Constraints of background risk on space use and learning in Trinidadian guppies

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Abstract

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Predators affect prey populations by driving prey to adopt costly antipredator strategies. Spatial neophobia mitigates the risks of novel spaces by reducing space use and is inducible through exposure to short-term elevated background risk. We hypothesized that background risk influences the ability to learn safe refuge sites through decreased exploration of novel habitats; specifically, high risk constrains the ability to learn safe refuge sites. We conducted three experiments using shoals of five female guppies pre-exposed to high or low background risk. The shoals explored an eight-arm radial maze reinforced by different combinations of foraging patches, predator models, or empty arms, then were exposed to chemical alarm cue or distilled water without reinforcements. We found no evidence to support that background risk affects the speed or accuracy of the initial decision to flee following cue exposure. We saw an overall preference for arms that previously contained food over arms that were either empty or contained predator models, suggesting that prey learned safe areas. We also showed that fish given alarm cue entered predator arms more than those given distilled water, suggesting that an acute threat leads prey to make more mistakes. While there is no evidence that background risk affects space use and learning, our results indicate that guppies can learn and use safety information, while predation threats compromise their ability to use this information. Our study provides insight into the complexity of behavioural trade-offs. This has implications for conservation initiatives seeking to understand prey habitat selection.

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Introduction

Predators affect prey population densities, not only through consumption, but through non-consumptive means such as intimidation (Preisser et al., 2005). Non-consumptive effects cause changes in prey adaptive traits, such as growth and development (Peckarsky et al., 2008). In addition, these non-consumptive effects can shape prey behaviour by driving them to adopt antipredator tactics that serve to reduce the immediate threat of predation (Preisser & Bolnick, 2008), but that may also reduce energy intake, reproduction, and long-term survival (Lima, 1998; Preisser et al., 2005). It is therefore imperative that prey adopt antipredator tactics only when appropriate, making trade-offs between the benefits of responding to a potential predation threat and the benefits of engaging in other fitness-related activities.

Complex behavioural trade-offs can be understood in the context of error management theory. Error management theory predicts that ambient predation risk experienced by prey will determine the intensity and type of costs to prey (Johnson et al., 2013). Type I errors include identifying and responding to something as risky when it is not. In this case, the individual is being cautious in responding, but misses other opportunities to forage, mate, and/or explore novel habitats, thus incurring opportunity costs. The accumulation of these opportunity costs can have long-term fitness consequences (Lima & Dill, 1990; Preisser et al., 2005). Type II errors include identifying and responding to something as not risky when it is. In this case, the individual is at a higher risk of having made a lethal mistake as they have failed to detect a predator. The cost of failing to respond increases in cases where prey experience higher levels of background risk (Crane et al., 2020a). Prey should be able to balance the risk of committing these two types of errors and making successful trade-offs between antipredator responses and fitness related activities if prey have reliable risk assessment cues. Within aquatic systems, conspecific damage-released alarm cues, an honest indicator released only when prey has been injured, and disturbance cues, an early-warning signal released as metabolic by-products when prey are disturbed, are known to be reliable indicators of risk and involved in real-time risk assessment where predation risk is highly variable (Chivers et al., 2012).

Since predation risk often varies in time and space (Sih et al., 2000), prey capable of altering their behaviour based on the level of perceived risk should have an advantage (Dingemanse et al., 2010). Background levels of predation risk shape the response to the immediate (acute) predation threats and how prey categorize specific predator types (Chivers et al., 2014). Individuals exposed to high background risk may exhibit neophobic predator avoidance and categorize novel cues as risky compared to individuals from low background risk which do not (Brown et al., 2013). Neophobia, the fear of novel stimuli, habitats, or social contexts, is an adaptive, phenotypically plastic generalized avoidance response to unpredictable and/or variable threats (Mettke-Hofmann, 2014). The avoidance of novel habitats is referred to as spatial neophobia (Elvidge et al., 2016). Neophobia is argued to be an adaptive response as it is expected to reduce the costs associated with elevated and variable predation risk by allowing the prey to respond to potential predation threats without prior experience (Brown et al., 2013, 2014).

Predation risk has been known to alter prey space use (Godin, 1997; Kramer et al., 1997). The dangerous niche hypothesis posits that species in dangerous habitats exhibit increased neophobia to reduce the costs of unknown dangers associated with habitat use (Greenberg, 2003; Greenberg & Mettke-Hofmann, 2001; Mettke-Hofmann et al., 2013). However, the dangerous

niche hypothesis has only been tested at larger habitat scales. For example, migrant blackbirds expressed more neophobic responses than resident blackbirds when novel objects were placed next to food patches (Mettke-Hofmann et al., 2013). Since resident blackbirds are restricted to a smaller area, the opportunity costs may be higher, thus lower neophobia allows them to secure access to resources faster. The impact of local predation risks is less well known over smaller spatial scales (i.e. microhabitats). Individuals that experience high levels of background risk exhibit greater spatial neophobia than those that experience low levels of background risk (Elvidge et al., 2016). Guppies pre-exposed to high levels of background risk exhibit more cautious exploratory behaviour when exposed to a novel chemical cue (Crane et al., *in press*). What is unknown is if neophobic responses come at a cost to the ability to learn and use space, including that of new potential refuge sites through reduced exploration of novel habitats.

The exploration of novel habitats allows prey to learn about the distribution of resources, such as mates and food patches, as well as the distribution of predation risks. Increasing the quantity of information regarding the distribution of risky vs. safe areas through exploration should be expected to reduce the risk of 'making a bad decision' (Inglis et al., 2001; Smith, 1997). More information about the surrounding habitat should increase an individual's ability to forage efficiently, identify the location of potential mates, establish and maintain territories, and avoid predators by quickly identifying shelters (Odling-Smee et al., 2006). Fish learn space by collecting information using a variety of cues, including visual landmarks, chemosensory cues, and socially informed conspecifics (Warburton, 2006). Spatial learning allows fish to develop "mind maps" based on experience, where individuals remember the spatial layout of their environment (Broglio et al., 2003; Rodriguez et al., 2006). Spatial memory also allows prey to associate habitat features with risk level through associative learning with chemical alarm cues to efficiently navigate their environment (Kelley & Magurran, 2006). In other words, in order to safely explore their environment, prey can use cues that indicate danger to learn that an area is risky.

Since prey exhibit distinct responses to predation threats and can associate a variety of cues to these threats, most studies focus on prey responses to danger cues but might not consider the role of safety cues. Contrary to danger cues that reliably indicate high levels of predation risk, safety cues reliably indicate low levels of predation risk (Luttbeg et al., 2020). Just as danger cues, safety cues can come in a variety of forms, such as audio, visual, or chemosensory. Often, seeing or hearing the behaviour of conspecifics or other prey can indicate risk level (Feyten et al., 2021). The active participation of other prey in conspicuous activities, such as foraging, courtship and mating can provide a reliable indication that risk levels are low (Crane et al., *in press*). Being able to identify relative safety in low risk levels is crucial to reduce the cumulative effects of type I errors. Prey should benefit from learning and committing to memory what and where 'safe' is through multiple sources of information in order to efficiently navigate their environment (Luttbeg et al., 2020).

Fish have the ability to use complex spatial learning and memory to orient themselves in their environment (Broglio et al., 2003; Odling-Smee et al., 2006). Since prey can learn spatial layouts, they should be able to learn which spaces are safe, thus, in the event of an acute threat, they should be able to flee to a known safe space. The level of background risk they experience is also known to influence their degree of spatial neophobia (Crane et al., *in press*). What is unknown is if this spatial neophobia constrains their spatial learning. We predict that if

background predation risk shapes the likelihood of exploring novel habitats, it will constrain the opportunity to learn safe refuge sites. Individuals from low-risk backgrounds should explore more compared to individuals from high-risk backgrounds, and therefore be better able to learn the spatial configuration of their environment, including the safe areas. As such, in the event of a predation threat, we expect individuals from low-risk backgrounds to escape to known safe areas, whereas individuals from high-risk backgrounds should escape at random. We also predict that those from low-risk backgrounds should show shorter latencies to flee to known safe refuges. However, the opposite might be true since ensuring that they make the correct decision may take more time.

Methods

General Methods

All three experiments followed the same general protocol. Initially, we manipulated background risk levels (high vs. low risk) in shoals of guppies. We did not feed the guppies in the day between background risk conditioning and testing to encourage shoals to look for food during the exploration phase. We then placed shoals of five guppies in the center of an eight-arm radial maze and allowed them to explore the layout. After the exploration period, we returned shoals to the center of the maze, exposed them to either alarm cue or distilled water cue and observed them for a period of 10 minutes (Figure 1).

Test Fish

Adult female Trinidadian guppies (*Poecilia reticulata*), sourced from a laboratory population, were used as they have been shown to exhibit induced neophobic responses through exposure to short-term elevated background risk (Brown et al., 2013). Guppies were reared in 110L glass aquaria lined with gravel substrate, and filled with continuously filtered dechlorinated tap water (26°C, pH ~ 7.2, 12:12 Light:Dark cycle). Guppies were fed until satiation, twice daily, with commercial flake food (Nutrafin™).

Alarm Cue Preparation

To condition fish as high background risk and to provide an acute threat cue (see below), we collected the damage-released chemical alarm cues from whole body extracts from male and female donor guppies (Appendix Table 1). We euthanized donors via cervical dislocation in accordance with the Concordia University Animal Care Protocol (AREC 30000355). We removed the head and tail from each donor and immediately placed them in a 100 mL beaker of chilled distilled water. We homogenized the tissue, filtered them through polyester floss, then diluted the solution to a final concentration of ~0.1 cm^2 mL^{-1} (Elvidge et al., 2016). Alarm cue made from male donors was filtered a second time to reduce pigmentation. The alarm cue was frozen at -20°C in 20 mL aliquots until required. Damage-released chemical alarm cues are known reliable risk assessment cues (Chivers & Smith, 1998). Short term exposure (i.e. over a period of a few days) to high levels of these cue are known to induce neophobic responses in Trinidadian guppies (Brown et al., 2015).

Disturbance Cue Preparation

To label predator arms as risky, we collected disturbance cue, an early warning signal of local risks (Crane et al., 2022), from 10 adult female guppies (mean ± SD standard length = 1.88 cm ± 0.30 cm). We placed the guppies in a 9.5 L aquarium filled with 4.7 L of dechlorinated tap water and equipped with an air stone for four hours to acclimate. We then removed the air stone and passed the predator model attached to a metal rod through the tank for one minute, taking care not to contact any of the fish in the process so as not to introduce damage-released chemical alarm cue (Crane et al., 2020b; Goldman et al., 2019, 2020). One minute after the last pass, the disturbance cue was frozen in 30 mL aliquots at -20˚C.

Background Risk Conditioning

To generate test shoals experienced with different levels of risk, we conditioned groups of 12 fish as either 'high risk' or 'low risk' in 5 L glass tanks, allowing us to conduct two trials per conditioning group using five fish per test shoal. All tanks contained gravel substrate, were equipped with a single air stone, and contained 3 L of dechlorinated tap water (~24°C, pH ~ 7.2, 12:12 Light:Dark cycle). We lined all tanks on three sides using opaque light blue plastic to prevent visual communication between shoals. We conditioned the fish four times a day at 10 am, 12 pm, 2 pm, and 4 pm for two days prior to testing to induce neophobic predator avoidance behaviour in Trinidadian guppies. For experiments 1 and 3, conditioning tanks received 2.5 mL of their respective stimuli in order to conserve fish while the concentration of alarm cue in the tank remained suprathreshold. For experiment 2, high-risk tanks received 5 mL of alarm cue, while low-risk tanks received 5 mL of distilled water. We withdrew 60 mL of tank water via airline placed at the back of the tank near the air stone. The cue was then slowly injected, followed by the 60 mL of tank water.

Predator Models

We used a 10 cm long Storm Wildeye Live Pike[TM] fishing lure as the predator model after removing all metal hooks. For experiments 1 and 3, we hung the model 5 cm above the bottom of the maze by clear fishing wire. For experiment 2, the model was secured to a 5 cm tall, 1 cm in diameter clear plastic support. The support was secured in the middle of a 13.5 cm by 5 cm piece of white corrugated plastic filled with sand and sealed (Appendix Image 1).

Food Patch Preparation

For experiments 1 and 3, we prepared the food patches in a rectangular plastic container by adding two-parts water to one-part Repashy Superfood Spawn and Grow Conditioning Gel Premix[TM]. We allowed the preparation to set at room temperature for two hours and then cut it into approximately 1 cm by 1 cm by 0.5 cm strips.

For experiment 2, we prepared the food patches in a rectangular plastic container by dissolving 30 ml of unflavoured Knox gelatin in ~125 ml of water, followed by an additional ~125 ml of boiling water. After mixing, we added 40 g of commercial flake food along with another ~250 ml of cold water. We allowed the preparation to set overnight, and then cut it into 2 cm^3 cubes. The food patch was placed on a 13.5 cm by 5 cm piece of white corrugated plastic filled with sand and sealed to match that of the predator model.

Test Arena

We constructed an eight-arm radial maze using corrugated plastic (Figure 2). The central chamber was 33 cm in diameter and each of the arms were 9 cm in width and 40 cm long. Each arm was 6 cm apart. The opening of each arm contained a partitioning that restricted access to only the bottom 5 cm of each arm. The partitioning was labelled with a 3 cm by 3 cm black or white circle, triangle, rectangle, or bowtie shape on a light blue background for the fish to use as spatial landmarks. The arms were equipped with positive (food patch), negative (predator model), or neutral (empty) reinforcements. Reinforcements were placed 7 cm from the back of each arm. The number of arms containing food patches, predator models, or left empty were adjusted for each experiment. The order of the reinforcements and spatial landmarks for the arms were randomized for each trial. The maze was submerged in a 150 cm diameter test arena. The water level was 15 cm high using dechlorinated water (~24°C ± 2°C, pH ~ 7.2).

Experimental Protocol

Guppies exposed to either high or low levels of background predation risk were placed in shoals of five in the center of an eight-arm radial maze. The shoal acclimated in the center chamber in an 8.5 cm diameter and 23 cm tall transparent cylinder for ten minutes before the cylinder was gently lifted to allow them to explore the spatial layout of the maze. After the exploration period, the guppies were regrouped in the clear cylinder placed in the central chamber, then all foraging patches, predator models, and doors were removed. After an acclimation period, the cylinder was removed as the shoals were exposed to either a damage-released chemical alarm cue or distilled water control cue. During the testing period, the behaviour of the individuals was monitored for a period of ten minutes where the location of each fish was recorded every 15 seconds. The latency to flee was measured as the time it takes for the first individual to leave the central chamber. The individual was considered to have left once half its body length had entered an arm. The first arm to which the individual fled was also recorded to measure choice accuracy. The number of fish in each arm was recorded to determine the distribution of the fish. The number of times a fish or a group of fish returned to the central chamber was recorded to measure exploration.

Experiment 1

We tested if background risk affected the decision making of prey following exposure to an acute predation threat. Additionally, we tested if prey could learn safe or non-risky areas and use this information when faced with an imminent predation threat. Reinforcements were randomly assigned to each arm so that two arms contained a plastic predator model, two contained a food patch, while the other four were left empty. The guppies were observed every 15 seconds for a period of ten minutes every 20 minutes over the course of one hour, for a total of three observation periods. At the beginning of each observation period, 5 mL of disturbance cue, followed by 60 mL of water from the arena, was injected via airline on top of the predator models. After the guppies were regrouped in the central chamber and the reinforcements were removed, the shoal was given ten minutes to acclimate before being exposed to 5 mL of cue. We also recorded the number of times a group of fish entered each arm in the same way we measured the number of returns to the center.

To assess the effects of background risk and arm type on the distribution of the fish throughout the maze prior to the acute cue injection, we performed a GLM on a quasi-Poisson regression for over dispersed data on the number of fish in each arm type using the maximum number of fish as an offset. In order to test the effects of background risk and cue type on the latency to flee the central chamber after the acute cue injection, we performed a GLM on a quasi-Poisson regression for over dispersed data. We performed a multinomial logistic regression to determine the effect of background risk and cue type on the first type of arm entered following the acute cue injection. We used a GLM on a quasi-Poisson regression to assess the effect of background risk and acute cue type on the number of times a fish or a group of fish returned to the central chamber and entered each arm following the acute cue injection. To assess the effects of background risk and acute cue type on the distribution of the fish in each arm type throughout the maze following the acute cue injection, we performed a GLM on a quasi-Poisson regression for over dispersed data on the number of fish in each arm type using the maximum number of fish as an offset. We excluded data from one trial because none of the fish left the central chamber, for a total sample size of ten.

We calculated the multinomial logistic regression using jamovi version 1.6 (R Core Team, 2019; The jamovi Project, 2021) and the nnet package (Ripley & Venables, 2016). We calculated the GLMs on quasi-Poisson regressions using R version 3.6.2 and the car package (Fox & Weisberg, 2019). Post hoc analyses of pairwise comparisons using Tukey's HSD test were conducted using the emmeans package (Lenth, 2020).

<u>Experiment 2</u>

While experiment 1 provided prey with an opportunity to learn two safe, four neutral, and two unsafe arms over the course of one hour, experiment 2 simplified these choices by providing prey with four safe (foraging patch) and four unsafe (predator model) arms assigned at random over the course of four hours. Prey were observed every 30 seconds for a period of ten minutes every half an hour over the course of four hours, for a total of eight observation periods. The following morning after the shoal was regrouped in the central chamber and the reinforcements were removed, the shoal was exposed to 10 mL of cue.

We analyzed the latency to flee the central chamber, the proportion of fish in each arm before and after the introduction of the acute cue, and the number of times a fish or a group of fish returned to the central chamber after the acute cue injection as described in experiment 1. To determine the effect of background risk and acute cue type on the first type of arm entered immediately after the acute cue was injected, we performed a binomial logistic regression. We collected a sample size of ten for each background risk and acute cue type, except for the low background risk and distilled water control treatment, which had a sample size of nine. We excluded one replicate because no fish had entered any arm after the acute cue injection.

We calculated the binomial logistic regression using jamovi version 1.6. We calculated the GLM on a quasi-Poisson regression using R version 3.6.2 and the car package. Pairwise post hoc analyses were conducted using the emmeans package.

<u>Experiment 3</u>

Where experiment 2 provided prey with the opportunity to learn and decide between four safe and four unsafe arms, experiment 3 only allowed access to one safe arm to see if they were able to learn where safe was. One arm, randomly selected, was open with a food patch, while the other seven arms were blocked by guillotine doors. The fish were observed every 15 seconds for a period of ten minutes every 20 minutes over the course of 30 minutes, for a total of two observation periods. After the guppies were regrouped in the central chamber and the reinforcements were removed, the shoal was given ten minutes to acclimate before being exposed to 5 mL of cue. The number of times a group of fish entered each arm was measured the same as experiment 1.

The arms were numbered from one to eight, where the arm that was open with food was arm one and the others were numbered in a counter-clockwise manner. To determine the effect of background risk on the proportion of fish in the open arm before the introduction of the acute cue, we conducted a Mann-Whitney U test, as the data were not normally distributed. We analyzed the latency to flee the central chamber, the proportion of fish in each arm after the introduction of the acute cue, the number of times a fish or a group of fish returned to the central chamber after the acute cue injection, and the number of times fish or a group of fish entered each arm following the acute cue injections as described in experiment 1. We performed a binomial logistic regression to determine the effect of background risk and acute cue type on if the fish correctly chose the previously open arm first after the injection of the acute cue. Data from three trials were excluded since none of the fish left the central chamber and one was excluded because one of the females gave birth during the trial, for a final sample size of 23. The number of times a group of fish entered each arm was not recorded for several trials, resulting in a lower sample size of 11 for each treatment combination, except for fish pre-exposed to high background risk given the distilled water cue, which had a sample size of 12.

We calculated the Mann-Whitney U test and the binomial logistic regression using jamovi version 1.6. We calculated the GLMs on quasi-Poisson regressions using R version 3.6.2 and the car package. Post hoc analyses were conducted using the emmeans package.

Results

<u>Experiment 1</u>

Initially, we examined how background risk level affected where fish spent most of their time prior to the introduction of the acute cue. In the final observation period prior to the introduction of the acute cue (alarm cue or distilled water), we found no evidence that background risk affected the distribution of fish in each arm type. The proportion of fish in each arm type prior to the acute cue exposure was not affected by background risk ($\chi_1 = 0.01$, $p = 0.90$) or the interaction between background risk and the type of reinforcement (food, predator model or empty) ($\chi_2 = 0.26$, $p = 0.88$). However, there was a significant effect of reinforcement on the distribution of the fish prior to the acute cue injection ($\chi_2 = 7.40$, $p = 0.02$) (<u>Appendix Figure 1</u>). A higher proportion of fish in arms containing food arms than arms containing predator models, suggesting that the fish were able to correctly identify which arms were safe, but this did not differ between background risk treatments.

Immediately following the introduction of the acute cue, the background risk and acute cue treatments did not affect the speed or accuracy of the initial decision to flee. The latency to flee the central chamber immediately following the introduction of the acute cue was not affected by background risk ($\chi_2 = 0.12$, $p = 0.73$), acute cue type ($\chi_2 = 0.35$, $p = 0.55$), or an interaction between background risk and acute cue type ($\chi_2 = 1.95$, $p = 0.16$) (Figure 3). Following the introduction of an acute threat (i.e. cue injection), there was also no significant effect of background risk ($Z = -1.29$, $p = 0.20$) or acute cue type ($Z = -0.12$, $p = 0.91$) on whether the fish first entered an arm that previously contained food or was empty (Figure 4). Likewise, there was no significant effect of background risk ($Z = 0.14$, $p = 0.89$) or acute cue type ($Z = 0.49$, $p = 0.62$) on whether the fish first entered an arm that previously contained a predator model or was empty, suggesting that their initial decision was random.

Following the introduction of the acute cue, we tested how background risk level and acute cue type affected the overall movement of the fish. We found a significant effect of background risk ($\chi_1 = 4.95$, $p = 0.03$) and the background risk by acute cue type interaction ($\chi_1 = 4.32$, $p = 0.04$), but not of acute cue type ($\chi_1 = 1.67$, $p = 0.20$) on the number of returns to the central chamber that a fish or a group of fish made following the acute cue injection (Appendix Figure 2). When given alarm cue, fish pre-exposed to high background risk returned more to the center than fish pre-exposed to low background risk, suggesting fish pre-exposed to low background risk are more likely to stay in their chosen arm in the event of an acute threat.

Additionally, following the acute cue injection, we tested how background risk level, acute cue type, and prior knowledge of the test arena affected the exploratory behaviour of the fish. Following the introduction of the acute cue, background risk ($\chi_1 = 1.67$, $p = 0.20$), acute cue type ($\chi_1 = 0.02$, $p = 0.90$), reinforcement [food vs. predator model vs. empty] ($\chi_2 = 1.35$, $p = 0.51$), and the interaction between background risk and reinforcement ($\chi_1 = 0.35$, $p = 0.84$) did not significantly affect the number of times a group of fish entered an arm, but the background risk by acute cue type interaction ($\chi_1 = 17.61$, $p < 0.01$) and acute cue type by reinforcement interaction ($\chi_2 = 8.47$, $p = 0.01$) did have a significant effect (Appendix Figure 3). When given alarm cue, fish pre-exposed to high background risk entered arms more than fish pre-exposed to low background risk, suggesting that fish pre-exposed to high background risk explored more after an acute threat. We also saw that guppies chose arms with predator models significantly more when given alarm cue than distilled water, suggesting that guppies were more prone to erroneous decision making when exposed to an acute threat.

Moreover, we analyzed how treatment (background risk level and acute cue type) and prior knowledge affected overall decision-making following exposure to the acute cue by looking at where the fish spent most of their time. The proportion of fish in each arm type following acute cue exposure was not affected by background risk ($\chi_1 = 0.07$, $p = 0.79$), acute cue type ($\chi_1 = 0.10$, $p = 0.75$), reinforcement ($\chi_2 = 0.70$, $p = 0.71$), or the interactions between background risk and acute cue ($\chi_1 = 0.18$, $p = 0.67$), background risk and reinforcement ($\chi_1 = 3.73$, $p = 0.15$), and acute cue and reinforcement ($\chi_1 = 5.67$, $p = 0.059$) (Figure 5). The lack of difference between treatments suggests that space use was random and that the fish did not learn which arms were safe.

Experiment 2

As above, we initially examined how background risk level affected where fish spent most of their time prior to the introduction of the acute cue. In the final observation period prior to the introduction of the acute cue, there was no evidence that background risk or reinforcement (food vs. predator) affected the distribution of fish in each arm. The proportion of fish in each arm type prior to the acute cue exposure was not affected by background risk ($\chi_1 < 0.01$, $p = 0.98$), reinforcement ($\chi_1 = 1.38$, $p = 0.24$), or the interaction between background risk and the type of reinforcement ($\chi_1 < 0.01$, $p = 0.98$). This suggests that background risk did not affect exploratory behaviour in a novel environment.

We found no difference between background risk and acute cue type treatments in the speed or accuracy of the initial decision to flee immediately following the acute cue injection. The latency to flee the central chamber immediately following the introduction of the acute cue was not significantly affected by background risk ($\chi_1 = 1.21$, $p = 0.27$), acute cue type ($\chi_1 = 0.13$, $p = 0.72$), or an interaction between background risk and acute cue type ($\chi_1 = 0.07$, $p = 0.79$) (Figure 6). Likewise, there was no effect of background risk ($Z = 0.81$, $p = 0.42$) or acute cue type ($Z = 0.19$, $p = 0.85$) on whether the fish first entered an arm previously containing food or predator model after the acute cue was introduced, indicating that the guppies first choice of arm after the acute cue injection was random (Figure 7).

Following the introduction of the acute cue, we tested how background risk level and acute cue type affected the overall movement of the fish. Background risk ($\chi_1 = 0.07$, $p = 0.80$) or an interaction between background risk and acute cue type ($\chi_1 < 0.01$, $p = 0.99$) did not have a significant effect on the number of returns to center a fish or a group of fish made following the introduction of the acute cue. However, guppies returned significantly less often to the center when given alarm cue vs. distilled water ($\chi_1 = 7.61$, $p = 0.01$) (Appendix Figure 4), suggesting that once the prey had made a choice, they were less likely to leave the arm.

We next examined how acute cue type, background risk level and prior knowledge affected overall decision-making following acute cue exposure by looking at where the fish spent most of their time. Following the introduction of the acute cue, we found an effect of reinforcement ($\chi_1 = 6.18$, $p = 0.01$) and a significant interaction between reinforcement and acute cue type ($\chi_1 = 4.88$, $p = 0.03$) (Figure 8). When given alarm cue, there was a significantly higher proportion of guppies in arms previously with food than arms previously with predator models ($Z = 2.39$, $p = 0.02$), suggesting that, under an acute threat, previous knowledge of safe areas influenced their decision making. No interactions between background risk and acute cue type ($\chi_1 = 0.08$, $p = 0.77$), background risk and reinforcement ($\chi_1 = 1.38$, $p = 0.24$) were significant. Further, we saw no significant effect of background risk ($\chi_1 = 0.07$, $p = 0.78$) or acute cue type ($\chi_1 = 1.55$, $p = 0.21$) on the proportion of fish in each arm type after the acute cue injection, suggesting that background risk level does not influence their decision of where to flee.

Experiment 3

As in experiment 1 and 2, we initially examined the impact of background risk on space use prior to the introduction of the acute cue. Immediately prior to the introduction of the acute cue, we found there was no difference between background risk treatments in the proportion of

fish in the open arm ($U = 829$, $p = 0.07$). This suggests that background risk did not affect the exploratory behaviour of the guppies in a novel environment.

Immediately following the introduction of the acute cue, we found that neither background risk nor acute cue type affected the speed or accuracy of the initial decision to flee. The latency to flee the central chamber immediately after the acute cue injection was not significantly affected by background risk ($\chi_1 = 1.77$, $p = 0.18$), acute cue type ($\chi_1 = 1.75$, $p = 0.19$), or an interaction between background risk and acute cue type ($\chi_1 = 0.57$, $p = 0.45$) (Figure 9). Moreover, there was no significant effect of background risk ($Z = -0.98$, $p = 0.33$) or acute cue type ($Z = -0.98$, $p = 0.33$) on whether the fish first entered the arm that was previously open and contained food following acute cue exposure, indicating that their initial decision when fleeing was random (Figure 10).

Likewise, following the introduction of the acute cue, we tested how background risk level and acute cue type affected the overall movement of the fish. The number of returns to the central chamber that a fish or a group of fish made following acute cue exposure was not significantly affected by background risk ($\chi_1 = 0.39$, $p = 0.54$), acute cue type ($\chi_1 = 0.09$, $p = 0.77$) or the background risk by acute cue interaction ($\chi_1 = 0.55$, $p = 0.46$), suggesting that regardless of background risk or threat level, all shoals explored the maze similarly after the acute cue injection.

Furthermore, we examined the exploratory behaviour of the fish to determine if there was an overall arm preference after exposure to the acute cue. Background risk ($\chi_1 = 0.17$, $p = 0.68$), acute cue type ($\chi_1 = 0.07$, $p = 0.79$), and which arm relative to the one that was previously open with food ($\chi_7 = 10.02$, $p = 0.19$) had no significant effect on the number of times a fish or a group of fish entered each arm. There was no significant interaction between background risk and which arm was entered ($\chi_7 = 6.03$, $p = 0.54$) or between the acute cue and which arm was entered ($\chi_7 = 3.16$, $p = 0.87$). The number of times a fish or a group of fish entered each arm following the introduction of the acute cue was significantly affected by the interaction between background risk and acute cue type ($\chi_1 = 6.03$, $p = 0.01$) (Appendix Figure 5). When given distilled water, fish pre-exposed to low background risk entered arms more than those pre-exposed to high background risk, suggesting that exploration is more likely to happen in the lowest risk settings.

We examined how background risk level, acute cue type, and prior knowledge affected decision making after the acute cue was introduced by looking at where the fish spent most of their time. Following the introduction of the acute cue, background risk ($\chi_1 = 0.41$, $p = 0.52$), acute cue type ($\chi_1 = 2.32$, $p = 0.13$), and the interactions between background risk and acute cue ($\chi_1 = 0.48$, $p = 0.49$), background risk and which arm was entered ($\chi_7 = 4.93$, $p = 0.67$) and acute cue and which arm was entered ($\chi_1 = 13.89$, $p = 0.053$) had no significant effect on the proportion of fish in each arm, suggesting that threat level does not impact decision making. However, the overall proportion of fish in each arm significantly differed ($\chi_7 = 36.12$, $p < 0.01$) (Figure 11). There was a significantly higher proportion of fish in arm 1 than any other arm, arm 2 more than 3, 4, 5, and 6, and arm 8 more than 3, 4, and 6 (Appendix Table 2). The tendency for most fish to spend their time in arm 1 and the arms directly adjacent to it suggests that they had learned arm 1 as a safe choice.

Summary

Across all three experiments, we found no differences in high vs. low background risk conditions on the level or patterns of exploration prior to the acute cue injection, though experiment 1 showed an overall preference for arms that contained food patches over those that contained predator models. We found no effect of background risk or acute cue type on the speed or accuracy of the initial decision to flee the central chamber immediately after the acute cue was introduced, suggesting that fleeing dangerous areas is more important than the relative safety of where they fled to. Following the introduction of the acute cue, we also saw an overall preference for arms that previously contained food over arms that were previously empty (experiments 1 and 3) or contained predator models (experiment 2), suggesting that there is evidence that prey learned where safe areas were (Figure 12).

We found evidence that background risk and acute cue type interact to affect prey movement; When given alarm cue, fish pre-exposed to low background risk showed decreased movement compared to those pre-exposed to high background risk in experiment 1. Additionally, experiment 2 showed an overall decrease in movement for fish exposed to alarm cue. However, experiment 3 demonstrated that of those given distilled water, fish pre-exposed to low background risk showed increased movement compared to those pre-exposed to high background risk.

We also looked at which arms prey chose to explore after the acute cue injection. Experiment 1 showed that fish given alarm cue entered predator arms more than those given distilled water, suggesting that an acute threat leads prey to make more mistakes when deciding on which areas to frequent.

Prey spent overall more time in the correct arms, suggesting they had learned where the safe areas were. Experiment 2 showed that, following acute cue exposure, prey exposed to alarm cue spent more time in arms that had previously contained food than predator models. Meanwhile, experiment 3 showed that, following acute cue exposure, prey spent more time in the arm that was previously open and reinforced with food and the arms immediately adjacent to it than all other arms. However, prey did not spend more time in any arm type after exposure to the acute cue in experiment 1.

Discussion

Overall, across the three experiments, we found no differences in high vs. low background risk conditions on the level or patterns of exploration prior to the acute cue injection. Both groups pre-exposed to high or low background risk exhibited similar exploratory behaviour prior to the acute cue injection; Shoals pre-exposed to high and low background risk levels moved between arms at a similar frequency and spent a comparable amount of time in each arm. Guppies in experiment one spent more time in arms containing food than those containing predator models regardless of their background risk. As such, we found no evidence that the level of background risk affected the exploratory behaviour of the shoals prior to the introduction of an acute threat despite our initial predictions that fish pre-exposed to high background risk should exhibit higher spatial neophobia and therefore explore less than those pre-exposed to low background risk.

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Although fish may be able to recognize where safe areas are, as evidenced by the preference of arms containing food patches over predator models, they may not use that learned information. Results across all three experiments showed that background risk and acute cue type did not affect decision making time or accuracy of initial decision. The lack of difference between background risk levels does not support our initial predictions that high background risk shoals should exhibit shorter latencies to flee and more errors in their initial decisions to flee. Short latencies and random decision-making suggest that prey were trading off between speed and accuracy in terms of decision-making (Chittka et al., 2009). In other words, fleeing the dangerous area regardless of potential costs may be more important than where they flee to. Randomly fleeing immediately after acute cue exposure may also suggest that learned information was not a priority when under a perceived threat. Learned safety information is beneficial when danger cues are absent because safety may not be simply the absence of danger (Luttbeg et al., 2020). Fish may require a cue to signal safety rather than simply rely on previously learned information, as it may not be reliable enough to reduce the individual's uncertainty about making a decision.

Ecological uncertainty is the lack of complete information regarding local habitat conditions (Dall et al., 2005). Under uncertain conditions, the costs of making a decision are expected to increase (Crane et al., 2020a). As such, prey may benefit from assessing and responding to both safety and risky cues, thus using all the available information to reduce this uncertainty (Luttbeg et al., 2020). One way to obtain relevant information, thereby reducing uncertainty, is through exploration. Reducing this uncertainty is crucial for prey to optimize their decision making and balancing trade-offs. Understanding how prey learn and use safety information in the face of ecological uncertainty can prove useful in understanding and predicting prey behaviour, including how prey use their space, under constantly changing environmental conditions.

Despite the lack of difference in time spent in each arm between fish pre-exposed to high vs. low background risk prior to the introduction of the acute cue, background risk and acute cue type affected how prey used their space following the introduction of the acute cue. Fish pre-exposed to low background risk moved more than those pre-exposed to high background risk when given distilled water (experiment three). This was expected as the lowest risk control. Meanwhile, when given alarm cue, guppies pre-exposed to high background risk entered and exited arms more frequently than those pre-exposed to low background risk (experiment one). The decrease in the movement of fish pre-exposed to low relative to high background risk is a more intense antipredator response and is inconsistent with what we would expect according to the dangerous niche hypothesis (Greenberg, 2003; Mettke-Hofmann et al., 2013). Rather, it is consistent with the risk allocation hypothesis (Ferrari et al., 2009; Lima & Bednekoff, 1999), which posits that individuals exposed to brief and infrequent periods of high predation risk should exhibit the most intense antipredator responses, whereas individuals exposed to frequent periods of high predation risk should exhibit lower intensity antipredator responses and allocate the majority of their adaptive behaviours to the brief periods of low risk in order to reduce the cumulative effect of opportunity costs. While we had initially predicted that fish pre-exposed to high background risk should explore less than those pre-exposed to low background risk, our results suggest that the decision to explore is more complex when considering both background and acute predation threats in terms of opportunity costs.

Furthermore, acute threats may influence decision-making more strongly than background risk as evidenced by the lack of difference between background risk treatments. The introduction of the acute alarm cue triggered a reduction in movement, an antipredator response, in prey regardless of background risk treatment (experiment two). When exposed to an acute threat, prey adopt antipredator responses to minimize the predation threat, thus incurring the costs of lost opportunities associated with the non-consumptive effects of predation (Peckarsky et al., 2008; Preisser et al., 2005; Preisser & Bolnick, 2008). Thus, exposure to the acute cue between the exploration period and the testing period changed the overall response pattern in the shoals as expected. However, when given alarm cue, guppies entered arms that had previously contained predator models more than when given the distilled water control (experiment one). This result suggests that guppies were more prone to errors in decision-making when given an acute threat, but these errors were not affected by background risk levels as initially predicted.

Additionally, while the background level of risk did not affect the initial exploratory behaviour as predicted, the background level of risk did not affect the overall amount of time spent in each arm type after the introduction of the acute cue either. There was no significant difference between shoals pre-exposed to high or low background risk in the amount of time spent in each arm. Thus, there is no evidence to support that background risk affected the ability to learn or choose 'safe' areas regardless of our initial prediction that fish pre-exposed to high background risk should spend less time in 'safe' arms than those pre-exposed to low background risk. This may likely be due to the lack of difference in exploratory behaviour between high and low background risk groups prior to the introduction of the acute cue. While background risk did not affect the distribution of the fish after the cue injection, shoals spent more time in arms that previously contained food patches and those directly adjacent (experiment 3), particularly when exposed to an acute alarm cue (experiment 2). This suggests that previous knowledge of safe spaces is important in the event of acute threat, but that background predation risk did not affect the ability to learn or use this knowledge of safe spaces.

Future experiments should seek to include safety cues, such as information produced by other individuals, known as social information (Brown & Laland, 2006; Kendal et al., 2005). This social information can include providing cues from other conspecifics (Hasenjager & Dugatkin, 2017), or eavesdropping on the safety cues provided by members of other species (Lilly et al., 2019). Social cues that provide safety information in real time may be invaluable since information that is more current may be more relevant than relying on past information.

Conclusion

Given that elevated background risk induces neophobic responses in prey fish, we predicted that fish pre-exposed to high background risk levels would explore less, thereby reducing their ability to learn safe refuge sites than fish pre-exposed to low background risk levels. As such, we expected to find longer latencies to flee and increased errors in decision making in the event of an acute predation threat. Our three experiments, each successively simplified to examine the effects of background risk on their exploration patterns, do not support our initial predictions. We have no evidence to support that background risk levels affected the exploration of novel spaces or the ability to learn safe refuge sites. Our results suggest that guppies can learn safe spaces regardless of background risk and that exposure to an acute threat leads to a decrease in movement and an increase in errors in decision-making.

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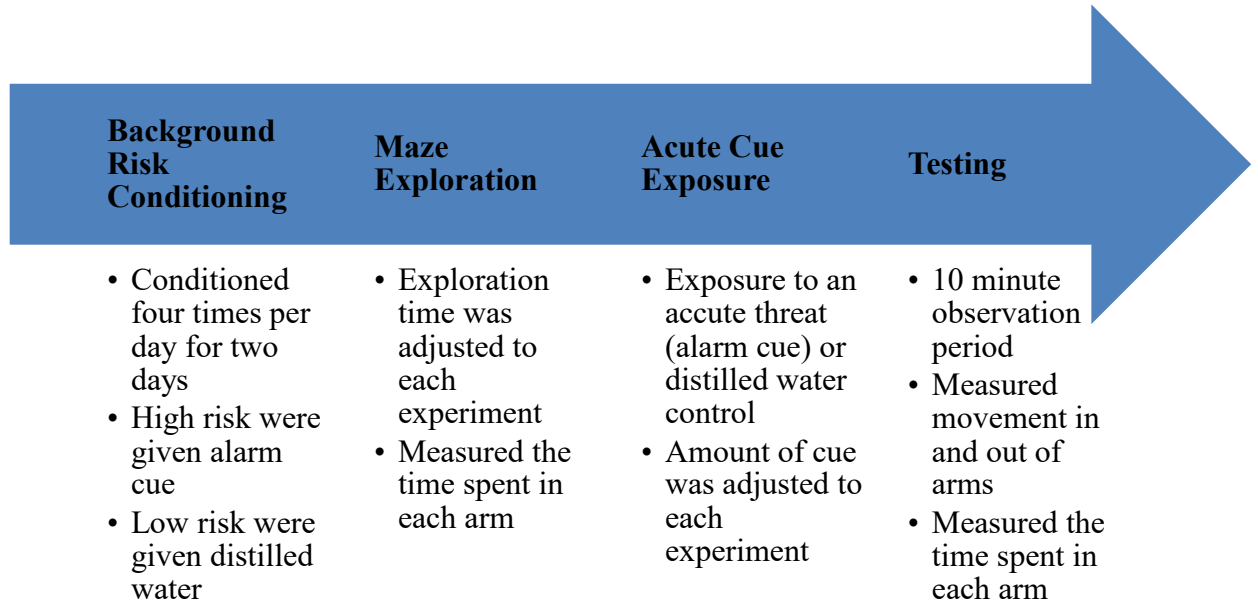
Figures

Background Risk Conditioning	Maze Exploration	Acute Cue Exposure	Testing
• Conditioned four times per day for two days • High risk were given alarm cue • Low risk were given distilled water	• Exploration time was adjusted to each experiment • Measured the time spent in each arm	• Exposure to an accute threat (alarm cue) or distilled water control • Amount of cue was adjusted to each experiment	• 10 minute observation period • Measured movement in and out of arms • Measured the time spent in each arm

Figure 1. Flowchart of experimental phases.
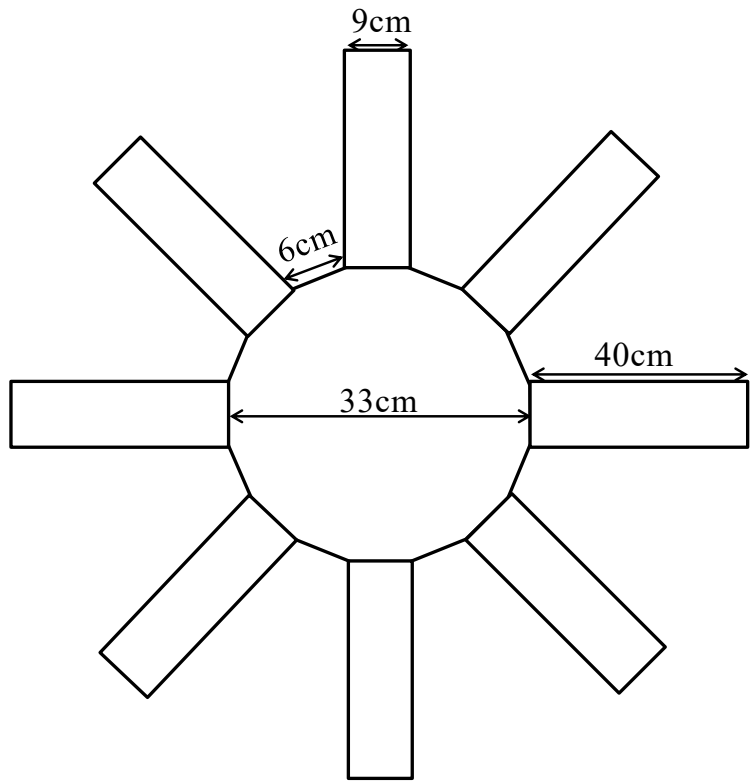
Figure 2. Diagram of the top-view of the eight-arm radial maze with dimensions.
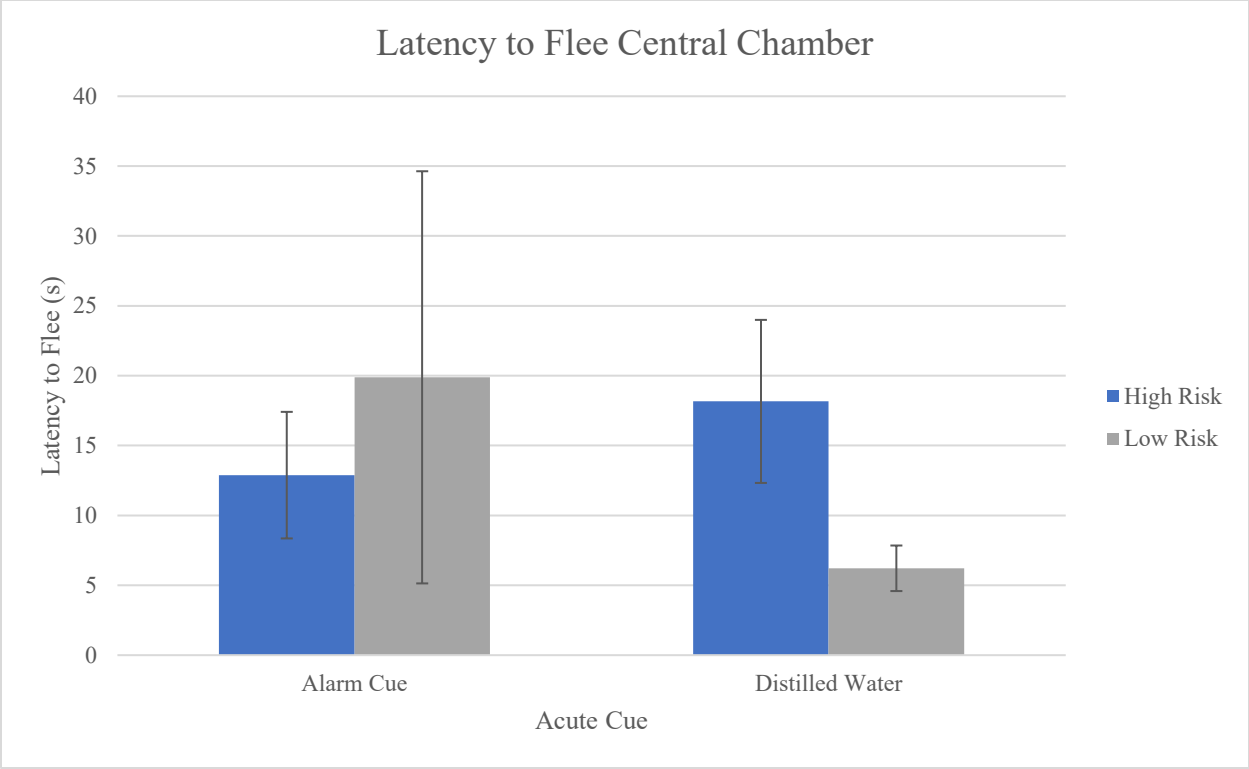
Figure 3. Mean (±SEM) latency to flee the central chamber (s) following the introduction of the acute cue for each background risk level (high risk/ low risk) and cue type (alarm cue/ distilled water) combination during experiment 1.
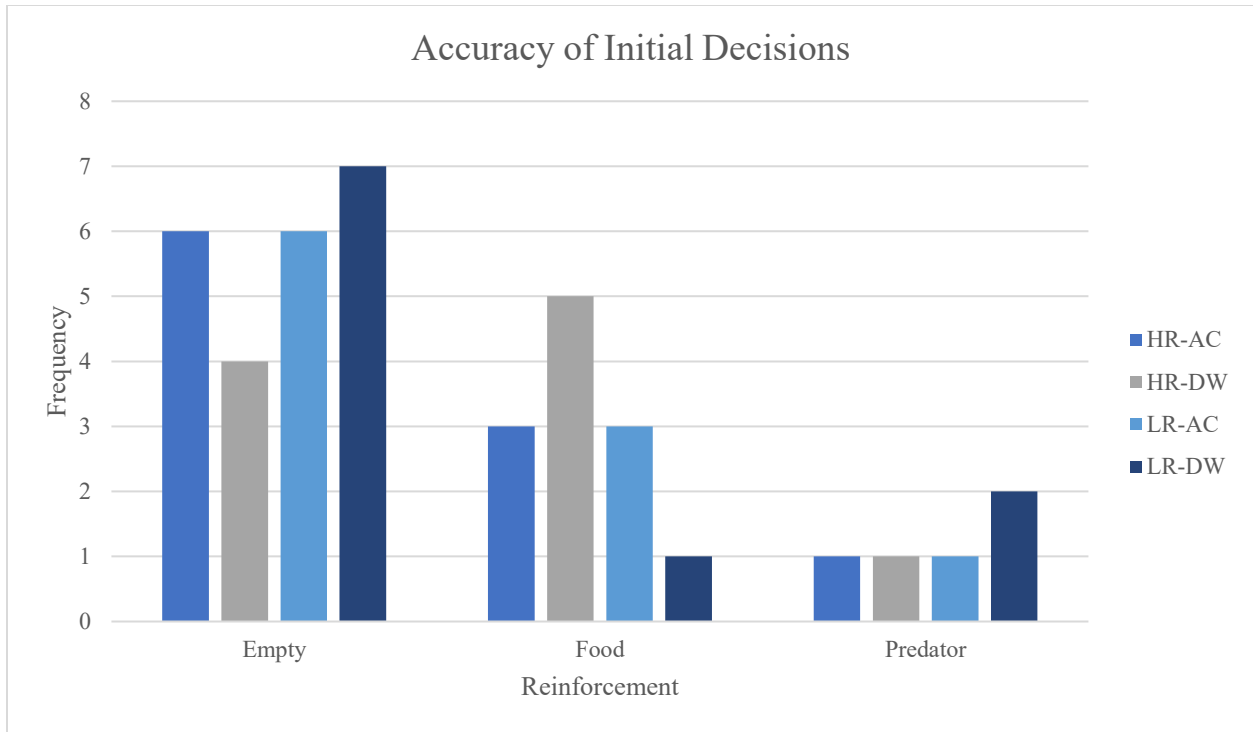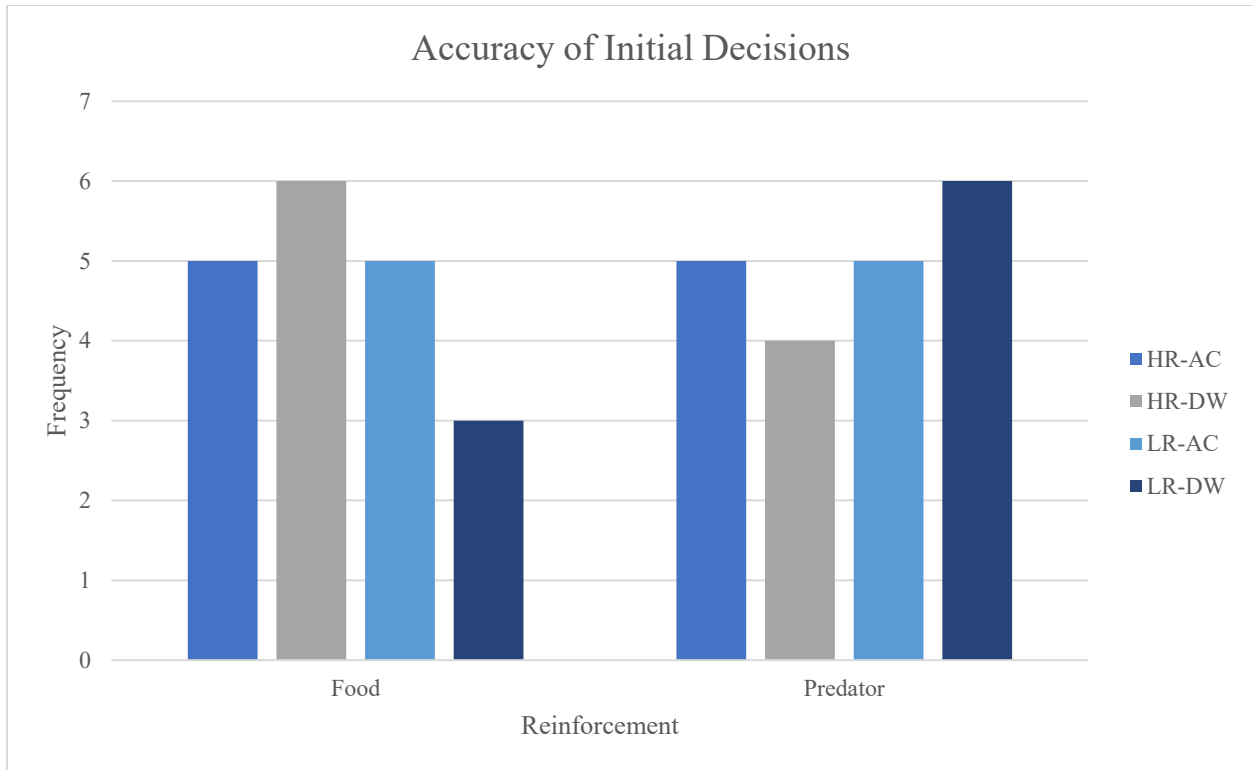
Figure 4. Number of times each reinforcement (empty/ food/ predator) was the first arm chosen when fleeing the central chamber following the introduction of the acute cue for each background risk level (high risk (HR)/ low risk (LR)) and cue type (alarm cue (AC)/ distilled water (DW)) combination during experiment 1.
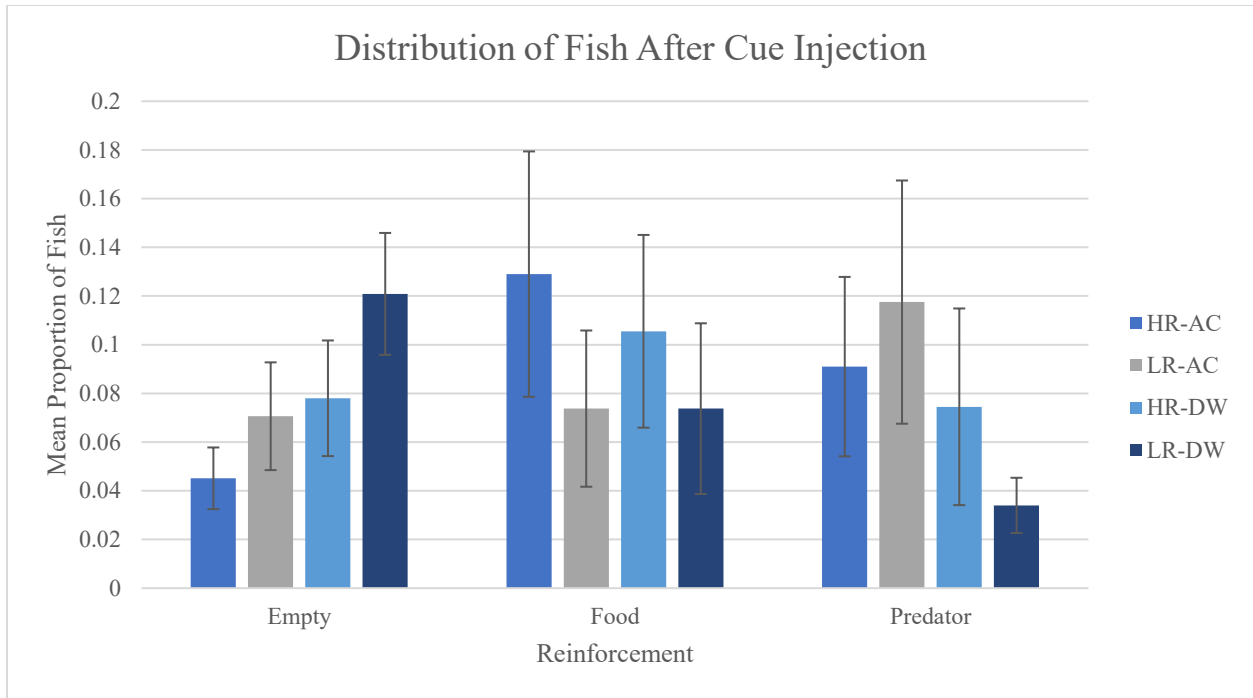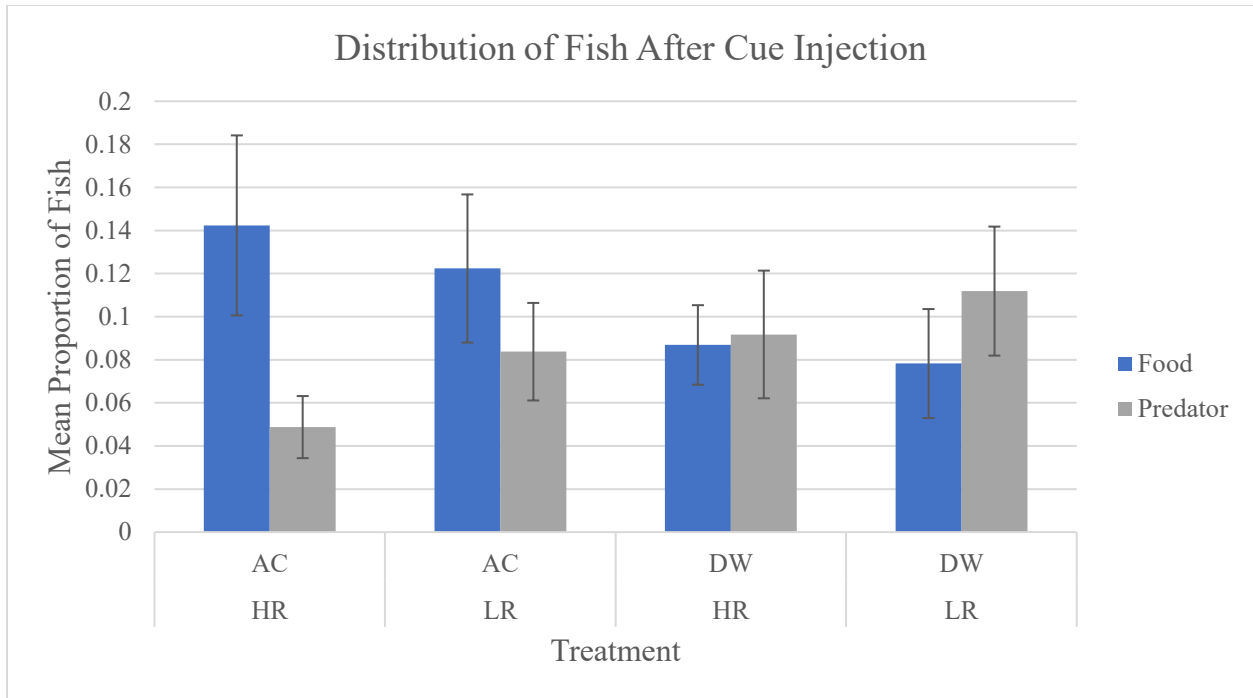
Figure 5. Mean (±SEM) proportion of fish in each arm type (empty/ food patch/ predator model) following the introduction of the acute cue for each background risk level (high risk (HR)/ low risk (LR)) and cue type (alarm cue (AC)/ distilled water (DW)) combination during experiment 1.
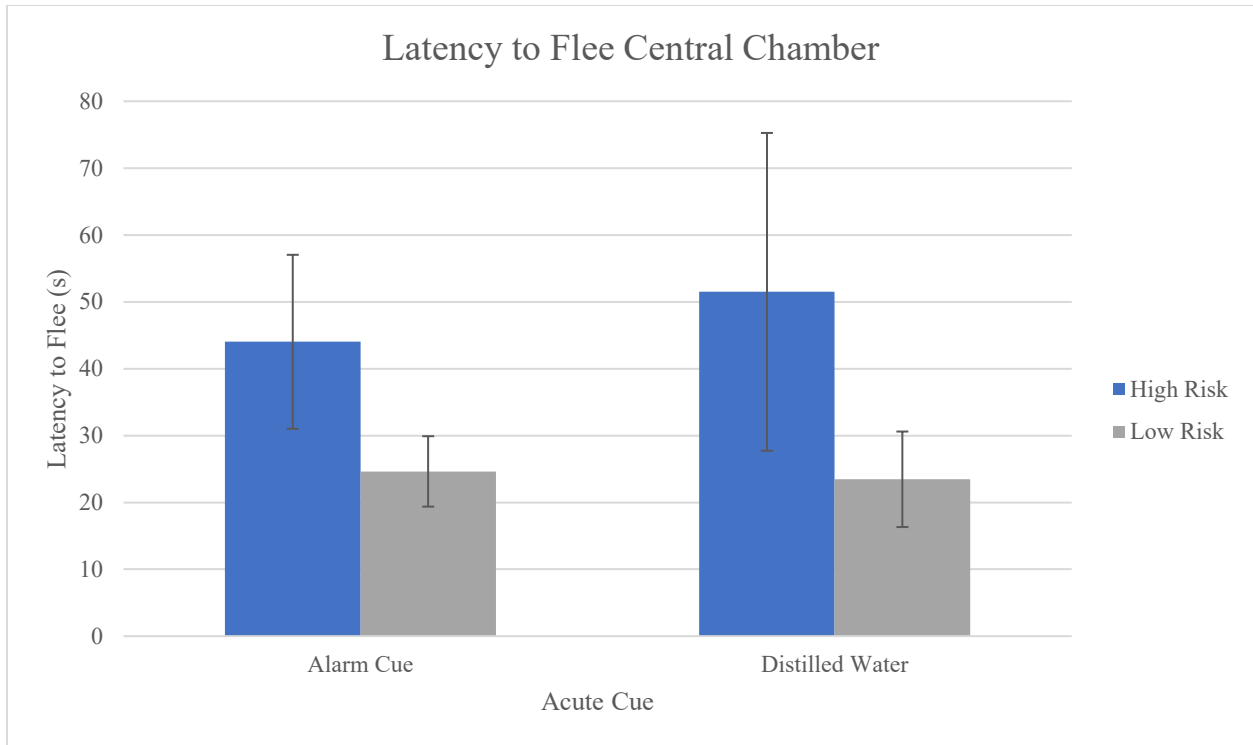
Figure 6. Mean (±SEM) latency to flee the central chamber (s) following the introduction of the acute cue for each background risk level (high risk/ low risk) and cue type (alarm cue/ distilled water) combination during experiment 2.
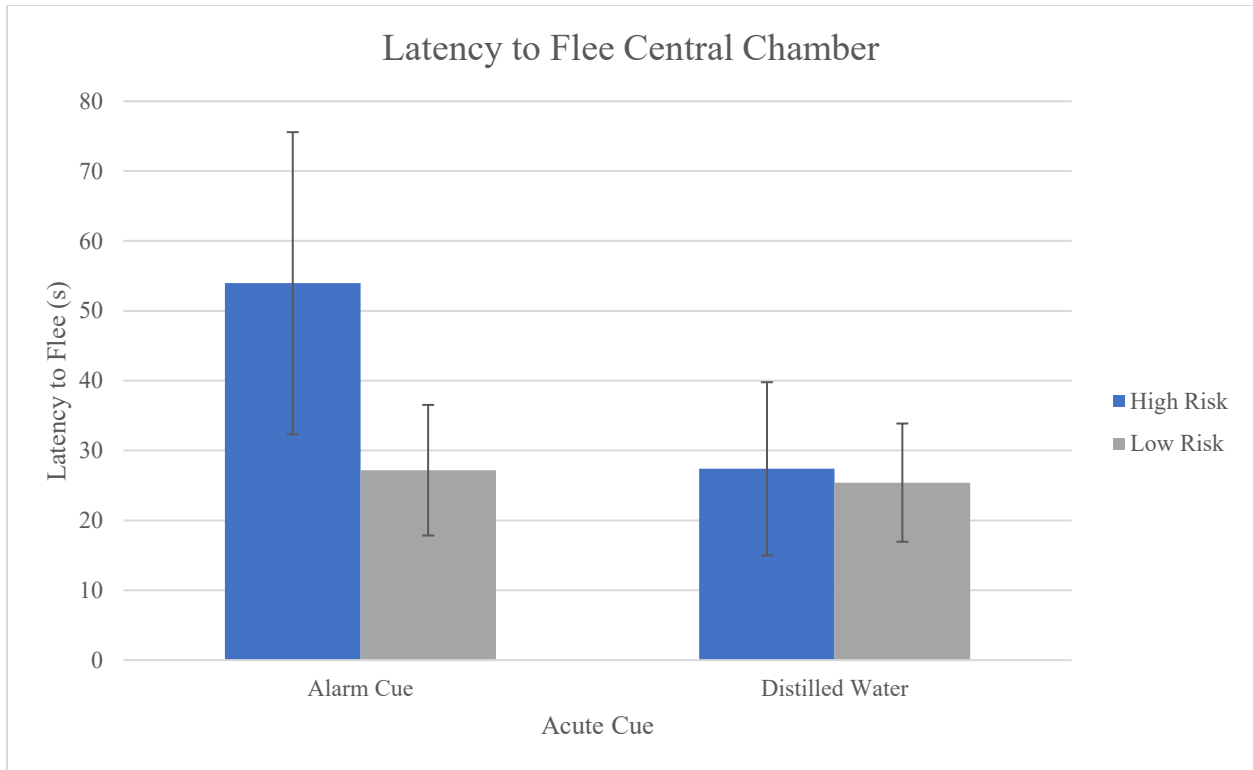
Figure 7. Number of times each reinforcement (food/ predator) was the first arm chosen when fleeing the central chamber following the introduction of the acute cue for each background risk level (high risk (HR)/ low risk (LR)) and cue type (alarm cue (AC)/ distilled water (DW)) combination during experiment 2.
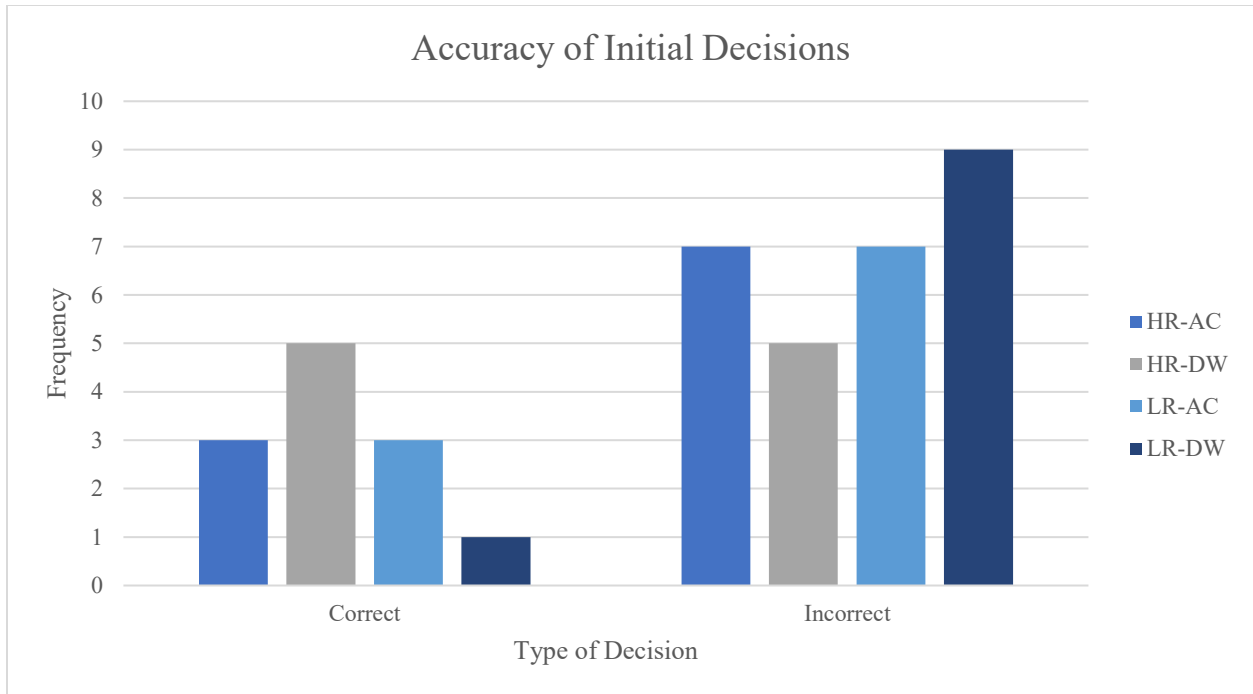
Figure 8. Mean (±SEM) proportion of fish in each arm type (food patch/ predator model) following the introduction of the acute cue for each background risk level (high risk (HR)/ low risk (LR)) and cue type (alarm cue (AC)/ distilled water (DW)) combination during experiment 2.
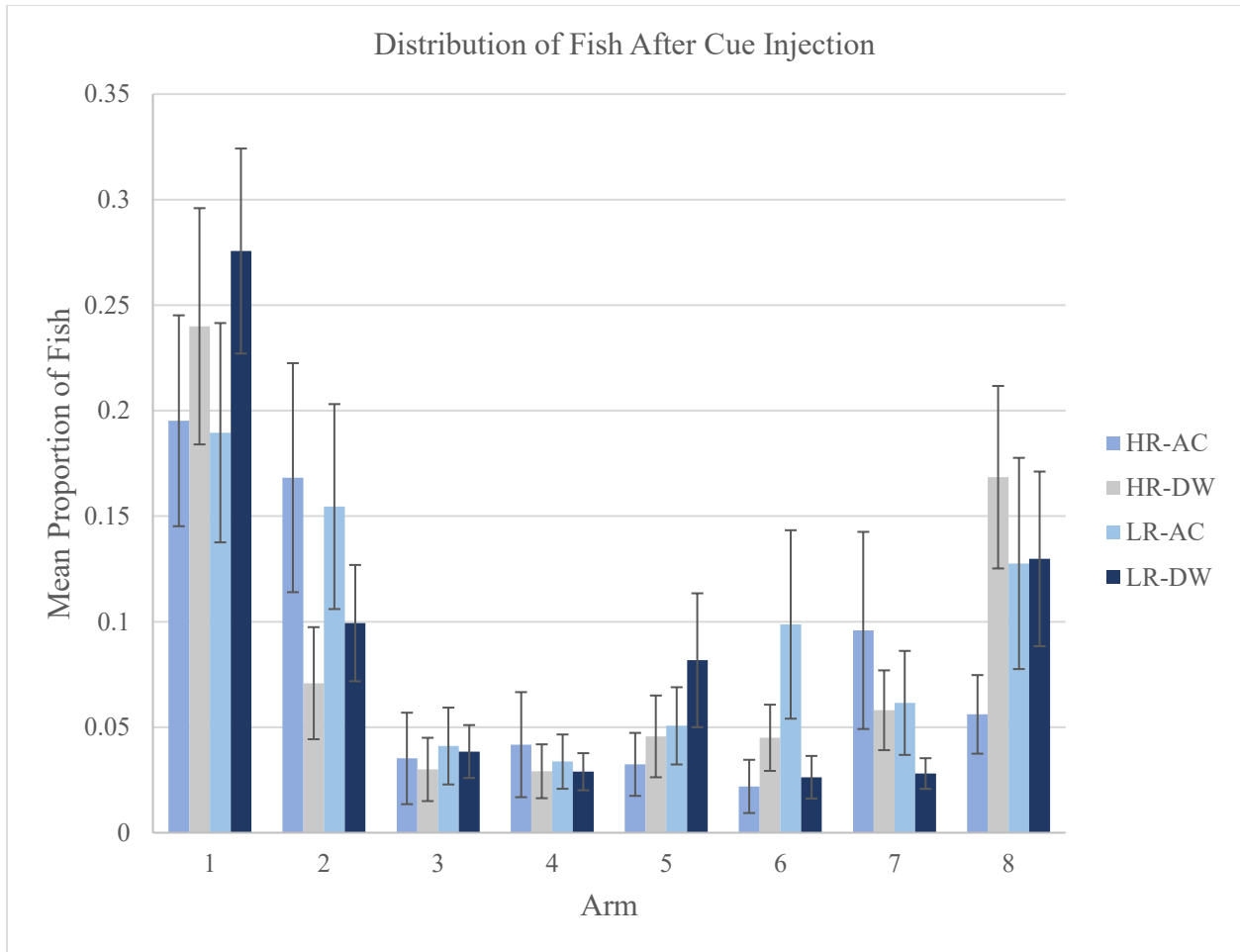
Figure 9. Mean (±SEM) latency to flee the central chamber (s) following the introduction of the acute cue for each background risk level (high risk/ low risk) and cue type (alarm cue/ distilled water) combination during experiment 3.

Figure 10. Number of times the correct (arm that was previously open with food) and incorrect (any other arm) arms were the first chosen when fleeing the central chamber following the introduction of the acute cue for each background risk level (high risk (HR)/ low risk (LR)) and cue type (alarm cue (AC)/ distilled water (DW)) combination during experiment 3.

Figure 11. Mean (±SEM) proportion of fish in each arm following the introduction of the acute cue for each background risk level (high risk (HR)/ low risk (LR)) and cue type (alarm cue (AC)/ distilled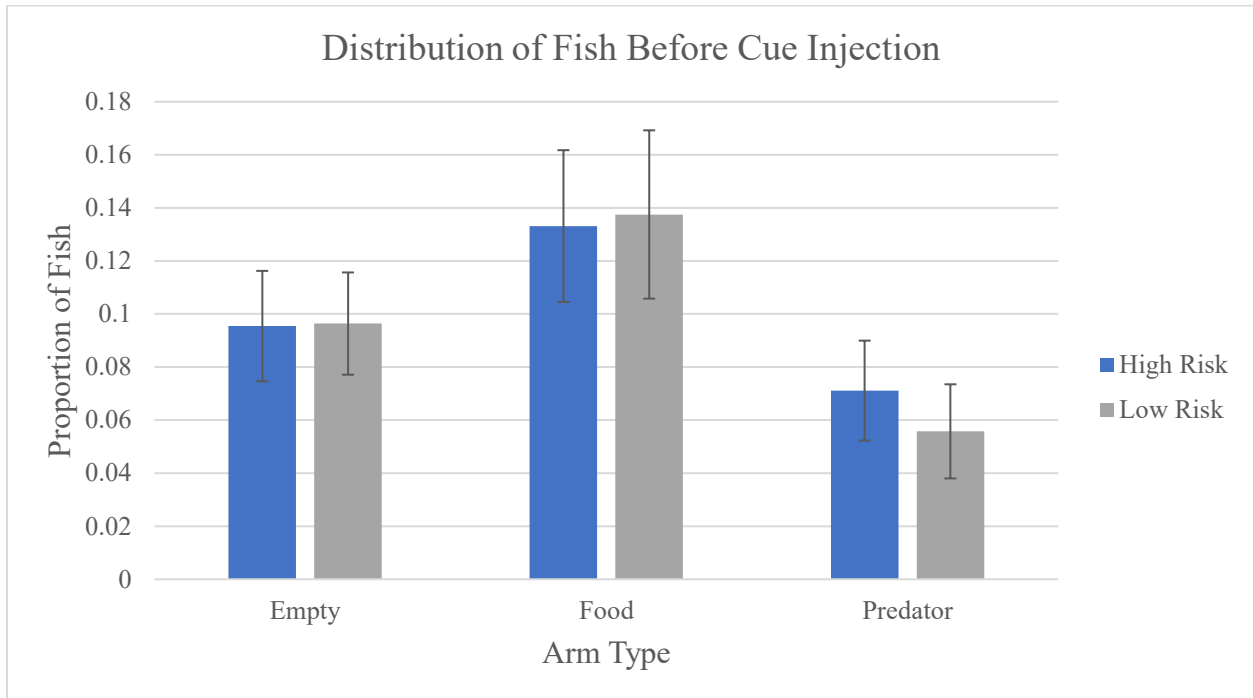 water (DW)) combination during experiment 3. Arm 1 was the only arm equipped with a food patch and available to the fish during the exploration period.

Experiment Prediction	1	2	3
Learning of safe spaces	Food > Predator	X	X
Speed/ Accuracy of initial escape	X	X	X
Exploration/ Movement	AC: HR > LR Predator: AC > DW	AC < DW	DW: LR > HR
Overall arm preference	X	AC: Food > Predator	Food > Empty

Figure 12. Summary of results for the main background risk predictions by experiment where X indicates no observed effect. Effects include background risk (high risk (HR)/ low risk (LR)), acute cue (alarm cue (AC)/ distilled water (DW)), and reinforcement (Empty, Food, Predator). Interactions are indicated by colons. Predictions: 'Learning of safe spaces': Background risk did not affect learning of safe spaces before the acute cue injection. Exp. 1: food arms were preferred over predator arms. 'Speed/ Accuracy of initial escape': Background risk did not affect the speed and accuracy of the initial escape after the acute cue injection. 'Exploration/ Movement': Background risk did not affect the exploration of guppies after the acute cue injection: Exp. 1: when given AC, HR moved more than LR and predator arms were chosen more by those given AC than DW. Exp. 2: fish moved more when given DW than AC. Exp. 3: when given DW, LR moved more than HR. 'Overall arm preference': Background risk did not affect the overall arm preference after the acute cue was injected: Exp. 2: when given AC, more time was spent in food arms than predator arms. Exp. 3: The open arm was chosen more than those that were closed.
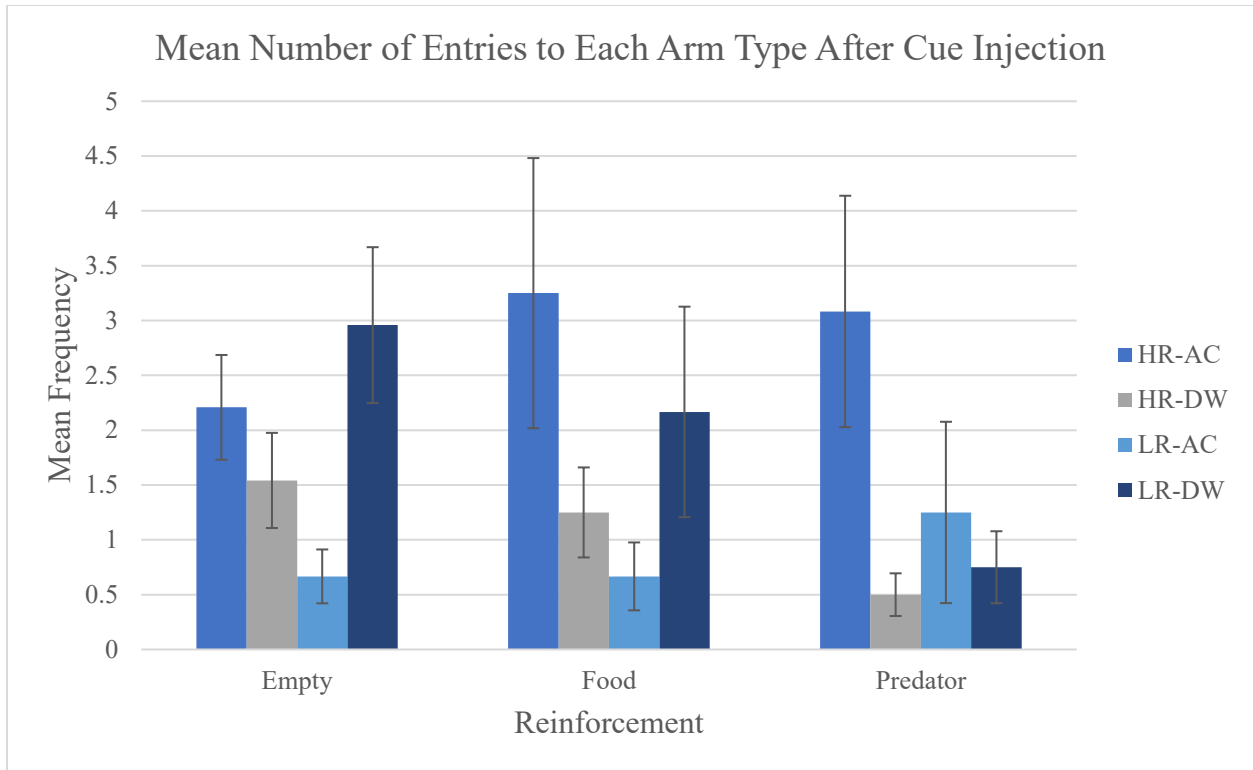
Appendix

Figure 1. Mean (±SEM) proportion of fish in each arm type (empty/ food patch/ predator model) prior to the introduction of the acute cue for each background risk level (high/ low) and cue type (alarm cue (AC)/ distilled water (DW)) combination during experiment 1.
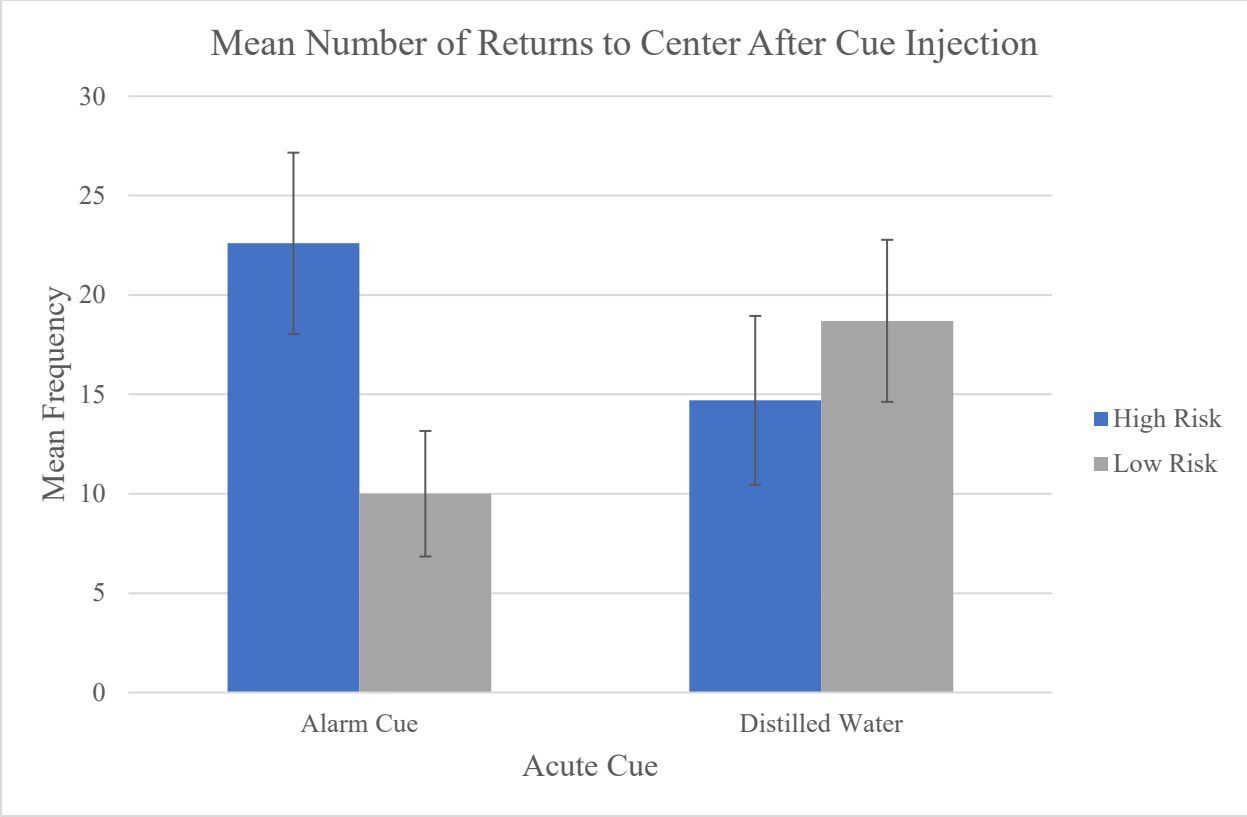
Figure 2. Mean (±SEM) number of returns to the central chamber made by a fish or a group of fish following the introduction of the acute cue for each background risk level (high/ low) and cue type (alarm cue/ distilled water) combination during experiment 1.
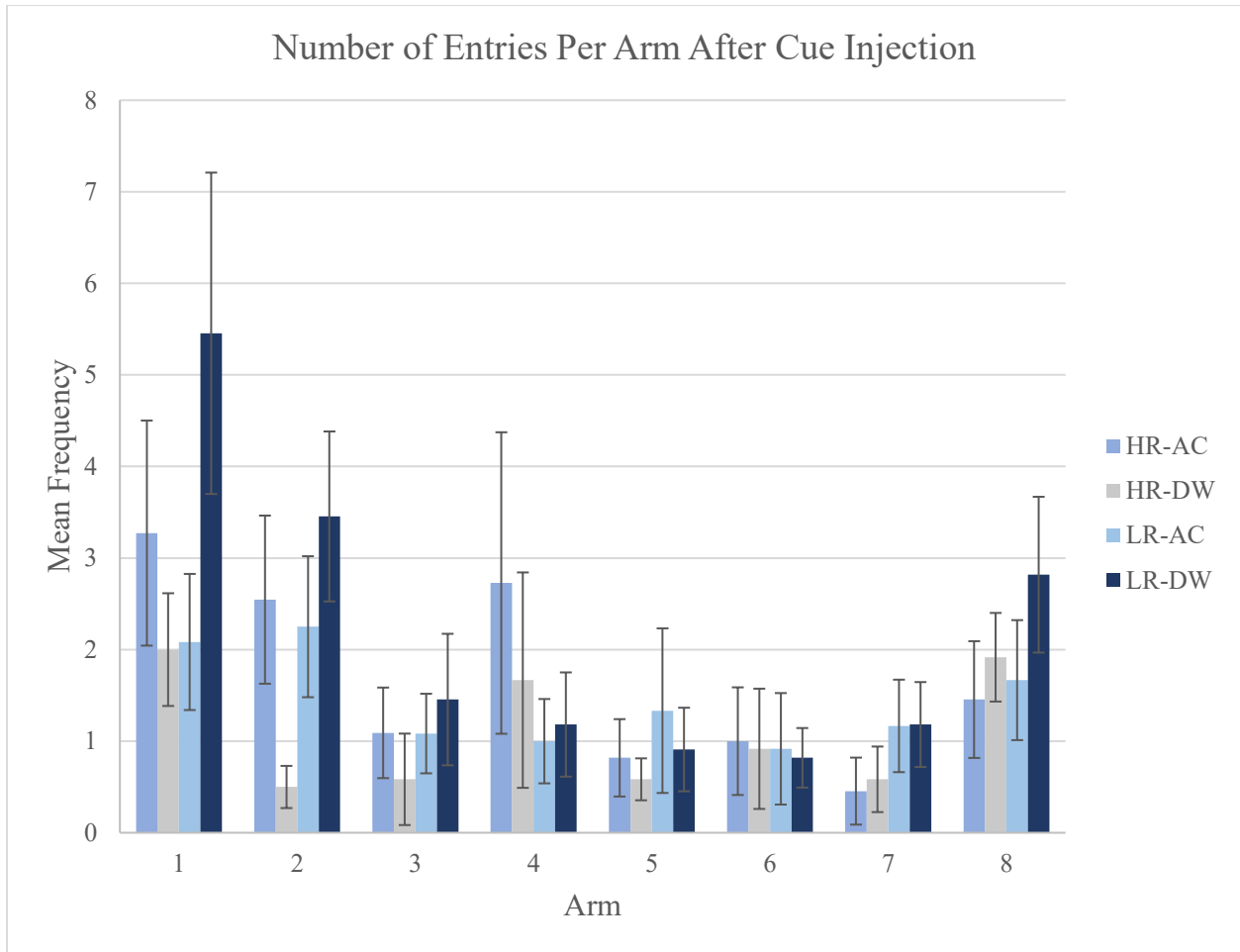
Figure 3. Mean (±SEM) number of entries into each arm type (empty/ food patch/ predator model) made by a fish or a group of fish following the introduction of the acute cue for each background risk level (high risk (HR)/ low risk (LR)) and cue (alarm cue (AC)/ distilled water (DW)) combination during experiment 1.
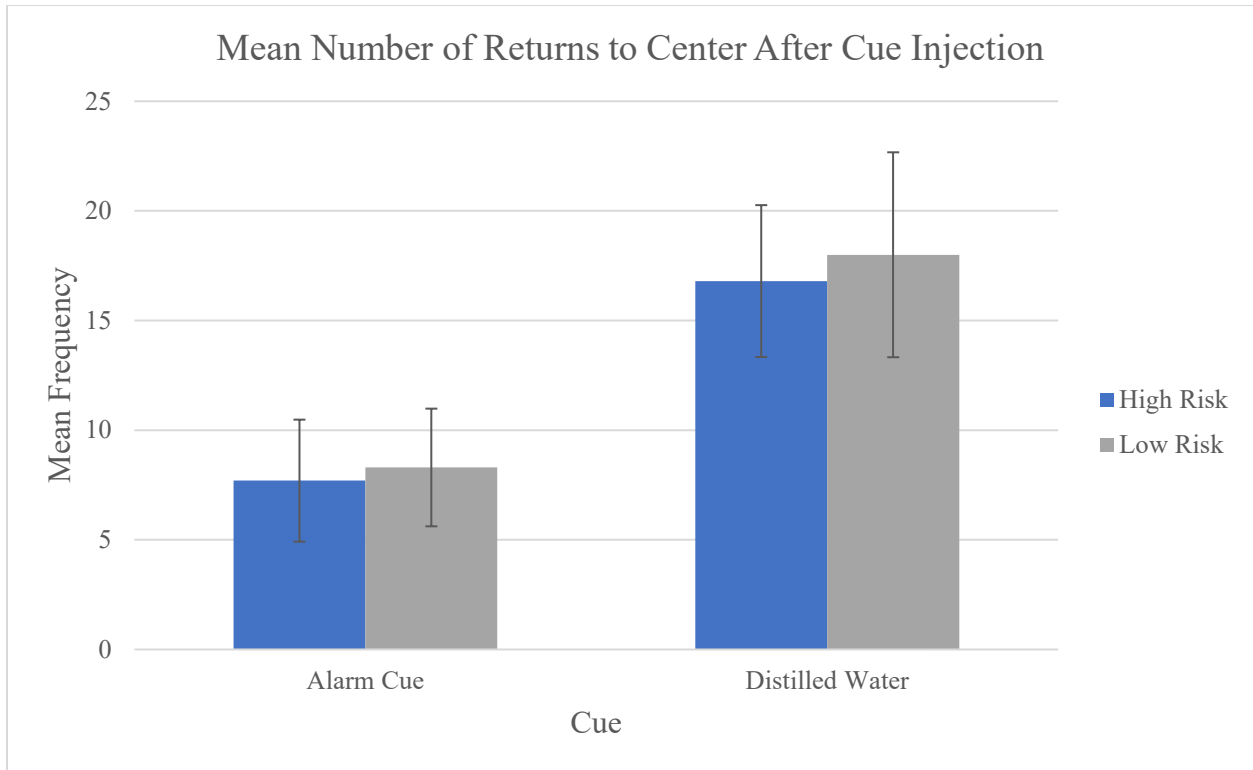
Figure 4. Mean (±SEM) number of returns to the central chamber made by a fish or a group of fish following the introduction of the acute cue for each background risk level (high risk/ low risk) and cue type (alarm cue/ distilled water) combination during experiment 2.

Figure 5. Mean (±SEM) number of entries into each arm made by a fish or a group of fish following the introduction of the acute cue for each background risk level (high risk (HR)/ low risk (LR)) and cue type (alarm cue (AC)/ distilled water (DW)) combination during experiment 3. Arm 1 was the only arm equipped with a food patch and available to the fish during the exploration period.

Experiment Number	Number of Donors	Mean Standard Length (cm)	Standard Deviation (cm)	Donor Sex
1, 3	105	1.59	0.15	Male
1	36	2.19	0.27	Female
2	35	1.85	0.28	Female
2	34	1.84	0.24	Female
3	51	1.85	0.21	Male
3	87	1.65	0.17	Male

Table 1. Number, sex, and mean standard length (± SD) of donor guppies used to prepare alarm cue.

Arm Contrast	Estimate	Standard Error	Z	p value
1 - 2	0.64	0.19	3.44	0.01
1 - 3	1.82	0.29	6.31	< 0.01
1 - 4	1.91	0.30	6.35	< 0.01
1 - 5	1.50	0.26	5.82	< 0.01
1 - 6	1.61	0.27	5.93	< 0.01
1 - 7	1.38	0.25	5.58	< 0.01
1 - 8	0.65	0.19	3.48	0.01
2 - 3	1.18	0.31	3.83	< 0.01
2 - 4	1.27	0.32	3.97	< 0.01
2 - 5	0.86	0.28	3.08	0.04
2 - 6	0.97	0.29	3.33	0.02
2 - 7	0.74	0.27	2.74	0.11
2 - 8	<0.01	0.21	0.02	1.00
3 - 4	0.09	0.39	0.22	1.00
3 - 5	-0.32	0.36	-0.90	0.99
3 - 6	-0.21	0.37	-0.57	1.00
3 - 7	-0.44	0.35	-1.27	0.91
3 - 8	-1.18	0.31	-3.83	< 0.01
4 - 5	-0.41	0.37	-1.11	0.95
4 - 6	-0.30	0.38	-0.79	0.99
4 - 7	-0.53	0.36	-1.47	0.82
4 - 8	-1.26	0.32	-3.97	< 0.01
5 - 6	0.11	0.34	0.33	1.00
5 - 7	-0.12	0.32	-0.37	1.00
5 - 8	-0.86	0.28	-3.07	0.04
6 - 7	-0.23	0.34	-0.69	1.00
6 - 8	-0.97	0.29	-3.32	0.02
7 - 8	-0.74	0.27	-2.73	0.11

Table 2. Tukey HSD pairwise comparisons for the distribution of fish in each arm following acute cue exposure for experiment 3.

<u>Image 1.</u> Plastic predator model used across all three experiments. The support and base shown in the picture were used in experiment 2.