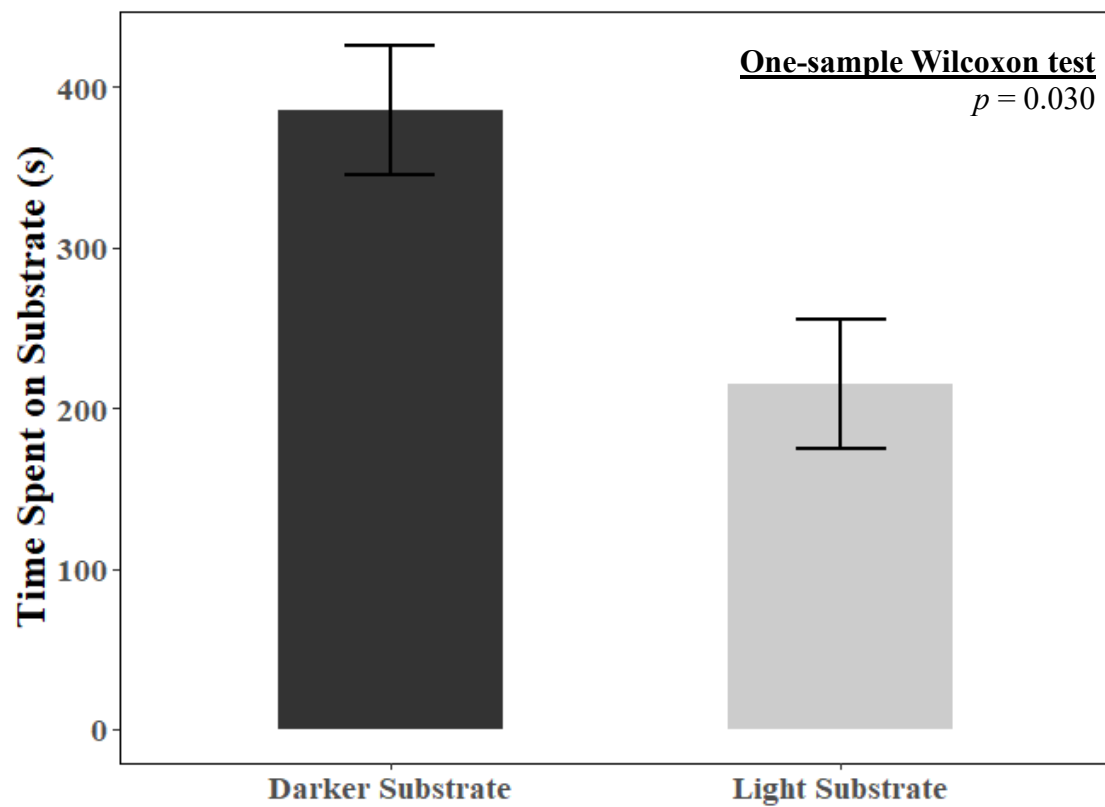


Figure 5. Mean (\pm SE) time spent by darters ($n = 16$ per treatment) between light and darker substrate.

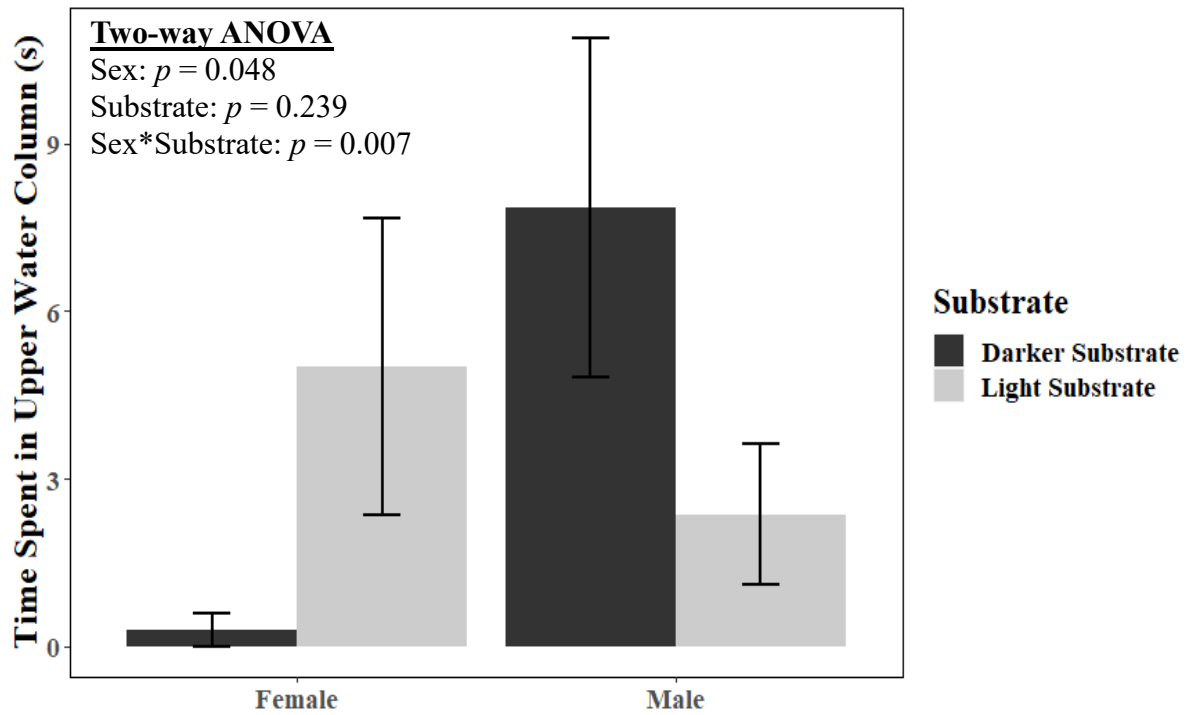


Figure 6. Mean (\pm SE) time spent in the upper water column by male (colored) and female (non-colored) darters ($n = 11$ per treatment) on the light and darker substrate types. All statistical analyses were completed using an align rank transformation of data. Untransformed data are shown.

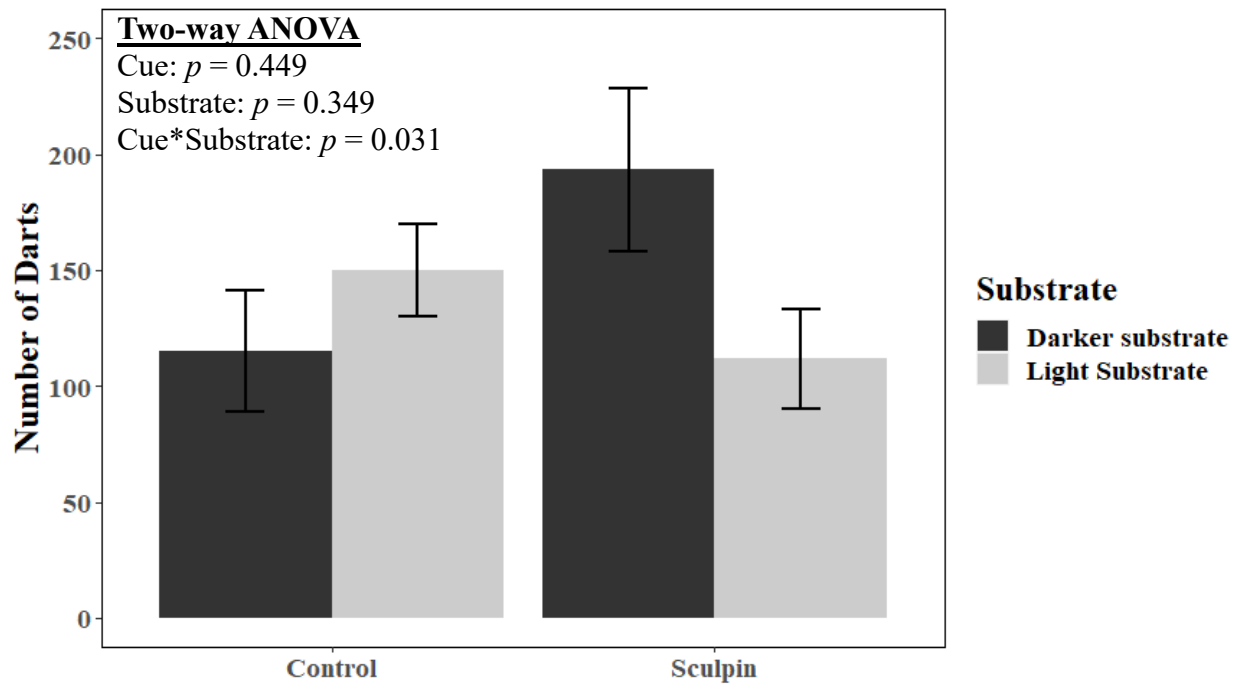


Figure 7. Number of darts mean (\pm SE) of darters ($n = 11$ per treatment) on the darker and light substrate types when exposed to different blank (control) and benthic predator (sculpin) treatments. All statistical analyses were completed using an align rank transformation of data. Untransformed data are shown.

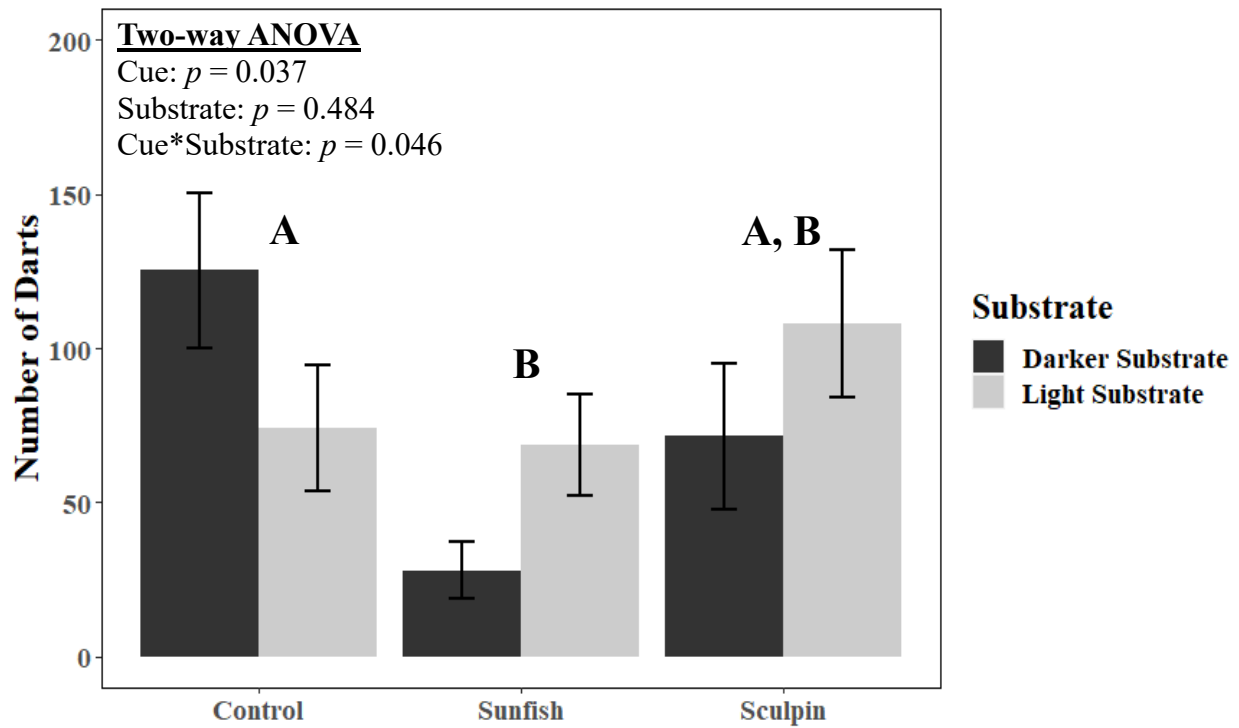


Figure 8. Number of darts (mean \pm SE) by darters ($n = 9$ per treatment) on darker (left) and light (right) substrates following exposure to blank (control), pelagic predator (sunfish), and benthic predator (sculpin). All statistical analyses were completed using an align rank transformation of data. Untransformed data are shown. Means that are not significantly different share a letter.

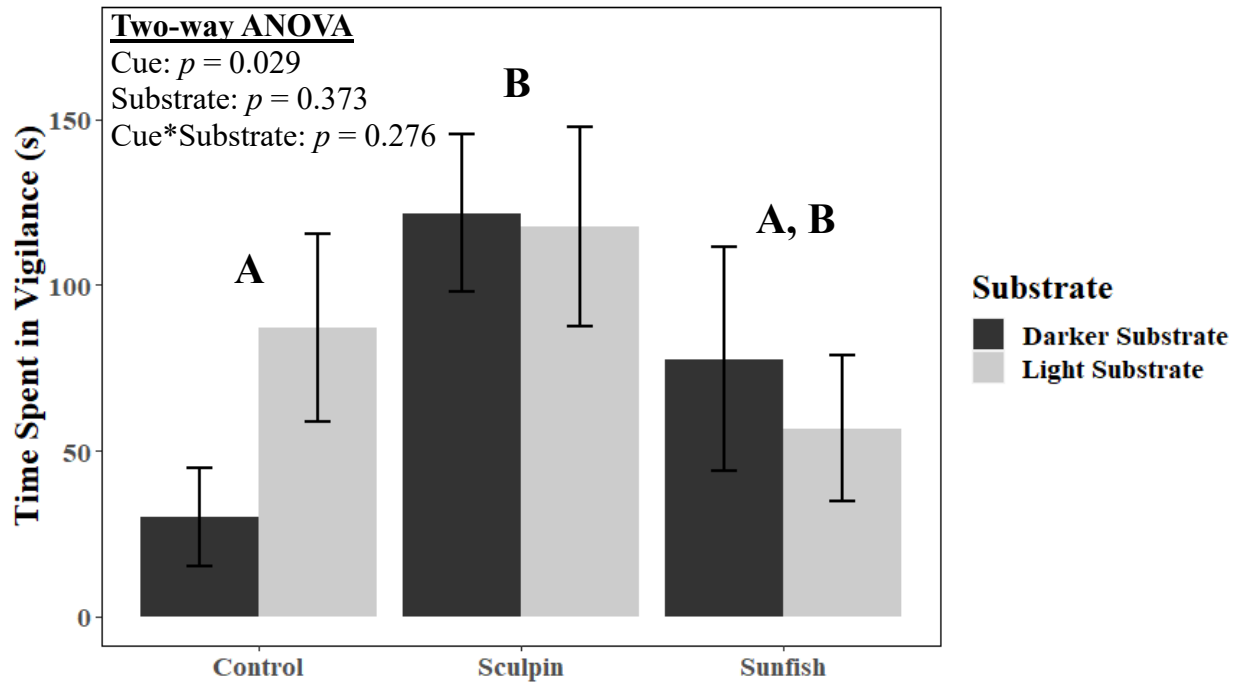


Figure 9. Time spent in vigilance (mean \pm SE) by darters ($n = 9$ per treatment) on darker (left) and light (right) substrates following exposure to blank (control), pelagic predator (sunfish), and benthic predator (sculpin). All statistical analyses were completed using an align rank transformation of data. Untransformed data are shown. Means that are not significantly different share a letter.

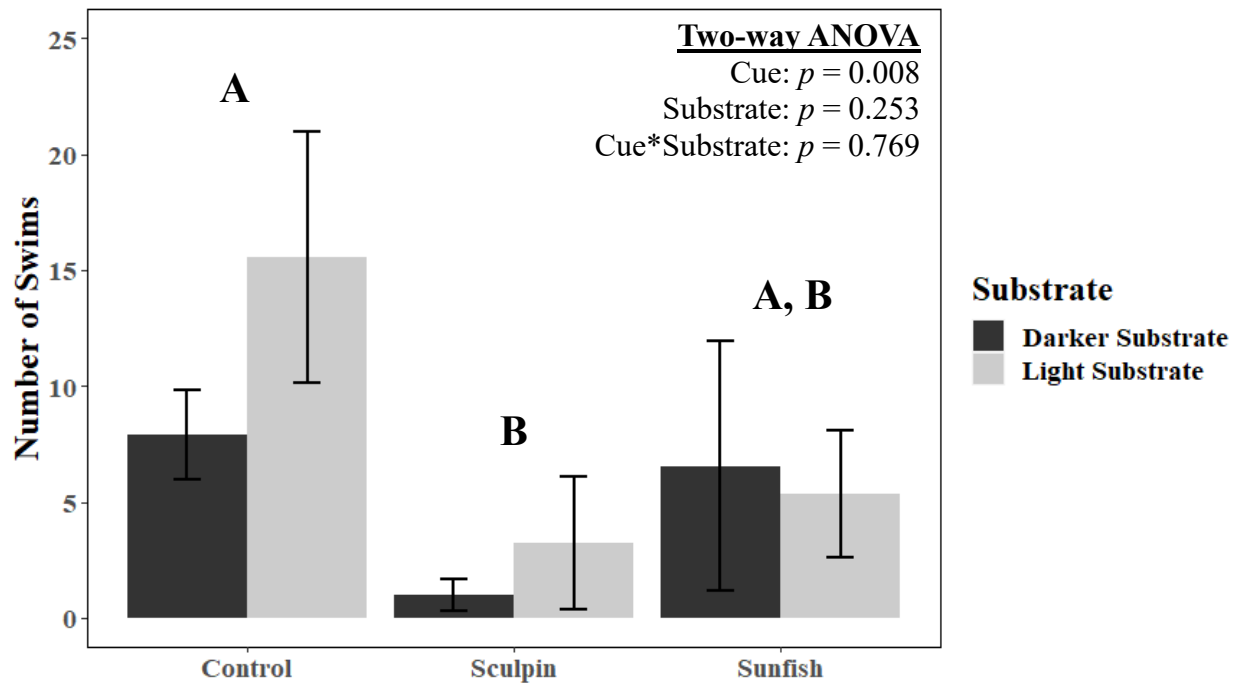


Figure 10. Mean \pm SE number of times darters ($n = 9$ per treatment) spent 2 s swimming on darker (left) and light (right) substrates following exposure to blank (control), pelagic predator (sunfish), and benthic predator (sculpin). All statistical analyses were completed using an align rank transformation of data. Untransformed data are shown. Means that are not significantly different share a letter.

DISCUSSION

Experiment 1: Effect of Risk from a Benthic Predator on Darter Substrate Preference in the Non-breeding Season

Spectrometry data confirmed that darter reflectance values had less contrast in naturally occurring, darker collection of rocks, at least in the non-breeding season. Darters against a darker substrate should thus be more cryptic than when against a light-colored rock background. As predicted, darters showed a preference for the darker substrate, spending 64% of their time on the side of the tank with the darker mixture of rocks. These results are consistent with previous studies in which animals in other taxa chose a background that is patterned or colored in a manner that allows for lower contrast and optimization of concealment (lizards: Salisbury & Peters, 2019; Vetter & Brodie, 1977; moths: Kang et al., 2012).

Unlike some fish species, predation risk did not influence choice of light vs. darker substrate by darters. For example, the upper water column dwelling Least Killifish, *Heterandria formosa*, preferred backgrounds that matched their bodies' striped patterns only when predation risk was high (Kjernsmo & Merlaita, 2012). Darters do show other threat sensitive behavior, most notably decreased activity (Commens & Mathis, 1999; Moran et al., 2014; Becker & Gabor, 2012). Movement between substrate patches is an inherently risky behavior because the risk of detection from movement may be higher than the potential of being spotted against the background. Some benthic fish species have plasticity in their expression of color patterns and brightness to enhance background matching on a substrate (Cox et al., 2009), which might minimize the cost of movement. However, it is not known whether rapid color change that enhances camouflage is present in darters.

Experiment 2: Effect of Substrate Type and Risk from a Benthic Predator on Darter Behavior in the Breeding Season

Substrate affected the amount of time darters spent swimming in the upper water column, which was different between males and females. While the bright colored individuals (considered “male” in this study) spent more time in the upper water column on the darker substrate, they had an inverse response on the light substrate. Dull-colored individuals (“females”) had a reverse response, spending more time in the upper water column on the light substrate and less time on the darker substrate. Though darters rarely venture far from the benthic zone in nature (Magoulick, 2004), some fish species have been observed altering their depth preference when substrate may not appear suitable (Blaser & Goldsteinholm, 2012). When nuptial coloration is expressed, reflectance values are highest when colors are bright, and male darters express these bright colors on their fins and parts of the body when in breeding condition. The reflectance from the darters’ fins produces the bright oranges and blues that maintain reflectance values in the short and long wavelengths (Zhou et al., 2015). Therefore, when in breeding condition, I predict that the reflectance values would be more similar to that of the light substrate, making light substrate more suitable for background matching. It is possible that, for the highly reflective male darters on the darker substrate, there are fewer cryptic opportunities and to remain against the substrate may not be favorable, causing an increase of time in the upper water column. Because female darters do not undergo a change in coloration, the reflectance spectra are likely similar for the females used in this experiment compared to the individuals in Experiment 1, making the light substrate less cryptic, which could explain their increased time spent in the upper water column. Though this study did not collect reflectance data for darters in

the breeding season, further research should be pursued to determine if this change in reflectance is consistent with this prediction.

The influence of substrate type on activity levels of darters was significantly influenced by predation risk in the breeding season. However, the direction of the difference was unexpected. Previous studies typically showed that darters responded to predator cues with decreased activity (Wisendon et al., 1995). We expected that decreased activity would be most pronounced on the darker background under predation risk, because decreased activity enhances the effects of crypticity. However, although darters did decrease their activity on the light background when predation risk was high, they increased their activity under high-threat levels on the darker substrate. I suspect that this difference was caused by the change in darter coloration and behavior that occurs in the breeding season.

Experiment 3: Effect of Substrate Type and Risk from Benthic and Pelagic Predators in the Non-breeding Season

The influence of substrate type on activity levels of darters was significantly influenced by predation risk in the non-breeding season. Under high levels of risk, darters showed a decrease in activity on the darker substrate, which offered the highest level of crypticity, and an increase in activity against the light background. These activity changes were evident in terms of number of darts. Decreased activity can enhance the effectiveness of crypticity (Martel & Dill, 1995). Decreased activity on the darker substrate was strongest in response to sculpin, the benthic, ambush predator. Sculpins are adept at using the lateral line system to target moving prey (Kanter & Coombs, 2003). In contrast, activity levels under predation risk tended to be higher on the highly reflective light substrate than the more cryptic darker substrate, which might

indicate a willingness to leave the area or to seek shelter. This higher level of activity on the light substrate was stronger for the active, pelagic predator (sunfish). Sunfish use visual cues to select prey (O'Brien et al., 1976), and sunfish retinal receptors are contrast sensitive (Hawryshyn et al., 1988). Thus, darters might be particularly vulnerable to sunfish on a contrasting substrate. A stronger reaction to the sculpin may be due to their shared preferred habitat in the benthic zone of streams. Both predators were present at the sites in the stream where the darters were collected (personal observation), but it is possible that darters, remaining amongst the substrate with the benthic sculpin, may have more experience with predation from sculpin than sunfish, who forage in a range of microhabitats (Schaefer et al., 1998). However, these results confirm that predator ecology and background availability are determinant in antipredator reactions and can vary given seasonal changes in prey.

This experiment also shows a shift of antipredator tactics between breeding and non-breeding seasons. Similar seasonal changes in antipredator behavior also have been reported for Orangethroat darters, *E. spectabile* in which non-breeding individuals freeze when threatened, but individuals in breeding coloration become less likely to freeze and more likely to flee (Radabaugh, 1989). These darters, like the Rainbow Darters in our study, show dramatic differences in coloration between breeding and non-breeding seasons. In Experiment 2 (breeding season), the darters in this study, having lost most of their higher-reflectance nuptial coloring, better matched the darker substrate and adjusted their antipredator behavior from freezing to flight. This plasticity in antipredator behavior can occur in environments where effects of seasonality are present and morphological crypsis and behavioral crypsis must operate in concert under these different conditions (Bergen & Beldade, 2019). Shifts in pigmentation and coloration have been observed as causing a changed reaction to predators on different

backgrounds by the individuals who possess these color characteristics (Polo-Cavia & Gomez-Mestre, 2017).

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APPENDICES



Completion Date	01-Oct-2019
Expiration Date	N/A
Record ID	33600325

This is to certify that:

Sarah White

Has completed the following CITI Program course:

Working With Fish in Research Settings
(Curriculum Group)

Working with Fish
(Course Learner Group)

1 - Lab Animal Research
(Stage)

Not valid for renewal of certification through CME.

Under requirements set by:

Missouri State University



Collaborative Institutional Training Initiative

Verify at www.citiprogram.org/verify/?w51c85965-7683-4a70-891e-7345d83a8b15-33600325

Appendix A. CITI program training approval to work with fish.

From: LabTracks <REDIPComputing@missouristate.edu>
Sent: Friday, December 20, 2019 12:14 PM
To: Mathis, S Alicia <AliciaMathis@MissouriState.edu>
Subject: LabTracks: Protocol Approved

Protocol 19-007.0 has been approved.

Appendix B. IACUC protocol 19-007.0 approval.