PUPFISHES AS A SYSTEM TO TEST THE PREDATOR NAIVETÉ HYPOTHESIS

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Pupfishes as a System to Test the Predator Naiveté Hypothesis

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ABSTRACT

Pupfishes are an ideal system to test this the predator naiveté hypothesis because they often occur in isolated springs across a gradient of predation pressure. A convenient tool for assaying antipredator competence are behavioral responses to chemical alarm cues released when the epidermis is damaged during a predation event. Behavioral responses of three pupfish species, Red River Pupfish (*Cyprinodon rubrofluviatilis*), Amargosa River Pupfish (*C. nevadensis amargosae*), and Shoshone Pupfish (*C. n. shoshone*), which occur across a gradient of community complexity and predation pressure, were evaluated to test the effect of community composition, including predator variety and density, on antipredator behaviors. All three species responded to alarm cues by either reducing activity and/or lowering position in the water column, regardless of respective isolation or predation risks. I found no support for the predator naiveté hypothesis in these populations, which suggests that pupfish can be managed in multi-species habitats.

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DEDICATION

For Gramy Eleanor.

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1. LITERATURE REVIEW

Predation pressure can drastically affect prey species, causing changes in life history traits (Reznick et al. 1990), morphology (Reznick et al. 1990; Walker 1997; Johnson 2001; Moore et al. 2016), and/or behavior (Magurran et al. 1992; White and Berger 2001; Ross et al. 2019). Predation impacts on life history traits have been well studied, especially for numerous live-bearing fishes (Family Poeciliidae). Specifically, these studies have shown that individuals from populations under intense predation pressure reach maturity earlier than individuals from populations that experience weak predation pressure (Reznick et al. 1990; Johnson 2001; Moore et al. 2016). These patterns were initially thought to be due to inter-population variation in size-specific mortality rates (Reznick and Bryga 1987; Reznick et al. 1990). For example, by reaching maturity at earlier ages, prey fish are able to outgrow certain predators that target smaller sizes, and therefore reach reproductive age more rapidly (Reznick and Bryga 1987). However, subsequent work showed that predation on all life stages can also select for early maturity to allow for increased reproductive opportunities, but this increases mortality of mature fish (Reznick et al. 1996).

Selection by predation has been shown to cause evolution of life history traits in a relatively short period of time. For example, female guppies (*Poecilia reticulata*) translocated from a location with intense predation to a site with weak predation pressure shifted over a period of 11 years to delayed sexual maturity (Reznick et al. 1990). This work included a common garden experiment that showed a genetic basis for these predation-induced changes in life history (Reznick 1982; Reznick and Bryga 1987; Reznick and Bryga 1996). Lab-reared F-2 guppies from the introduction site were older and larger at maturity compared to guppies from the donor population. These findings were supported by data from both field and laboratory studies (Reznick 1982; Reznick et al. 1996) showing that predators play a direct role in shaping life history traits of prey species (see also Reznick et al. 2019).

Morphological responses to predation are also observed across predation risk gradients. For example, Threespine Stickleback (*Gasterosteus aculeatus*) living in habitats without fish predators had slightly smaller bodies, shorter median fins, and more posteriorly positioned dorsal spines compared to sticklebacks found in sympatry with fish predators (Walker 1997). In predator-free populations, there was less selection for predator defenses than in populations exposed to predators, and therefore median fins and dorsal spines were shorter (Walker 1997). Morphological responses can also occur over the span of a few weeks. For example, chemical cues released from damaged epidermal tissues of Crucian Carp (*Carassius carassius*) induces shifts in growth trajectory of carp (Brönmark and Miner 1992). After 12 weeks of exposure to the odor of Northern Pike (*Esox lucius*) feeding on Crucian Carp, carp developed significantly deeper body depth, making them more difficult to swallow by gape-limited predators (Brönmark and Miner 1992).

Behavioral changes in response to predators have also been widely documented. These observations include both comparative studies as well as work where predation risk was experimentally altered. Comparative studies have included numerous systems with strong gradients in predation pressure (Fu et al. 2015). For instance, guppies from high predation-risk habitats schooled more cohesively than guppies from low predation sites (Magurran et al. 1992). Guppies from high-predation habitats also maintained further distance from the predator when performing predator-inspection behavior compared to guppies from low-predation habitats (Magurran et al. 1992). These differences clearly implicate predation risk as a driver of antipredator behaviors. In another example, Pale Chub (*Zacco platypus*) individuals sourced from high predation sites were less active and spent less time in an experimental risk area compared to individuals from low predation sites (Fu et al. 2015).

Antipredator behavior has been documented in aquatic organisms across multiple taxonomic groups (Chivers and Smith 1998; Kats and Dill 1998), and many aquatic organisms rely on chemical cues for information about predation risk (Ferrari et al. 2010). This information is obtained through chemical cues such as kairomones, which are released by predators and detected by prey, and dietary cues, which are released from the digestive system of predators (Ferrari et al. 2010). In addition, aquatic organisms often detect and respond to chemical alarm cues released when epithelium of a conspecific is damaged during a predator attack (Chivers and Smith 1998; Ferrari et al. 2010; but see Wisenden et al. 2022). Such cues effectively signal that a predator is foraging nearby (Pfeiffer 1977; Chivers and Smith 1998). The release of these damage-released cues benefits the receiver, not the sender, because the sender is a victim of attempted or successful predation (Wisenden 2015). Furthermore, the receiver may ignore damage-released cues if responding incurs more cost than benefit. For example, fish do not respond to alarm cue with antipredator behavior when they have been deprived food for 24h (Brown and Smith 1996), or if the concentration of alarm cue is too low, or in some cases, when alarm cue is derived from conspecifics from a different ontogenetic stage (Chivers and Smith 1998; Ferrari et al. 2010).

Antipredator behavior of freshwater fish in response to damage-released alarm cues has been widely studied (Chivers and Smith 1998; Ferrari et al. 2010). Alarm cue is released from epidermal club cells when these cells are ruptured, providing public information to the surrounding water column of a predation event (Ferrari et al. 2010). However, Ferrari et al. (2010) also noted that club cell presence may not be necessary for the production and response to damaged epithelial tissue. Antipredator responses in fish are mediated by cue concentration, time elapsed since cue release, and group size if an individual is a member of a group (Ferrari et al. 2010). Wisenden (2008) illustrated that the concentration of alarm cue plays a role in intensity of response to alarm cue. He found that minnow traps scented with alarm cue caught the fewest Fathead Minnows (*Pimephales promelas*), while traps scented with blank water placed 2m from a source of alarm cue caught an intermediate number of fish and traps 8m away from a source of alarm cue caught the most fish. In another study, Wisenden et al. (2004) demonstrated that density of Blacknose Shiner (*Notropis heterolepis*) and other cyprinids significantly decreased in the littoral zone of field sites where Blacknose Shiner alarm cue was introduced to the water. This demonstrated that both conspecifics and heterospecifics avoided areas chemically-labeled with alarm cue (Wisenden et al. 2004).

When predation pressure is reduced or removed for many generations, selection to maintain costly antipredator behaviors is relaxed, leading eventually to loss of capacity to mount an effective antipredator response (Lima and Dill 1990; Cox and Lima 2006). When antipredator traits are lost, prey may be highly vulnerable to predators if predators are subsequently encountered (Cox and Lima 2006). For example, removal of Gray Wolves (*Canis lupus*) for decades from Yellowstone National Park, USA, resulted in decreased vigilance in Elk (*Cervus elaphus*) compared to vigilance levels prior to wolf reintroduction (Laundré et al. 2001). Elk that were more vigilant spent less time foraging, which was a cost associated with maintenance of antipredator behavior. However, one year after wolf reintroduction, female elk with calves showed restored levels of vigilance in wolf areas in response to the threat of wolf predation (Laundré et al. 2001).

A lack of antipredator behaviors over many generations can lead to evolutionary naiveté, which occurs when a prey species is unable to recognize or appropriately respond to a novel predator with which it has not co-occurred (Cox and Lima 2006; Anton et al. 2020). This can occur when prey species retain adequate predator responses to native predators but do not recognize novel predators (Macdonald and Harrington 2003; Cox and Lima 2006; Ellender et al. 2018; Anton et al. 2020). Evolutionary naiveté can also result from a prey species having an appropriate but ineffective antipredator response, either morphologically, physiologically, or behaviorally, against an introduced predator (Cox and Lima 2006; Anton et al. 2020), or from overresponding to an introduced predator (Anton et al. 2020).

Inadequate antipredator responses can result from evolutionary naiveté (Cox and Lima 2006). For example, Water Voles (*Arvicola amphibius*) in England effectively coexist with native predators but were vulnerable to predation by non-native American Mink (*Neogale vison*) (Macdonald and Harrington 2003). Antipredator responses by voles to native predators was to hide in burrows, but this behavior was ineffective against female American Mink, which were small enough to enter vole burrows (Macdonald and Harrington 2003). Eastern Cape Redfin (*Pseudobarbus afer*) exhibited appropriate antipredator behavior by remaining in the water column and avoiding benthic habitat when native nocturnal predators were active and remained near vegetation during the day (Ellender et al. 2018). However, this antipredator behavior increased risk of predation by introduced black basses (*Micropterus sp.*), which are ambush predators, resulting in elimination of redfin from stream reaches where bass occurred (Ellender et al. 2018).

Some or all antipredator behaviors can be lost after isolation from predators due to relaxed selection to maintain costly antipredator behaviors (Blumstein and Daniel 2005). For example, group size effects, where predator vigilance behavior by each individual in a group declines as group size increases, were not maintained in island macropodid populations compared to continental macropodid populations (Blumstein and Daniel 2005). Similarly, prey fish in the Atlantic Ocean fail to recognize invasive Lionfish (*Pterois volitans*) as predators and maladaptively approach lionfish and are eaten (Anton et al. 2016).

Predator naiveté is particularly common for insular populations of fishes, reptiles, and amphibians compared to species that occur in species-rich, complex communities (Cox and Lima 2006, Anton et al. 2020). The impacts of predator naiveté are particularly relevant for island prey populations exposed to non-native predators. For instance, Brown Tree Snakes (*Boiga irregularis*) introduced to Guam after World War II eliminated native vertebrates from some areas of the island (Rodda and Savidge 2007). Throughout Guam, brown tree snakes persist at much higher densities than in their native range and transitioned to consuming introduced prey species after decimating native species on the island (Rodda and Savidge 2007). Another example of an introduced predator having detrimental effects on an island prey species is non-native House Mice (*Mus musculus*) preying on the eggs of burrowing Atlantic Petrels (*Pterodroma incerta*) (Dilley et al. 2015). Adult petrels failed to recognize mice as predators and therefore did not protect their eggs or chicks from mice predation (Dilley et al. 2015).

Like terrestrial islands, the deserts of the southwestern United States surround small “aquatic islands”. At the end of the Pleistocene, vast interconnected lakes in the Death Valley system desiccated, stranding fish populations in insular habitats (Miller 1948, Miller 1950; Soltz and Naimann 1978). As a consequence, the so-called *desert fishes* evolved in small, simple, isolated and distinct habitats with limited competition and few fish predators. This evolutionary history may have led to the loss of costly antipredator traits, which in turn made desert fishes vulnerable to non-native predators (Minckley and Deacon 1968).

Predation by non-native species has been documented as a primary cause of decline for desert fishes (Deacon et al. 1964; Minckley and Deacon 1968; Meffe 1985). For example, the extinction of endemic Monkey Springs Pupfish (*Cyprinodon* sp.), occurred shortly after the introduction of non-native Largemouth Bass (*Micropterus salmoides*) to Monkey Spring (Miller et al. 1989). Similarly, the introduction of non-native Shortfin Mollies (*Poecilia mexicana*) resulted in the decline of both Moapa Dace (*Moapae coriacea*) and Moapa White River Springfish (*Crenichthys baileyi moapae*) (Minckley and Deacon 1968). Western Mosquitofish (*Gambusia affinis*), also an introduced predator, replaced numerous populations of the Sonoran Topminnow (*Poeciliopsis occidentalis*) in as little as four months post-introduction (Meffe 1985).

Invasive species have had particularly strong impacts on poolfishes (*Empetrichthys* sp.) of southern Nevada. In fact, the extinction of Ash Meadows Poolfish (*Empethrichthys merriami*) was likely due to predation by two non-native species: Red Swamp Crayfish (*Procambarus clarkii*) and American Bullfrogs (*Lithobates catesbeianus*) (Miller et al. 1989). Further, invasive species have led to the rapid decline of three refuge populations of the endangered Pahrump Poolfish (*E. latos*) (Kevin Guadalupe, Nevada Department of Wildlife, pers. comm.). Most notably, the un-authorized introduction of Red Swamp Crayfish and Western Mosquitofish to Lake Harriet within Spring Mountain Ranch State Park, Nevada, were associated with the rapid collapse of the poolfish population from over 10,000 individuals to a few hundred individuals after 3- and 1-years post-introduction, respectively (Kevin Guadalupe, Nevada Department of Wildlife, pers. comm.).

Experimental work has confirmed the vulnerability of selected desert fishes to non-native predators. For example, experimental populations of the Sonoran Topminnow failed to recruit juveniles because of mosquitofish predation on larval topminnows (Meffe 1985). Least Chub (*Iotichthys phlegothontis*) young-of-year exhibited decreased survivorship and growth in the presence of mosquitofish (Mills et al. 2004). Small young-of-year chub were readily eaten by adult mosquitofish, and chub responded to mosquitofish with reduced foraging and increased time spent within vegetation refugia (Mills et al. 2004). Mosquitofish also preyed on larval Mohave Tui Chub (*Siphateles bicolor mohavensis*) and reduced recruitment (Henkanaththegedara and Stockwell 2014). However, in an unexpected twist, adult tui chub preyed on smaller-bodied mosquitofish (Henkanaththegedara and Stockwell 2013; Henkanaththegedara and Stockwell 2014). Mosquitofish also eliminated recruitment of Pahrump Poolfish when held in sympatry in mesocosm experiments (Goodchild and Stockwell 2016; Paulson 2019). In all of these cases, experimental findings are consistent with observations of non-native species impacting wild populations of associated fish species.

These previously mentioned impacts of invasive predators on desert fish populations have been hypothesized to result from predator naiveté (Miller 1961; Minckley and Deacon 1968). However, the role of evolutionary naiveté in determining the fate of desert fishes in response to invasive predators has received limited attention. The history of isolation of aquatic habitats in the American southwest make desert fish an ideal study system for evaluating if and how the predator naiveté hypothesis affects prey in isolated habitats.

Prey in complex communities experience and therefore recognize more predator archetypes than prey from simple or single-species communities (Anton et al. 2020). Prey response to introduced predators is dependent on familiarity of the prey with similar predators

(Sih et al. 2010). Prior exposure to predators increases the chance of predator recognition in prey species, especially if predators already exist within the biological community (Sih et al. 2010). For some desert fishes, a gradient exists between insular species in simple, isolated communities and those existing in large river systems with complex communities (Soltz and Naimann 1978). For example, species such as the Pahrump Poolfish and Sonoran Topminnow, which evolved in insular habitats, were decimated by introduced Western Mosquitofish (Goodchild and Stockwell 2016; Meffe 1985). By contrast, some species such as the Amargosa Pupfish have co-evolved with other fish have been able to co-persist with non-native mosquitofish (Goodchild and Stockwell 2016). Similarly, Moapa Dace and Moapa White River Springfish, which occur in more complex communities, were able to co-persist with introduced species (Minckley and Deacon 1968). In larger systems, such as the Colorado River, communities are complex with both native and non-native piscivorous fish species (Soltz and Naimann 1978). Thus, it is important to study antipredator responses of desert fishes across varying levels of community complexity.

Pupfishes offer such an opportunity to assess antipredator behaviors among populations that differ in community composition and evolutionary history. For example, Red River Pupfish (*Cyprinodon rubrofluviatilis*) (Figure A-1) range throughout the Brazos River in Texas and the Red River, which flows through both Texas and Oklahoma (Echelle et al. 1972). Many populations of Red River pupfish are found in complex communities that include numerous fish predators, while some populations occur in simple communities (Echelle et al. 1972; Ruppel 2019). By contrast, several species of pupfishes (*Cyprinodon* sp.) found near Death Valley, California, such as the Amargosa Pupfish (*C. nevadensis amargosae*) (Figure A-2) and the Shoshone Pupfish (*C. n. shoshone*) (Figure A-3) evolved in isolated springs with simple communities (Miller 1948; Soltz and Naimann 1978). These species evolved with limited predation pressure, and therefore are less likely to recognize introduced predators (Sih et al. 2010). Amargosa Pupfish evolved in sympatry with only one other fish species, the Amargosa Canyon Speckled Dace (Distinct Population Segment of *Rhinichthys osculus nevadensis;* Mussman et al. 2020), and has co-persisted with non-native Western Mosquitofish for decades (Miller 1969). By contrast, the Shoshone Pupfish evolved in the absence of any other fish species (Miller 1948). The wide range of habitats and community compositions, geographic locations, and evolutionary history of pupfishes provide a system to evaluate evolutionary naiveté within fish occurring in isolation versus connected habitats.

Evaluation of response to alarm cue can provide insight into general antipredator competence of each species that may be based on evolutionary history. This behavioral assay is a convenient assessment tool to test if species have become evolutionary naïve due to isolation and/or completely incompetent in coping with predators of any kind. I tested whether ecological history affects antipredator responses to alarm cue by Red River Pupfish, Amargosa Pupfish, and Shoshone Pupfish. Specifically, I evaluated whether populations of Red River pupfish sourced from five different sites with varying species and predator composition show a corresponding gradient in behavioral response intensity to conspecific alarm cue. I also evaluated conspecific alarm cue responses for Amargosa River Pupfish and Shoshone Pupfish, which evolved in relatively simple communities. I then evaluated responses of Amargosa River Pupfish and Shoshone Pupfish to heterospecific alarm cue from Amargosa Canyon Speckled Dace, which co-occur with Amargosa River Pupfish in the Amargosa Canyon, but do not co-occur with Shoshone pupfish (Miller 1969; Scoppettone et al. 2011).

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2. EVALUATION OF ANTIPREDATOR BEHAVIOR OF THREE PUPFISH SPECIES

2.1. Abstract

Following the desiccation of Pleistocene Lakes, many desert fishes evolved in isolated "aquatic island" habitats with limited predation-associated selection pressure. The strong negative impacts of non-native predators on desert fishes may be explained by the predator naiveté hypothesis (Cox and Lima 2006; Anton et al. 2020). This work evaluated antipredator behavior of three pupfishes that exist across a gradient of isolation and predation risk: Red River Pupfish (*Cyprinodon rubrofluviatilis*), Amargosa River Pupfish (*C. nevadensis amargosae*), and Shoshone Pupfish (*C. n. shoshone*). I evaluated behavioral responses to chemical alarm cues released when the epidermis is damaged by a predator. This assessment allowed me to determine if predator naiveté is enhanced in pupfishes based on isolation and predation risk. All three species exhibited antipredator behavior in response to alarm cue, regardless of isolation or predation risk. This demonstrates pupfishes are able to respond to predators and can be managed in multi-species habitats.

2.2. Introduction

Many desert fish that evolved with limited predation risk have been severely impacted by non-native predators (Minckley and Deacon 1968). Approximately 10,000 years ago, the desiccation of the Pleistocene Lakes stranded fishes in isolated systems with simple community structure and limited predation pressure (Miller 1950; Miller 1961; Minckley and Deacon 1968; Miller et al. 1989). This evolutionary history may have resulted in the loss of antipredator traits (Sih et al. 2010), which in turn may have made desert fishes vulnerable to non-native predators. In fact, non-native species have caused the decline of many desert fishes (Deacon et al. 1964; Minckley and Deacon 1968; Meffe 1985), and these declines have been attributed to evolutionary naiveté towards the introduced predators (Miller 1961: Minckley and Deacon 1968).

Evolutionary naiveté has been extensively reported for insular populations that lack evolutionary history with a given predator and is therefore unable to recognize or adequately respond to an introduced predator (Cox and Lima 2006). Inadequate antipredator responses can be behavioral, morphological or physiological, or result from appropriate but inadequate antipredator behavior (Cox and Lima 2006; Anton et al. 2020). Furthermore, freshwater fishes are more likely to exhibit naiveté towards introduced predators due to isolation in aquatic environments and lack of experience with the introduced predator archetype (Anton et al. 2020). For example, Eastern Cape Redfin (*Pseudobarbus afer*) populations were heavily impacted due to predation by non-native black basses (*Micropterus sp.*) (Ellender et al. 2018). Performance of antipredator behaviors towards native predators, which are nocturnal and feed on the stream bottom, increased predation risk from black basses, which are ambush predators, and resulted in the eradication of redfin from reaches inhabited by bass (Ellender et al. 2018).

Evolutionary naiveté may explain the detrimental impacts of non-native predators on desert fishes (Miller 1961; Minckley and Deacon 1968). For example, Sonoran Topminnow (*Poeciliopsis occidentalis*) larvae were heavily preyed upon by Western Mosquitofish (*Gambusia affinis*), causing extirpation of some populations within a few months (Meffe 1985). Similarly, extinction of the Ash Meadows Poolfish (*Empetrichthys merriami*) was likely caused by the introduction of Red Swamp Crayfish (*Procambarus clarkii*) and American Bullfrog (*Rana* [*Lithobates*] *catesbeianus*) (Miller et al. 1989). Further, multiple refuge populations of the endangered Pahrump Poolfish (*Empetrichthys latos*), have been decimated by the introduction of crayfish and mosquitofish (Kevin Guadalupe, Nevada Department of Wildlife, pers. comm).

Experimental work in mesocosms has corroborated decreased survival of desert fish in sympatry with introduced predators (Meffe 1985; Mills et al. 2004; Rogowski and Stockwell 2006; Henkanaththegedara and Stockwell 2014; Goodchild and Stockwell 2016; Paulson and Stockwell 2020). For example, Sonoran Topminnow larvae were heavily preyed upon by Western Mosquitofish in a laboratory setting (Meffe 1985). Pahrump Poolfish larval production was virtually eliminated by the presence of Western Mosquitofish (Goodchild and Stockwell 2016). Additionally, Pahrump Poolfish adult survival was reduced in the presence of Red Swamp Crayfish (Paulson 2019). On the other hand, Amargosa River Pupfish (*Cyprinodon nevadensis amargosae*) (Figure A-2) juvenile recruitment was reduced in the presence of Western Mosquitofish, but the two species were able to co-persist in sympatry (Goodchild and Stockwell 2016).

The mechanisms explaining the differential responses of desert fishes to non-native predators have not been evaluated. One tool for testing antipredator behavior in fishes has been to evaluate behavioral responses to conspecific alarm cues, which are damaged released cues from the epidermis of prey species (Chivers and Smith 1998, Ferrari et al. 2010a). Chemical composition of the alarm substance is currently unknown, but the substance released from epidermal club cells, which also play a role in immune response of fish (Ferrari et al. 2010a, Pandey et al. 2021). These cues alert receivers to the nearby presence of active predators (Wisenden 2015). Responses to alarm cues may vary depending on whether a response is deemed too costly (Chivers and Smith 1998, Ferrari et al. 2010a). Laboratory and field studies have demonstrated that fish respond to conspecific and heterospecific alarm cues through changing behavior, often by reducing activity and vertical position in the water column (Ferrari et al. 2010a). By contrast, Pahrump Poolfish did not respond to conspecific alarm cues

(Stockwell et al. in review) providing a baseline for evaluating alarm cue responses. Thus, such behavioral assays provide a promising approach to compare antipredator behavioral responses among fishes that occupy divergent predation regimes.

An ideal examination of the predator naiveté hypothesis would be within a system with closely related species or populations that occur across a wide gradient of predation risk (Nosil and Crespi 2006; Langerhans et al. 2007; Marchinko 2009). Such is the case with Pupfishes (*Cyprinodon sp.*), which occupy ecologically divergent habitats (Miller 1948, Soltz and Naiman 1978). Pupfishes occur in habitats ranging from large river systems such as the Red River of Texas and Oklahoma (Echelle et al. 1972), as well as isolated habitats in the southwestern United States. (Miller 1948; Soltz and Naimann 1978).

Red River pupfish (*Cyprinodon rubrofluviatilis*) (Figure A-1) are found throughout the Brazos River of Texas and Red River of Texas and Oklahoma, USA (Echelle et al. 1972; Ruppel 2019). Red River Pupfish are commonly found in communities including Plains Killifish (*Fundulus zebrinus*), Red River Shiner (*Notropis bairdi*) and Plains Minnow (*Hypognathus placitus*) (Echelle et al. 1972). Red River Pupfish also occur in habitats with numerous fish species including predatory species such as Western Mosquitofish and sunfishes (*Lepomis sp.)* (Echelle et al. 1972). Salinity is a significant determinate for fish communities throughout the Red River, where many piscine predators of pupfish are generally not found at salinities greater than 10ppt (Echelle et al. 1972). However, salinity likely does not affect alarm cue signals as shown by extensive alarm cue research in both marine and freshwater habitats (Ferrari et al. 2010a).

In contrast to the Red River Pupfish, both the Shoshone Pupfish (*C. n. shoshone*) (Figure A-3) and Amargosa River Pupfish evolved in relatively simple, isolated communities within the

Death Valley System (Miller 1948). Shoshone Pupfish evolved in allopatry as the only fish species within the biological community, whereas Amargosa River Pupfish evolved in sympatry with Amargosa Canyon Speckled Dace (*Rhinichthys osculus nevadensis*) (Miller 1948, Soltz and Naimann 1978) and currently co-persist with Western Mosquitofish (Miller 1969).

Therefore, pupfishes provide a system to evaluate antipredator behavior among populations/species that have divergent predation regimes. I evaluated pupfish antipredator behavioral responses to conspecific alarm cue and compared population and species from different predation risk regimes. I tested three pupfish species including Red River Pupfish, Amargosa River Pupfish, and Shoshone Pupfish. Red River Pupfish were sourced from five populations with varying community composition and predation pressure to test whether there were any differences in antipredator behavior that could be correlated with high or low community predation pressure. Shoshone Pupfish and Amargosa River Pupfish were tested against both conspecific alarm cue and cue from Amargosa Canyon Speckled Dace because this species evolved in sympatry with Amargosa River Pupfish, and it is well documented that cyprinids produce and respond to conspecific alarm cue (Ferrari et al. 2010a).

Based on the predator naiveté hypothesis, I anticipated that Amargosa Pupfish and Shoshone Pupfish responses would be limited due to isolation in aquatic island habitats. On the other hand, I expected that Red River Pupfish would exhibit a strong response to conspecific alarm cue due to increased community complexity throughout the river system. Furthermore, I expected that Red River Pupfish populations under moderate to high predation risk would display stronger antipredator responses than populations exposed to low predation risk.

2.3. Methods

2.3.1. Population Selection and Fish Collection

Experiment 1 evaluated antipredator responses to conspecific alarm cue of female pupfish originating from five different populations of Red River pupfish. Populations were selected based on fish community composition outlined in Ruppel (2019) to test the effects of duration of isolation and community complexity on antipredator response intensity. The Fishes of Texas database (Hendrickson and Cohen 2015) provided additional information on community composition and history of isolation. Red River Pupfish in populations 1 and 5 co-occurred in simple communities with only Plains Killifish (*Fundulus zebrinus)*, and these populations were classified as having low predation risk (Table 2.1). Fish communities for populations 3 and 4 included two to three other species but no large predators and were designated as having moderate predation risk because of possible predation on pupfish eggs and larvae (Table 2.1). Population 2 was classified as having high predation risk due to complex community structure including known pupfish predators Largemouth Bass (*Micropterus salmoides*), Orange Spotted Sunfish (*Lepomis humilis*), Western Mosquitofish (*Gambusia affinis*), and Red Shiner (*Cyprinella lutrensis*) (Table 2.1). Female Red River Pupfish were collected at the identified sites by a contractor from June 1-3, 2020, and shipped to NDSU overnight via FedEx on the day of capture. Upon arrival, fish were transported to a field site on the North Dakota Agricultural Experiment Station where fish were acclimated to 1135L population-specific holding tanks that had been set up two weeks prior with salinity set at 10 ppt. Salinity was set to 10ppt to reflect a moderate salinity level based on the variation of salinities measured at sampling sites (Table 2.1) and to standardize salinity across all trials. Trials began on June 15, 2020, to allow for adequate acclimation time for all fish to 10ppt salinity.

For experiment two, Shoshone Pupfish and Amargosa River pupfish were selected for comparison of antipredator response to both conspecific and heterospecific alarm cues. Female Amargosa River Pupfish and Amargosa River Speckled Dace (not sexed) were captured using Gee minnow traps in the Amargosa River near Tecopa, California on May 5, 2021 (Table 2). Female Shoshone Pupfish were collected from two sites in the Shoshone wetlands between May 4-7, 2021, using standard Gee minnow traps (Table 2). Fish were shipped overnight via FedEx on the day of capture and upon arrival, fish were acclimated to 378L population-specific holding tanks on the Minnesota State University Moorhead (MSUM) campus. All holding tanks and experimental work at MSUM used freshwater (approximately 0ppt salinity) to reflect natural conditions at sample sites.

2.3.2. Preparation of Alarm Cues and Evaluation of Fish Behavior

Antipredator behavior in response to conspecific alarm cue was evaluated by observing behavior both before and after exposure to either a treatment or a control stimulus; water and alarm cue, respectively (see Wisenden 2011). Chemical alarm cues were prepared following the protocol provided by Wisenden (2011). Donor fish were euthanized with MS-222 (tricaine mesthanesulfonate) and cervical dislocation before the epidermis of each fish was removed (NDSU IACUC Protocols A18054 and A21042). Skin fillets from each side of the fish were laid on a flat surface and measured for total skin area, then placed in a beaker of deionized water resting on a bed of crushed ice. Once skin fillets were removed from all donor fish, the skin was blended with a handheld blender for 3 min and diluted to a final concentration of 1 cm^2 of skin per 10 mL concentration. Chemical alarm cue was then aliquoted into 10-mL doses in individual 10-mL mailing tubes, then stored at -20 °C until needed for trials.

Trials were conducted by testing single focal fish in 37-L glass aquaria with a 5 x 5 cm grid drawn on the short side of the tank (Figure 2.1). Opaque dividers were placed between adjacent aquaria to visually isolate the focal fish. An air stone supplied oxygen to the trial tanks and a separate stimulus delivery tubing secured to the air stone housing apparatus for stability was used to deliver test stimuli to the tank (alarm cue or water as a control) (Figure 2.1). Focal fish were acclimated for a minimum acclimation period of 20 h. Each fish was fed at least 20 min before the start of the trial to reduce overall stress.

All observations were recorded using a Canon VIZIA HF R700 video camera positioned in front of the test tank. For each trial, activity was measured by counting the total number of lines crossed by the eye of the focal fish during 5-min pre- and 5-min post-stimulus observation periods (Wisenden 2011). Vertical position was recorded every 10 s for both pre- and post-stimulus periods by noting the horizontal row in the grid occupied by the test subject, where 1 was the row at the tank bottom and 5 was the surface row. For each trial, the test stimulus (water or alarm cue) was delivered to the tank after the pre-observation period via aquarium tubing inserted with the air stone.

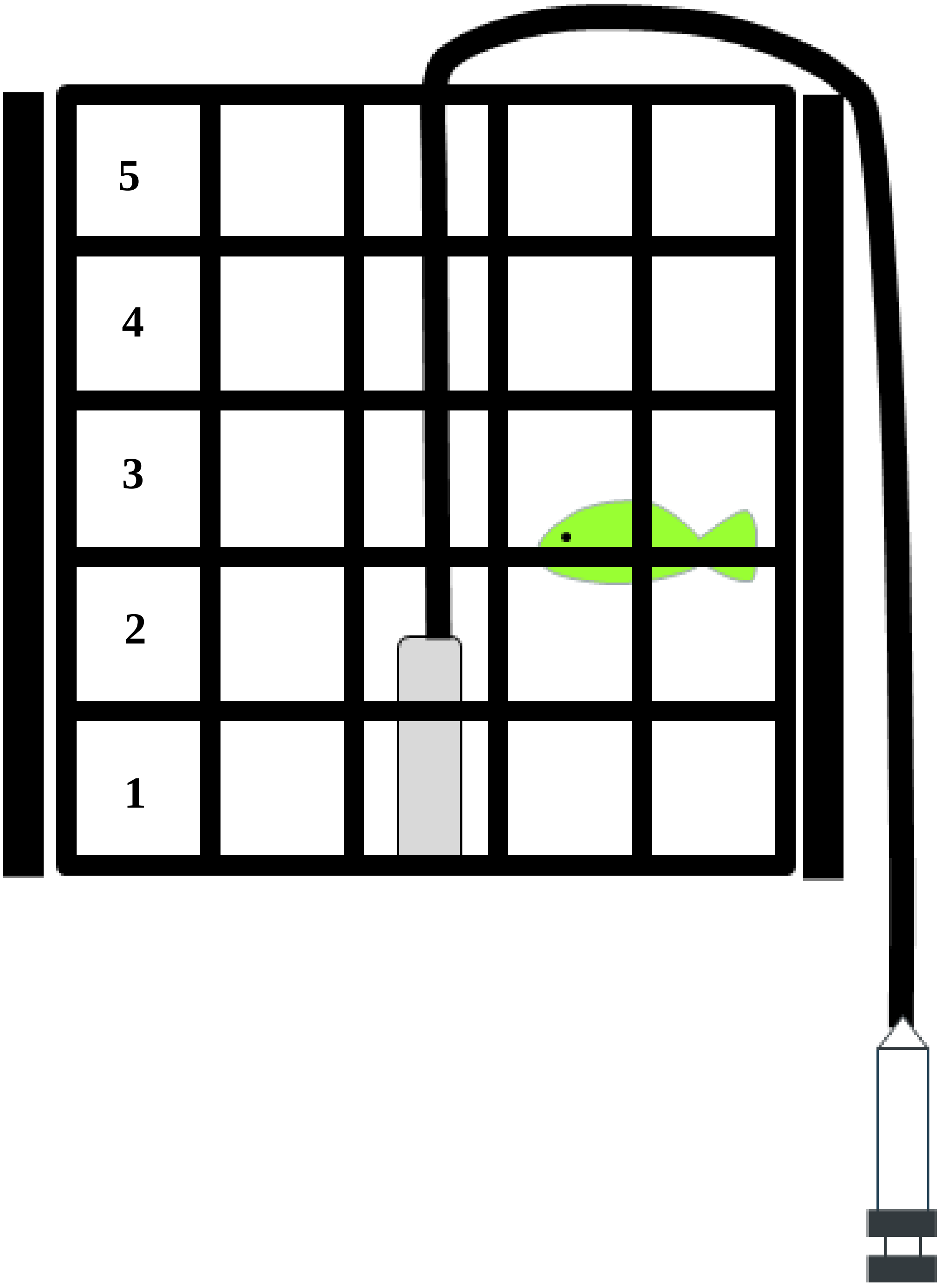

Figure 2.1: Experimental tanks setup for all alarm cue trials.

2.4. Experiment 1: Evaluation of Five Red River Pupfish Populations to Conspecific Alarm Cue

Trials were conducted using the technique outlined in the previous section. Dechlorinated tap water was used for all trials and salinity level was kept consistent at 10 ppt using Instant Ocean aquarium salt (Spectrum Brand, Blacksburg VA) across all trials. All trials were completed at room temperature as experiments occurred from June to August 2020. Lighting was set for a 16h:8h light:dark setting in the trial room to match outdoor lighting conditions because fish were held in outdoor tanks.

A randomized block design was used to standardize evaluation of all five populations across time for the duration of the experiment, with each block composed of 10 aquaria. Within each block two females from each of the five populations were randomly assigned the two treatments. A randomized block design also allowed each block of populations to be tested within a single day, which controlled for variation due to factors such as time spent in captivity. Fish that were not consistently active in the "pre-stimulus" observation period were not exhibiting normal behavior of comfortably swimming about the tank, and therefore would not provide an adequate assessment of behavioral changes between the pre- and post-stimulus observation periods. Any trial that included a "0" score of activity during any measured 1-min interval during the pre-observation period was excluded from analysis. Data were analyzed using Analysis of Covariance (ANCOVA; JMP Pro 15.0 software), with a categorical predictor of Treatment (alarm cue or water) and Population (1, 2, 3, 4, or 5) as categorical predictors and a covariate of Pre-stimulus behavior (activity or vertical position). Block effects were tested for and were not included in final model if Block was not significant.

Table 2.1: Red River Pupfish populations, sampling locations, predation risk, community composition, and experimental sample sizes.

Population	**Site Description**	**Location (Lat/Long)**	**Predation Risk**	**Conductivity (µS/cm)**	**Other Fish Species Present**	**Treatment Sample Sizes**
Site 1	Prairie Dog Town Fork of Red River at Hwy 256/70	*Lat = 34.628348;* *Long = -100.942*	Low	18159	Plains Killifish (*Fundulus zebrinus)*	Alarm Cue: 20 Water: 14
Site 2	Pease River at Hwy 283	*Lat = 34.179296;* *Long = -99.2784*	High	13847	Plains Killifish Red River Shiner (*Notropis bairdi*) Red Shiner (*Cyprinella lutrensis*) Largemouth Bass (*Micropterus salmoides*) Orange spotted sunfish (*Lepomis humilis*) Western Mosquitofish (*Gambusia affinis*) Bullhead minnow (*Pimephales vigilax*)	Alarm Cue: 12 Water: 10
Site 3	Pease River Hwy 62/68	*Lat =34.194236;* *Long =-100.251*	Moderate	24151	Plains Killifish Red River Shiner Plains minnow (*Hypognathus placitus*)	Alarm Cue: 21 Water: 17
Site 4	Prairie Dog Town Fork of Red River at Hwy 207	*Lat = 34.837054;* *Long= -101.416*	Moderate	25403	Plains Killifish Red River Shiner	Alarm cue: 18 Water: 20
Site 5	Prairie Dog Town Fork of Red River at Hwy 62/83	*Lat = 34.566653;* *Long = -100.196*	Low	82466	Plains Killifish	Alarm Cue: 14 Water: 15

2.5. Experiment 2: Evaluation of Shoshone Pupfish and Amargosa River Pupfish to Conspecific and Heterospecific Alarm Cues

Test procedures followed the protocols described in Section 2.3 in terms of trial tanks and alarm cue preparation. In addition to preparing conspecific alarm cue for both Shoshone Pupfish and Amargosa River Pupfish, alarm cue was also prepared using Amargosa Canyon Speckled Dace collected from the same collection site as Amargosa pupfish. All trials were completed using freshwater, and overhead lights were set to a 12h:12h light dark cycle because these fish were held indoors. Amargosa River pupfish were tested with the following treatment combinations: a) Amargosa River Pupfish alarm cue, b) Amargosa Speckled Dace alarm cue and c) dechlorinated tap water (as a control). Shoshone Pupfish were tested with the following treatment combinations: a) Shoshone Pupfish alarm cue, b) Amargosa Canyon Speckled Dace alarm cue and c) dechlorinated tap water (as a control). Both Amargosa River Pupfish and Shoshone pupfish were tested at the same time using a randomized block design with a block size of n = 6 so that all treatment combinations were tested within each block, allowing for uniform testing conditions of each treatment group over time. Fish that were not consistently active in the “pre-stimulus” observation period were not exhibiting normal behavior of comfortably swimming about the tank, and therefore would not provide an adequate assessment of behavioral changes between the pre- and post-stimulus observation periods. Thus, any trial that included a “0” score of activity during any of the pre-stimulus one-minute intervals was excluded from analyses.

Data were analyzed via an Analysis of Covariance (ANCOVA) using JMP Pro 15.0 software, with a categorical predictor of Treatment (conspecific alarm cue, dace cue, or water) and a covariate of Pre-stimulus behavior (activity or vertical position). The initial analyses included “Block”, but this term was excluded from final models when it was not significant. For the Amargosa Pupfish and Shoshone Pupfish data sets post-hoc pairwise comparisons were completed to test slope differences among the three treatments for the pre-stimulus versus post-stimulus behavior using t-tests. Significance was determined with a sequential Bonferroni test (Rice 1989).

Table 2.2: Shoshone Pupfish and Amargosa River Pupfish sampling locations and experimental sample sizes.

Species	**Location**	**Latitude/Longitude**	**Sample Size Per Treatment**		
			Alarm Cue	**Dace Cue**	**Water**
Shoshone Pupfish	Blue Trail Pond	Lat: 35°58'33.75"N Long: 116°15'55.63"W	25	28	27
	Rosa's Pond	Lat: 35°58'31.03"N Long: 116°15'53.73"W			
	Caliche Ponds	Lat: 35°58'24.21"N Long: 116°15'49.05"W			
Amargosa River Pupfish	Amargosa River	Lat: 35°50'56.78"N Long: 116°13'49.69"W	24	19	20
Amargosa Canyon Speckled Dace	Amargosa River	Lat: 35°50'56.78"N Long: 116°13'49.69"W	–	–	–

2.6. Results

2.6.1. Experiment 1: Evaluation of Five Red River Pupfish Populations to Conspecific Alarm Cue

Red River Pupfish post-stimulus activity differed significantly in reaction to stimulus type (alarm cue vs. control). Additionally, Post-stimulus activity was closely correlated with pre-stimulus activity, whereas fish that were active during the pre-stimulus observation period were generally more active during the post-stimulus observation period. Block was not significant (Table A-1) and was therefore not included in the final model (Table 2.3). The effect of population was not significant, meaning that activity levels were notably consistent among populations and showed reduced activity in response to alarm cue (Table 2.3, Figures 2.2 and 2.3; Tables A-3 through A-12). This effect was strongest for highly active fish (Figure 2.2).

Red River Pupfish reduced post-stimulus vertical position in response to treatment (Figures 2.4, 2.5), but this was correlated with pre-stimulus vertical position (Figures 2.4, 2.5). Block was not significant and was therefore not included in the final model (Table A-2). The effect of population was not significant, meaning that the vertical position responses were notably consistent among populations and showed decreased vertical position in the water column in response to alarm cue (Table 2.4; Figure 2.5; Tables A-13 through A-22).

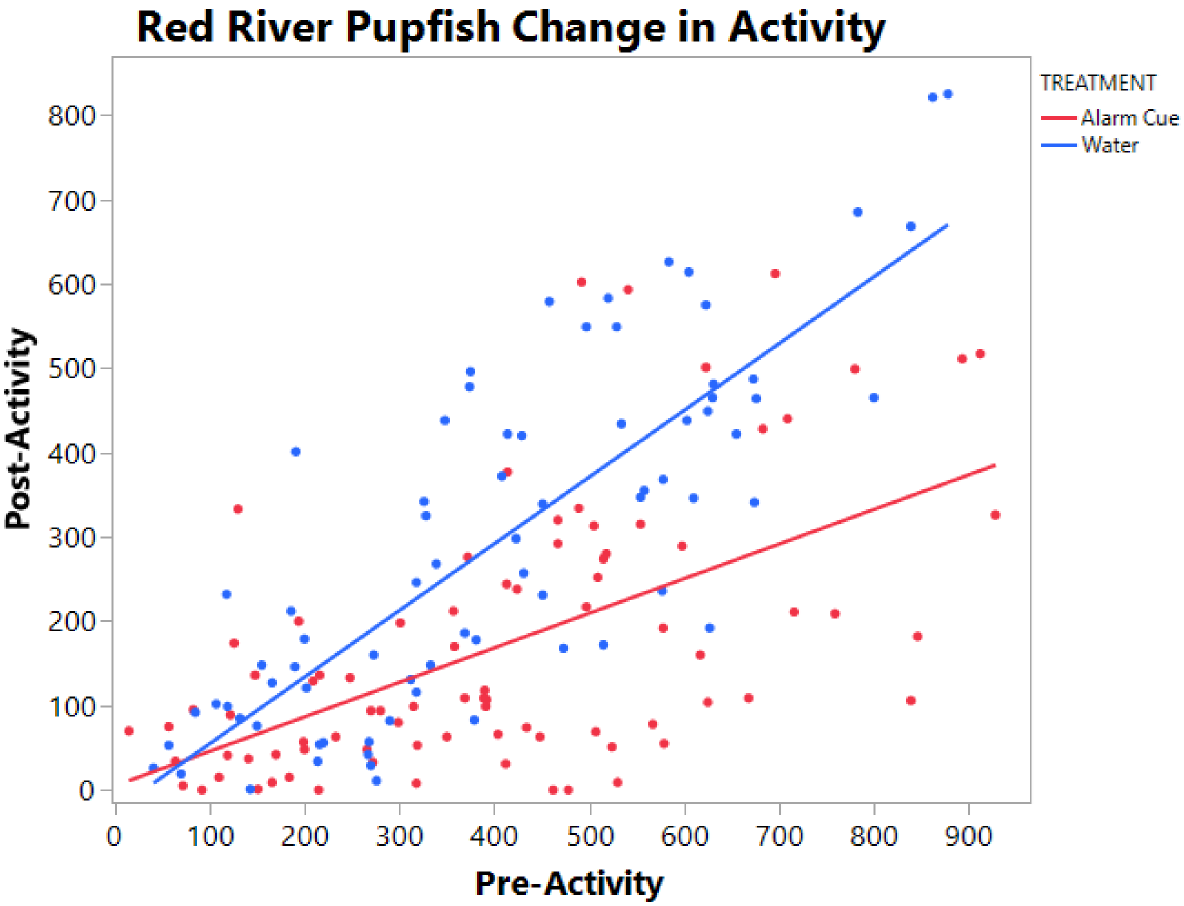

Figure 2.2: Red River Pupfish change in activity after introduction of test cue relative to activity before introduction of test cue.
Populations are combined because this predictor was not a significant term in the ANCOVA (P>0.05).

Table 2.3: Red River Pupfish change in activity level in response to conspecific alarm cue.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
POPULATION	1, 152	12821.4	0.8088	0.3699
TREATMENT	1, 152	617392.2	38.9478	**<.0001***
POPULATION*TREATMENT	1, 152	2965.2	0.1871	0.6660
PRE-ACTIVITY	1. 152	2458073.2	155.0660	**<.0001***
POPULATION*PRE-ACTIVITY	1, 152	25809.5	1.6282	0.2039
TREATMENT*PRE-ACTIVITY	1, 152	230897.9	14.5660	**0.0002***
POPULATION*TREATMENT*PRE-ACTIVITY	1, 152	56010.7	3.5334	0.0621

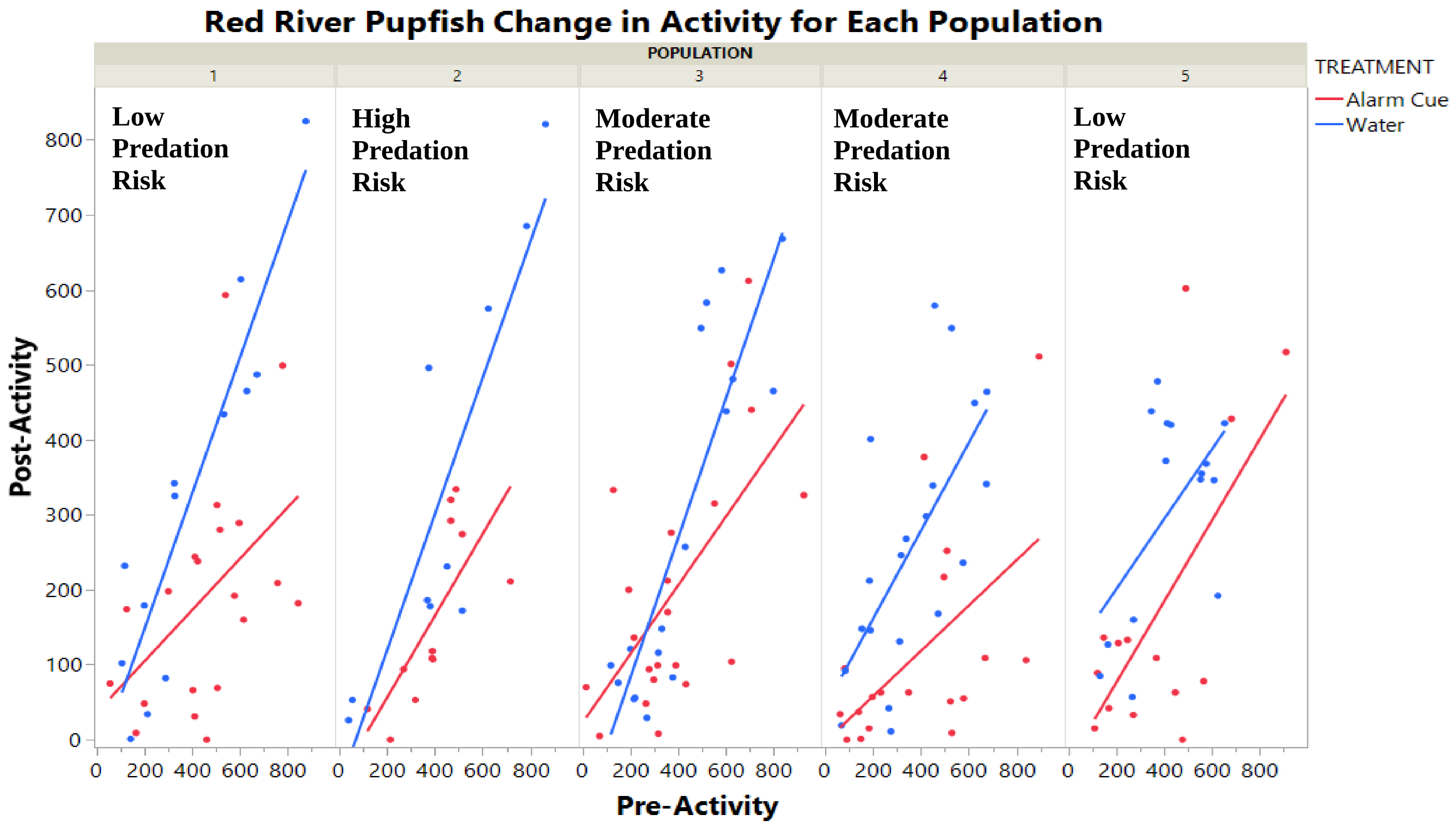

Figure 2.3: Change in activity of each population of Red River Pupfish in response to conspecific alarm cue. Populations 1 and 5 occurred in simple communities with only one other fish and thus categorized as low predation risk. Populations 3 and 4 occurred in fish communities with 2-3 other fish species but no major predators and therefore categorized as moderate predation risk. Population 2 co-occurred with numerous other species, including several known pupfish predators, and categorized as high predation risk (see Table 2.2).

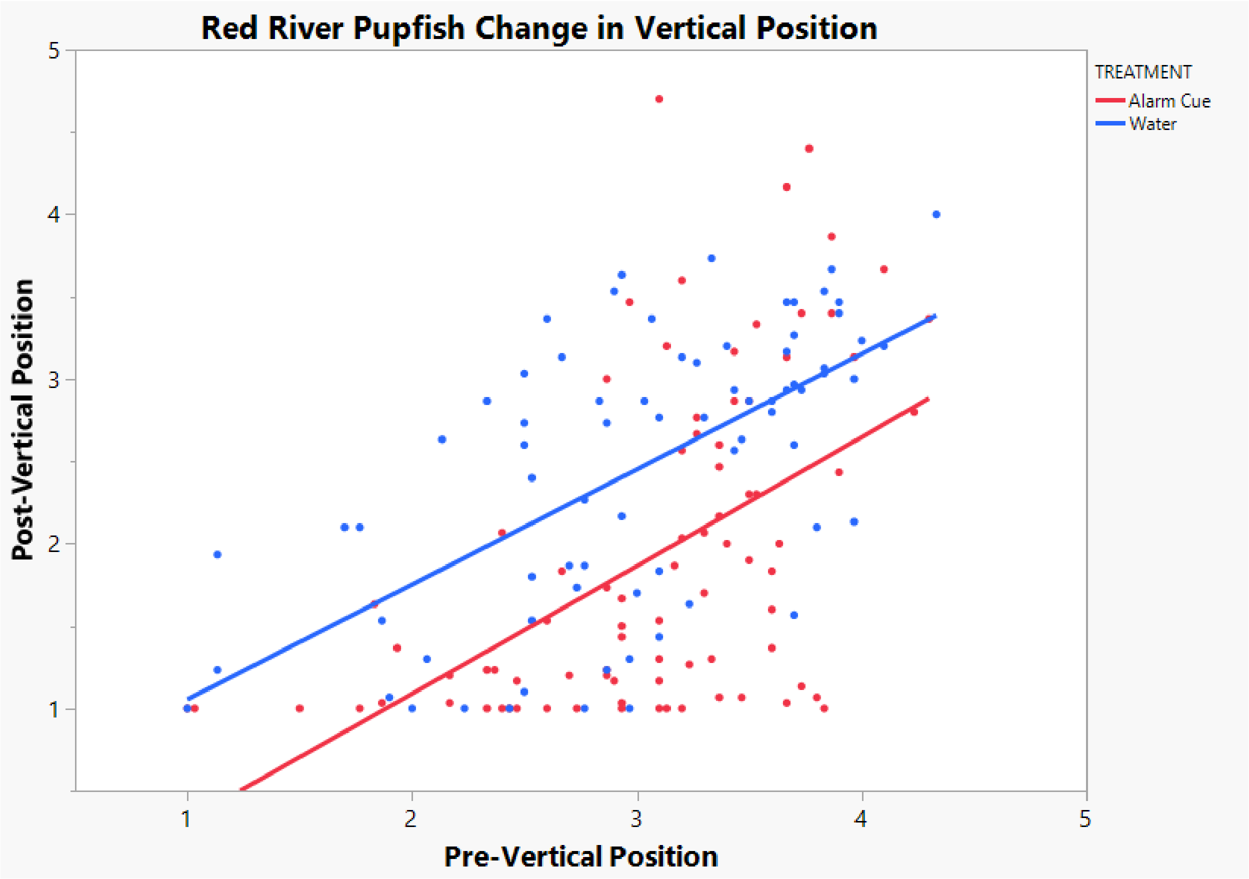

Figure 2.4: Red River Pupfish change in vertical position after introduction of test cue relative to activity before introduction of test cue.
Populations are combined because this predictor was not a significant term in the ANCOVA (P>0.05).

Table 2.4: Red River Pupfish change in vertical position in response to conspecific alarm cue.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
POPULATION	1, 152	0.006816	0.0117	0.9140
TREATMENT	1, 152	13.984056	23.9952	**<.0001***
POPULATION*TREATMENT	1, 152	0.795608	1.3652	0.2445
PRE-VERTICAL	1, 152	41.158541	70.6238	**<.0001***
POPULATION*PRE-VERTICAL	1, 152	1.287775	2.2097	0.1392
TREATMENT*PRE-VERTICAL	1, 152	0.071099	0.1220	0.7274
POPULATION*TREATMENT*PRE-VERTICAL	1, 152	0.054901	0.0942	0.7593

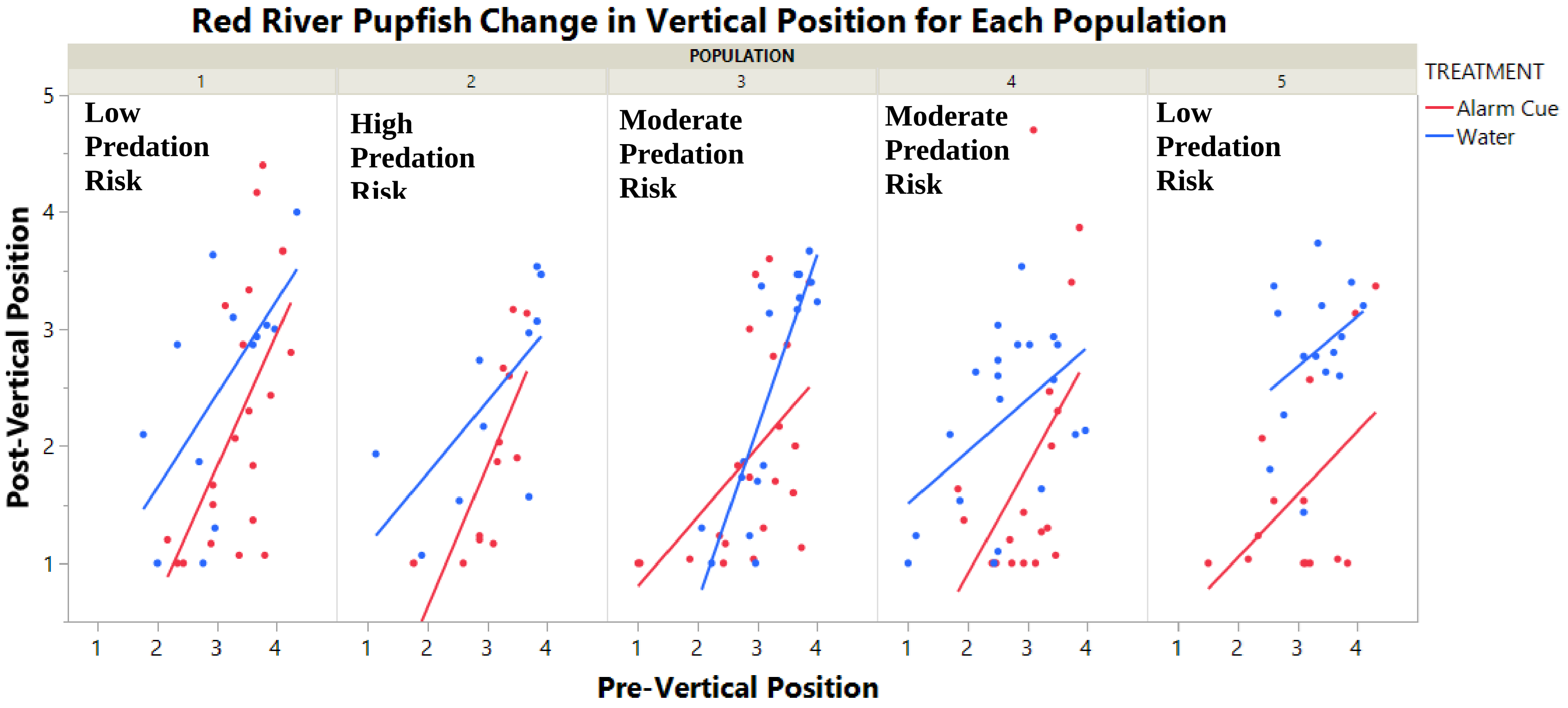

Figure 2.5: Change in vertical position of each population of Red River Pupfish in response to conspecific alarm cue. Populations 1 and 5 occurred in communities with only one other fish and thus categorized as low predation risk. Populations 3 and 4 occurred in fish communities with 2-3 other species but no major predators and therefore categorized as moderate predation risk. Population 2 co-occurred with numerous other species, including several known pupfish predators, and thus categorized as high predation risk (see Table 2.2).

2.6.2. Experiment 2: Evaluation of Amargosa River Pupfish and Shoshone Pupfish to Conspecific and Heterospecific Alarm Cues

Amargosa River Pupfish post-stimulus activity was not significantly affected by the direct or interactive effects of Pre-vertical position and Treatment (Figure 2.6; Table 2.5).
By contrast, Amargosa River Pupfish post-vertical position was significantly affected by Pre-vertical position and the interactive effect of Pre-Vertical position and Treatment (Figure 2.7; Table 2.6). Block was not significant for activity or vertical position and was not included in the final model (Tables A-23 and A-24, respectively). Post-hoc pairwise analyses detected a significant reduction in vertical position for the conspecific alarm cue treatment compared to the control (P=0.0018; Table A-25), as well as conspecific alarm cue compared to the dace cue treatment (P = 0.0062; Table A-25). However, there was no significant differences in post-stimulus vertical position between control and dace cue treatments (P=0.7391; Table A-25).

Shoshone Pupfish post-stimulus activity was significantly affected by Pre-activity and by the interactive effect of Pre-activity and Treatment (Figure 2.8; Table 2.7). Block was not significant and was not included in the final model (Tables A-26). A post-hoc pairwise analysis showed a significant change in activity for the conspecific alarm cue compared to both the control and when compared to the dace cue treatments (P=0.0063 and P=0.0124, respectively; Table A-27). However, there was no significant differences in post-stimulus activity between control and dace cue treatments (P=0.7881; Table A-27).

Shoshone Pupfish post-stimulus vertical position was significantly affected by Pre-vertical position and the interactive effects of Pre-vertical position and Treatment (Figure 2.9; Table 2.8). Block was not significant and was not included in the final model (Table A-28). A post-hoc pairwise analysis showed Shoshone pupfish had had a tendency toward a reduction in water column position in response to conspecific alarm cue and dace cue when each was compared to the control (P = 0.0379, P=0.0185, respectively; both significant after Sequential Bonferroni adjustment but with alpha = 0.10; Table A-29). However, there was no significant difference change in vertical position between conspecific alarm cue and dace cue treatments (P=0.7885; Table A-29).

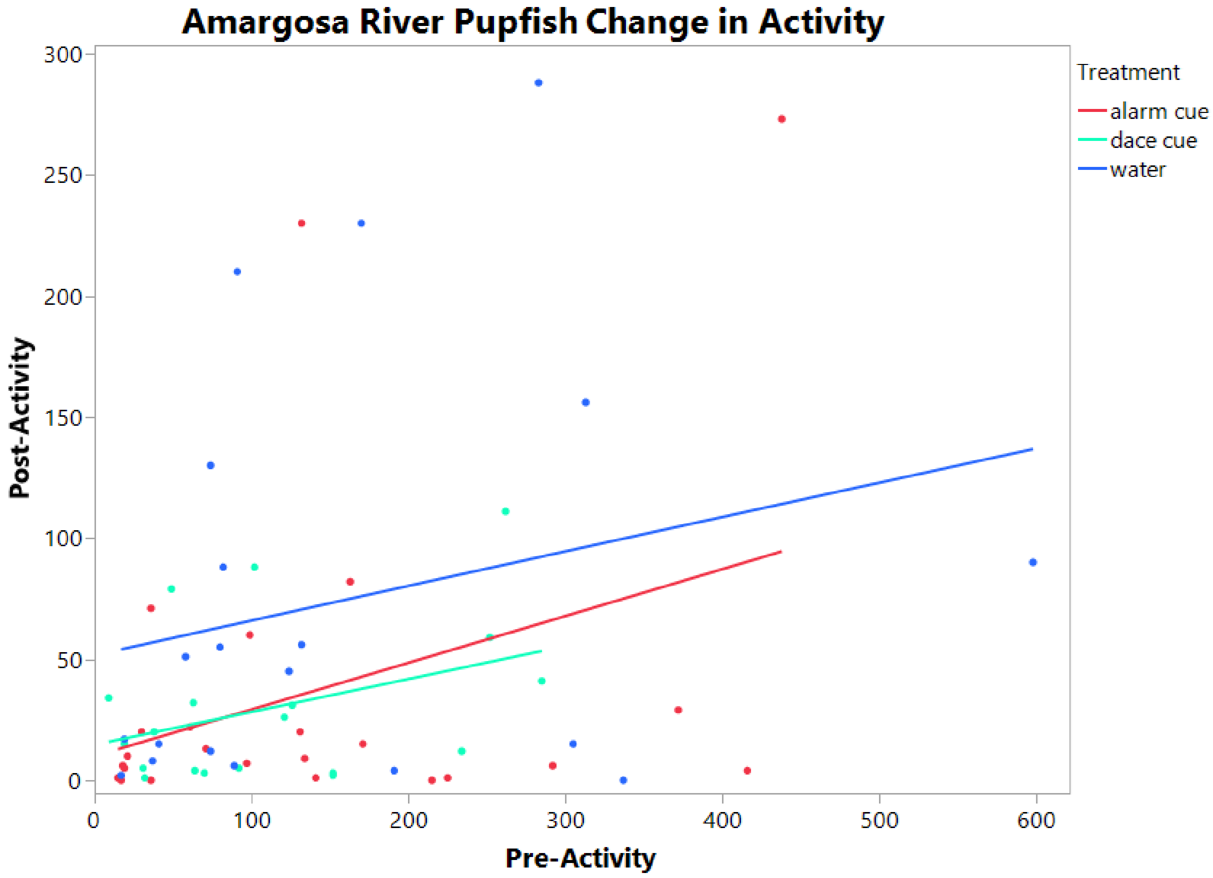

Figure 2.6: Amargosa River Pupfish change in activity in response to conspecific alarm cue, Amargosa Canyon Speckled Dace alarm cue, and water.

Table 2.5: Amargosa River Pupfish change in activity in response to conspecific alarm cue, Amargosa Canyon Speckled Dace alarm cue, and water (control).

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	2, 57	17570.225	2.0296	0.1408
PRE-ACTIVITY	1, 57	18039.731	4.1676	**0.0458***
TREATMENT*PRE-ACTIVITY	2, 57	614.726	0.0710	0.9315

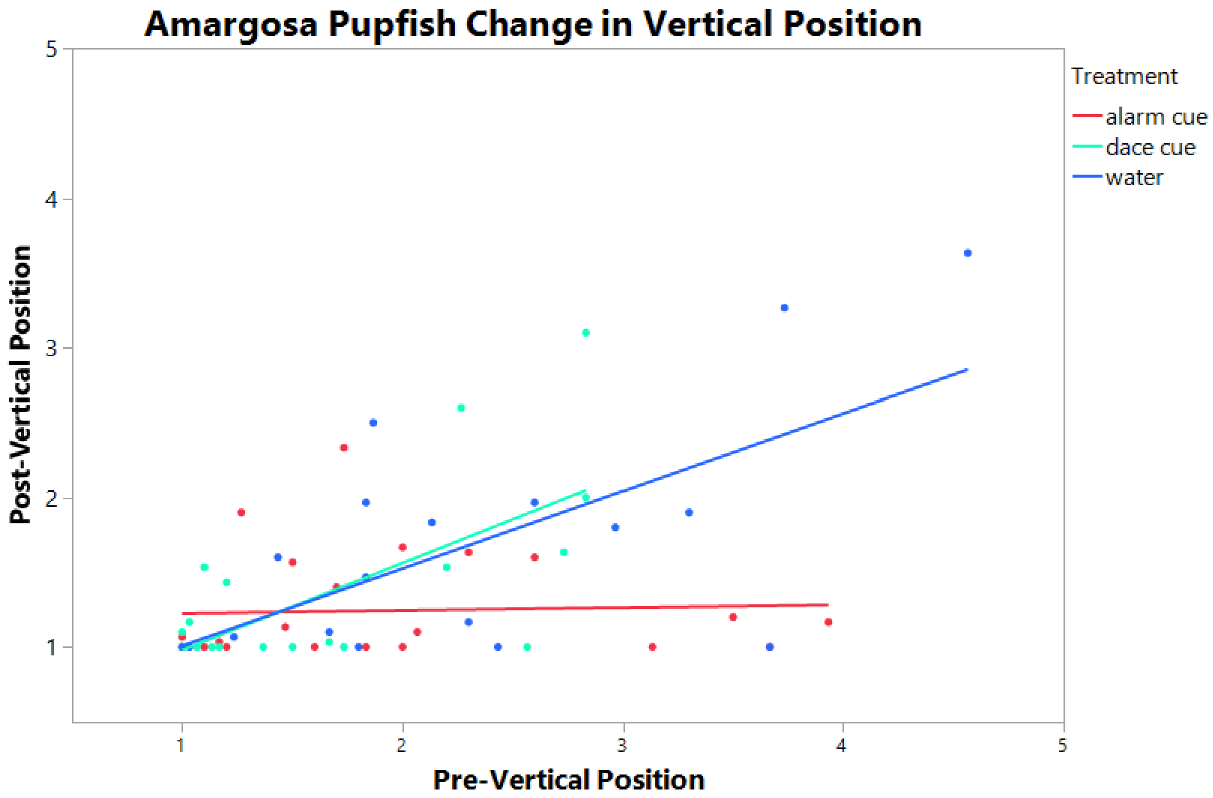

Figure 2.7: Amargosa River Pupfish change in vertical position in response to conspecific and Amargosa Canyon Speckled Dace alarm cues.

Table 2.6: Amargosa River Pupfish change in vertical position in response to conspecific and Amargosa Canyon Speckled Dace alarm cues.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	2, 57	0.9033795	2.0256	0.1413
PRE-VERTICAL	1, 57	5.5479750	24.8803	**<.0001***
TREATMENT*PRE-VERTICAL	2, 57	3.0185487	6.7684	**0.0023***

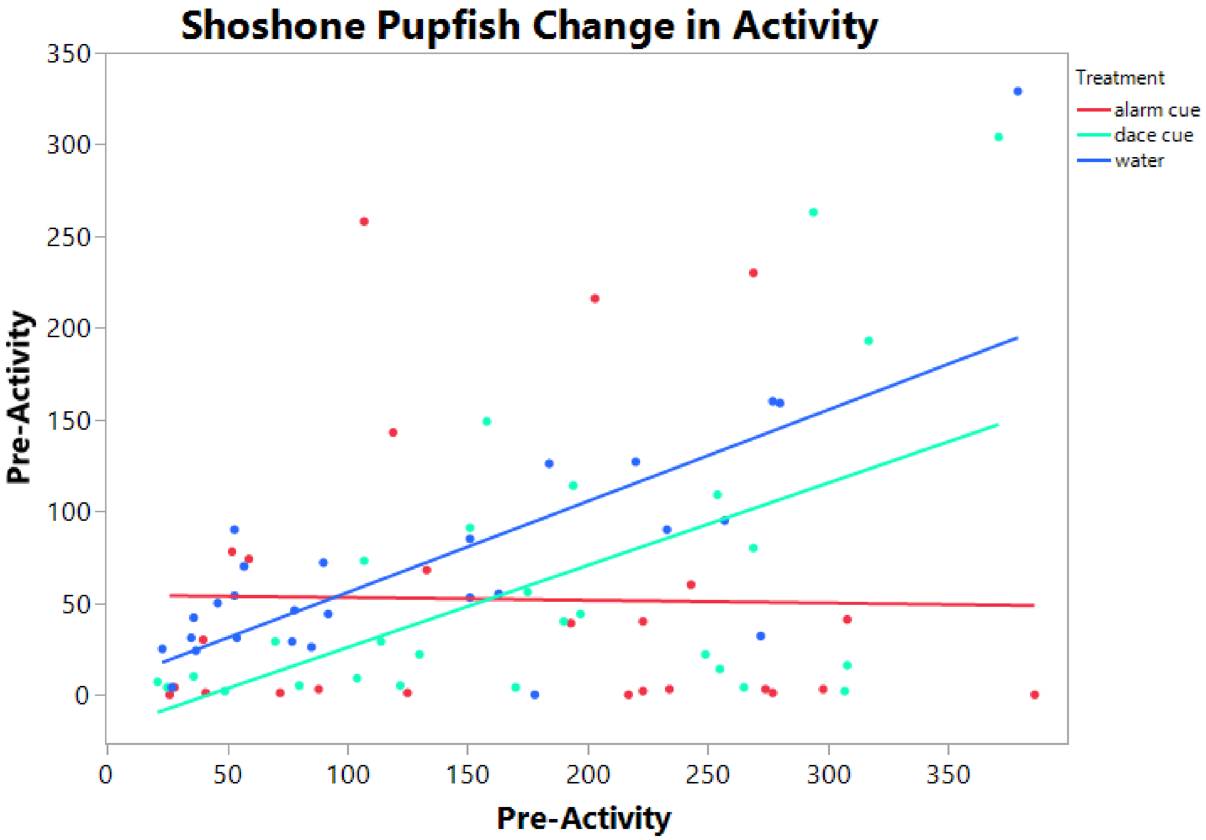

Figure 2.8: Shoshone Pupfish change in activity in response to conspecific and Amargosa Canyon Speckled Dace alarm cues.

Table 2.7: Shoshone Pupfish change in activity in response to conspecific alarm cue and Amargsoa Canyon Speckled Dace alarm cue.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	2, 74	18724.145	2.1893	0.1192
PRE-ACTIVITY	1, 74	75213.224	17.5888	**<.0001***
TREATMENT*PRE-ACTIVITY	2, 74	41441.957	4.8457	**0.0105***

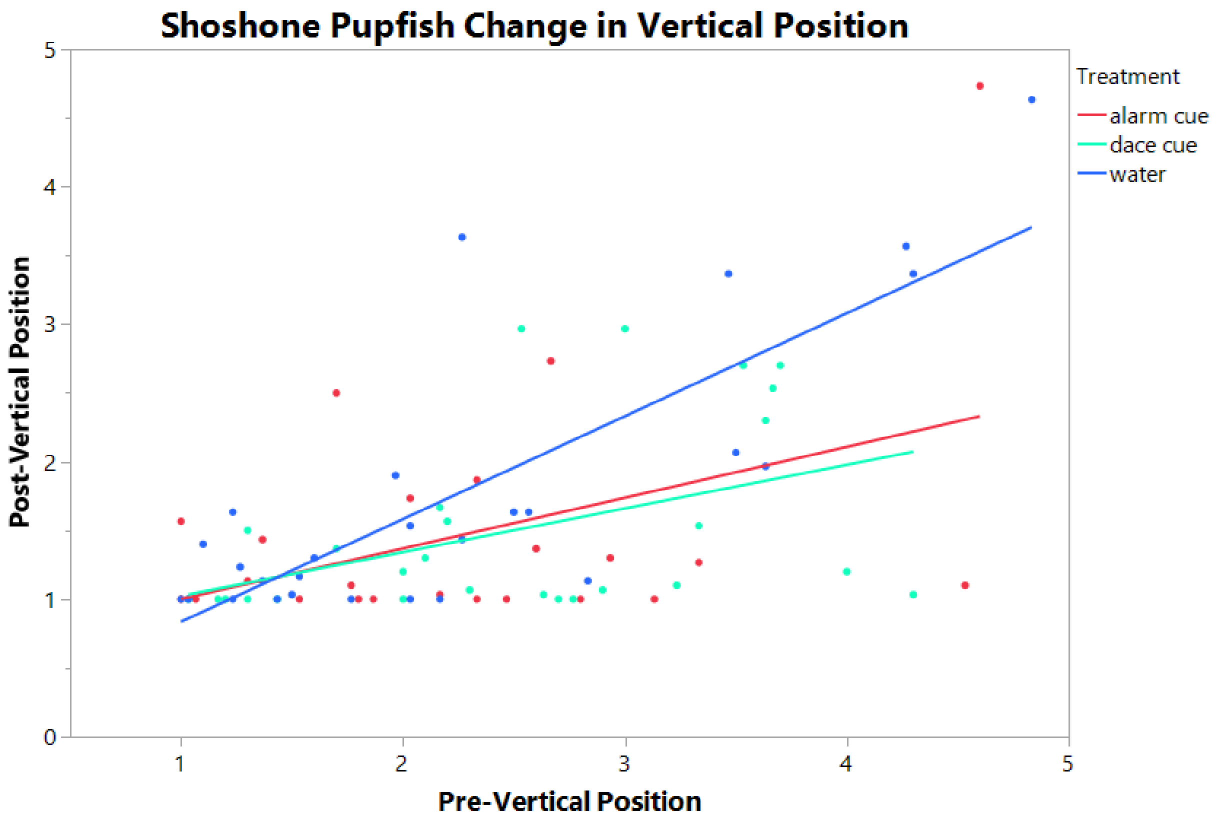

Figure 2.9: Shoshone Pupfish change in vertical in response to conspecific and Amargosa Canyon Speckled Dace alarm cues.

Table 2.8: Shoshone Pupfish change in vertical in response to conspecific alarm cue and Amargosa Canyon Speckled Dace alarm cues using Student's t test.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	2, 74	2.215940	2.5160	0.0877
PRE-VERTICAL	1, 74	18.128816	41.1673	**<.0001***
TREATMENT*PRE-VERTICAL	2, 74	3.160953	3.5890	**0.0325***

2.7. Discussion

In general, all three pupfish species showed some level of response to chemical alarm cues. Red River Pupfish significantly reduced activity and water column vertical position in response to conspecific alarm cue. However, community complexity (predation risk) was not a significant predictor for behavioral responses. Amargosa River Pupfish did not change activity levels in response to alarm cue, but this species did reduce vertical position in response to both conspecific alarm cue and when comparing conspecific alarm cue and dace cue. Amargosa Pupfish did not change vertical position in response to dace cue. Shoshone Pupfish displayed a significant change in activity in response to conspecific alarm cue and dace cue, and a significant change in vertical position in response to dace cue. However, Shoshone Pupfish did not significantly change activity in response to dace cue.

The species I tested were selected to encompass a range of community complexity and predation pressure. Red River Pupfish occur in both simple and complex communities throughout the Red River watershed, while both Amargosa Pupfish and Shoshone Pupfish occur in isolated habitats. Amargosa Pupfish co-evolved with Amargosa Canyon Speckled Dace, while Shoshone Pupfish evolved without other fish species present. The evolutionary naiveté hypothesis predicts that species or populations evolving in the absence of predators or with limited predation pressure may behave naïvely towards introduced predators (Cox and Lima 2006). Testing these species allowed for evaluation of pupfish species across a gradient of community complexity and duration of isolation from fish predators. Based on the predator naiveté hypothesis, I anticipated that Amargosa Pupfish and Shoshone Pupfish responses would be limited due to isolation in aquatic island habitats. On the other hand, I expected that Red River Pupfish would exhibit a strong response to conspecific alarm cue due to increased community complexity throughout the river system. However, I expected that Red River Pupfish populations under moderate to high predation risk would display stronger antipredator responses than populations exposed to low predation risk.

Red River Pupfish response to conspecific alarm cue was uniformly strong across all five populations for both change in activity and vertical position. There was no evidence that increased predator pressure resulted in a stronger response. These changes in behavior are consistent with antipredator responses of other fishes to conspecific alarm cue described in numerous studies (Ferrari et al. 2010a).

The findings from the Red River Pupfish experiment could result from several non-mutually exclusive factors. First, it is possible that my classification system of predation pressure did not reflect actual predation pressure. Communities defined as having low predation pressure occurred with only Plains Killifish (*Fundulus zebrinus*) in addition to Red River Pupfish. Plains Killifish may directly compete with Red River Pupfish and are possible egg predators (Echelle et al. 1972). The species found within the moderate predation risk communities coincide with species commonly co-occurring in communities with Red River Pupfish (Echelle et al. 1972). Second, fish communities may rapidly change during and after high flow events when fish are likely to move (Stoffels et al. 2016). Thus, community structure may not be as static as I inferred. Third, high flow regimes would also facilitate dispersal among populations (Stoffels et al. 2016), and the associated gene flow could limit predator-driven evolutionary divergence among populations (Hendry et al. 2007; Lenormand 2002). Fourth, other sources of predation, such as odonate larvae and birds, which I did not quantify, could influence maintenance and variation of antipredator responses. Fifth, ecological factors such as salinity may be confounded with predation pressure. However, salinity is unlikely to affect alarm cue signals because alarm reactions have been reported from both freshwater and marine systems (Ferrari et al. 2010a). Finally, variation in predator responses could be present for additional behaviors that I did not measure.

The responses to heterospecific alarm cue by Shoshone Pupfish and Amargosa Pupfish were also contrary to predictions from the predator naiveté hypothesis. I expected a stronger response by Amargosa Pupfish to dace cue because these two species co-occur and thus likely share similar predation risk. In fact, other fish species respond to heterospecific alarm cues from other similar sized prey species (Wisenden et al. 2004; Ferrari et al. 2010a). These findings suggest additional factors may mediate pupfish responses to heterospecific alarm cue. For example, dace cue may actually be a feeding cue (sensu Wisenden et al. 1999) for pupfish and could possibly explain the limited antipredator response of Amargosa River Pupfish to dace cue. It is possible that Amargosa River Pupfish have habituated to dace alarm cue released due to frequent attacks by non-native crayfish and mosquitofish that have co-persisted with Amargosa Pupfish for decades (Miller 1969). However, cichlids exposed to frequent high predation risk from different stimuli responded with decreased antipredator behaviors, indicating adaptive risk management and not habituation (Ferrari et al. 2010b).

My findings for pupfish contrast with work with another insular desert fish, Pahrump Poolfish, which did not respond to conspecific alarm cue (Stockwell et al. In Review). These three species share a similar history of evolving with limited piscivorous predation pressure since the end of the Pleistocene. However, findings reported here corroborate field reports and mesocosm experiments showing that pupfish can co-persist with non-native predators such as mosquitofish and/or crayfish (Scoppettone et al. 2011; Goodchild and Stockwell 2016; Paulson 2019; Paulson and Stockwell 2020). Thus, I argue that isolation from piscivorous fish may not be sufficient to result in evolutionary naiveté. Further, pupfishes likely have a deeper co-evolutionary history with large fish predators that occupied the Pleistocene lakes . Pupfish likely co-occurred with large fish species such as tui chub (*Siphateles bicolor ssp.*), which have been shown to prey on small-bodied fish (Henkanaththegedara and Stockwell 2013; Henkanaththegedara and Stockwell 2014).

Finally, loss of alarm cue responses may be very rare, making the Pahrump Poolfish findings an exception. Snider (2019) hypothesized that limited alarm cue responses of poolfish could be due to a weak alarm cue signal due to a low densities of club cells in poolfish epidermis (Snider 2019). Epidermal club cells are hypothesized to be a source of chemical alarm cues of fish (Ferrari et al. 2010a, Pandey et al. 2021). However, previous research has also shown that Amargosa Pupfish have both low prevalence and low densities of epidermal club cells (Snider 2019). My findings show a response of Amargosa Pupfish to alarm cue, despite reduced epidermal club cell prevalence and density. Thus, club cells may not be necessary for the production and/or detection of alarm cue or antipredator behavioral responses. Furthermore, the phylogeny presented by Stockwell et al. (In Review) showed Pahrump Poolfish to be the only species lacking a response to alarm cue among the many killifish species tested (Order Cyprinodontiformes; the order that includes the pupfishes).

This work opens the door to additional behavioral questions concerning the antipredator behavior of pupfish. For instance, while this work focused on the presence of piscivorous fish species, I did not examine the effects of aquatic insects or terrestrial predators on fish behavior. Such predation pressure may be sufficient to maintain generalized antipredator behaviors such as alarm cue responses. This work also focused on adult pupfish. Additional work evaluating antipredator responses by juveniles may provide insights to the findings of Goodchild and Stockwell (2016) and the general wide-spread impact of non-native predators on desert fishes. In fact, some non-native predators, such as mosquitofish that commonly infiltrate desert ecosystems, prey primarily on juvenile fish (Henkanaththegedara and Stockwell 2013; Henkanaththegedara and Stockwell 2014; Goodchild and Stockwell 2016). Thus, future research should focus on identification of additional sources of predation pressure that could contribute to retention of antipredator responses in isolated fish species.

Although other desert fish may be evolutionarily naïve, behavioral responses to alarm cues appears to be conserved in pupfishes even after prolonged periods of isolation in simple communities with relaxed selection from fish predators. Experimental work showed pupfish can co-persist in sympatry with non-native predators in mesocosms (Goodchild and Stockwell 2016; Paulson 2019; Paulson and Stockwell 2020), while field observations also verify that pupfish co-persist with introduced predators (Miller et al.1969; Scoppettone et al. 2011). These findings indicate that pupfish may be effectively managed in multi-species habitats.

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APPENDIX

Table A-1: Effects test results for Red River Pupfish change in activity with block included.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
BLOCK	23	375161.2	1.0343	0.4286
POPULATION	1	6160.1	0.3906	0.5331
TREATMENT	1	626389.0	39.7207	**<.0001***
POPULATION*TREATMENT	1	4105.2	0.2603	0.6108
PRE-ACTIVITY	1	1884532.9	119.5023	**<.0001***
POPULATION* PRE-ACTIVITY	1	19503.9	1.2368	0.2682
TREATMENT* PRE-ACTIVITY	1	279503.4	17.7239	**<.0001***
POPULATION*TREATMENT* PRE-ACTIVITY	1	71003.9	4.5025	**0.0358***

Table A-2: Effects test results for Red River Pupfish change in vertical position with block included.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
BLOCK	23	13.614882	1.0186	0.4475
POPULATION	1	0.000261	0.0004	0.9831
TREATMENT	1	13.239954	22.7823	**<.0001***
POPULATION*TREATMENT	1	0.364203	0.6267	0.4300
PRE-VERTICAL	1	33.702753	57.9931	**<.0001***
POPULATION*PRE-VERTICAL	1	0.454336	0.7818	0.3782
TREATMENT* PRE-VERTICAL	1	0.035007	0.0602	0.8065
POPULATION*TREATMENT* PRE-VERTICAL	1	0.029898	0.0514	0.8209

Table A-3: Analysis of variance results for Red River Pupfish Population 1 change in activity level.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	829824.2	276608	17.2579
Error	29	464810.1	16028	Prob > F
C. Total	32	1294634.2		**<.0001***

Table A-4: Effects test results for Red River Pupfish Population 1 change in activity level.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	1, 31	229795.61	14.3372	**0.0007***
PRE-ACTIVITY	1, 31	623454.37	38.8980	**<.0001***
TREATMENT*PRE-ACTIVITY	1, 31	127167.81	7.9341	**0.0086***

Table A-5: Analysis of variance results for Red River Pupfish Population 2 change in activity level.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	799546.9	266516	20.9701
Error	18	228768.2	12709	Prob > F
C. Total	21	1028315.1		**<.0001***

Table A-6: Effects test results for Red River Pupfish Population 2 change in activity level.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	1, 20	108464.44	8.5342	**0.0091***
PRE-ACTIVITY	1, 20	403741.14	31.7673	**<.0001***
TREATMENT*PRE-ACTIVITY	1, 20	25143.18	1.9783	0.1766

Table A-7: Analysis of variance results for Red River Pupfish Population 3 change in activity level.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	958025.1	319342	19.6386
Error	34	552872.7	16261	Prob > F
C. Total	37	1510897.7		**<.0001***

Table A-8: Effects Test Results for Red River Pupfish Population 3 change in activity level.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	1, 36	37661.93	2.3161	0.1373
PRE-ACTIVITY	1, 36	858281.41	52.7817	**<.0001***
TREATMENT*PRE-ACTIVITY	1, 36	99315.60	6.1076	**0.0186***

Table A-9: Analysis of variance results for Red River Pupfish Population 4 change in activity level.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	537460.0	179153	11.4066
Error	34	534008.9	15706	Prob > F
C. Total	37	1071468.9		**<.0001***

Table A-10: Effects test results for Red River Pupfish Population 4 change in activity level.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	1, 36	220469.50	14.0372	**0.0007***
PRE-ACTIVITY	1, 36	342065.34	21.7791	**<.0001***
TREATMENT*PRE-ACTIVITY	1, 36	34864.31	2.2198	0.1455

Table A-11: Analysis of variance results for Red River Pupfish Population 5 change in activity level.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	433230.42	144410	7.5928
Error	25	475486.27	19019	Prob > F
C. Total	28	908716.69		**0.0009***

Table A-12: Effects test results for Red River Pupfish Population 5 change in activity level.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	1, 27	85571.00	4.4991	0.0440*
PRE-ACTIVITY	1, 27	259081.40	13.6219	0.0011*
TREATMENT*PRE-ACTIVITY	1, 27	1421.35	0.0747	0.7868

Table A-13: Analysis of variance results for Red River Pupfish Population 1 change in vertical position.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	13.392604	4.46420	5.9116
Error	29	21.899517	0.75516	Prob > F
C. Total	32	35.292121		**0.0028***

Table A-14: Effects test results for Red River Pupfish Population 1 change in vertical position.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	1, 31	2.214825	2.9329	0.0975
PRE-VERTICAL	1, 31	12.528250	16.5903	**0.0003***
TREATMENT*PRE-VERTICAL	1, 31	0.377912	0.5004	0.4850

Table A-15: Analysis of variance results for Red River Pupfish Population 2 change in vertical position.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	8.511753	2.83725	7.4000
Error	18	6.901378	0.38341	Prob > F
C. Total	21	15.413131		**0.0020***

Table A-16: Effects test results for Red River Pupfish Population 2 change in vertical position.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	1, 20	1.4680148	3.8288	0.0661
PRE-VERTICAL	1, 20	6.9811841	18.2081	**0.0005***
TREATMENT*PRE-VERTICAL	1, 20	0.7398645	1.9297	0.1817

Table A-17: Analysis of variance results for Red River Pupfish Population 3 change in vertical position.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	19.100135	6.36671	12.9340
Error	34	16.736365	0.49225	Prob > F
C. Total	37	35.836500		**<.0001***

Table A-18: Effects test results for Red River Pupfish Population 3 change in vertical position.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	1, 36	0.274572	0.5578	0.4603
PRE-VERTICAL	1, 36	16.198146	32.9066	**<.0001***
TREATMENT*PRE-VERTICAL	1, 36	2.959903	6.0131	**0.0195***

Table A-19: Analysis of variance results for Red River Pupfish Population 4 change in vertical position.

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	8.797948	2.93265	4.0435
Error	34	24.659245	0.72527	Prob > F
C. Total	37	33.457193		**0.0146***

Table A-20: Effects test results for Red River Pupfish Population 4 change in vertical position.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	1, 36	3.7968233	5.2350	**0.0285***
PRE-VERTICAL	1, 36	7.1935292	9.9184	**0.0034***
TREATMENT*PRE-VERTICAL	1, 36	0.8736509	1.2046	0.2801

Table A-21: Analysis of variance results for Red River Pupfish Population 5 change in vertical position

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Ratio**
Model	3	13.210469	4.40349	9.5196
Error	25	11.564326	0.46257	Prob > F
C. Total	28	24.774795		**0.0002***

Table A-22: Effects test results for Red River Pupfish Population 5 change in vertical position.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
TREATMENT	1, 27	7.9589184	17.2058	**0.0003***
PRE-VERTICAL	1, 27	2.1799869	4.7127	**0.0396***
TREATMENT*PRE-VERTICAL	1, 27	0.0277549	0.0600	0.8085

Table A-23: Effects test for Amargosa River Pupfish change in activity with block included.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Block	36	141977.82	0.7906	0.7387
Treatment	2	3990.80	0.4000	0.6753
Pre_Activity	1	1112.04	0.2229	0.6417
Treatment*Pre-Activity	2	4315.45	0.4326	0.6545

Table A-24: Effects test for Amargosa River Pupfish change in vertical position with block included.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Block	36	7.0618067	0.7293	0.8022
Treatment	2	0.3146927	0.5850	0.5659
Pre-Vertical	1	0.1912447	0.7110	0.4086
Treatment*Pre-Vertical	2	0.4649990	0.8644	0.4358

Table A-25: Pairwise post-hoc analyses among the three treatments are shown for the slope comparisons of pre-stimulus vs. post-stimulus vertical position in Amargosa Pupfish.

Treatment * PreVert Slope Comparisons	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Control vs. Alarm Cue	-0.498522	0.152132	-3.28	0.0018**
Control vs. Dace Cue	0.0655119	0.195744	0.33	0.7391
Alarm Cue vs. Dace Cue	0.5640336	0.198283	2.84	0.0062**

** Significant after sequential Bonferroni correction with experimental wise alpha set at 0.05.

Table A-26: Effects test for Shoshone Pupfish change in activity with block included.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Block	30	138849.97	1.1467	0.3340
Treatment	2	14600.24	1.8087	0.1758
Pre-Activity	1	4539.79	1.1248	0.2947
Treatment*Pre-Activity	2	41185.74	5.1022	**0.0102***

Table A-27: Pairwise post-hoc analyses among the three treatments are shown for the slope comparisons of pre-stimulus vs. post-stimulus activity in Shoshone Pupfish

Treatment * Pre-Activity Slope Comparisons	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Control vs. Alarm Cue	-0.511645	0.181853	-2.81	0.0063*
Control vs. Dace Cue	-0.048848	0.181112	-0.27	0.7881
Alarm Cue vs. Dace Cue	0.4627971	0.180627	2.56	0.0124*

** Significant after sequential Bonferroni correction with experimental wise alpha set at 0.05.

Table A-28: Effects test for Shoshone Pupfish change in vertical position with block included.

Source	**DF**	**Sum of Squares**	**F Ratio**	**Prob > F**
Block	30	13.648905	1.0570	0.4262
Treatment	2	2.298249	2.6698	0.0805
Pre-Vertical	1	9.620900	22.3524	**<.0001***
Treatment* Pre-Vertical	2	3.228609	3.7505	**0.0313***

Table A-29: Pairwise post-hoc analyses among the three treatments are shown for the slope comparisons of pre-stimulus vs. post-stimulus vertical position in Shoshone Pupfish.

Treatment * PreVertical Slope Comparisons	**Estimate**	**Std Error**	**t Ratio**	**Prob>|t|**
Control vs. Alarm Cue	0.3795417	0.179585	2.11	0.0379*
Control vs. Dace Cue	-0.43058	0.178749	-2.41	0.0185*
Alarm Cue vs. Dace Cue	-0.051038	0.189578	-0.27	0.7885

** Significant after sequential Bonferroni correction with experimental wise alpha set at 0.10.

Figure A-1: Red River Pupfish (*Cyprinodon rubrofluviatilis*) female.
Photo Credit: Cody Anderson

Figure A-2: Amargosa River Pupfish (*Cyprinodon nevadensis amargosae*) female.
Photo Credit: Cody Anderson

Figure A-3: Shoshone Pupfish (*Cyprinodon nevadensis shoshone*) female.
Photo Credit: Cody Anderson

Figure A-4: Amargosa Canyon Speckled Dace (*Rhinichthys osculus spp.*).
Photo Credit: Cody Anderson