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**EFFECTS OF STRESSORS ON THE DEVELOPMENT AND RESILIENCE OF
ADULT FISH BEHAVIOR**

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Biology

by

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ABSTRACT

Stressful experiences, whether encountered at certain stages of development or throughout life, can have a profound impact on an animal's future behavior. How these stressors affect the animal can be influenced by the future environments an animal finds itself in. If an animal experiences an environment that matches its stressful rearing environment, the animal may have an advantage because it is already physiologically and behaviorally adapted to these conditions. On the other hand, a mismatched environment (one without stressors) may put a developmentally stressed individual at a disadvantage. This concept, known as the match/mismatch hypothesis, has recently been investigated in mammals, but little is known about these kinds of processes in fish. With their relatively simpler nervous system, and the ability to study populations in the field and in captivity, fish could be an interesting taxonomic group in which to investigate the effects of the match/mismatch phenomenon. My dissertation investigates how a fish's developmental and evolutionary ecology affect its future temperament and coping style in different environmental contexts.

First, I investigated the effect of a brief exposure to stress in early-life on future, adult exploration behavior in guppies (*Poecilia reticulata*). I found that fish descended from a wild-caught Trinidad population behaved differently to fish bred from a domesticated population. Exposing guppies to stressors during development changed how the fish explored a novel environment as adults, and their behaviors varied depending on whether they were tested in a threatening, or a non-threatening context. Together, these results demonstrate that adult behavior can be influenced both by an animal's background (descended from a wild or a domesticated strain) as well as its early life experience.

Following on from this, I conducted a field project with wild Panamanian bishop fish (*Brachyrhaphis episcopi*) to examine context-dependent stress-related behavioral responses.

Using populations that varied in how much experience they had with predation throughout life, I investigated anxiety-related behaviors in bishop fish from high and low predation sites. The fish were tested in a novel tank diving test before or after exposure to an acute confinement stressor. I found that low predation fish behaved more anxiously compared to high predation fish and that this was consistent after exposure to the acute stressor. In a second study, I examined how varying levels of predation exposure affected exploration behavior in terms of how bishop fish responded towards two different kinds of object. Depending on stream origin, populations were found to differ in how they explored a novel arena and objects within it. Together, these results highlight how local ecology and contrasting experiences of predation threat promote different levels of anxiety, and they also affect how the fish respond to novelty.

In a final study, I investigated how the appraisal of specific stimuli influences zebrafish (*Danio reio*) behavior. Here, I tested whether the fish could learn to discriminate between two humans and associate them with either a positive or a negative experience. I found that zebrafish not only distinguished between two different people, but they were also able to correctly assign a particular role to each person. These results suggest that giving human caretakers specific roles in fish research laboratories could help the fish prepare for different kinds of experience, e.g. feeding versus handling.

My dissertation illustrates how factors, such as evolutionary background and stress exposure affect future adult behavior and stress-coping responses. Moreover, the context in which a fish finds itself; i.e., safe or threatening, rewarding or aversive, can alter how it responds based on its previous experience. My results also have implications for the welfare of captive populations, and indicate that the captive environment can be made more predictable by giving individual caretakers specific husbandry roles.

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“For he who has once seen the intimate beauty of nature cannot tear himself away from it again. He must become either a poet or a naturalist and if his eyes are good and his powers of observation sharp enough, he may well become both.”

— **Konrad Lorenz**
King Solomon's Ring

Lorenz, K. (1952). King Solomon's Ring. New York: Crowell.

Chapter 1

Introduction: Behavior and Stressors, Factors Affecting the Stress Response, and Research Objectives Addressed in this Thesis

Many of the experiences an animal has during different stages of development go on to influence how the animal behaves as an adult, and this is the case whether the animal is in a captive or a natural environment. For example, juvenile, captive cod (*Gadus morhua*) exposed to physical enrichment (i.e., objects placed inside their holding tank that they could interact with and manipulate) developed more complex behavior compared to fish reared without enrichment (Salvanes *et al.* 2007). Cod that experienced tank enrichment tightly shoaled when exposed to an open test tank, but split up and hid amongst objects when provided with a more complex environment. Fish only exposed to a barren environment during early life; however, did not modify their behavior between the open, or complex testing environments schooling tightly under both conditions (Salvanes *et al.* 2007). Cod with enrichment experience, however, were better at adapting their schooling responses to fit the test environment more appropriately, i.e., schooling tightly when in an open, exposed space, or moving apart and using the landscape for shelter in a more complex environment.

Experiencing social stimuli during development also affects future adult behavior (De Medeiros *et al.* 2012, Caruso *et al.* 2014). For example, male rodents raised with mostly female siblings behaved differently when engaging in reproductive behavior as adults, compared to males raised with primarily male siblings (De Medeiros *et al.* 2012). Specifically, males reared with more sisters engaged in less reproductive behavior (e.g., fewer female mounts) compared to males raised with more brothers.

Experiencing unpredictable, or stressful situations during early life has also been found to result in long-lasting changes in behavior. The term “stress,” is a widely used and broadly defined term, so it needs to be carefully defined with regard to the context or situation where it occurs. In the broadest of definitions, Hans Selye (for many the father of stress research) considered stress to be "the non-specific response of the body to any demand upon it" (Barton 2002, Szabo *et al.* 2012). Today, however, stress is thought of as a multi-dimensional concept involving a challenging stimulus (a stressor) that an organism is exposed to. In response to this challenge, behavioral and physiological processes are initiated within the body (the stress response) that help an organism better cope and survive (Barton 2002, Oliveira and Galhardo 2009, Koolhaas *et al.* 2011, Trompeter and Langkilde 2011, Hau *et al.* 2016). When an organism is unable to cope with the stressor, however, it enters a state of "distress," where it is more vulnerable to harmful consequences, such as cardiovascular disease, memory loss, immune system suppression, and insulin resistance (Barton 2002, McEwen 2005, Hau *et al.* 2016). Animals will, as part of their natural life, face times when they are challenged. Sometimes, these situations are relatively short-lived; for instance, animals may directly compete with one another for access to a limited resource, but in other situations, the stressor may operate over a more extended timescale, for example, a prey animal living sympatrically with many predator species.

The way in which an organism responds and copes with a stressor is further affected by a number of additional factors, such as how an animal appraises a stressor (e.g., perceiving an event to be unpredictable or predictable), an animal's temperament (e.g., being risk prone or risk averse) and its overall coping style (e.g., being risk-averse *and* having a highly reactive "fight or flight" response). An animal's population history (e.g., domesticated versus wild), and whether it experienced stressful contexts in early life can also influence the coping process (Koolhaas *et al.* 1999, Réale *et al.* 2007, Oliveira and Galhardo 2009, Archard and Braithwaite 2010, Hau *et al.* 2016).

Traditionally, it was proposed that stress during early life often results in maladaptive consequences in later life (Hau *et al.* 2016, Vindas *et al.* 2017). However, recent evidence suggests that the context of an animal's future environment affects the role that an animal's past experiences play. If an organism's future environment is challenging, and their prior rearing environment was stressful, then they cope better compared to individuals that have a relatively stress-free developmental period, but then find themselves in unpredictable, challenging environments as adults (Schmidt 2011, Chaby *et al.* 2015a, Hau *et al.* 2016). For example, Atlantic salmon (*Salmo salar*) exposed to a period of mild stress during early life expressed a physiological response that allowed better coping when exposed to acute stressors in later life, compared to control fish that were kept in constant conditions (Vindas *et al.* 2017). Salmon stressed during development also had a reduced “fight or flight” stress response, and produced fewer stress-related hormones compared to control fish. A reduced stress response could be advantageous as it could help the fish to cope with environmental challenges, and avoid reaching a state of “distress,” and the harmful effects that repeated activation of the stress response can trigger (Archard *et al.* 2012, Hau *et al.* 2016). The context of an animal’s past environment, therefore, can influence how an animal adjusts its behavior and physiology to cope with stress in the future.

In this dissertation, I have chosen to investigate how different factors such as the appraisal of a stressor, temperament, coping style, population history, timing of prior stress exposure, and context of the future environment (i.e., non-stressful vs. stressful) influence how an animal adjusts its future behavior in response to adverse challenges. To understand how these different kinds of factors affect behavior, it is necessary to describe the role of both short- and long-term physiological stress responses. These processes underlie the observable behavioral phenotype, and understanding how these two stress responses operate is essential for recognizing what an organism undergoes when a stressor challenges it.

Homeostasis, Allostasis, and The Vertebrate Stress Response

Homeostasis refers to the internal state of an organism where its underlying physiological parameters, e.g., pH, temperature, are at the ideal "set points" for the body to function properly and efficiently (McEwen and Wingfield 2010, Koolhaas *et al.* 2011, Hau *et al.* 2016). When an organism faces a stressor, this results in a disruption in homeostasis causing the organism to switch into "survival mode" (Wingfield *et al.* 1998, Hau *et al.* 2016). This switch results in the body adjusting different processes, such as increasing its heart rate and blood pressure to help it prepare to fight or flee from the situation, and also to help it cope with the stressful experience in general (McEwen and Wingfield 2010, Monaghan and Hausmann 2015, Hau *et al.* 2016). This "survival mode" is known as the "emergency life history stage" of an organism, where processes such as reproduction and glucose storage are deterred, and the body's energy is focused on other processes that immediately benefit survival (Wingfield *et al.* 1998, Monaghan and Hausmann 2015, Hau *et al.* 2016).

The stressor itself can be considered to be an uncontrollable and unpredictable stimulus, where an organism perceives that it has no (or limited) control (Oliveira and Galhardo 2009, Koolhaas *et al.* 2011). It is also important to note that stressors can vary in their duration; where acute stress is short-lived, chronic stress is associated with repeated or ongoing exposure to challenges (Brachetta *et al.* 2014). The field of stress-related research has traditionally utilized rodent models to investigate the effects of stress exposure (Champagne *et al.* 2010). This research has found that in mammals the stress response is composed of two neuroendocrine pathways. The Sympathetic-Adrenal-Medullary (SAM) is most active in response to acute stress experiences, and the Hypothalamic-Pituitary-Adrenal (HPA) axis is more involved with the response to chronic stress (Barton 2002, Hau *et al.* 2016).

In the last two decades, zebrafishes (*Danio rerio*) have become a popular model to address stress-related research questions (Guo 2004, Egan *et al.* 2009, Champagne *et al.* 2010). Many species of small fishes have shorter developmental stages, faster generation times, and a higher reproductive output compared to rodents that help to promote studies addressing the effects of early rearing environments (Guo 2004, Egan *et al.* 2009). For a number of fish species, research questions can also be investigated in both an animal's natural field setting as well as in more artificial, captive, laboratory environments. Examining animals in both natural as well as captive environments can inform research with regards to biologically and ecologically meaningful stressors to help focus research questions around relevant factors (Archard and Braithwaite 2010). In this dissertation, I focus on small, freshwater fish species and study these in either the controlled environment of the laboratory, in more natural field conditions, or in both. Although there are several similarities between the stress response in fishes and mammals, there are some key differences. I discuss some of these in the following section.

In fishes, the equivalent of the mammalian SAM response is the HSC (Hypothalamus-Sympathetic-Chromaffin) response (Barton 2002, Oliveira and Galhardo 2009). Unlike mammals, fish do not have an adrenal gland, but rather the anterior kidney produces catecholamines that induce the “fight or flight” response (Barton 2002, Oliveira and Galhardo 2009, Champagne *et al.* 2010). Both the SAM and HSC pathways produce catecholamines (epinephrine and norepinephrine) which promote increases in heart rate, respiratory rate, the suppression of the parasympathetic nervous system (“rest and digest”), as well as other related reactions (Oliveira and Galhardo 2009). The equivalent of the mammalian HPA response in fish is the Hypothalamic-Pituitary-Interrenal (HPI) axis (Oliveira and Galhardo 2009). The end product of both processes is a glucocorticoid; cortisol, in humans and fishes, or corticosterone in rodents, birds, reptiles and amphibians (Egan *et al.* 2009, Oliveira and Galhardo 2009, Champagne *et al.* 2010, Piato *et al.* 2011, Trompeter and Langkilde 2011, Hau *et al.* 2016). Glucocorticoids have a

variety of different functions, such as the mobilization of sugars and lipids into the bloodstream to supply energy to various sites throughout the body (Hau *et al.* 2016).

In addition to coping and surviving when facing a stressor, the body also tries to return to a less energetically costly state, or back to its homeostatic state (Barton 2002, McEwen 2005, Hau *et al.* 2016). When the body moves away from homeostasis it enters a state of allostasis where several adaptive processes work together, e.g., cytokines of the immune response fight off pathogens and glucocorticoids of the stress response act as an inhibitor to this, helping the body to return to a homeostatic state. However, if the body is unable to return to a homeostatic state and continues to be in a state of heightened stress, then it becomes victim to “allostatic overload” and enters a state of “distress.” When the body is in a state of "distress," it becomes more vulnerable to the adverse effects of long-term glucocorticoid circulation, e.g., neuron degradation resulting in memory loss, and muscle depletion that can lead to heart disease.

Appraisal of a Stressor

One way to mitigate the effects of a stressor, and thus avoid allostatic overload, is to increase a stressor’s predictability. When an animal can predict the onset of a stressor, they can better prepare behaviorally and physiologically for the impending threat (Bassett and Buchanan-Smith 2007, Oliveira and Galhardo 2009, Galhardo *et al.* 2011). By reducing a stressor's unpredictability, this reduces the overall impact of the stressor on the animal. This process of appraising whether a stimulus is a true threat has been well studied in humans and other mammals, and recently identified in fishes as well (Oliveira and Galhardo 2009, Galhardo *et al.* 2011).

Galhardo (2011), for example, examined the behavior and physiology between groups of a cichlid species (*Oreochromis mossambicus*) that were either exposed to a predictable or an unpredictable aversive event (Galhardo *et al.* 2011). Cichlids in the predictable treatment group were consistently shown a visual cue card before the onset of a confinement stressor, while the unpredictable group was exposed to the cue card randomly before or after the event. It was found that fish exposed to the predictable cue engaged in less freezing behavior during the cue's presentation, and produced less cortisol after the confinement stressor, compared to the unpredictable treatment group. Cichlids that were provided with a predictable cue, therefore, were more prepared when facing a threat which generated an attenuated behavioral and physiological stress response. This reduced response is presumably beneficial in that it allows the fishes to refocus their attention and energy on other tasks, such as foraging or reproduction (Archard *et al.* 2012). Furthermore, a diminished stress response would also decrease the likelihood that the fishes would enter a state of allostatic overload (Hau *et al.* 2016).

Similar results were also found in another study where researchers tested whether rainbow trout (*Oncorhynchus mykiss*) could prepare for a stressful event by learning that a neutral stimulus (switching off tank water flow) warned of an upcoming aversive stimulus, i.e., the presentation of a large and aggressive conspecific (Carpenter and Summers 2009). Initially, the presentation of the conspecific alone elicited a fear response, but shutting off the tank water flow did not. However, after repeated pairing, the subject gradually learned to associate the water flow stopping with the exposure to the aggressive conspecific (Lissek *et al.* 2005). This process is known as classical fear conditioning (Lissek *et al.* 2005, Carpenter and Summers 2009). Once the trout learned the association they had a choice: they could either escape from the aggressor by exiting through a hole into a "safe area," or they could stay near the aggressor and surrender, exhibiting social defeat (Carpenter and Summers 2009). It was found that "escapers" produced less cortisol in response to the water shutting off compared to non-escapers. Escapers also took

less time to leave the aversive part of the tank and move into the "safe area" as the trials progressed, indicating that the fish learned the value of escaping out of the aversive area.

For the non-escaping fish, when the water was switched off the fish froze at the bottom of the tank and were found to increase their cortisol production (Carpenter and Summers 2009). These results suggest that non-escapers also learned the classical fear conditioning paradigm by associating the water being turned off as a signal for the upcoming aversive stimulus, but rather than physically escaping, they physiologically prepared themselves for the aversive event by increasing cortisol production.

It is interesting to note that within this single population of trout, individuals acted differently by expressing one of two separate stress coping strategies when facing a potential threat (Carpenter and Summers 2009). Non-escapers stayed within the conspecific-aggressor area and utilized the water shut-off a signal to prepare their body for the upcoming stressful event. This is likely to help non-escapers to divert energy to their respiratory and cardiovascular systems during the threatening event (Wingfield *et al.* 1998, Carpenter and Summers 2009, Monaghan and Haussmann 2015, Hau *et al.* 2016). Escapers, however, had a reduced stress response and chose a more proactive approach by leaving the aggressor area and physically removing themselves from the threat (Carpenter and Summers 2009).

In both the cichlid and the trout study, it can be seen that giving an animal some form of predictability regarding anticipating an aversive event helped prepare the fishes for an aversive event (Carpenter and Summers 2009, Galhardo *et al.* 2011). Thus, providing animals with an opportunity to learn or appraise a warning stimulus could potentially be used as a tool to help the animal prepare for the onset of a stressful experience.

Temperament and Coping Style

Over the decades and across numerous scientific disciplines, different theories have emerged to address why there is individual variation in how an animal behaves, such as when facing a potential threat (Coppens *et al.* 2010). Within the field of applied animal behavior, Koolhaas (1999) observed that individual laboratory and farm animals responded differently when encountering a stressful stimulus. Koolhaas termed this phenomenon an animal's coping style, or a coherent and related set of behaviors and physiologies in response to a threat (Koolhaas *et al.* 1999, Coppens *et al.* 2010, Koolhaas *et al.* 2010).

Moreover, Koolhaas noticed two coping styles occurring most often: proactivity and reactivity (Koolhaas *et al.* 1999, Coppens *et al.* 2010, Koolhaas *et al.* 2010). Proactive individuals are defined as being bold or being more likely to respond to a situation before something occurs, e.g. quickly entering into a novel area to investigate it, and also usually have lower HPA/HPI activity (less stress responsivity) (Koolhaas *et al.* 1999, Koolhaas *et al.* 2010, Ruiz-Gomez *et al.* 2010, Vindas *et al.* 2017). While reactive individuals are the opposite as they are shy and tend to wait until something happens before they respond, and typically they also have higher HPA/HPI activity (higher responsivity).

Similarly, within the field of behavioral ecology, Réale (2007) proposed that individual variation is underpinned by differences in an animal's temperament. Réale defined temperament as a behavioral attribute that varies within a group of individuals, that is displayed across many situations including a "novel, risky, or challenging setting." Although temperament and coping style emerged from two different disciplines and focused on different populations, captive animals in Koolhaas' work versus wild animals in Réale's, both concepts are similar to each other. For example, proactive animals are usually bold and have lower HPA/HPI activity; "bold" represents an animal's temperament, and temperament *coupled with* lower HPA/HPI activity

describes the proactive coping style (Koolhaas 1999, Ruiz-Gomez 2010, Vindas *et al.* 2017). Temperament, therefore, is nested within an animal's coping style and it is likely both of these phenomena together contribute to individual variation. Although my dissertation considers stressors and stressful events, I take an approach that focuses more on an animal's behavior with less emphasis on physiology, so my research is more directed at interpreting individual differences in temperament.

Réale (2007) describes several temperament traits; however, for my work, I have chosen to focus on two: exploration and social behavior. The reason I have decided to examine these two traits is because of their importance for survival and navigation within the natural environment; fish must explore to search for food or a mate, and, for species that school with conspecifics, social interactions and how cohesive an individual is within its school can affect how well predators are avoided (Oliveira *et al.* 2015, Graham *et al.* 2018). I will also address anxiety within my dissertation, another temperament trait which is the propensity for an individual to react to stressful/frightening stimuli, such as an attack by a predator (Maximino *et al.* 2012). Anxiety also influences other fear-related behaviors. For example, if an animal has high anxiety, then it will be less likely to explore its environment (exploration) and more likely to shoal with conspecifics for protection (social behavior).

The role of temperament in animal behavior has received considerable interest over the past decade. One problem that has arisen over time is that a number of studies have used behavioral assays to infer temperament traits, but the assays have not been properly validated, or definitions have been confounded (Réale *et al.* 2007, Burns 2008, Carter *et al.* 2013). For example, in an open field arena with a sheltered area, the test animal has a choice; it can hide or explore, but it has been hard to distinguish whether the observed behavior is, in fact, exploration, or anxiety/fear-related behavior. In Chapter 2 of this dissertation, I decided to use a traditional open field set-up which lacks a shelter (see Réale *et al.* 2007). I did this so that the behaviors I

monitored would be indicative of fear or anxiety (Réale *et al.* 2007). I have also chosen to follow the definitions laid out by Réale and colleagues (2007) as their comprehensive review provides a clear framework for testing and interpreting temperament traits.

Measuring Temperament

Exploration is defined by Réale and colleagues (2007) as an individual's reaction to novelty (i.e., a new environment, or unfamiliar objects), which can be inherently risky or stressful. Exploratory behavior can be measured using apparatus called an open field arena, and it allows an observer to note variables such as the amount of area that the subject travels over (Réale *et al.* 2007, Carlson and Langkilde 2013). A trial begins by placing an individual in the middle of the open field arena, where they are then allowed to explore the environment for the duration of the trial; for example, in Panamanian Bishop fish (*Brachyrhaphis episcopi*) it was found that fish reared in a high-stress environment had a higher rate of exploration and covered more area in a given time, compared to low-stress exposed fish (Archard *et al.* 2012). Variations on this assay can include novel objects that are included within the arena. Here, exploratory behavior can also be investigated by determining the latency with which an animal approaches the novel objects, and also by measuring the amount of time an individual spends near them (Réale *et al.* 2007, Cavigelli *et al.* 2011).

Réale and colleagues (2007) also propose that social behavior can be assessed by quantifying the response of a test individual to the presence or absence of conspecifics. Measurements of social behavior can also involve other components depending on the research question addressed. Shoaling preference in fish, for example, can be measured by isolating an individual from conspecifics and then measuring the latency before the fish tries to rejoin the

group (Réale *et al.* 2007). Social behavior can also be investigated by determining an individual's preference in group or shoal size; for example, Thünken and colleagues (2014) examined shoal size preference by measuring the amount of time three-spined stickleback (*Gasterosteus aculeatus*) fish spent adjacent to compartments that contained different sized groups. It was found that fish preferred to shoal with a larger group, but this decreased over time likely due to no visible threat being detected.

Anxiety can also be measured in various ways; for example, the elevated plus maze is one of the assays commonly utilized in rodents (File 2001, Frye and Walf 2007). The apparatus is in the shape of a plus sign, which consists of two open arms exposed to the outside environment and two arms that are surrounded or enclosed by walls. A rodent is defined to be less anxious the more often it enters, and the more time it spends in the open arms because of their innate fear of open spaces.

In fish, alternative assays have been designed. One well-validated assay is the novel tank diving test (Egan *et al.* 2009). This assay is based on the fact that zebrafish (*Danio reio*) have a strong preference to avoid unprotected, open areas and so, immediately dive to the bottom of a novel and plain tank. Eventually, when the fish gain confidence, they swim towards the surface of the water. The fish are considered to be more anxious when they spend more time at the bottom of the tank (Levin *et al.* 2006, Egan *et al.* 2009).

I make use of the novel tank diving test to measure the response of individual fish to a novel environment in chapter 3. In this case, I used wild Panamanian bishop fish tested in the field. These fish show a reversed diving response to that shown by zebrafish which is not surprising given that for Bishop fish most of their predation threat come from other, larger fish that live towards the bottom of the streams and pools where the Bishop fish are found (Brown & Braithwaite 2004). Avoidance of deeper water areas where predators frequent has similarly been seen in guppies (Liesenjohann and Krause 2012, Swaney *et al.* 2015). Thus, for the novel tank

diving test used in chapter 3, I use latency to dive down to the bottom of the tank as a measure of anxiety.

Population Evolutionary History

The evolutionary background of an animal can impact its temperament and coping style, whether the animal is living in the wild or within captivity, or if the animal is descended from wild or domesticated lines. For example, environments with a low vs. high abundance of predators are known to promote differences in prey behavior and physiology. This is due to the varying degree of frequency and intensity of stressful encounters between these two environments, i.e. predatory attacks (Brown and Braithwaite 2004, Brown *et al.* 2005a, Brown *et al.* 2005b, Brown *et al.* 2007a, Brown *et al.* 2007b, Archard and Braithwaite 2011, Archard *et al.* 2012, DePasquale *et al.* 2014, Beri *et al.* 2014). Panamanian bishop fish (*Brachyrhaphis episcopi*) exposed to low vs. high predatory environments have been found to differ in their long-term stress response, and also in their activity, exploration and boldness behavior (physiological stress response: Archard *et al.* 2012; exploration and activity behavior: Archard and Braithwaite 2011; boldness: Brown and Braithwaite 2004 and Brown *et al.* 2007a and b). Similar phenomena have also been shown in populations of Trinidadian guppies with regard to life-history related traits (review: Endler 1995), and also in the terrestrial, eastern fence lizards (*Sceloporus undulatus*) with regard to stress-related temperament traits (exploration: Trompeter and Langkilde 2011; physiological stress response: McCormick *et al.* 2017).

This pattern is even found when wild animals are brought into captivity and are bred over successive generations. Captive breeding can result in particular traits being selected for through artificial selection (Price 1999, Künzl *et al.* 2003, Zeder 2012). A captive environment is different from an organism's natural environment because the animal goes from having complete

independence (e.g., foraging to acquire food, or a need to locate safe areas and shelter), to being entirely dependent on a human caretaker. When a population is brought into safe, captive environments, the natural selection pressures it faced in the wild are relaxed, and behaviors and traits that were previously important for survival can change. Time spent in captivity can, for example, influence the ability of an animal to cope with a stressor compared to populations that remain in their natural, wild habitat.

In rainbowfish (*Melanoteania duboulayi*), fish tested from a captive population released less cortisol after exposure to a mild acute stressor (being chased), compared to wild caught rainbowfish (Zuberi *et al.* 2011). This pattern was also seen in a different study with wild or captive parrots; after experiencing an acute stressor, and /or being briefly restrained, wild birds released corticosterone for longer than captive individuals (Cabezas *et al.* 2013). Why captive-reared populations have attenuated or weakened stress responses, is not clear, it may be due to their now living in a more predictable, safer environment that leads to overall reduced fear, or a decreased ability to understand or perceive a threat. Alternatively, it may, in fact, be that the captive environment is inherently more stressful as the animals are unable to escape frequent handling or brightly lit environments, and thus develop a diminished response to a stressor to avoid the consequences that come with constant activation (e.g., higher risk of disease) (Barton 2002, McEwen 2005, Archard & Braithwaite 2010, Hau *et al.* 2016).

In a study investigating differences in domesticated and wild populations of guppies (*Poecilia reticulata*), compared to domesticated populations, feral populations were found to shoal more in general, but particularly in the presence of a predator (Swaney *et al.* 2015). Shoaling is a well-known anti-predator defense in fish as it helps to increase the efficacy of vigilance, and individuals are better able to evade attack by group coordinated movement (Magurran 1990, Seghers and Magurran 1995, Swaney *et al.* 2015). Here, it is likely that feral guppies shoaled even during the absence of a predator because experiencing a new environment

can be perceived as risky, by, e.g., containing unknown and unfamiliar threats (Réale *et al.* 2007). However, when a threat *is* detected, i.e., a predator, then this can increase an animal's anxiety due to the confirmation of an identified threat. Thus, again we see that when facing a challenge, domesticated populations can respond differently, both behaviorally and physiologically, compared to their wild counterparts.

Stress Exposure During Development

In addition to population history, another factor that can affect an animal's ability to cope with future stress is previous experience, particularly during sensitive stages of development in early-life. Animals that are exposed to stress during development are especially sensitive to the long-lasting effects of stress due to changes that become established in the brain and nervous system; its underlying pathways (e.g. the stress response), and its synaptic connections for neural communication (Jankord *et al.* 2011, Kindsvatter and Geroski 2014, Monaghan and Hausman 2015). During development, for example, frequent and persistent activation of the stress response can lead to the stabilization and strengthening of neuronal connections involved in the stress-coping process. (Schmidt 2011, Kindsvatter and Geroski 2014, Chaby *et al.* 2015c, Colson *et al.* 2015).

Early-life stress exposure can come in many forms, such as a mother's experiences that can impact the future performance and behavior of her offspring. In birds, the stress hormone corticosterone can be passed on to offspring prenatally via the egg, and exposure to stress hormones in this fashion has been shown to affect future development (Hayward and Wingfield 2004, Saino *et al.* 2005). Saino (2005), for example, found that barn swallows (*Hirundo rustica*) injected with corticosterone during the egg-stage had a lower probability of hatching, and those that did hatch were smaller in size compared to groups injected with a control (oil) or handled but

did not receive an injection. Similarly, in damselfish (*Pomacentrus amboinensis*), fish exposed to injected cortisol in the field produced smaller offspring compared to fish injected with a control (cocoa butter) or those not exposed to an injection (McCormick 1998).

Other methods of elevating a stress response can also induce changes in offspring. Overcrowded damselfish nests where the number of breeding females were manipulated, resulted in smaller offspring that produced higher amounts of cortisol, compared to offspring from nests under natural/control conditions, i.e., no added females (McCormick 2006). In a study with rodents, mothers exposed to a period of chronic stress during pregnancy produced offspring that were less explorative, and also were worse at caring for their offspring as adults (Champagne and Meaney 2006). All of these studies, emphasize the power of the early environment and experience in terms of how it affects future offspring life-history and behavior.

Experiencing stress beyond very early developmental stages can also impact future adult behavior. This has mostly been studied in mammals, especially with regard to stress exposure during adolescence (see Chaby 2016 for review). Adolescence is regarded as a transformative period where an animal undergoes an assortment of different changes (e.g., physiological, behavioral, neurological, morphological), and often during this stage of development an animal is more sensitive to the impact of stressful stimuli (Jankord *et al.* 2011, Buwalda *et al.* 2013, Hau *et al.* 2016). Rodents, for instance, that were exposed to a period of chronic stress during adolescence and later tested as adults showed more caution to novel situations than non-stressed control rats (Chaby *et al.* 2015b).

In a different study, Buwalda and colleagues (2013) exposed rodents to a brief period of social defeat during adolescence and again during adulthood (Buwalda *et al.* 2013). It was found that rodents that experienced losing in an altercation with a larger conspecific during adolescence had a higher latency to attack an aggressive conspecific during adulthood, compared to controls. Here, it is possible that individuals that experienced social defeat during development were better

prepared during adulthood when facing this threat again, by possibly learning from their previous experience. In contrast, experiencing stress during early life can also produce higher levels of anxiety in adulthood, which can lead to a decreased ability to forage efficiently in a controlled laboratory environment (Chaby *et al.* 2015b). Thus early-life stress experience can influence future adult behavior in a number of different ways.

The Importance of Testing Context

Traditionally, stress research studies investigate the effects of early-life stress exposure on future behavior and physiology under a single testing context that is typically controlled, laboratory conditions. However, if an animal was exposed to a stressful upbringing and is then tested in a controlled environment, the results may be misleading because this scenario does not explore how the animal responds under threatening situations. For example, when investigating the effects of early life stress exposure in a population that was reared with a high abundance of predators, it would be valuable to investigate an individual's behavior under both a non-risk and a risky context, to investigate the animal's behavior under both contexts. In other words, it is important to test animals in a range of contexts, particularly one that matches the subject's rearing environment.

In Schmidt (2011) the match/mismatch hypothesis was proposed; if a subject's rearing environment, whether beneficial or aversive, matches the environment they experience in the future, then they will have an adaptive advantage in coping with future challenges. For example, if the adult offspring in the Champagne and Meaney (2006) study discussed above were tested under standard laboratory conditions, *and* under a stressful context that matched their earlier rearing environment, would adult offspring be more explorative if they came from stressed

mothers?

Studies have begun to examine animal behavior in multiple contexts and specifically, contexts relevant to an animal's earlier rearing environment. In Panamanian bishop fish, cortisol levels were compared between populations that were either exposed to a stressful or a non-stressful rearing environment. It was found that there were no differences in baseline cortisol levels between populations; however, there was a difference in fish stress responsivity *after* being exposed to a stressor. Specifically, populations that experienced more stress during development excreted less cortisol than fish that experienced less stress (Archard *et al.* 2012). In a study with rats, researchers examined foraging ability under high and low-threat testing conditions in individuals that experienced chronic stress as adolescents versus non-stressed control animals (Chaby *et al.* 2015c). Individuals exposed to chronic stress took longer to forage under a low-threat context, but under a high-threat context these individuals began foraging sooner and consumed more food compared to controls. Thus, the context in which animals are tested (i.e., whether this is a match or a mismatch with previous experiences) is an important consideration for experimental designs.

Thesis Approach and Rationale

By examining animals from different evolutionary backgrounds (e.g., captive versus wild) that face diverse environmental contexts in the future (i.e., non-stressful versus stressful), my dissertation investigates ways in which experiencing stress, or unpredictability during early-life can impact an animal's future behavior. I also take into consideration how an animal appraises a stressor and its temperament as these additional factors can also influence stress-coping behavior.

My first project investigated how early-life stress exposure affects future adult behavior under low and high-stress conditions, both in captive-reared wild-type and domesticated guppies (*Poecilia reticulata*). Domesticated fish were descended from individuals purchased from a pet store, whereas the wild guppies descended from wild-caught fish that lived with a low abundance of predators in a stream in Trinidad (Fischer *et al.* 2014). These populations were used to examine the effect of domestication and captivity on stress-coping behavior in different contexts. Juvenile fish were exposed to a brief period of chronic stress as juveniles (i.e., net chasing), and then later tested as adults to assess their exploration behavior in the presence or absence of a predator.

In chapter 3, I switched my focus to investigating stress-coping behavior in wild populations of fish in a field setting. In central Panama, the Panamanian bishop fish (*Brachyrhaphis episcopi*) live within rivers that contain barrier waterfalls that block the movement of fish predators (Brown and Braithwaite 2004, Brown *et al.* 2005a, Brown *et al.* 2005b, Brown *et al.* 2007a, Brown *et al.* 2007b, Archard and Braithwaite 2011, Archard *et al.* 2012, DePasquale *et al.* 2014, Beri *et al.* 2014). Bishop fish that live above the falls live with very few predators, while fish below the falls live with many predators. I investigated the anxiety responses of these populations both before and after exposure to an acute stressor to assess context-dependent anxiety responses. In chapter 4, I wanted to examine bishop fish exploration behavior, in fish from low and high predation populations. Here, I compared responses to novel objects in an open field arena. My original goal for this experiment was to test fish before and after experiencing a mild, acute, confinement stressor, however, as I explain later, I was only able to test the fish in one context (without exposure to an acute stressor).

In chapter 5, I describe a study with zebrafish (*Danio reio*), that was designed to assess whether the fish could learn to use specific cues to help them predict what would shortly happen to them. Here, I investigated whether the fish were able to recognize individual human caretakers that were associated with specific husbandry-related tasks. A capacity for individual human

recognition could be beneficial as it could be used as a tool to introduce predictability into the captive environment, for example, recognizing somebody associated with a handling, or cleaning procedure could help the fish prepare behaviorally and/or physiologically for a stressful experience and possibly reduce the impact that procedure has on the fish.

Together, all of the studies detailed within my dissertation provide novel insights into the way different factors affect an animal's ability to cope with a stressor. Using three different species of fish and investigating these in a range of contexts, I explore how experience and life history influence anxiety, exploration and social behaviors.

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Chapter 2

Controlled exposure to stress in early life: effects on adult anti-predator behavior and exploration in domesticated and wild guppies

Abstract

In mammals, the experience of stress during development has been found to have long term effects on adult behavior. Some of the changes induced by stress exposure during development appear to help the animal cope when it faces stressful situations as an adult, indicating that context matters. Whether this phenomenon also occurs in fish, and if a domesticated or wild-type background influences how sensitive a fish may be to experiences in early life has yet to be examined. To investigate this, domesticated and wild-descendant juvenile guppies (*Poecilia reticulata*) were exposed to stressful challenges and then, as adults, they were tested in different contexts (low or high-threat). When tested in a low-threat open field arena, wild fish exposed to stress during development were more cautious than wild controls; however, stressed domesticated fish behaved oppositely to this. When compared across different contexts, guppies were more active when a predator was present, but showed less explorative behavior under low threat conditions. Fish that had experienced stress during development also spent less time interacting with their mirror image compared to non-stressed fish, and fish spent less time engaging in this behavior when a predator was present. These results illustrate how an individual's adult behavioral phenotype is a product of gene and environment interactions, and highlight how exposure to period of acute stress during development can have long-lasting effects into adulthood.

Introduction

Animals that come from stressful environments tend to develop changes in behavior that appear to help them cope with a range of environmental challenges. Rodents, for example, exposed to chronic unpredictable stress during adolescence become more sensitive to novel environments and are more hesitant to consume a familiar food reward, compared to siblings reared in less stressful housing conditions (Chaby *et al.* 2015b). Similarly, Brydges (2014) found that rodents stressed as young pups spend less time exploring areas of a maze as adults compared to control animals. These studies highlight how experiencing stress during development can induce long-term effects that affect an animal's behavior by influencing how much time and energy an animal devotes to essential tasks, e.g., foraging or exploring new, or threatening environments (Hau *et al.* 2016).

Stress exposure during development, however, does not always lead to maladaptive consequences in later life. This observation especially rings true if an animal's future environment matches their earlier rearing environment. Sometimes referred to as the match/mismatch hypothesis (Schmidt 2011). This idea proposes that individuals are especially sensitive to the effects of stress exposure in development because physiological and neurological systems are still maturing, and stressful, or challenging conditions can lead to better coping strategies and behaviors. Once these processes develop, they can then promote long-term adaptation to future stressful environments. Such processes presumably promote survival in future challenging situations, but what happens if the environment encountered by the adult animal is not stressful? In this situation, the animal may now express responses and behaviors that are mismatched with what the animal experiences.

Chaby *et al.* (2015a), for example, described how rodents exposed to chronic unpredictable stress during adolescence out-performed non-stressed control animals as adults, in a

foraging task in a high-threat environment. Specifically, animals visited more foraging patches and consumed more food rewards than controls while a silhouette of a hawk predator with accompanying vocalizations, moved above the arena. Similar findings have also been observed in other taxonomic groups as well. Populations of the Panamanian bishop fish (*Brachyrhaphis episcopi*) are naturally exposed to either many or few predators because of where they live within streams (Brown and Braithwaite 2004, Brown *et al.* 2005a, Brown *et al.* 2005b, Brown *et al.* 2007). Bishop fish living in areas with many predator species have to exist in a high-risk environment, whereas, fish exposed to little predation occupy low-stress environments. This natural contrast in predation pressure has influenced a suite of different behaviors and physiological responses in the fish. For example, *B. episcopi* from high predation sites produce less of the stress hormone cortisol after experiencing an acute stressor, compared to fish from low-stress, low predation environments (Archard *et al.* 2012). Interestingly, there were no differences in the amount of cortisol excreted under baseline conditions, i.e., before the fish were exposed to acute stress.

The studies above suggest that early experience primes an animal for facing similar future environments. However, this is only beneficial when the future environment matches the one experienced during development. Studies addressing matched versus mismatched environments have focused on the effects of traits related to life-history and/or physiology (Tsalafouta *et al.* 2014, Vindas *et al.* 2017), far fewer have looked specifically at behavioral traits. Also, the studies investigating match versus mismatch effects have tended to test animals in low-threat contexts (Liesenjohn and Krause 2012, Brydges *et al.* 2014, DePasquale *et al.* 2016). To truly address the match versus mismatch hypothesis, however, animals should be investigated under both non-threatening and threatening scenarios.

In this chapter, I have chosen to investigate the match / mismatch hypothesis in guppies; I did this by rearing guppies with or without exposure to unpredictable stress during development.

After the fish had become adults, I tested how the same individual behaved under both low and high-threat testing conditions. As discussed in Chapter 1, temperament, in its broadest sense, is defined as a behavioral trait (or traits) that is characteristic to and varies within, a group (Réale *et al.* 2007, Burns 2008, Carter *et al.* 2013). Moreover, an animal's underlying temperament can affect how an animal reacts to novel or risky stimuli, such as a predator (Réale *et al.* 2007, Carter *et al.* 2013). In my study, I investigated differences in behaviors related to exploration and anxiety, because these represent well-recognized temperament traits (Maximino *et al.* 2012, Carter *et al.* 2013, Burns *et al.* 2016). According to Réale (2007), measuring behavior in a novel, and risky environment explicitly tests exploration behavior. Two kinds of assays were performed. In the first, temperament was gauged in an open-field arena to measure behavior under controlled, low-threat laboratory conditions. In the second assay, the behavior of individual fish was tested twice: once in a low-threat and once in a high-threat context.

In addition, I compared two different strains of guppy; a population recently brought into captivity from a natural river in Trinidad, and domesticated fish that had been bred for the pet trade. I wanted to compare the behaviors of these two groups and to investigate the effect of population background on stress responsivity. Domestication is generally believed to result in changes in behavior, morphology, and genetics owing to a relaxation of selective pressures faced in the wild (Künzl and Sachser 1999, Price 1999, Zeder 2012). I hypothesized that domesticated guppies would behave less cautiously regardless of context compared to wild fish. Domestication has long been implicated in an animal's attenuated response to stressful stimuli due to fewer interactions with stressors in the "safe," captive environment, and also due to the advantage of a diminished stress response which promotes ease of handling in captivity (Price 1999, Zeder 2012). Therefore, I predicted that domesticated fish would behave more naively even if in a threatening environment, and would express more explorative and less anxious behaviors during testing.

Before testing began, half of the domesticated and wild fish were exposed to chronic stress during development, and the remaining guppies remained in standard, low-stress laboratory conditions. Fish were later tested as adults to determine stress-related behavior under different conditions. I predicted that fish exposed to stress in early life would behave more robustly as adults when assessed under stressful conditions, due to the rearing environment that they had already experienced matching the future test environment (Schmidt 2011).

Methods

Study Animal and Treatments

Lab-bred wild-descendant guppies were utilized for both assays. These fish originated from a population living with few predators within the Naranjo tributary of the Aripo River in the northern mountains of Trinidad (Magurran and Seghers 1990, Fischer *et al.* 2014). This population had been bred for more than ten generations in captivity. In contrast, domesticated fish were from a stock population purchased at a pet store and then bred in the laboratory for a similar number of generations.

Sixty fish from each population were housed by dividing the guppies into groups of 5 across 12 population-specific tanks (35 x 20 x 27 cm). Each tank was equipped with a bio-filter, a heater, gravel, and plastic plants to provide enrichment. Commercial fish flake was given to fish daily, and the tanks were maintained on a 12-hour light: dark cycle at a water temperature of $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$. After a two-week acclimation period, three replicate tanks from each population were randomly exposed to two environmental challenges for 14 consecutive days, and the remaining three tanks remained as controls. The groups that were challenged experienced net chasing, i.e., a dip net was swept through the upper and lower parts of the tank in a figure-eight

pattern, or the water level was reduced to a third of the original depth for 15 minutes. Both of these stimuli have been successfully used as stressors in the past (Verbeek *et al.* 2008, Depasquale *et al.* 2016).

Fish were exposed to these challenges either in the morning (8 am to noon) or in the afternoon (noon to 4 pm) with one stressor occurring only once per day. This protocol was used to make the challenges unpredictable regarding which and when a challenge would occur, to fit the definition of a stressor (Oliveira and Galhardo 2009). A random number generator was used to determine the time of day and the type of challenge the fish received, but this was managed so that equal numbers of water reduction or net chasing occurred over the 14 days. Control groups were only handled during tank cleaning which occurred once every two weeks. Stress-treated groups were also exposed to bi-weekly husbandry duties as well.

After 14 days, all fish were returned to pre-stressor conditions and were allowed to grow for four more weeks by which point they reached sexual maturity. At this time, it was possible to identify sex. Females were only used in experimental assays because there are known sex differences in guppy behavior and also females tend to behave more consistently (Lucon-Xiccato and Bisazza 2016). By pooling females from each of the replicate tanks, there were two stress groups, i.e., wild offspring $n = 10$ and domesticated offspring $n = 10$, and two control groups, i.e., wild offspring $n = 10$, and domesticated offspring $n = 10$. However, owing to an unidentified infection sample sizes were reduced to the following before testing began: Wild stress $n = 6$, Wild control $n = 8$, Domesticated control $n = 9$, and Domesticated stress $n = 10$.

Individual Tagging

Fish were tagged with a unique identifying elastomer implant. Individual fish were first gently netted and placed in a buffered solution of Tricaine-S (MS-222, Sigma). As they became sedated, they were marked on the left and right side near the caudal fin. All fish were given two weeks to recover from the anesthesia, handling, and tagging before testing.

Ethical Note

All rearing, experimental design, handling, and tagging procedures were approved under IACUC Protocol # 45265.

Open Field: Experimental Setup

Fish were tested in a clear plastic arena (51.5 x 33 x 35 cm³ with water depth at 12 cm, see Figure 2-1) with black plastic surrounding all sides (Archard and Braithwaite 2011, Archard *et al.* 2012). A video camera placed above the arena recorded the movements of each fish, and a trial began once an individual was gently netted and placed in an opaque cylinder in the center of the test arena. After a 2 min settling period, the cylinder was raised, and a 5-min trial began. The arena floor was divided into a grid made of 48 evenly spaced squares and a line 6 cm from the walls of the arena on all sides marked the edge of the open field. Rate of movement (the number of line crosses divided by total time moving) latency to the border, the proportion of time spent in the center (adjusted with latency to the border), and time spent frozen were noted. Freezing behavior was defined as an individual not moving for greater than two seconds. Line crosses were

considered to occur when half of an individual's body was over a line. All videos were analyzed with the BORIS behavioral coding software (Friard *et al.* 2016).

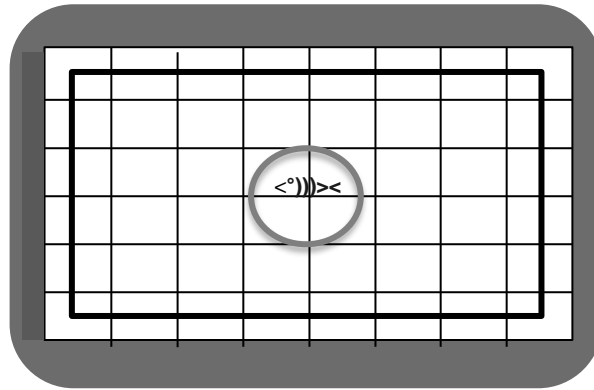


Figure 2-1: Schematic diagram of the experimental tank set-up for the open field arena. A subject was placed in the center of the arena inside of an opaque cylinder for 2 min. A trial began once the cylinder was lifted from behind a curtain, and then the fish was free to explore the arena for 5 min. A camera was placed above the arena to record and view each trial.

Testing with or without a Threatening Context: Experimental Setup

Fish were tested in a novel tank (60 x 30 x 30 cm³ with water depth at 24 cm, see Figure 2-2) divided into two compartments and surrounded by black plastic on all sides to minimize outside disturbances. The test compartment contained the start and predator areas and was immediately adjacent to a second compartment separated by a translucent, porous Plexiglas divider that housed a natural predator of the guppy, a blue acara cichlid (*Aequidens pulcher*) in half of the trials (Botham *et al.* 2008; Figure 2-2).

A trial began by gently netting and transferring a fish into the start area of the test compartment (15 cm x 30 cm), where it was allowed to acclimate for 10 seconds before an opaque divider was then lifted to expose the second half of the test compartment, which was either empty, or it contained a predator.

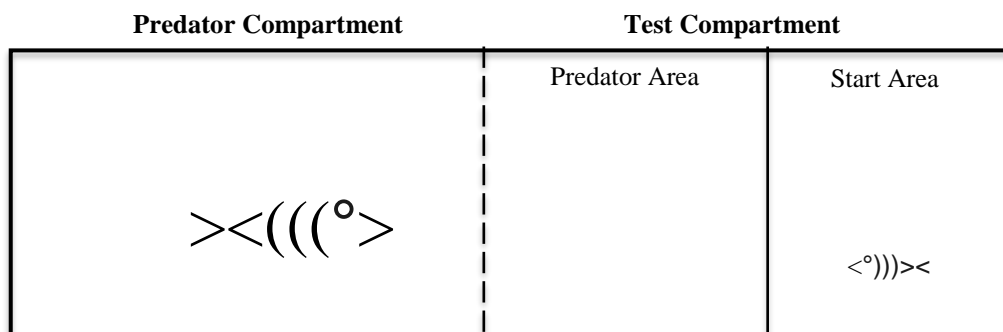


Figure 2-2: Schematic drawing of the two-compartment test arena to assess guppy behavior during both the absence and presence of a cichlid in the second compartment. The test compartment was split in half by an opaque moveable divider creating the predator and start areas. The test and predator compartments were permanently separated with a clear, porous Plexiglas divider. Once the opaque divider was lifted, fish had 5 minutes to navigate the entire test compartment freely.

Fish behavior was filmed remotely using a video camera suspended above the test tank. The guppy had 5 minutes to move around and explore the test compartment after the divider was lifted. Each guppy was tested twice in a repeated-measures design and order of which trial they experienced first was randomized. Between each trial, the predator compartment environment was altered (i.e., plants moved, addition or removal of the predator) to maintain novelty. The clear, and porous plexiglass divider separating the two compartments allowed for both visual and olfactory cues of the predator to be detected by the guppies when the blue acara was present. Tank water was replaced in between predator presence and absence trials.

Latency for an individual to leave the start area and enter the predator area, the proportion of time spent in the predator area of the test compartment, the number of times the guppy crossed between the start area and predator area of the test compartment, and the proportion of time spent frozen were noted for both the predator presence and absence trials. Freezing behavior was defined as an individual not moving for greater than two seconds. Crosses between start and

predator areas were counted when the front half of an individual's body crossed over into the start of the predator area.

Lastly, within the arena reflective surfaces were created by the black plastic surrounding the outside of the tank walls; this allowed fish to interact with their mirror image. It has been suggested that mirror image interaction behavior is indicative of an individual's desire to engage in social behavior (Desjardins and Fernald 2010, Cattelan *et al.* 2017). Guppies are a very social species, especially when a threat is present, e.g. schooling for protection from a potential predator (Magurran 1990). Thus, this behavior was examined to determine if it was affected by the presence of a fish predator within the predator context assay. An individual was considered to be interacting with their mirror image once they were oriented toward the tank walls and nudged at their image with their snout. All videos were analyzed with the BORIS behavioral coding software (Friard *et al.* 2016)

Statistical Analyses

To check that data conformed to the assumptions of analyses of variance (ANOVA), the data were tested for equality of variance and transformations were performed as needed. ANOVA was used with significance tested at $\alpha = 0.05$. Main factors of population (domesticated vs. wild-descendant), and treatment (stress during development vs. no stress) were compared in all analyses. For the open field experiment latency to the border was log transformed, and proportion of time spent in center data was arcsine square root transformed. When a transformation could not achieve equality of variance, a non-parametric Mann Whitney U test was used. For the predator context assay, a repeated measures ANOVA compared fish behavior during predator presence and absence trials. The proportion of time spent frozen, the proportion

of time spent interacting with mirror image, and the proportion of time spent in the predator area of the test compartment were all arcsine square root transformed.

Results

Open Field

Movement rate There was a main effect of population ($F_{1,28} = 6.13$, $p = 0.01$; Figure 2-3); however, there was no effect of treatment ($F_{1,28} = 0.61$, $p = 0.44$), and no interaction between population and treatment ($F_{1,28} = 0.90$, $p = 0.34$).

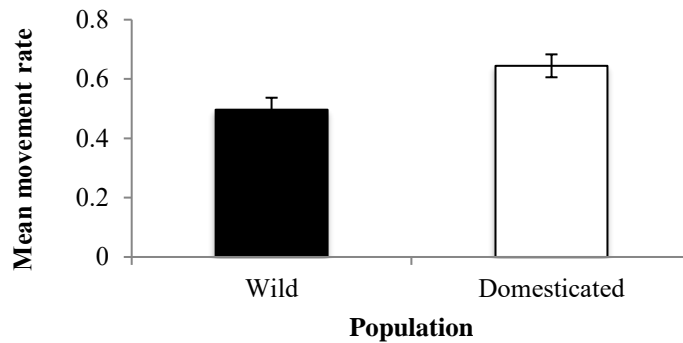


Figure 2-3: Mean movement rate (\pm s.e) between wild-descendant and domesticated populations.

Latency to border There was no effect of population ($F_{1,28} = 0.01$, $p = 0.89$) or treatment ($F_{1,28} = 1.08$, $p = 0.30$). However, there was an interaction ($F_{1,28} = 8.51$, $p < 0.05$) and a post-hoc test revealed that the stressed, wild-descendant fish took longer to approach the border of the arena compared to their control counterparts ($t_{1,11} = 2.50$, $p = 0.02$; Figure 2-4).

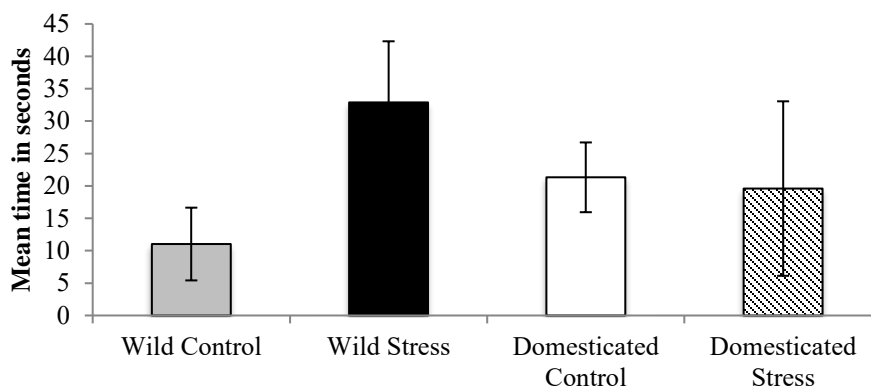


Figure 2-4: Mean time to reach the border (\pm s.e) for the different treatment groups, including the outlier in the domesticated stress group. Raw data are shown.

On looking more closely at the raw data, however, an outlier was found in the domesticated stress group. This fish had a latency score of 140 seconds, which was more than two standard deviations ($SD = 42.58$) away from the sample mean (Mean = 19.58). A further analysis was therefore done with this outlier removed. The new analysis found a significant difference between domesticated, stressed, and control fish ($t_{1,16} = -2.70$, $p < 0.05$; Figure 2-5), and the interaction between population and treatment was still significant ($F_{1,27} = 11.11$, $p < 0.01$).

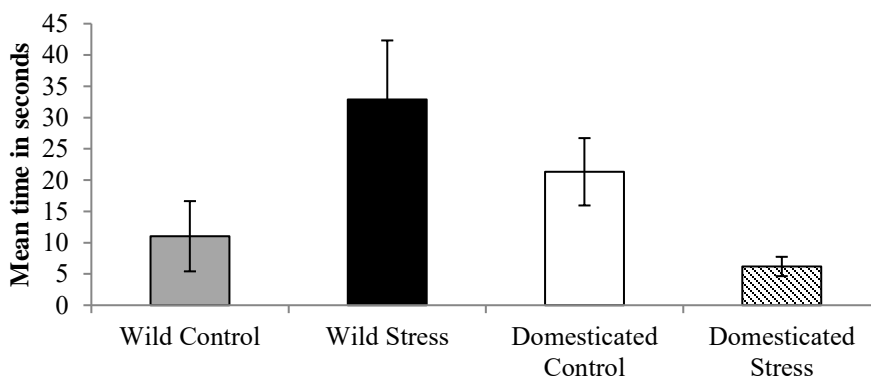


Figure 2-5: Mean time to reach the border (\pm s.e) for the different treatment groups with the outlier in the domesticated stress group removed.

Proportion of time spent in the center of the arena There was a main effect of population ($F_{1,28} = 4.39$, $p < 0.05$; Figure 2-6) and treatment ($F_{1,28} = 4.11$, $p = 0.05$; Figure 2-7). There was no interaction ($F_{1,28} = 0.28$, $p = 0.59$).

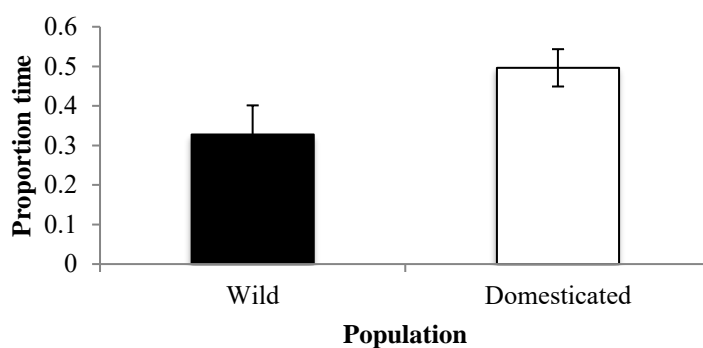


Figure 2-6: Mean proportion of time spent in the center (\pm s.e) between wild-descendant and domesticated populations. Raw data shown, and time was measured in seconds.

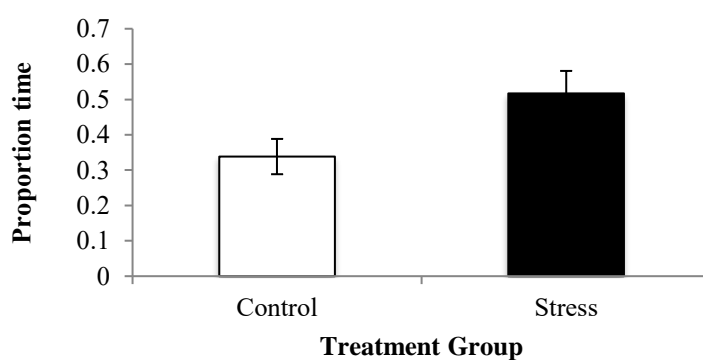


Figure 2-7: Mean proportion of time spent in the center (\pm s.e) between control and stress treatment groups. Raw data shown, and time was measured in seconds.

Proportion of time spent frozen There was an effect of population ($Z = -3.16$, $p < 0.01$) with wild descended fish freezing more than domesticated fish. There was no effect of treatment ($Z = -0.37$, $p = 0.70$).

Predator Context Assay

Latency to leave the start area and enter the predator area There was a main effect of context ($F_{1,28} = 4.79$, $p = 0.03$), and an almost significant effect of population ($F_{1,28} = 3.68$, $p = 0.06$; Figure 2-8). There were no effects of stress during development ($F_{1,28} = 0.03$, $p = 0.84$), and no interactions.

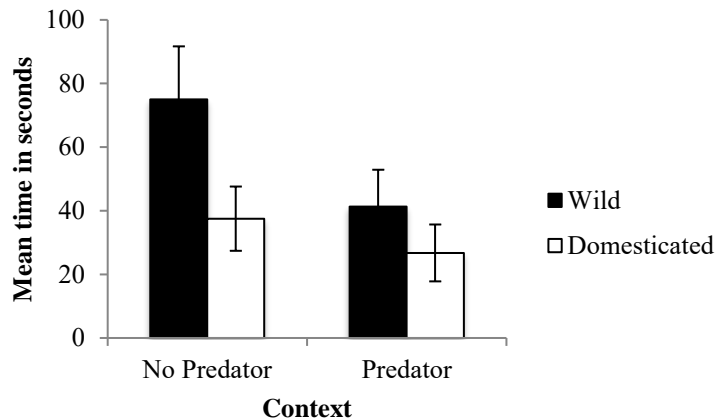


Figure 2-8: Mean time for fish to leave the start area and move into the predator area of the arena (\pm s.e.).

Proportion of time spent in the predator area of the test compartment There was no effect of population ($F_{1,28} = 1.25$, $p = 0.27$), treatment ($F_{1,28} = 4.0 \times 10^{-3}$, $p = 0.94$), or context ($F_{1,28} = 1.29$, $p = 0.26$), and no interactions.

Number of line crosses between the start and predator area There was a main effect of population, ($F_{1,28} = 6.89$, $p < 0.05$; Figure 2-9). However, there was no effect of treatment ($F_{1,28} = 0.19$, $p = 0.66$), and context ($F_{1,28} = 2.68$, $p = 0.11$). There was an interaction between population and context ($F_{1,28} = 5.51$, $p = 0.02$) and there was a three-way interaction between context, population, and treatment ($F_{1,28} = 15.24$, $p < 0.01$; Figure 2-10). None of the other interactions were significant.

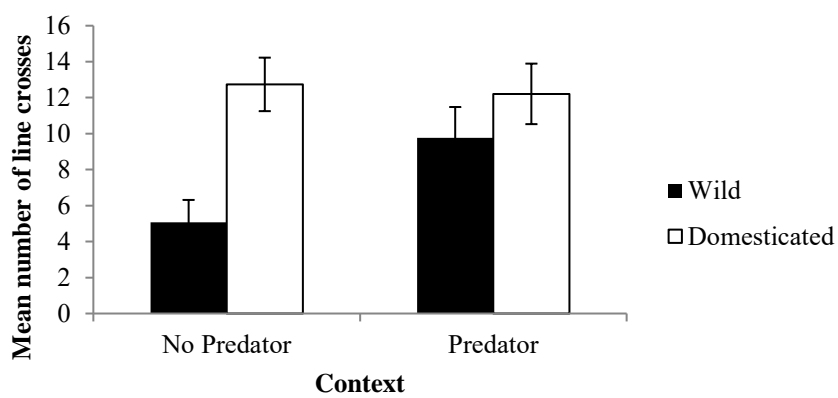


Figure 2-9: Mean number of line crosses (\pm s.e) between the two halves of the test arena in the presence or absence of a predator.

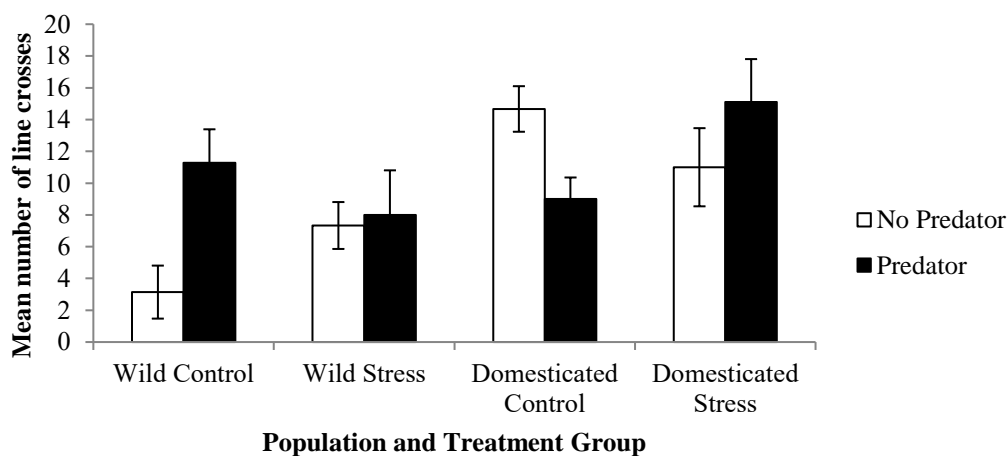


Figure 2-10: Mean number of line crosses (\pm s.e) between the two halves of the test arena of all treatment groups.

Proportion of time spent frozen There was a main effect of population ($F_{1,28} = 13.11$, $p < 0.01$; Figure 2-11). There was no effect of stress during development ($F_{1,28} = 1.51$, $p = 0.22$), no effect of context ($F_{1,28} = 2.42$, $p = 0.13$), and no significant interactions.

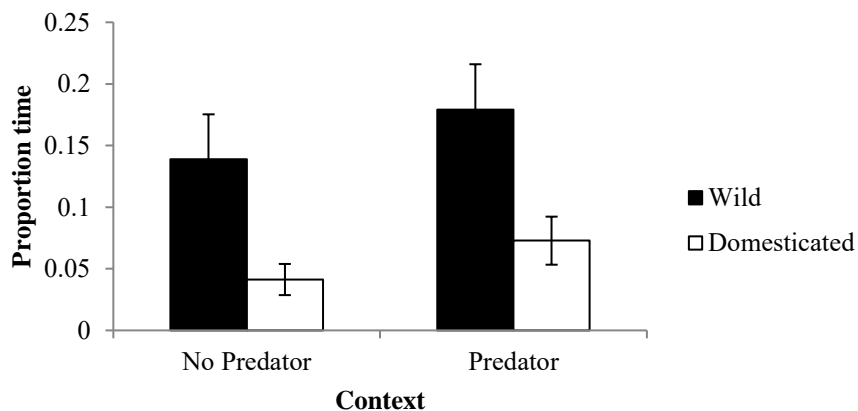


Figure 2-11: Mean proportion of time spent frozen (\pm s.e) during the absence and presence of a predator. Raw data shown, and time was measured in seconds.

Proportion of time interacting with mirror image There was a main effect of treatment ($F_{1,28} = 7.72$, $p < 0.01$; Figure 2-12) and context ($F_{1,28} = 22.83$, $p < 0.01$). There was no effect of population ($F_{1,28} = 1.18$, $p = 0.28$), or any interactions.

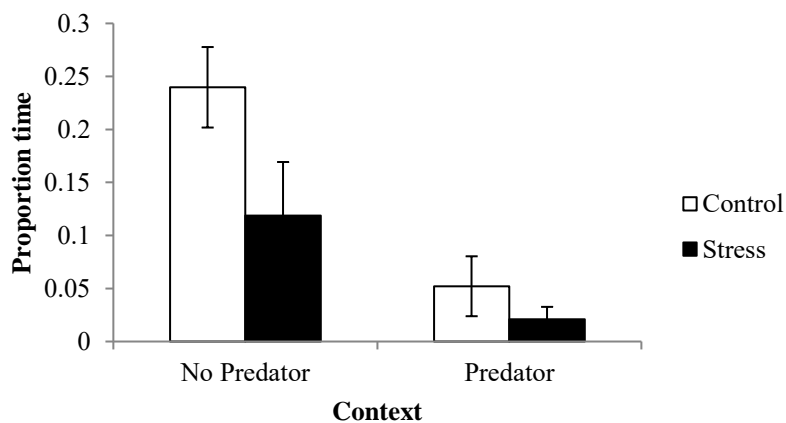


Figure 2-12: Mean proportion of time spent interacting with mirror image (\pm s.e) during the absence and presence of a predator. Raw data shown, and time was measured in seconds.

Discussion

I hypothesized that wild-descended fish would tend to be more sensitive and cautious in threatening, or novel situations, whereas I expected domesticated fish to be less sensitive owing to artificial selection of attenuated stress-related traits (Price 1999, Zeder 2012). Within both behavioral assays, coming from a wild or domesticated background did have an effect. In the open field assay domesticated fish froze less, had a higher movement rate, and spent more time in the center of the arena, i.e. an open area where fish are more exposed to a potential threat. In the predator context trials, domesticated fish moved more frequently between the two halves of the test compartment, they were quicker to leave the start area at the beginning of the trial, and they spent less time frozen compared to wild-descended guppies.

Population Differences

My results suggest that in both assays domesticated fish behaved less anxiously when facing stressful stimuli, compared to wild-descendant fish; when tested within a novel and potentially risky environment, domesticated fish behaved with less caution spending more time exploring and less time freezing. Spending more time frozen is considered to indicate higher levels of anxiety as freezing behavior is considered to be a fear-related defense mechanism (Oliveira and Galhardo 2009, Campler *et al.* 2009, Redfern *et al.* 2017). Being released into an unfamiliar environment (the open field arena), and visual and chemical predator cues were used to induce potential risk-related behaviors; both are validated methods for doing so (Réale *et al.* 2007, Archard *et al.* 2012).

Domesticated animals expressing decreased anxiety compared to their wild counterparts has also been found in other species. Verbeek and colleagues (2008), for example, investigated

potential differences in stress responsivity between wild and domesticated populations of Siamese fighting fish (*Betta splendens*). Here, cortisol levels were compared before and after populations were exposed to novelty stress, showing that compared to wild fighting fish, domesticated fish excreted less cortisol when transferred from their home tank to a novel, unfamiliar environment.

Population differences between domesticated and wild-descended animals is most likely a result of the domestication process itself. This multi-generational process involves relaxation in selective pressures due to a shift from a random and chaotic, wild environment to an artificial, more controlled setting (Price 1999 and Zeder 2012). In captivity, humans control an animal's daily life, such as feeding, reproduction, protection, and overall welfare. Traits for reduced stress responsivity are often selected for as these traits are considered desirable because they make animals easier to handle and house. Reduced stress responsivity in domesticated populations also results in less frequent activation of the stress response. Increased cortisol production can be detrimental because, if prolonged, it leads to chronic stress that can cause negative effects such as a weakened immune system and the development of behavioral stereotypies (Hau *et al.* 2016, Zeder 2012). A capacity to dampen stress responsivity allows captive animals to adapt to their environment (Archard *et al.* 2012).

In an earlier study, Burns and colleagues (2016) examined temperament traits in different wild Trinidadian guppy populations and found that fish from low predation areas were more explorative than guppies sampled in high predation sites. The wild-descendent fish I used in my study originated from a population considered to experience low levels of predation, but compared to domesticated guppies, I found the wild-descendent fish behaved more like the high predation exposed fish in Burns *et al.* (2016). My results are consistent with the observations of Burns *et al.* (2016) in that compared to the domesticated fish, my wild descendent-guppies will have historically been exposed to more novelty and some predation threat even if these instances were relatively low.

Burns *et al.* (2016) only examined temperament differences at baseline, i.e., low-stress conditions. However, to understand the full range of an animal's behavior it is necessary to examine an animal in multiple contexts, e.g., under low versus high stress. It would be interesting to determine if the differences in exploration traits that Burns *et al.* (2016) observed in a low threat scenario remained consistent if wild high and low predation fish were tested under conditions where their stress response was activated.

Environmental Context

Investigating how exposure to stressors in early life affects adult behavior under low or high threat conditions has recently been attracting attention (Colson *et al.* 2015, Chaby *et al.* 2015a and b, Vindas *et al.* 2017). In rainbow trout (*Oncorhynchus mykiss*), for example, fish exposed to cortisol during development reacted differently to a novel disturbance as adults, compared to control fish (Colson *et al.* 2015). Similarly, Vindas *et al.* (2017) found that adult Atlantic salmon (*Salmo salar*) exposed to chronic stress during development had a mitigated catecholaminergic response after experiencing an acute stressor, compared to unexposed, control salmon.

The results of the predator context assay described in this chapter, suggest that stressful experiences during development influenced some future adult behaviors. Adult wild-descendent guppies that had been exposed to stress during early life moved more frequently compared to non-stressed, control wild-descendent fish, but only when the predator was absent. Context was also seen to affect the general movement, and space use of the guppies, in that fish, were slower to leave the start area and enter the predator area of the test compartment when there was no predator present. And in terms of fish interacting with their mirror image, they did this more

when the predator was absent. These results again highlight the value of screening individual behavioral responses under different conditions (i.e., high versus low threat).

Early Life Stress

My results support a number of other studies that have found an animal's rearing environment influences how it responds to a future potential threat (Oliveira and Galhardo 2009; Schmidt 2011; Kindsvatter and Geroski 2014; Chaby *et al.* 2015a, b, and c; Colson *et al.* 2015). In my study, fish exposed to stress in early life behaved more robustly in a future stressful environment, compared to control fish. In the open field experiment, fish exposed to stress during development spent more time in the arena's center than control fish, and wild-descended fish that were stressed had a higher latency to reach the border of the arena. While domesticated fish that were stressed had a lower latency to reach the border of the arena compared to their control counterparts.

The results for the wild-descended fish in the stress-treated group were consistent with the hypothesis that early life challenges can help promote resilience in adulthood. These fish carried out more line crosses in the predator context assay, spent more time in the center, and took longer to reach the border of the open field assay. These behaviors are consistent with a more robust physiological and behavioral phenotype as the fish did not immediately flee to seek cover or try to escape (Archard and Braithwaite 2011). However, the results from the domesticated fish exposed to stress showed that this result did not generalize to another guppy strain. Rather, the domesticated fish displayed an increase in fear-related behavior if they had been stressed during development. It seems that the experience of stress in early life for the domesticated fish made them more sensitive to threat in later life.

Regarding how the fish interacted with their mirror-image, there were again differences between control and stress-treated fish. The motivation for guppies to perform interactions with their image is not clear, it has been suggested that for territorial species, mirror-interactions are displays of aggression, but in the case of the guppy and other Poecilids, this behavior appears to have very little to do with aggression as there is no lunging or biting (Desjardins and Fernald 2010, Cattelan *et al.* 2017). An alternative explanation is that the interactions are more relevant to social-behavior with the fish attempting to gain access to a conspecific. This interpretation seems more likely, because these fish have a strong schooling tendency (Magurran 1990), and mirror image interactions are most often observed when guppies are individually housed (Desjardins and Fernald 2010, Cattelan *et al.* 2017).

I hypothesized that stress-treated fish would interact with their mirror image more, and particularly in the presence of a predator since the company of conspecifics are likely to be more valuable in a threatening environment (Magurran 1990). However, my results showed that fish exposed to stress in early life engaged less in interacting with their image compared to control fish. This may be because fish exposed to stress in early-life were more attentive to the potential threat that a novel environment imposed, compared to stress-naive control fish. Future studies could examine this further by testing for a correlation in the amount of time fish spend interacting with their mirror image and time spent trying to gain access to a school of conspecifics behind a transparent barrier.

Conclusion

My results indicate that similar to research on various taxa, but mainly mammals, early-life stress exposure can affect future adult behavior in guppies. I also found consistent differences

in the way domesticated and wild-descended fish are affected as adults by early-life stress experience. Moreover, that the context under which the guppies were tested affected how they behaved, highlighting the value of testing animals in a range of contexts (e.g., threatening or safe) if we are to more fully understand how experience during development influence future adult behavior.

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Chapter 3

Naturally living with high or low levels of stress: streamside assay of anxiety in wild populations of the Panamanian bishop fish

Abstract

Multiple studies have investigated adaptations in prey fish from populations experiencing different levels of predation threat, but this generally occurs after the fish have been caught, handled, and transported back to a laboratory where they are maintained in captivity. Periods in captivity, however, can alter an animal's behavior and physiology, so an alternative approach to understanding the effects that predator presence has on prey species is to test fish '*in situ*.' Owing to the difficulties that come with working in the field, this kind of investigation is undertaken much less frequently. In this chapter, I describe a study where I compared anxiety-like behavior in six different populations of the Panamanian bishop fish (*Brachyrhaphis episcopi*) from three high and low predation sites where the fish were tested stream-side. Using a novel tank diving test, both before and after exposure to an acute stressor, I found that low predation fish took longer to dive, and spent more time at the surface compared to high-predation fish. After confinement, low predation fish continued to take longer to dive, and spent less time at the bottom compared to high predation fish. Stream and sex differences were also detected, with males taking longer to dive than females, and high-predation males spending more time at the bottom than low-predation males. The results of these experiments highlight how fish populations that live just a few hundred meters apart express contrasting levels of anxiety that appear to be related to how much predation pressure they experience.

Introduction

Prey species that live sympatrically with predators typically need to adjust their behavior and physiology to cope with stressful challenges (Archard *et al.* 2012). Studies with populations of prey fish have described how different levels of predation pressure lead to changes in a variety of life-history related traits. This has been particularly well-studied in Trinidadian guppies (*Poecilia reticulata*), where the fish live within several rivers that contain waterfall barriers that block the upstream movement of most predatory fish, but the guppy populations are found above and below the falls (Magurran and Seghers 1990, Seghers and Magurran 1994, Botham *et al.* 2008, Fischer *et al.* 2014, Burns *et al.* 2016). Guppies below waterfalls live with a high abundance of different predators, e.g., the blue acara (*Aequidens pulcher*) and pike cichlids (*Crenicichla alta*), which creates a high-predation pressure environment. Above the falls, guppies live with a low abundance of gape-limited predators, creating a lower-predation environment. Guppies from high predation sites maintain a greater distance when investigating a predator (Magurran and Seghers 1990), spend less time foraging (Seghers and Magurran 1994) and engage in schooling behavior more in the presence of a predator (Seghers and Magurran 1991, Seghers and Magurran 1994) compared to low predation conspecifics. Thus, individuals across different populations vary considerably in how they behave.

Over much of the last decade, there has been a growing interest in determining what underlies individual variation in behavior (Réale *et al.* 2007, Burns 2008, Archard and Braithwaite 2011, Carter *et al.* 2013). Réale (2007) proposed that an animal's underlying temperament, i.e., inherent behavioral traits that are characteristic of all or some individuals within a group or species, could explain why variation in behavioral responses exists, and why some animals react differently from others when, for example, facing a threatening or stressful situation. Temperament research has, therefore, become particularly appealing to ecologists and

evolutionary biologists because of the growing recognition that an animal's temperament can contribute to its survival and overall fitness.

It is also important to note that the degree to which these temperament traits are expressed depend on the different selective pressures faced by an animal within its environment (Smith and Blumstein 2008). Examining the effects of stress exposure in different contexts, i.e., low versus high-threat, however, has only recently gained attention with studies in rodents, and more recently in fish (Chaby *et al.* 2015, Colson *et al.* 2015, Vindas *et al.* 2017, Redfern *et al.* 2017). In rainbow trout (*Oncorhynchus mykiss*), for example, eggs exposed to the stress hormone cortisol during fertilization produced juveniles that were more active both before and more so after experiencing an acute stressor, in comparison to control, un-manipulated fish (Colson *et al.* 2015). A suppressed physiological response in the presence of a threat can be advantageous in situations where it helps an animal to focus its energy on essential functions, such as reproduction, and foraging (Botham *et al.* 2006, Archard *et al.* 2012). And an attenuated stress response can also decrease the negative consequences that come with frequent stress response activation, such as a weakened ability to fight off infection or disease (McEwen 2005, Oliveira and Galhardo 2009, Hau *et al.* 2016).

A reduced physiological response when facing threat has also been detected in the Panamanian bishop fish (*Brachyrhaphis episcopi*), a small, live-bearing poeciliid that like the guppy are found in sites separated by waterfalls that act as barriers to predatory fish species (Brown and Braithwaite 2004, Brown *et al.* 2005, Archard and Braithwaite 2011, Archard *et al.* 2012, DePasquale *et al.* 2014). Studies with low and high-predation bishop fish have found differences in respiratory and physiological responses to acute stress, as well as a suite of stress-related behaviors (boldness behavior: Brown and Braithwaite 2004 and Brown *et al.* 2007; exploration and activity behavior: Archard and Braithwaite 2011; respiratory rate: Brown *et al.*

2005; cortisol release rate: Archard *et al.* 2012). Anxiety related behaviors are, therefore, predicted to vary across populations; however, these have not been previously investigated.

Anxiety is considered to be a temperament trait directly related to stress coping behavior and is defined as a behavioral response to a potential threat as opposed to fear which is a response to immediate and imminent danger (Maximino *et al.* 2012). In fish, a validated technique to induce and measure anxiety-related behavior, i.e., diving, freezing, erratic movement, is the novel tank diving test (Levin *et al.* 2006, Egan *et al.* 2009, Cachat *et al.* 2010, Maximino *et al.* 2012, Redfern *et al.* 2017). In species where this assay has been commonly used; zebrafish (*Danio reiro*) and largemouth bass (*Micropterus salmoides*), fish typically respond to being placed into a novel tank environment by quickly diving to the bottom. This is presumed to mimic a response in the animal to seek shelter or escape (Carlson and Langkilde 2013). Gradually, however, as the fish become more confident, i.e., there is no imminent threat to their safety, they start to swim up and explore the surface water. Such diving behavior has been shown to be related specifically to anxiety, because it can be reversed when anxiolytic, or anxiety-inhibiting drugs such as fluoxetine, are given to the fish (Egan *et al.* 2009).

The nature of the diving response, however, appears to be ecologically and experience sensitive. Botham *et al.* (2008), for example, noted that Trinidadian guppies respond differently in the novel tank diving test by remaining closer to the water surface and avoiding the bottom of the tank. Botham *et al.* (2008) concluded that guppies behaved this way because they would normally be safer in the upper parts of the water column because predatory fish species tend to lurk in deeper areas closer to the substrate. Thus here, fish spending more time at the surface likely indicates higher levels of anxiety as opposed to diving down to the bottom of the test tank.

In this chapter, I extend the work from Botham *et al.* (2008) by examining anxiety-like behavior in populations facing different levels of stress, and across different environmental contexts. I also examined this behavior immediately after sampling fish from their natural

environment, to avoid any unwanted behavioral changes associated with capture and transport into a captive environment. I predicted that high-predation fish would behave less anxiously (e.g., less time at the surface) after exposure to an acute stressor compared to low-predation fish. Secondly, I predicted that this difference in behavior would not be evident at low-stress, i.e., low-risk conditions. I also hypothesized that females would behave more anxiously than males due to previous work showing female bishop fish to be less explorative compared to males (Archard *et al.* 2012). As anxiety is likely to be underpinned by the exploration-avoidance axis, decreased exploration should likely co-occur with increased anxiety (Maximino *et al.* 2012).

Methods

Subjects

During the dry season of 2016, Panamanian bishop fish in the Soberania National Park were tested stream-side to assess their anxiety levels. Fish from low predation (LP) and high predation (HP) sites of the Quebrada Juan Grande (QJG), Rio Limbo (RL), and Rio Macho (RM) were tested (see Fig. 3-1 and Table 3-1). All three streams run independently into the Panama Canal, and the populations within these streams are separated by barrier waterfalls. The three rivers were chosen because these sites have been used in previous work, and predator surveys have confirmed a low abundance of predators in sites above the barrier waterfalls, but a high abundance below the falls (Brown and Braithwaite 2004).

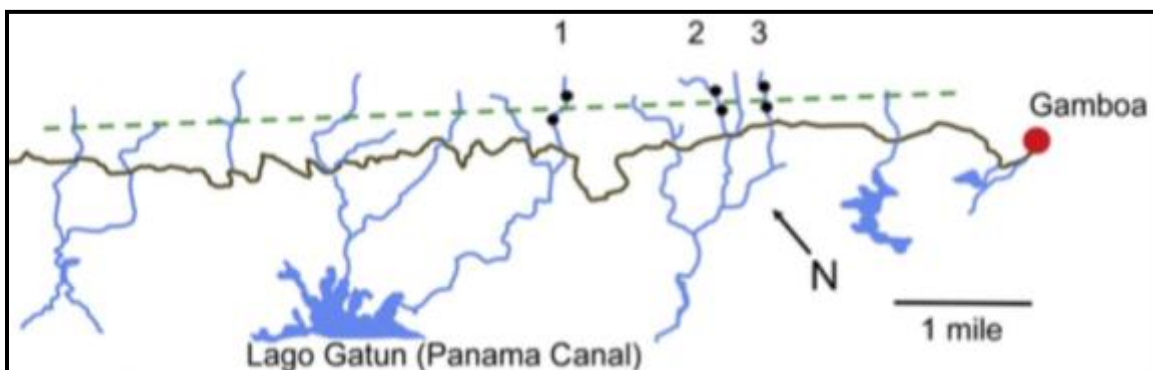


Figure 3-1: Distribution of low and high predation field sites of Rio Macho (1), Rio Limbo (2), and Quebrada Juan Grande (3). The dashed green line represents the separation of the low and high predation sites by the barrier waterfalls. The solid brown line represents Pipeline Road, which was utilized to access the field sites within Soberania National Park. (Image courtesy of V.A. Braithwaite)

Stream	Population	GPS Coordinates
QJG	LP	9° 8' 31.704" N, 79° 42' 53.964" W
QJG	HP	9° 8' 34.44" N, 79° 42' 57.24" W
RL	LP	9° 9' 54.792" N, 79° 44' 25.98" W
RL	HP	9° 9' 29.52" N, 79° 44' 14.28" W
RM	LP	9° 10' 52.32" N, 79° 45' 38.88" W
RM	HP	9° 10' 58.944" N, 79° 45' 24.048" W

Table 3-1: The GPS (Global Positioning System) coordinates of each sample site.

Novel Tank Diving Test

Fish were tested in a novel tank diving arena, and all tests were conducted next to the site where the fish were caught. Water within the tank was collected from the stream, and the whole tank of water was replaced after ten individuals were tested. A total of 97 fish were tested (Table 3-2). The test tank was covered in black plastic along the sides and back to minimize disturbing fish during trials, but the front side of the tank was left open so the fish could be filmed. A webcam was placed 2.5 feet in front of the tank and was connected to a PC that was operated by an observer several feet behind the back of the tank. The test tank (36 x 20 x 28 cm³) had a 4 x 4 grid drawn onto the front so that different zones could be identified during each trial, e.g., surface versus bottom (see Figure 3-2). A neutral area in the middle of the tank was included to make a clear distinction between fish choosing to be either at the surface or the bottom of the tank. Although the fish moved through this neutral area, the time spent in this zone was discounted (Figure 3-2).

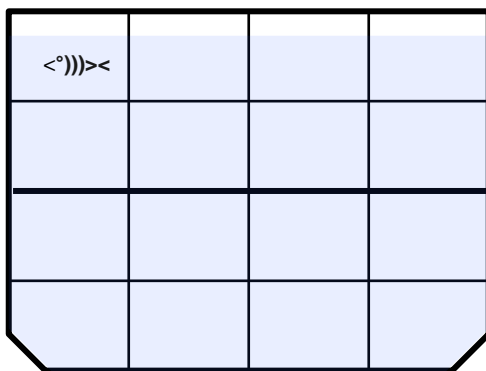


Figure 3-2: Schematic drawing of the novel diving tank. The bold middle line represents the center of the tank. Fish in the 4 upper most surface grid squares were considered to be at the surface, and those in the bottom 4 grid squares were categorized as being in the bottom of the tank.

Experimental Set Up: Baseline Experiment

Trials were conducted during the day from 9.00 to 16.00, with only one population tested per day. Upon reaching a site, fish were gently captured with a dip net, sex was determined to collect an even number of males to females, and then fish were placed in a covered bucket before trials began.

A trial started by netting and transferring an individual and gently placing the fish into the top left-hand corner of the novel diving tank. Latency to dive down past the red line dividing the bottom and top of the tank was noted, and the movements of the fish were then monitored for 5 min (Figure 3-2). At the end of the trials, the fish were returned to a covered bucket and were eventually transported back to an aquarium facility in a research building in Gamboa for later behavioral assays that were performed in captivity.

Experimental Set Up: Confinement Stressor Experiment

After all baseline measurements were completed for all low and high predation populations, the sites were returned to, to test the behavior of the fish after they had been exposed to an acute stressor. Fish were gently netted, collected, and sexed as described above. To stress the fish, an individual was isolated and placed into a small, covered container (51.48 cm³) where they were confined for 5 min. Isolation and confinement is a commonly used stressor and has been validated in a previous study with *B. episcopi* populations (Brown *et al.* 2005). After 5 minutes of confinement, individual fish were then placed into the novel tank diving test, and a trial commenced, as described above. Here, a total of 113 fish were tested (Table 3-2).

Sample Sizes							
<u>Baseline</u>				<u>Confinement Stressor</u>			
River	Site	Males	Females	River	Site	Males	Females
QJG	LP	9	8	QJG	LP	10	11
	HP	10	5		HP	9	12
RL	LP	11	8	RL	LP	7	12
	HP	7	8		HP	3	12
RM	LP	10	7	RM	LP	6	11
	HP	10	4		HP	11	9

Table 3-2: Sample size of females and males per low predation (LP) and high predation (HP) sample sites for Quebrada Juan Grande (QJG), Rio Limbo (RL), and Rio Macho (RM).

Quantifying Behavior

Once placed in the novel diving tank, bishop fish took a considerable amount of time to dive to the bottom, expressing similar behavior as the guppies studied by Botham *et al.* (2008). Latency for fish to dive down below the red line was measured to determine the point at which the fish overcame their anxiety. The proportion of time spent at the surface and tank bottom were also noted. The proportion of time fish spent frozen, and the number of grid-line crosses completed during each trial was used to gauge activity. The proportion of time fish chose to spend at the water's surface was adjusted by subtracting the amount of time the fish spent in this area initially when placed in the tank by the observer. A dive was defined as completed once an individual crossed the red line dividing the top half from the bottom half of the tank. Lastly,

freezing behavior was defined as an individual not moving for more than 2 seconds. All videos were analyzed with the BORIS behavioral coding software (Friard *et al.* 2016).

Statistical Analyses

Main factors of stream origin, predation level (low vs. high), context (pre vs. post-confinement stressor) and sex were considered in all analyses. Latency to dive, proportion of time spent at the tank bottom and surface, proportion of time spent frozen, and number of line crosses were compared for all groups. Analyses of variance (ANOVA) were used, and assumptions of equality of variance were tested, with transformations used when needed. For data that did not conform to the assumptions of ANOVA, non-parametric analyses were used instead. Significance was tested at $\alpha = 0.05$, and post-hoc Fisher's PLSD tests were used to identify which factors were responsible for significant differences that were found.

Latency to dive data was \log_{10} transformed, and proportion of time spent at the bottom data were arcsine square root transformed. Proportion of time spent at the surface, proportion of time spent frozen, and the number of lines crosses completed were compared with a Mann Whitney U test, and median values with interquartile values are reported.

Results

Latency to dive There was a main effect of predation ($F_{1,186} = 12.32$, $p < 0.01$; Figure 3-3), and stream origin ($F_{2,186} = 3.09$, $p = 0.04$). A Fisher's PLSD determined that Rio Macho fish behaved differently than Quebrada Juan Grande and Rio Limbo fish There was also a main effect

of sex ($F_{1,186} = 4.44$, $p = 0.03$); however, there was no overall effect of context, i.e., being tested before or after a stressor ($F_{1,186} = 2.89$, $p = 0.09$).

There was a significant two-way interaction between stream origin and sex that was significant ($F_{2,186} = 6.04$, $p < 0.01$), and an almost significant interaction between predation level and context ($F_{2,186} = 3.11$, $p = 0.07$). A four-way interaction between stream origin, predation level, sex, and context also showed a tendency towards significance ($F_{2,186} = 2.65$, $p = 0.07$), but all other interactions, summarized below in Table 3-3, were not significant.

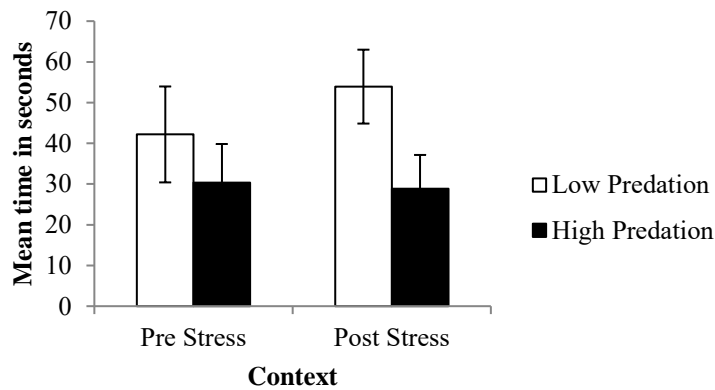


Figure 3-3: Mean latency for fish to dive (\pm s.e) between pre and post-confinement stressor groups of low and high predation sites. Raw data shown.

Independent variables	F ratio	<i>df.</i>	P
Stream x Predation	0.17	2,186	0.83
Stream x Context	2.02	2,186	0.13
Predation x Sex	0.21	1,186	0.64
Sex x Context	1.74	1,186	0.18
Stream x Predation x Sex	1.74	2,186	0.17
Stream x Predation x Context	0.39	2,186	0.67
Stream x Sex x Context	0.97	2,186	0.37
Predation x Sex x Context	0.34	1,186	0.56

Table 3-3: Non-significant main effects and interactions of independent variables for the latency for individual fish to dive from the surface to the bottom of the test tank.

Proportion of time spent at the bottom There was a main effect of predation ($F_{1,186} = 4.40$, $p < 0.05$; Figure 3-4) and context ($F_{1,186} = 6.49$, $p = 0.01$). However, there was no effect of stream origin ($F_{2,186} = 1.54$, $p = 0.21$) or sex ($F_{1,186} = 0.81$, $p = 0.36$).

The interaction between predation level and context was significant ($F_{2,186} = 6.78$, $p < 0.01$), as was the interaction between stream of origin and predation level. There was also an interaction between predation level and sex ($F_{2,186} = 8.11$, $p < 0.01$; Figure 3-5). Non-significant interactions from analyses are summarized in Table 3-4.

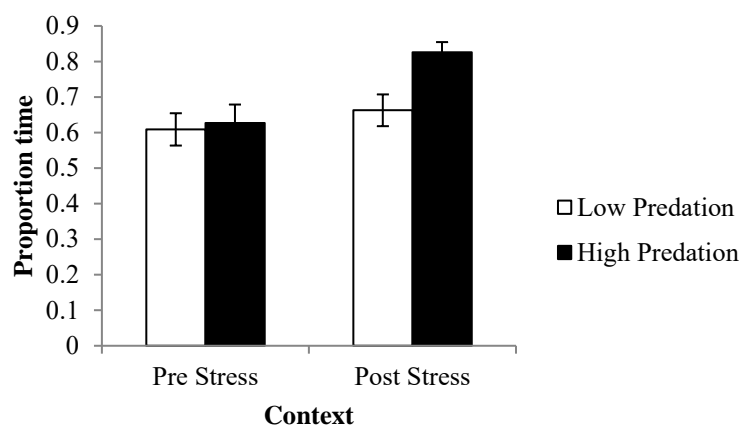


Figure 3-4: Mean proportion of time spent at the bottom (\pm s.e) between pre and post-confinement stressor groups of the low and high predation sites. Raw data shown, and time was measured in seconds.

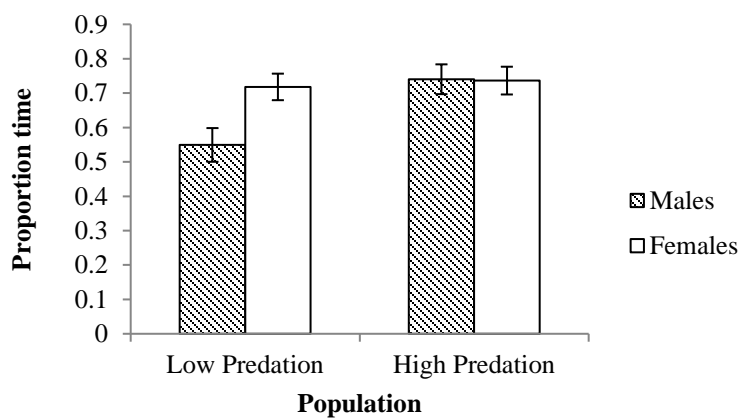


Figure 3-5: Mean proportion of time spent at the tank bottom (\pm s.e) between males and females of the low and high predation sites. Raw data shown, and time was measured in seconds.

Independent variables	F ratio	<i>d.f.</i>	P
Stream x Sex	2.14	2,186	0.12
Stream x Context	0.92	2,186	0.40
Sex x Context	<0.01	1,186	0.96
Stream x Predation x Sex	0.77	2,186	0.46
Stream x Predation x Context	1.26	2,186	0.28
Stream x Sex x Context	0.23	2,186	0.78
Predation x Sex x Context	1.89	1,186	0.17
Stream x Predation x Sex x Context	0.51	2,186	0.59

Table 3-4: Non-significant main effects and interactions of independent variables for proportion of time individual fish spent at the bottom of the test tank.

Proportion of time spent near surface There was an effect of predation level ($Z = -2.09$, $p < 0.05$; Figure 3-6) and context ($Z = -2.16$, $p < 0.05$; Figure 3-6).

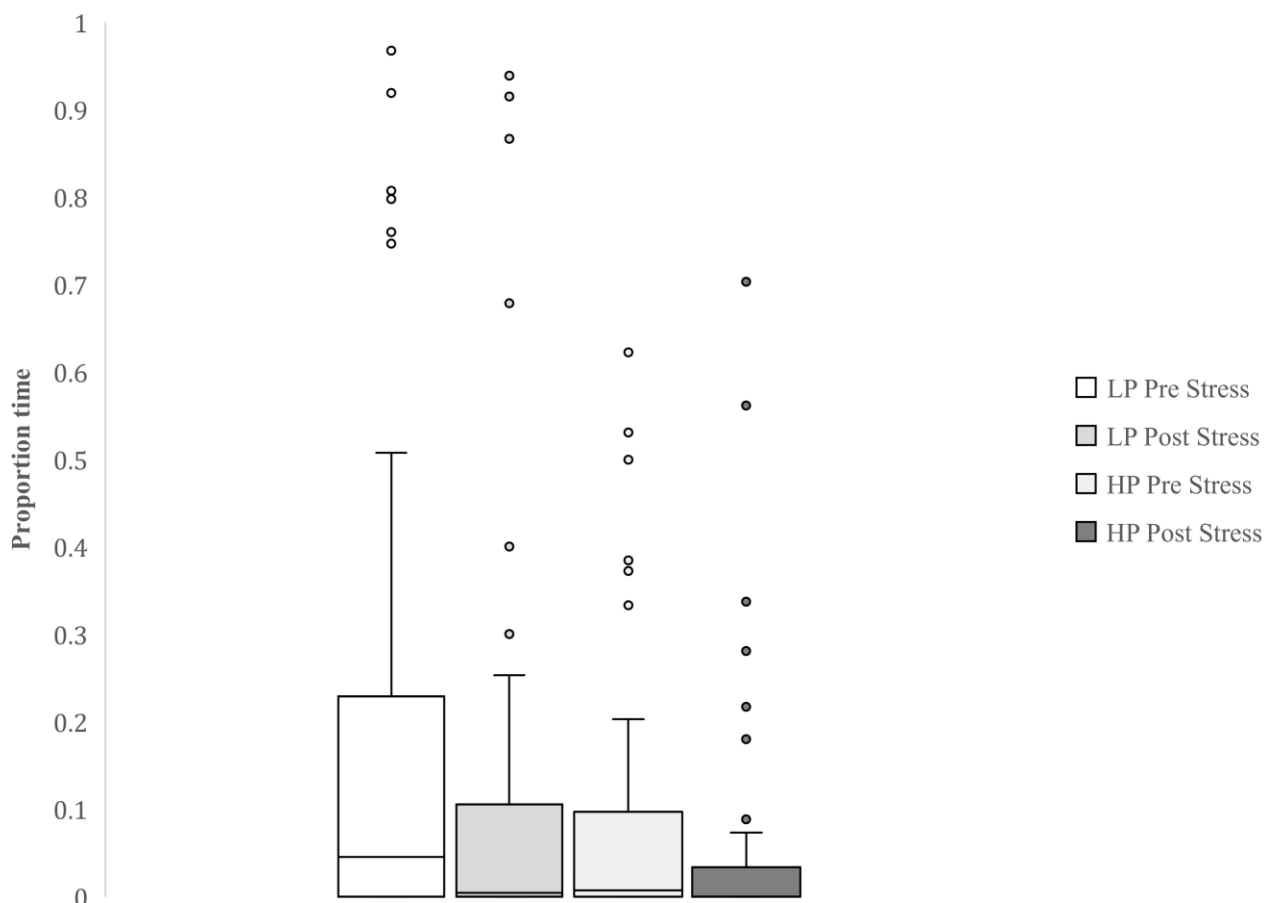


Figure 3-6: Median proportion of time spent at the water's surface (\pm max or minimum between pre and post-confinement stressor groups of low and high predation sites. Circles indicate outliers.

Proportion of time spent frozen There was no effect of predation level ($Z = -1.43$, $p = 0.15$); however, there was an effect of context ($Z = -1.96$, $p = 0.05$; Figure 3-7).

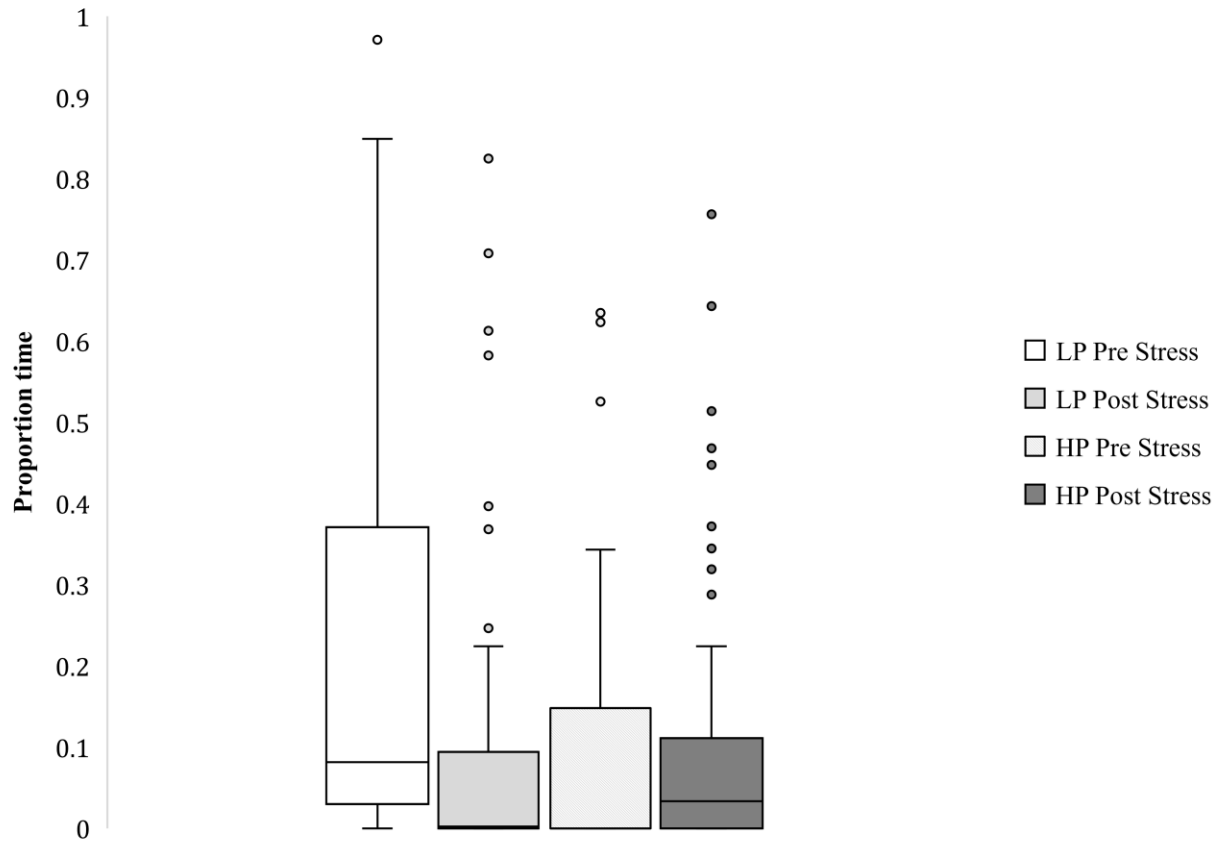


Figure 3-7: Median proportion of time spent frozen (\pm max or minimum) between pre and post-confinement stressor groups of low and high predation sites. Circles indicate outliers.

Number of line crosses There was no effect of predation ($Z = -0.14$, $p = 0.88$; Figure 3-8) or context ($Z = -1.16$, $p = 0.24$) on this movement.

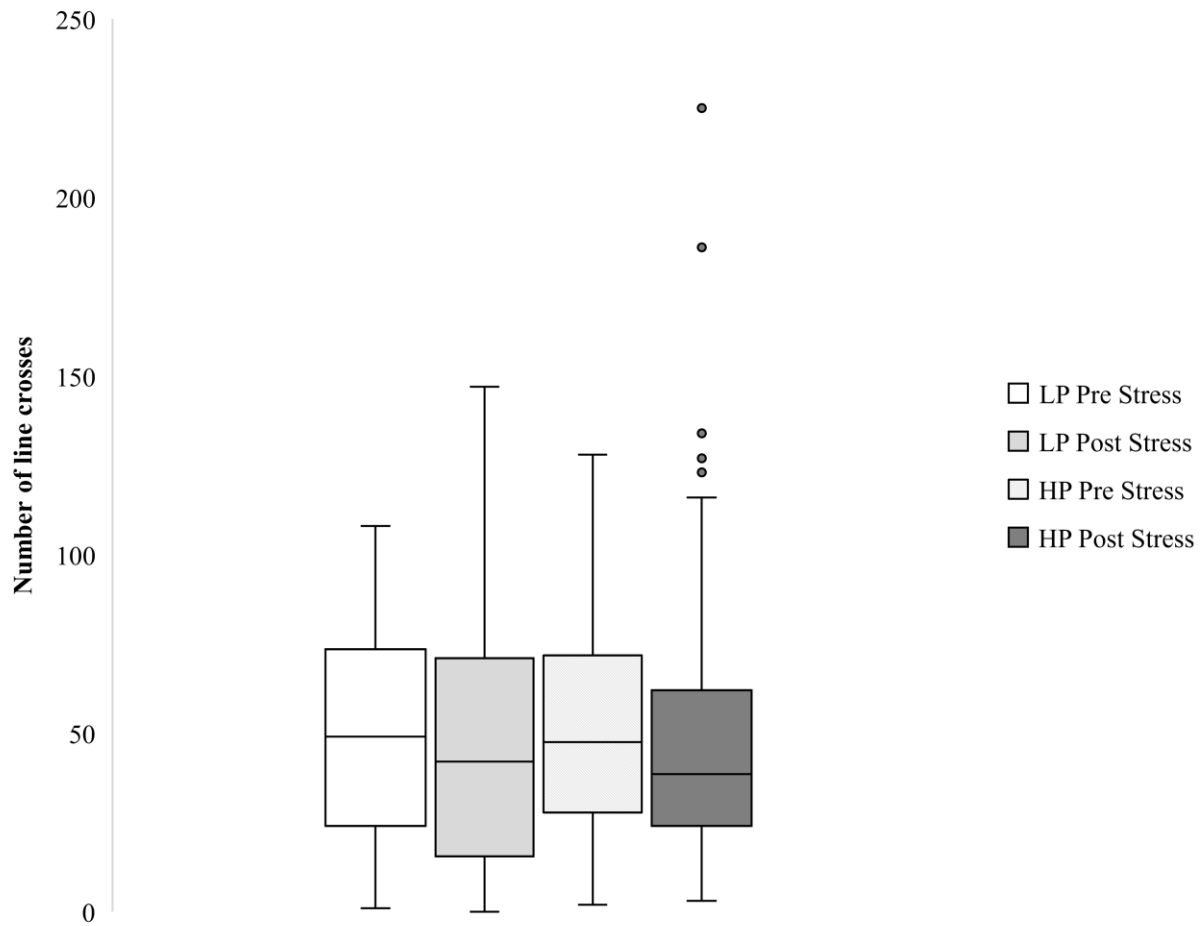


Figure 3-8: Median number of line crosses (\pm max or minimum) between pre and post-confinement stressor groups of low and high predation sites. Circles indicate outliers.

Discussion

With the novel tank diving test several differences between populations of Panamanian bishop fish and the context in which they were tested were revealed. Using the natural history of the system to make inferences about how the fish would respond, it was assumed surface areas would be perceived as being less dangerous as piscine predators lurk lower in the water column. The results of the novel tank diving test found that high predation populations were quicker to dive down in the tank, that they spent less time at the water's surface, and, after their initial dive, they spent more time at the tank bottom compared to low predation populations. This last result seems to be driven specifically by high predation fish after experiencing the confinement stressor (Figure 3-4).

The behaviors described above align well with previous research that has suggested high predation fish display a more proactive coping style compared to low predation fish. An animal's "coping style" is a set of behavioral and physiological responses to a stressor that is characteristic to a group of individuals (Koolhaas *et al.* 1999, Coppens *et al.* 2010, Koolhaas *et al.* 2010, Archard *et al.* 2012). Within bishop fish, high predation populations have previously been shown to excrete less cortisol when tested under stressful conditions, while being more active, explorative, and bold compared to low predation populations (e.g., physiological stress response: Archard *et al.* 2012; exploration and activity: Archard and Braithwaite 2011; boldness: Brown and Braithwaite 2004 and Brown *et al.* 2007). These heightened behaviors and a dampened physiological response to stress are all characteristics of a proactive coping style (Koolhaas *et al.* 1999, Coppens *et al.* 2010, Koolhaas *et al.* 2010, Archard *et al.* 2012). Furthermore, proactive individuals are also characterized as expressing lower levels of anxiety compared to those that display a reactive coping style (Koolhaas *et al.* 1999, Maximino *et al.* 2012).

Effect of Context

The tests reported in this chapter were conducted in a field setting where the fish were removed from their natural environment just shortly before being tested. Many similar behavioral studies have tested animals under controlled, standardized conditions after they have been in captivity for some time. Captive conditions are very different from the conditions animals experience in their natural environment, and such differences can influence and change behaviors, particularly those associated with stress responses, such as fear or anxiety (Oliveira and Galhardo 2009, Archard and Braithwaite 2010, Schmidt 2011, Redfern *et al.* 2017). Thus, examining an animal under multiple contexts, and where possible in a natural setting, helps to provide a more comprehensive assessment of behavioral responses.

When animals have been tested across different kinds of environment or context, the background of the animal, and their early life experiences affect how they respond to different kinds of assays. In rainbow trout (*Oncorhynchus mykiss*) for example, fish that have been artificially selected to have either a high or a low physiological response to stressors (HR and LR, respectively) differ in a suite of stress-related behaviors (Pottinger and Carrick 1999, Pottinger and Carrick 2001, Øverli *et al.* 2002, Øverli *et al.* 2007, Coppens *et al.* 2010, Ruiz-Gomez *et al.* 2011, Basic *et al.* 2012). For instance, Øverli *et al.* (2002) investigated the activity of HR and LR trout both during the absence and presence of a threat. They found that in the presence of a conspecific intruder, HR fish were more active than LR fish, but this difference disappeared when there was no threat present (Øverli *et al.* 2002). Context, therefore, matters, with the trout, in this case, responding differently when threatened. Similarly, Archard *et al.* (2012) detected differences in stress responsivity in Panamanian bishop fish after they had experienced an acute stressor; high predation fish excreted less cortisol compared to low predation fish, but no differences in cortisol release were detected during control conditions.

At the beginning of this chapter, I predicted that high predation populations would display an attenuated anxiety response after exposure to the confinement stressor, compared to low predation fish. Several of my results support this with high predation fish spending more time at the bottom of the novel test tank. These populations also tended to dive more quickly and were less reactive to the acute stressor compared to low predation fish (Figure 3-3). Furthermore, high predation fish also had a more constrained behavioral stress response by spending a similar amount of time at the water surface and also time frozen before and after confinement, compared to low predation fish (Figures 3-6 and 3-7).

Thus, my study suggests that high predation fish behaved less anxiously compared to low predation populations. These results complement the physiological results of Archard *et al.* (2012) by high predation bishop fish displaying an attenuated stress response after experiencing an acute stressor compared to low predation fish. With high predation fish displaying a more constrained physiological stress response before and after stressor exposure. Decreased stress responsivity is likely to be advantageous in that fish from high-risk environments will be able to focus their behavior on tasks such as foraging and reproduction despite experiencing high risks of predation (Archard *et al.* 2012, Hau *et al.* 2016).

As discussed in Chapter 2, immobility or freezing behavior is considered to be indicative of anxiety (Koolhaas *et al.* 1999, Botham *et al.* 2008, Maximino *et al.* 2012); however, other authors have considered erratic movement to also be indicative of heightened anxiety (Carpenter and Summers 2009, Archard and Braithwaite 2011, Maximino *et al.* 2012). Erratic movement can be interpreted as an individual frantically looking for an escape from a stressful situation or potential threat. In support of this interpretation, I frequently observed bishop fish swimming erratically after experiencing the confinement stressor as I tracked fish behaviors in the video analyses (personal observation). This may explain the results for bishop fish freezing behavior. For my assay, I intentionally kept the tank empty so that I could observe fish throughout their

trial. However, it would be interesting to know how bishop fish would respond if enrichment or areas of shelter were provided. Given the results I obtained, it seems likely the fish would have used these areas and remained in them particularly after experiencing the confinement stressor.

Sex Differences

Previous studies have found differences in the behavior and physiology of male and female bishop fish with male fish expressing more robust responses, i.e., males were more explorative in an open-field arena and excreted relatively lower levels of cortisol after experiencing a stressor compared to females (Brown *et al.* 2007, Archard *et al.* 2012). Given these earlier observations, I expected males in this study to behave less anxiously than females, i.e., males would be faster to dive to the tank bottom, and would spend more time at the bottom of the tank. My results, however, were not consistent with these predictions; males took longer to make their initial dive compared to females, and low-predation males spent less time at the bottom compared to low-predation females. Further experiments are needed to gain a better understanding of these sex differences in behavior, although one possibility might be the clear size sexual dimorphism (males are considerably smaller than females). This may make males more vulnerable to predators in novel, unknown conditions.

Conclusion

The results of my study show that when tested in a stream-side assay in the field differences in the relative exposure to predators affects the behavior of Panamanian bishop fish. Low predation fish tended to express higher levels of anxiety related behavior (i.e., spent more

time at the surface of the test tank); however high predation fish behaved in the opposite manner by, for example, altering their responses after exposure to a stressor, i.e., increased the time they spent at the bottom of the tank after experiencing a potential threat. In comparison with earlier bishop fish studies, my results provide further evidence that high predation fish generally display a proactive coping style. Furthermore, my data highlight the importance of the context under which a behavioral assay is quantified and then relating this back to an animal's natural history when interpreting observed behavioral responses.

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Chapter 4

A comparison of exploration behavior across populations of Panamanian bishop fish sampled from high and low predation areas

Abstract

A willingness to explore an environment affects many aspects of an animal's behavior. Individuals that explore more will be quicker to learn about the distribution of key resources such as food or shelter. However, moving around novel places increases an individual's vulnerability to unknown threats such as predators or becoming lost in unfamiliar areas. Whether an individual expresses an exploratory phenotype or not is likely to be influenced by previous experience, particularly during early life when assessments about threat can be developed. In this study, I compare the exploratory tendencies of Panamanian bishop fish sampled from three different streams that contain paired low and high predation areas. Using an experimental design to assess exploration traits, I compared how fish from different populations approached and interacted with two different novel objects in a novel environment. Fish from the three streams were found to differ in how quickly they moved into the center of a novel arena, in how long they took to approach a novel object, and how long they spent frozen during the trials. There were some tendencies for predation effects, and several interactions between stream of origin and predation level were also found. Overall, the results indicate that local ecology appears to affect exploration traits, and exposure to different levels of predation further influence this.

Introduction

Exploration is a recognized personality trait that influences how animals move around and interact with objects and other individuals in their environment (Réale *et al.* 2007, Archard & Braithwaite 2010, Graham *et al.* 2018). There are multiple ways to examine this temperament trait; the most common involve assessing behavior in open field arenas or investigating willingness to approach novel objects (Réale *et al.* 2007, Burns 2008, Cavigelli *et al.* 2011, Burns *et al.* 2016). Previously, studies addressing the closely related personality trait of boldness have used similar assays to measure either boldness or exploration; however this has led to considerable confusion in the literature (Réale *et al.* 2007, Carter *et al.* 2013, Roche *et al.* 2016). A comprehensive review by Réale and colleagues (2007) laid out a suite of definitions and methods for quantifying different temperament traits. For exploration, they pointed out that this temperament trait should be thought about in terms of an animal's reaction to novelty. Whereas boldness is associated to a risky environment that is familiar. Both these temperament traits will contribute to how an animal responds to stressful stimuli (Koolhaas *et al.* 1999, 2011; Réale *et al.* 2007), and will influence their ability to find food, mates, and shelter.

Both boldness and exploration have been studied in natural populations of poeciliid fish, both in the field and in more controlled laboratory settings. Particularly for populations with contrasting predation experiences. For example, Burns and colleagues (2016) tested the exploratory behavior of guppies from three high and low predation sites and found that fish from high predation sites were less exploratory in a novel open field arena. Similar results were found regardless of whether the fish were tested in a field or captive setting. In contrast to these results, a test of boldness in the Panamanian bishop fish found that fish from high predation sites were bolder than low predation (Brown and Braithwaite 2004, Brown *et al.* 2007). A later study

focusing on exploration found Panamanian bishop fish from high predation areas expressed heightened exploration traits (Archard and Braithwaite 2011a).

The reason that the guppy and Panamanian bishop fish differ in their exploration and boldness remains unclear; however, the biology and behavior of these two species are distinct, whereas their ecologies with respect to predation are quite similar (Magurran 2005). Guppies are morphologically smaller than bishop fish, they show strong schooling tendencies, and they develop strong social relationships (Seghers and Magurran 1991, Magurran 2005). Panamanian bishop fish, in contrast, are larger, particularly the females, and this species tends to behave more independently (Brown and Braithwaite 2004, Archard and Braithwaite 2011a and b). However, the experimental designs used to quantify boldness and exploration in both guppies and bishop fish have varied considerably which makes it hard to compare these temperament traits directly.

In this chapter, I have decided to focus specifically on exploration behavior in Panamanian bishop fish. Previous work has attempted to study this using a comparison between a social stimulus and a physical object; measuring the latency of bishop fish to approach and interact with either a pair of familiar conspecifics or a brightly colored sphere (Brown *et al.* 2007). The results showed no differences between populations from high or low predation sites, which was surprising because other studies have reported differences in activity levels, boldness and aggression (Brown and Braithwaite 2004, Archard *et al.* 2011a and b).

A later study looked at the willingness of a group of fish to approach either the reflective or the reverse opaque side of a mirror introduced into a familiar home tank (Archard and Braithwaite 2011b). This study also found differences in the way low and high predation fish interacted with a novel stimulus. Although these studies were trying to assess bishop fish exploratory behavior, they both confound social behavior with exploration itself. A better method to quantify exploration according to Réale *et al.* (2007) is to examine individual responses within a novel context.

For this chapter, I examined exploration of novel objects in captive populations of Panamanian bishop fish from three streams with paired high and low predation sites. Based on my results from chapter 3, I predicted that fish from high predation sites would show increased exploratory behaviors because these fish behaved in a less anxious manner compared to fish from low predation sites. Similarly, I expected to find lower freezing responses in fish from high predation sites.

The original design for this experiment was to investigate exploration behaviors across contexts by testing the fish before and after experiencing a mild stressor. Unfortunately, owing to contracting Zika virus and Dengue fever during my field season, I was only able to complete the first stage of this experiment. The results reported in this chapter therefore only involve fish that were tested without exposure to a stressor.

Methods

Subjects

Panamanian bishop fish from three low predation (LP) and three high predation (HP) sites in the Quebrada Juan Grande (QJG), Rio Limbo (RL), and Rio Macho (RM) rivers were captured using hand-held dip nets. The fish were carefully transferred to containers filled with stream water. These were enclosed in darkness and carried initially on foot and then by vehicle back to a laboratory at the Smithsonian field station in Gamboa, Panama. The water in each container was replaced halfway during the return trip to the laboratory facility to ensure the fish had access to oxygenated water. A schematic drawing and GPS coordinates of the sites used for fish collection can be viewed in Chapter 3 (Figure 3-1 and Table 3-1 respectively). A total of 51 fish were collected: 12 fish from Quebrada Juan Grande (low predation $n=7$ and high predation n

= 5), 22 fish from Rio Limbo (low predation $n = 13$ and high predation $n = 9$), and 17 fish from Rio Macho (low predation $n = 6$ and high predation $n = 11$).

Once in the laboratory at the Smithsonian Gamboa field station, fish tank water was controlled by the ambient temperature, which was approximately 25 to 27 °C. Each population was maintained in a separate tank (60 x 30 x 40 cm³) that contained gravel and a filter. Three sides of each tank were covered with black plastic, and each tank was covered with a lid to prevent fish from jumping out.

Experimental Setup: Novel Object Test

For the novel object trials, fish were individually tested in a small opaque wading pool (81 cm diameter x 20 cm height). The pool contained two novel objects that were chosen such that fish would not have encountered these items in their natural environment, i.e., a soda can (6 cm diameter x 12 cm height) and a small, triangular block of cement (10 x 10 x 7 cm³). The novel objects were placed roughly 20 cm to the left and 20 cm to the right of the central point of the wading pool (see Figure 4-1), and bishop fish were tested approximately one week after capture.

A trial began by gently netting and releasing an individual into a fixed position at the edge of the pool, equidistant from either of the novel objects. An acclimation period and start cylinder were not used to maintain novelty of the test environment (Redfern *et al.* 2017). A webcam placed 2.5 feet above the pool was connected to a PC where an observer positioned 2 m behind an opaque curtain monitored each subject during a 5 min trial.

Once all trials were completed, each video was transferred into an editing program where circles were drawn onto the video image to mark the bounds of the object areas and the center of the pool (Figure 4-1). A circle was overlaid on top of both objects to reflect approximately one

fish body length around each object. Another circle bounded the edges of both objects to create the center area of the pool, so a distinction between center and edge could be made.

Latency to reach the center of the pool, the proportion of time spent in the center, and proportion of time spent frozen were noted. The latency to approach each object and time spent within one body-length of the objects was also examined. An approach to an object and time spent around an object were defined as when at least half of the individual's body crossed over the circle representing one body length around the objects.

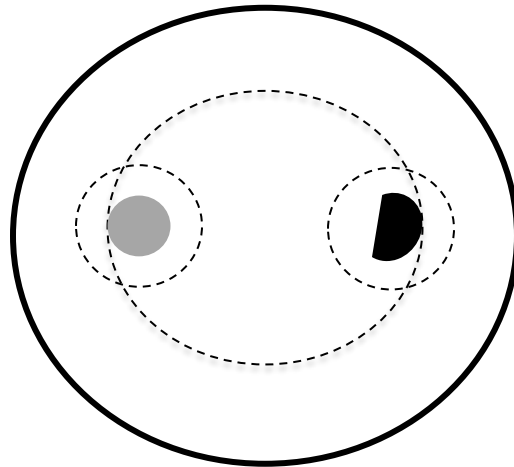


Figure 4-1: Schematic drawing of the exploration assay. The novel objects, i.e., the soda can on the left (grey circle), and cement block on the right (black symbol), were 20 cm from the central point of the pool. The large dashed circle represents the center area of the pool, which is bounded by the outermost edge of each novel object. The smaller dashed circles represent the immediate area around the novel object, which was determined by the estimated body length of each fish during each video trial.

Ethical Note

The experimental design and all handling procedures were approved under IACUC Protocol # 45265.

Statistical Analyses

Data were checked against the assumptions of analyses of variance (ANOVA), and were tested for equality of variance and transformations were performed as needed. Significance was tested at $\alpha = 0.05$. Main factors of stream origin and predation level (low and high) were compared in all analyses. Latency to the center of the pool, the proportion of time spent in the center, latency to approach either object were compared for all groups. Latency to the center was \log_{10} transformed, and all proportional data were arcsine square root transformed. Any data that did not meet the assumptions for ANOVA were tested with Mann Whitney U tests. All videos were analyzed with the BORIS behavioral coding software (Friard *et al.* 2016).

Results

Latency to center There was no difference in the amount of time low, or high predation fish took to reach the center of the arena ($F_{1, 45} = 2.11, p = 0.15$; Figure 4-2). However, there was a main effect of stream ($F_{2, 45} = 11.52, p < 0.01$). There was no significant interaction between stream origin and predation level ($F_{2, 45} = 0.43, p = 0.65$).

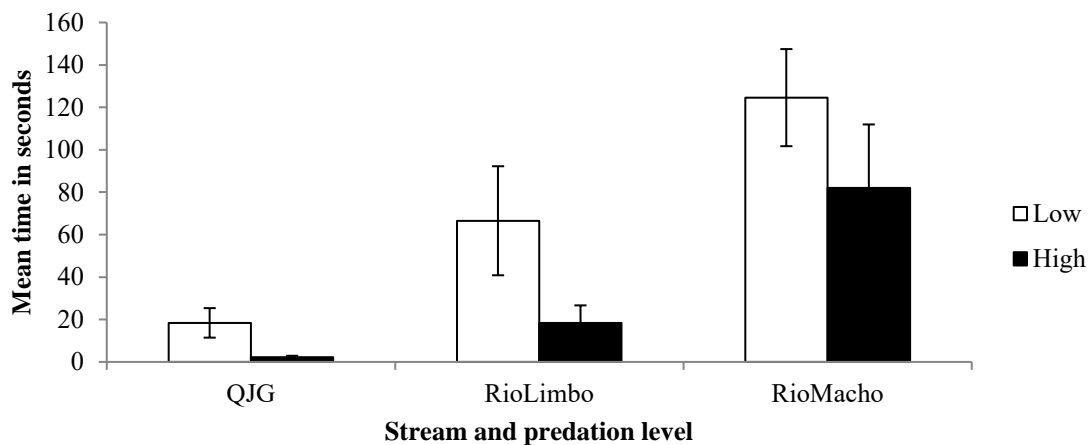


Figure 4-2: Mean latency to enter the center of the arena (\pm s.e) between low and high predation sites for all three streams. Raw data shown.

The proportion of time spent in the center There was no main effect of stream origin ($F_{2, 45} = 1.86, p = 0.16$; Figure 4-3), but there was a trend in the predation data ($F_{1, 45} = 3.70, p = 0.06$), and also a trend for an interaction between stream origin and predation level ($F_{2, 45} = 2.89, p = 0.06$).

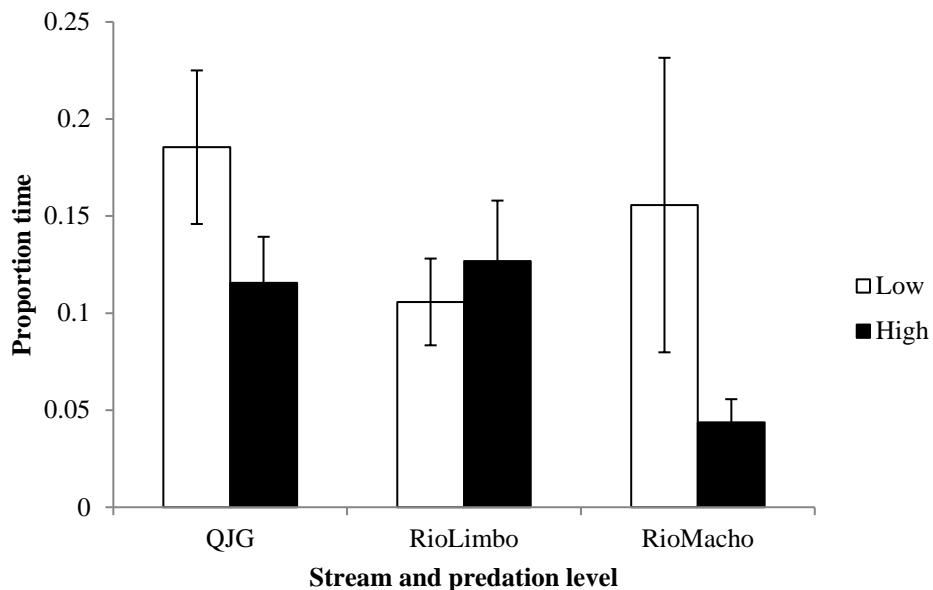


Figure 4-3: Mean proportion of time spent in the center of the arena (\pm s.e) between low and high predation sites for all three streams. Raw data shown, and time was measured in seconds.

Latency to approach the first object There was no difference in the amount of time for low or high predation fish to approach either the can or the block ($F_{1,45} = 0.07$, $p = 0.78$; Figure 4-4). However, there was an effect of stream ($F_{2,45} = 3.70$, $p = 0.03$). There was a trend for an interaction between stream origin and predation level ($F_{2,45} = 2.79$, $p = 0.07$). From Figure 4-4, the data show that Quebrada Juan Grande low predation fish were quicker than high predation fish to approach an object; however, Rio Limbo low and high predation fish behaved oppositely.

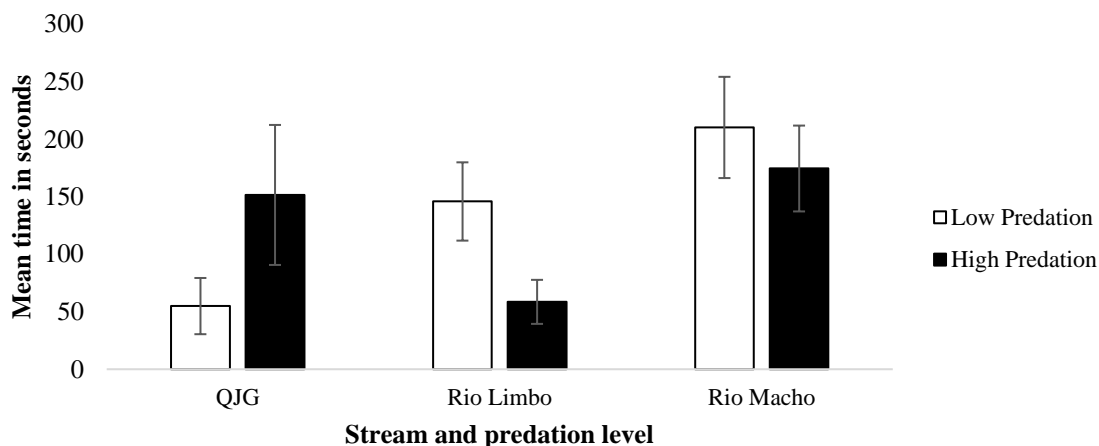


Figure 4-4: Mean latency to approach an object (\pm s.e) between all three low and high predation stream sites.

Latency to approach the can There was no main effect of stream origin ($F_{2,45} = 2.21$, $p = 0.12$) or predation level ($F_{1,45} = 0.31$, $p = 0.57$); however, there was an interaction between stream origin and predation level ($F_{2,45} = 7.64$, $p < 0.01$; Figure 4-5). Here, Quebrada Juan Grande low predation fish were quicker to approach the object compared to high predation fish, but Rio Limbo fish showed the opposite pattern

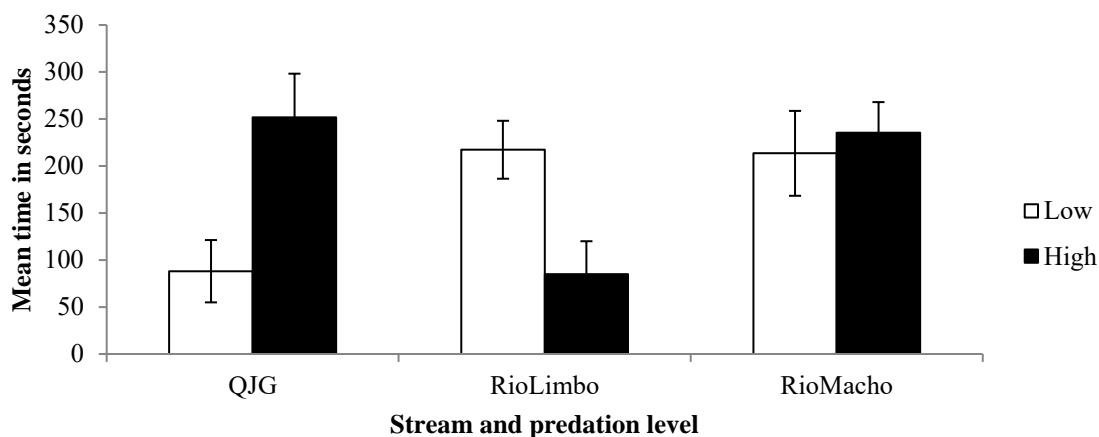


Figure 4-5: Mean latency to approach the can (\pm s.e) between all stream low and high predation sites.

Proportion of time spent around the can Mann Whitney U Tests showed no differences between the streams (Quebrada Juan Grande vs. Rio Limbo $Z = -0.23$, $p = 0.81$; Rio Limbo vs. Rio Macho $Z = -1.12$, $p = 0.26$ and $Z = -0.60$, $p=0.54$; Quebrada Juan Grande and Rio Macho $Z = -1.30$, $p=0.19$). For the predation level, there was only a difference for one stream comparison (Quebrada Juan Grande vs. Rio Limbo fish $Z = -0.07$, $p = 0.94$; Rio Limbo vs. Rio Macho $Z = -0.60$, $p = 0.54$; and Quebrada Juan Grande and Rio Macho $Z = -2.30$, $p = 0.02$).

However, on looking at the raw data, there appeared to be an outlier in the Quebrada Juan Grande low predation group. This fish had spent 21% (63 / 300 second trial) of their time around this object, while the rest of the sample spent between 0.7% to 7% of their time around it. The 21% data point is more than two standard deviations ($SD = 0.075$) away from the sample mean (Mean = 0.056). Once this outlier was removed, the data conformed to the assumptions of an ANOVA. This analysis revealed no effect of stream origin ($F_{2,44} = 1.30$, $p = 0.28$), no effect of predation level ($F_{1,44} = 1.71$, $p = 0.19$), but there was an interaction between stream origin and predation level ($F_{2,44} = 7.47$, $p < 0.01$; Figure 4-6).

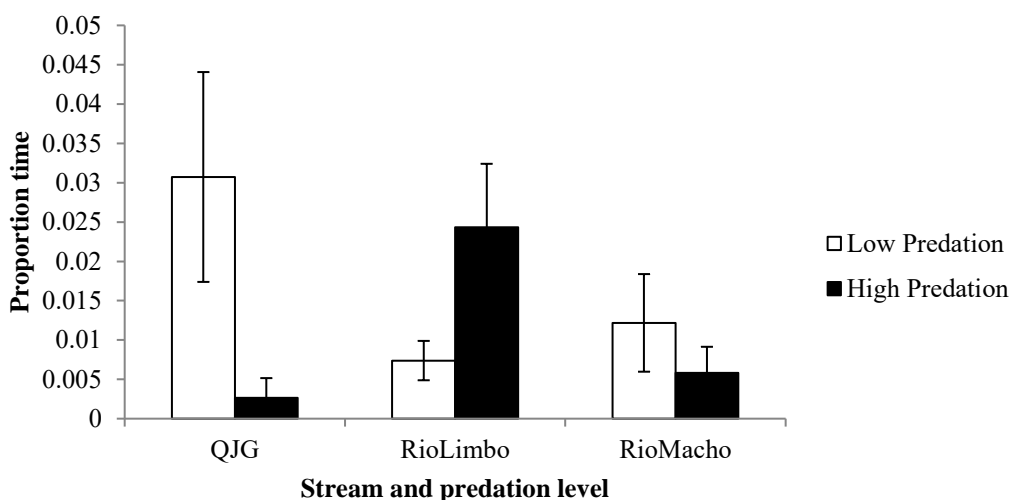


Figure 4-6: Mean proportion of time spent around the can (\pm s.e) between all stream low and high predation sites. Raw data shown, and time was measured in seconds.

Latency to approach the block There was no effect of stream origin ($F_{2,45} = 0.78$, $p = 0.46$), or predation level ($F_{1,45} = 0.28$, $p = 0.59$) on latency to approach the cement block, and there was no interaction between these two factors ($F_{2,45} = 0.86$, $p = 0.42$).

Proportion of time spent around the block There was no effect of stream origin on the proportion of time spent around the block ($F_{2,45} = 0.38$, $p = 0.68$), no effect of predation level ($F_{1,45} = 0.16$, $p = 0.68$), and no interaction ($F_{2,45} = 1.46$, $p = 0.24$)

Proportion of time spent frozen There was a main effect of stream origin ($F_{2,45} = 4.36$, $p < 0.05$; Figure 4-8), and a trend in the predation effect ($F_{1,45} = 3.12$, $p = 0.08$; Figure 4-7). There was no interaction between stream origin and predation level ($F_{2,45} = 1.19$, $p = 0.31$).

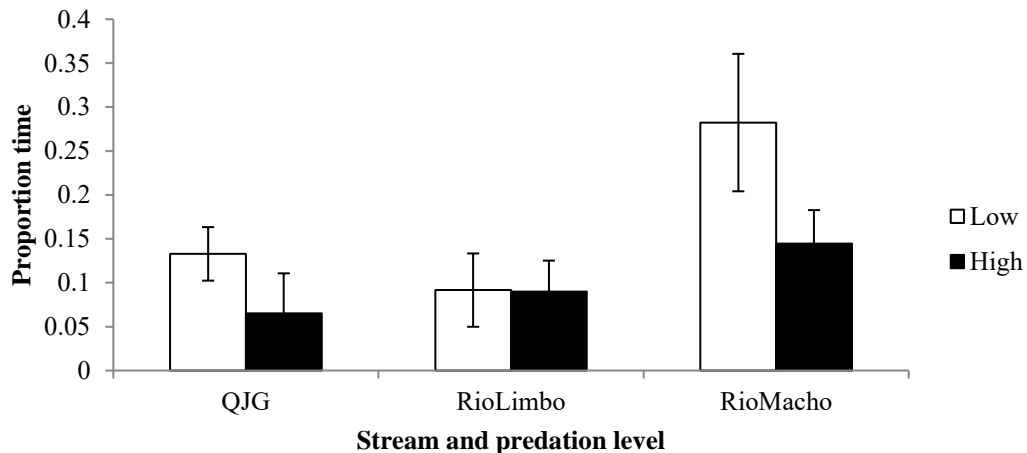


Figure 4-7: Mean proportion of time spent frozen (\pm s.e) between all stream low and high predation sites. Time was measured in seconds.

Discussion

I hypothesized that high predation fish would be more explorative than low predation fish because this would be consistent with the results reported in Chapter 3, and also with previous work detailing high predation fish usually displaying a proactive coping style. However, my results in the current chapter seem to have generated a more complex picture. First, there was a tendency for high predation fish to spend less time frozen than low predation populations, but it was also found that high predation fish tended to spend less time in the center of the arena. While the former implies decreased anxiety, the latter implies that high predation fish spent more time at the edge of the arena, presumably searching for shelter or an escape (Archard *et al.* 2012, Carlson and Langkilde 2013). Unfortunately, some of the sample sizes in the populations tested in this experiment were relatively low, and this may have contributed to some of the trends rather than significant differences in the data. It would be interesting to determine whether predation effects become more evident with larger sample sizes. Another way to test for this would be to use power analyses with the current data set.

Interestingly, it appears that stream origin plays a strong role in influencing the direction of exploration-avoidance behavior of the Panamanian bishop fish. Specifically, Rio Macho fish were found to be consistently less explorative and more cautious than Quebrada Juan Grande and Rio Limbo fish. Rio Macho fish also took the longest to reach the center of the arena (Figure 4-2) and spent the most time frozen out of all three streams (Figure 4-7). Furthermore, Rio Macho high predation fish appeared to spend less time in the center than Quebrada Juan Grande high predation fish (Figure 4-3) and took longer to approach an object compared to Rio Limbo high predation fish (Figure 4-4). The factors that differ between the streams which generate such differences in exploration is unclear. Earlier surveys on the same streams have indicated some variation in fish species (both non-predatory and predatory) across sites and rivers, but other

physical factors such as stream depth, canopy coverage, were broadly similar (Brown and Braithwaite 2004).

Previous work with the Panamanian bishop fish system have identified differences between low and high predation populations, as well as differences between streams (Brown *et al.* 2005, Archard *et al.* 2012, Beri *et al.* 2014). For example, a study looking at how quickly fish make decisions and the speed with which they react showed faster responses for high compared to low predation fish, but Quebrada Juan Grande fish also made more rapid responses compared with Rio Macho fish (Beri *et al.* 2014). The results reported by Beri *et al.* (2014) are similar to my observations with Quebrada Juan Grande fish being more exploratory than Rio Macho fish. In a different study investigating respiratory rate, at rest, low predation fish had a lower rate compared to high predation fish, but this was reversed after experiencing a stressor (Brown *et al.* 2005). This study also revealed differences in respiratory rates depending on stream origin, with Quebrada Juan Grande fish showing a higher rate overall compared to Rio Limbo and Rio Macho fish (Brown *et al.* 2005). It is interesting that it is often the Quebrada Juan Grande fish that were responding differently, as this appears to mirror some of the stream effects I report in this chapter.

Exploration behavior can be examined by quantifying an animal's responsiveness to a novel object by measuring the time it takes for an animal to approach an object, the amount of time the animal spends around it and so forth (Réale *et al.* 2007, Burns 2008, Archard and Braithwaite 2011a, Graham *et al.* 2018). Studies have examined exploration behavior to address a variety of questions, particularly ecological questions because of the effect of exploration on survival and fitness, however, many fewer studies have focused on investigating an animal's response to novelty in the form of physical stimuli (e.g., birds: Carere *et al.* 2005; fish: Galhardo *et al.* 2012; rodents: Cavigelli *et al.* 2011). Where it has been investigated, exploration behavior in the form of novel object responsiveness have unfortunately found mixed results (Roselini and Widman 1989 and Saul *et al.* 2012). While Roselini and Widman (1989) found a significant

effect of stress exposure on future novel object exploration behavior in rodents, Saul and colleagues (2012) did not. However, both studies differ in their study design; the former really investigates boldness because the authors allowed their study animals to habituate to the novel object with repeated testing, while the latter only exposed individuals to the novel object once. Roselini and Widman (1989) also exposed rodents to a total of 80 acute stress events, while Saul and colleagues (2012) only exposed rodents to a total of 6 events. The differences in experimental design make it difficult to directly compare these two kinds of study. Thus, there is not much in the current literature that has tested how stressors (acute, or experienced during earlier stages in life) influence exploration traits. My results suggest that stressors (in this case predation pressure) do not have as strong an effect on exploration traits as they do on other temperament traits such as anxiety or boldness (see chapter 3, Brown and Braithwaite 2004).

An animal's physiological response to stress coupled with its temperament comprise an animal's overall coping style, and this influences how it reacts to stressful situations (Koolhaas *et al.* 1999, Coppens *et al.* 2010, Koolhaas *et al.* 2011, Archard *et al.* 2012). The novel object exploration work of this chapter, coupled with previous results and past bishop fish temperament work, indicate that contrasting predatory exposure can influence how an animal copes with stress (boldness behavior: Brown and Braithwaite 2004; exploration and activity behavior: Archard and Braithwaite 2011a; respiratory rate: Brown *et al.* 2005; cortisol release rate: Archard *et al.* 2012). My results, however, also highlight something else is generating differences in temperament, behavior, and physiology; the streams used in my thesis, and in previous experiments, consistently show effects of their own (Brown *et al.* 2005, Archard *et al.* 2012, Beri *et al.* 2014). What is generating these stream differences remains unclear. There are some gradient differences between the sites that could affect flow rates and some differences in the fish species living in each stream. However, for many other measured physical characteristics, there do not appear to be obvious differences that explain the consistent stream effects (Brown *et al.* 2005). A study

focusing on what might be driving differences between streams would be useful because a better understanding of the field system as a whole would allow for an improved interpretation of the way in which different factors contribute to stream and population differences. It is interesting to note that the stream effects appear to be real and consistent, and yet they are often over-looked compared to population effects that focus on differences in predation. Determining what might be underlying stream effects could help refine predictions and hypotheses regarding this system for future studies.

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Chapter 5

Can we increase predictability in the captive zebrafish environment in the form of human facial recognition?

Abstract

Living within a captive environment can deliver certain advantages; such as access to food and protection from predation. However, captivity also removes an animal's capacity for autonomy, which can lead to harmful effects, such as abnormal behavior, altered physiological stress responses and suppressed immune function. One successful method to alleviate stress exposure within captivity is to provide some predictability for captive animals, such as husbandry tasks fish perceive as positive, e.g., feeding, as well as events that are likely to be stress-inducing, e.g., handling. By doing this, animals might be able to prepare behaviorally and physiologically for impending disturbances. In this chapter, I investigated the effects of providing predictability to captive zebrafish. Using visually distinct human caretakers, predictability was manipulated by giving different caretakers distinct roles. In the first experiment, fish experienced one caregiver providing food, while the second person's role was to present a novel object. In a second experiment, caretakers fed fish or induced a stressor in the form of net-chasing. Several behaviors were noted to determine if fish behavior changed in accordance to which caretaker was involved.

During the final stages of both experiments, after fish had experienced multiple interactions with the caretakers, the zebrafish were found to move into feeding areas when they saw the caretaker associated with feeding, or they moved out of the feeding area when presented with a caretaker associated with the presentation of a novel object or net-chasing. These data show that zebrafish are capable of the facial recognition of individual humans and that this is a potentially an easy way to introduce more predictability into a captive environment.

Introduction

Within the last decade, zebrafish have increasingly been used to address a wide range of research questions (Guo 2004, Colwill *et al.* 2005, Egan *et al.* 2009, Champagne *et al.* 2010, Oliveira *et al.* 2015, Hamilton *et al.* 2016, May *et al.* 2016, Meshalkina *et al.* 2017, Graham *et al.* 2018 a and b). As such, they are a widely used model species, but one for which little is known concerning their overall welfare, and their ability to cope in captivity (Ebbesson & Braithwaite 2012). These mysteries surrounding zebrafish stem from the difficulties that come with conducting *in-situ* studies within the turbid streams of their natural environment (Ebbesson & Braithwaite 2012, Graham *et al.* 2018 a and b).

Despite this, researchers have made best efforts to examine zebrafish behavior within a laboratory setting to better understand how to improve their welfare in captivity (Graham *et al.* 2018b). As a result, researchers have discovered that zebrafish are capable of various complex cognitive tasks, such as discriminating between objects of different shapes and colors (Colwill *et al.* 2005, May *et al.* 2015, Aoki *et al.* 2015, Hamilton *et al.* 2016, Graham *et al.* 2018a and b). Moreover, it is well known that adding different objects or structures into a captive animal's environment can improve their welfare (Salvanes *et al.* 2007, Depasquale *et al.* 2016, Graham *et al.* 2018a).

However, solely adding environmental enrichment into the captive zebrafish environment may not be enough to improve their overall welfare. Captive animals are frequently exposed to stressful stimuli (e.g., handling and cleaning of enrichment), which can lead to changes in the animal's physiology and behavior (Bassett and Buchanan-Smith 2007, Morgan and Tromberg 2007). Although captive animals are often perceived to be living in a much safer environment compared to the wild, they are still at risk of chronic stress exposure due to frequently encountering stimuli that would not normally be encountered in the wild (Morgan and Tromberg

2007, Bassett and Buchanan-Smith 2007). Minimizing stress exposure within a captive environment is desirable if we are to promote welfare and well-being of captive animals.

One way to reduce the effects of a stressor might be to introduce predictability into an animal's environment due to stressors being defined as stimuli that are perceived to be unpredictable and uncontrollable (Oliveira and Galhardo 2009, Morgan and Tromberg 2007, Hau *et al.* 2016). Increased predictability, however, could help an animal to prepare both behaviorally and physiologically for the upcoming stressor (Badia *et al.* 1979, Davis and Levine 1982, Bassett and Buchanan-Smith 2007, Galhardo *et al.* 2011, Madaro *et al.* 2016). This is known as the preparatory response hypothesis (Badia *et al.* 1979). Predictability is also tightly linked with control, in that introducing predictability into an animal's environment can increase an individual's perceived control of a stimulus, which may further minimize the power of the stressor (Bassett and Buchanan-Smith 2007, Oliveira and Galhardo 2009).

One method to introduce predictability into a captive animal's environment is to provide a warning signal before the onset of an event, especially if aversive (Bassett and Buchanan-Smith 2007, Galhardo *et al.* 2011, Madaro *et al.* 2016). The additional benefit of utilizing signaled predictability is that when the signal is not in use, this indicates to the captive animal that the environment is safe. This is known as the safety hypothesis (Badia *et al.* 1979, Bassett and Buchanan-Smith 2007, Galhardo *et al.* 2011, Gottlieb *et al.* 2013). Benefits of introducing predictable positive events such as feeding regimes are less clear (Bassett and Buchanan-Smith 2007). While some studies show beneficial effects such as increased social behavior, other studies highlight negative effects such as heightened anxiety and anticipatory behaviors (Ulyan *et al.* 2006, Bassett and Buchanan-Smith 2007, Galhardo *et al.* 2011). Therefore, it seems that there is more promise in introducing predictable adverse events into a captive environment, rather than positive.

Work surrounding the signaled predictability concept stems back as early as the 1950s, with researchers examining potential differences in behavior and stress physiology between laboratory rats presented with predictable versus unpredictable shocks (Badia *et al.* 1971, Badia *et al.* 1979, Davis and Levine 1982, Bassett and Buchannan-Smith 2007). Despite discrepancies across various studies, such as inconsistencies in behavioral or physiological variables examined, it has been shown that study animals prefer signaled and temporal predictable aversive events over unpredictable ones (Badia *et al.* 1971, Badia *et al.* 1979, Bassett and Buchannan-Smith 2007).

Over the last decade, preference in signaled predictable aversive events has been examined in the context of animal husbandry routines (e.g., daily cleaning, feeding, enrichment modification) conducted within a captive environment (Ulyan *et al.* 2006, Greiveldinger *et al.* 2007, Gottlieb *et al.* 2013, Rimpley and Buchanan-Smith 2013). Several of these studies have identified physiological and behavioral benefits of predictable aversive events such as reduced anxiety and cortisol production before the event's onset (sheep: Greiveldinger *et al.* 2007; capuchin monkeys: Rimpley and Buchanan-Smith 2013; cichlid fish: Galhardo *et al.* 2011). These studies also utilized cost-effective signals, such as a cue card or a light being switched on. However, considering that within a captive environment an animal likely interacts with the same staff daily for feeding, cleaning, and other general husbandry duties, the caretakers themselves could be another potential route for providing predictability (Bassett and Buchannan-Smith 2007, Rimpley and Buchanan-Smith 2013). For this to work, however, captive animals would need the ability to recognize individual human caretakers.

In humans, the ability to recognize specific faces is processed by parts of the neocortex, which is a unique six-layered structure composed of cortical neurons (Tong *et al.* 2000, Hoffman *et al.* 2007, Kaas 2011). However, the ability to discriminate between human faces has also been identified in non-human species that lack a sophisticated neocortex, such as cattle, sheep, dogs,

pigs and even crows (cattle: Taylor and Davis 1998, sheep: Peirce *et al.* 2001, dogs: Huber *et al.* 2013, pigs: Wondrak *et al.* 2018, crows: Marzluff *et al.* 2010). Therefore, it seems that facial recognition may be possible through processes such as object recognition (Mandeville *et al.* 2003, Hoffman *et al.* 2007, Jüttner *et al.* 2016).

Furthermore, evidence of discrimination between pictures of human faces has also been recently reported in fish (Newport *et al.* 2016). However, it has yet to be seen if fish can recognize real, three-dimensional human beings as well as recognize them in regards to specific roles they may have. Determining that fish can discriminate between different caregivers could potentially be a method to help captive fish to better predict and cope with different events that may be about to happen to their captive environment.

In this chapter, I describe two experiments that investigated whether zebrafish could discriminate between different human caretakers and their respective roles. In the first, two kinds of positive reinforcement were used; feeding, or access to investigate a novel object. These tasks were chosen as both are considered rewarding within a captive environment, and both are directly applicable to a daily husbandry routine (Morgan and Tromberg 2007, Bassett and Buchanan-Smith 2007). In the second experiment, fish were tested to see if they could distinguish between a caretaker that always fed the fish, versus a caretaker associated with an aversive stimulus; a chase with a small, hand-held dip-net within the feeding area.

Methods

Subjects

Adult wild-type zebrafish (*Danio rerio*) were acquired from Pennsylvania State University's College of Medicine, Hershey, PA (n = 100). The fish were divided across ten different tanks (91 x 30 x 30 cm³ with a water depth of 25 cm) such that there were ten fish per tank. Each tank contained a heater, a biofilter, and various plastic plants for enrichment. The tanks were housed in two different rooms, five in Laboratory 1, and five in Laboratory 2. Experimental tanks were visually isolated from one another, and black plastic was wrapped on the back and one side of the tank to minimize outside disturbance.

For the second experiment, new adult wild-type zebrafish from the College of Medicine (n = 120) were housed as above, but this time across twelve tanks (91 x 30 x 30 cm³ with 25 cm water depth). All tanks were located in the same laboratory, but six tanks were located in the front half of the lab (Row 1), and six tanks were located in the back of the lab (Row 2). An opaque partition separated the first and second rows, so again the experimental tanks were visually isolated from one another.

All tank temperatures were maintained between 25°C ± 1°C, and the outward laboratory environment was set on a 12:12 light-dark cycle. The fish were fed with standard commercial fish flake daily according to the experimental protocol below.

Facial Recognition Experiment 1: Experimental Set Up

During January 2017, after an initial two-week acclimation period, contents within all ten zebrafish tanks were re-arranged to create a central enrichment area (25 cm long), and two end

barren areas (each 20 cm long, see Figure 5-1). The central enrichment area contained plastic plants of different sizes, the tank heater, and biofilter. Each empty end-area was used for a specific kind of interaction with the fish; either feeding or the presentation of a novel object. On the end associated with the novel object presentation, the black plastic was partially pulled back so that the novel object could be presented from the side, outside of the tank. At the opposite end, the food area contained a floating, plastic ring that was attached to the end wall toward the front of the tank such that the ring remained in a fixed position at the surface of the water. A small pinch of commercial fish flake was placed within the food ring when fish were being fed during the experiment.

For 26 days novel objects were presented to the fish, or food was delivered to the food ring by two caretakers (A and B) that looked different to each other (Figure 5-2). The experiment was designed so that the role of the caretakers was counterbalanced across the two rooms, so Caretaker A fed the tanks in Laboratory 1, but presented novel objects in Laboratory 2, whereas Caretaker B presented novel objects in Laboratory 1, and fed in Laboratory 2. Furthermore, the layout of the experimental tanks was counterbalanced with five tanks having the food end on the left and novel object end on the right with the remaining five tanks having the opposite.

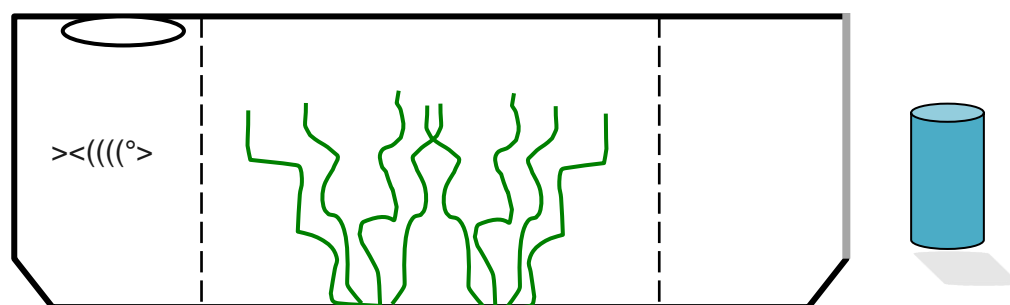


Figure 5-1: Schematic drawing of the experimental set-up for the first facial recognition experiment. The dashed lines represent the imaginary partitioning of the tank to create three distinct areas. (A) The feeding area containing a food ring (circle) at the top of the water column. (B) A central enrichment filled area. (C) An empty area containing an uncovered wall (indicated by the grey line) allowing fish to see novel objects through the side glass wall (the blue cylinder represents a novel object).

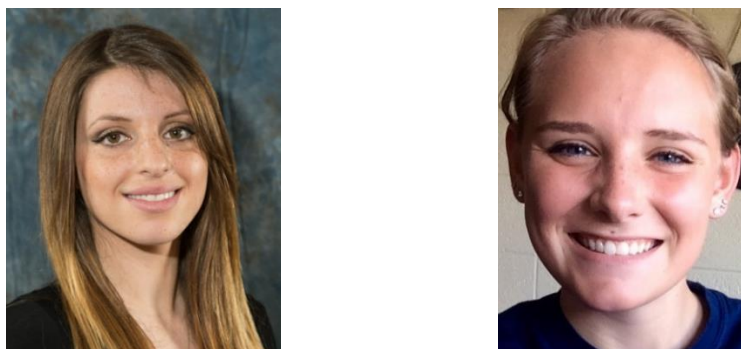


Figure 5-2: Pictures of Caretaker A (Alisha Pushinsky) and Caretaker B (Faith Leri) who were assigned specific tasks during both experiments 1 and 2.

Facial Recognition Experiment 1: Experimental Protocol

A trial began by a caretaker quietly entering a laboratory and noting baseline fish behavior from a distance. The baseline measurements quantified cohesion of the fish, their coordination, any aggression, and the position of the majority of the fish within each tank. Cohesion, coordination, and aggression were scored on an ordinal scale (Table 5-1), and the position was noted as either ‘food,’ ‘enrichment,’ or ‘object’ area.

After baseline measures were noted for all five tanks, a caretaker would then approach the first of the five tanks and present their face for 30 seconds in the front center of the tank. This process involved turning the head to the left for ten seconds, turning to the right for ten seconds, and staring head-on at the fish for ten seconds to present all sides of the face of both caretakers. After doing this for one tank, it was then repeated until all the tanks within that lab had experienced this process.

Next, the caretaker returned to the first tank and noted the position of the fish to determine if the fish had moved to one tank end or the other. After position was noted for all five

tanks, a caretaker would then either feed fish in the food area or present a novel object for 10 seconds. This process was repeated for all five tanks. A new object was presented every day to maintain novelty. The objects varied in size, shape, and color and included items such as a small purple umbrella, a medium sized white bowl, a pair of scissors, etc.

Cohesion, coordination, and aggressive behavior of the fish were scored again during a 10 second period during the time the fish were either fed or had the novel object presented. When this protocol was completed for one laboratory, the caretaker visited the other laboratory and used the same protocol to complete the behavior scoring and tasks for that laboratory.

Cohesion, coordination, and aggression are considered to be valid indicators of interest of positive emotion (affect) when animals are in the absence of threatening stimuli (Graham *et al.* 2018a). Therefore, these measures were used to determine which task fish found less aversive. It was predicted that feeding would be found to be more appealing due to its necessity for survival, i.e., group cohesion and coordination were expected to be higher, and aggressive behavior to be lower during feeding (Oliveira *et al.* 2015, Graham *et al.* 2018a).

Behavior	Score	Description	Definition
Cohesion	0	None	Fish scattered throughout the tank evenly
	1	Low	Fish scattered throughout the tank for most of the coding period
	2	Moderate	Fish occasionally grouping/becoming cohesive and within four body lengths of each other (BL)
	3	High	Majority of fish grouping together throughout the coding period (up to 2 BLs)
	4	Very high	Majority of fish in a very tight group for >50% of the coding period (being less than 2 BLs apart)
Coordination	0	None	Fish pointing in all different directions
	1	Low	Fish mostly pointing in different directions for most of the coding period (some brief periods of synchronized coordination)
	2	Moderate	Some fish occasionally seen to be pointing in the same direction and coordinated in their swimming
	3	High	Majority of fish becoming very synchronized/coordinated with each other for periods throughout the coding period
	4	Very high	Majority of fish highly synchronized for extended periods throughout the entire coding period
Aggression	0	None	No aggressive behavior
	1	Any agonism observed	At least a single occurrence of aggressive behavior, such as chasing, charges, lateral displays, nips, bites, etc.

Table 5-1: This ordinal scale of cohesion, coordination, and aggressive behavior was scored by Caretaker A and B to quantify group zebrafish behavior. Cohesion behavior was defined as the degree to which the fish within each tank were tightly grouped, coordination detailed the degree to which fish were swimming in the same direction, and aggressive behavior was indicated by a single occurrence of chasing, biting, etc.

Facial Recognition Experiment 2: Experimental Set Up

During September 2017 a follow-up experiment was performed with new fish. After a two-week acclimation period, 12 zebrafish tanks (91 x 30 x 30 cm³ at a water depth of 25 cm) were arranged such that there was a central empty area (25 cm long), and then two smaller areas (each 20 cm) at either end of the tank (Figure 5-3). One end of the tank was designated the enrichment area which contained various plastic plants, a tank heater, and biofilter, and the opposite end of the tank was designated the feeding area. Again, this area contained a plastic ring where commercial fish flake was placed when fish were fed during the experiment.

For 35 days, Caretakers A and B (Figure 5-2) were assigned specific tasks, but for this experiment, the tasks were to either feed the fish, or to stress the fish by rapidly moving a small dip-net around in the feeding area for 10 seconds. This experimental protocol was again counterbalanced with Caretaker A feeding the fish in the food area for Row 1 tanks and stressing the fish with a dip-net in Row 2. Caretaker B stressed the fish in Row 1 but fed the fish in Row 2. Rows 1 and 2 were completely visually isolated from each other. As before, the layout of all tanks was counterbalanced with six tanks having the food area on the left and enrichment area on the right, and the remaining six tanks set up in the opposite way.

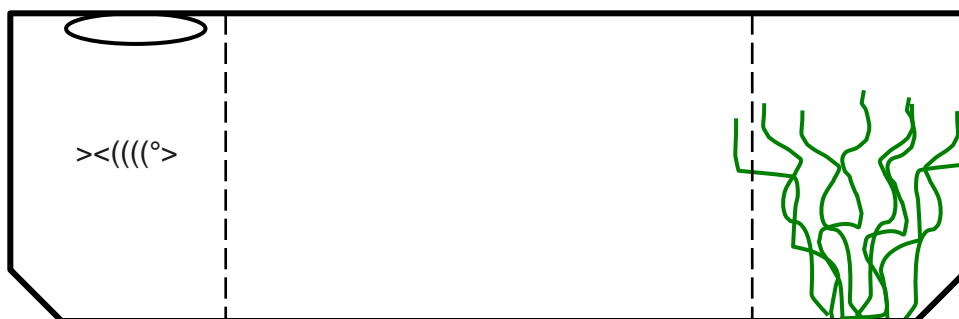


Figure 5-3: Schematic drawing of the experimental set-up for experiment 2. (A) The feeding area of the tank containing a plastic food ring (circle) at the top of the tank. (B) Empty middle area. (C) Enrichment area.

Facial Recognition Experiment 2: Experimental Protocol

A trial began when a caretaker entered the lab and quietly noted baseline measures at a distance to quantify fish cohesion, coordination, aggression, the position of the majority of the fish, and the number of fish in the food area within each tank for Row 1. For both experiments, it was predicted that more fish would be in the food area after being presented with the caretaker associated with this task, compared to the caretaker associated with the novel object presentation or net-chasing.

Cohesion, coordination, and aggression were scored on an ordinal scale (Table 5-1), and the position was noted as either ‘food,’ ‘enrichment,’ or ‘middle’ area. After baseline measures were noted, the caretaker then approached one tank at a time and presented their face in the front of the middle of the tank for 30 seconds. Again, this process involved turning the head to the left for ten seconds, turning to the right for ten seconds, and then staring head-on at the fish for ten seconds to present all sides of the face.

Next, the caretaker took a step back and noted over a 10 second period the position of the fish and the number of fish in the food area. This scoring was done to determine whether the fish could predict where they should move to, i.e., to the food area, or away from the food area (impending stressor). Cohesion, coordination, and aggressive behavior of the fish were also scored again during this period (Table 5-1). For this experiment since these behavioral measures were examined in a threatening context, they were now indicative of stress-related behaviors (Graham *et al.* 2018a). It was predicted that there would be increased cohesion, coordination, and decreased aggression when fish were presented with the caretaker associated with the net-chasing stressor. After noting these measurements, a caretaker then fed fish in the designated food area or chased the fish with a dip-net in the food area for 10 seconds. This process was repeated for the rest of the tanks within Row 1, and the entire protocol was then repeated for Row 2.

Statistical Analyses

For Experiment 1, all measurements were taken at two-time points; (i) baseline measures of cohesion, coordination, and aggression, and (ii) these were repeated during Caretaker/tank interactions, i.e., feeding or object presentation. These values were used to generate a difference score for each of the ten tanks on all 26 days of the experiment (i.e., the baseline measurement was subtracted from the second measurement to determine a potential change or difference from the start of the trials to the point when the fish were feeding or being presented with the novel object). The difference scores were then averaged across all tanks over days 1 to 26 to create a single measurement for cohesion, coordination, and aggression for the fish as they interacted with food, or with a novel object (see Table 5-2 as an example). Average values across all tanks were calculated because each tank was used as a replicate.

For Experiment 2, the same analysis (i.e., calculating average values across tanks) was used for cohesion, coordination, and aggression data over the 35 days of the experiment. However, the second measurement was carried out following the facial presentation and before any tank interaction. This change in methodology was used to determine whether group social behavior was altered at all by the fish recognizing the identity of the two caretakers and their roles.

The caretakers also measured the position of fish in Experiments 1 and 2. The first measurement was taken at baseline and the second was noted following the facial presentation but before tank interaction. These measures were based on where in the tank the fish were schooling. Locations were coded regarding whether the fish were moving into the feeding area when they saw the caretaker associated with food or moving away from the feeding area when they saw the caretaker associated with the novel object (Experiment 1) or net chasing (Experiment 2).

If fish started in a non-food area at baseline and moved into the food area for the second measurement, this was coded as +1. If fish started in the food area at baseline and moved into a non-food area (e.g., the area associated with novel object presentation) for the second measurement, this was coded as -1. However, this method does not account for the fish that potentially waited in the food area. Therefore, fish that started in the food area at baseline and stayed there for the second measurement were coded as +1 for the first position analysis. A second position analysis was also conducted where the data for fish that stayed within the feeding area both during the baseline and the second measurement, was excluded by coding this phenomenon as 0. This second analysis was undertaken to solely account for fish that made an active choice to enter into the feeding area.

The position codes were averaged across all tanks for days 1 through 26 for Experiment 1 and days 1 through 35 for Experiment 2. Lastly, for Experiment 2, the specific number of fish was noted both at baseline and following facial presentation, and before tank, interaction to further investigate a potential change in fish position. Both measurements were conducted as not all fish swam consistently within the school; therefore measuring school position alone could potentially miss the movements of individuals.

After all measures were averaged across all tanks for the duration of the experiments, and unpaired t-tests were used to compare the behavior of the fish at baseline and after seeing a caretaker. A Bonferroni correction was also used to decrease the risk of a Type I error or finding false-positives due to conducting multiple t-tests (Kao and Green 2008). Overall, ten t-tests were conducted for the first experiment so, and an alpha value of 0.005 was used (0.05 divided by 10). For the second experiment, a total of 12 t-tests were conducted, so an alpha value of 0.0041 was adopted.

For all t-tests, nominal variables of the task (e.g., feeding or object presentation) and day of the experiment were analyzed. Day of the experiment was examined by taking the total number

of days and then dividing this by 3 to create three groups of data or a beginning, middle, and end group of the experiment. For example, for Experiment 1, there were 26 days of observations, as 26 divided by three is roughly nine, the analyses focused explicitly on the first nine days and the last nine days of the experiment to determine whether the fish were learning and changing their behavior in response to seeing the caretakers as the experiments progressed. To examine differences in group social behavior (cohesion, coordination, and aggressive behavior), the measures were again compared for the first and last nine days of the experiment. In Experiment 2, owing to the longer number of days over which the fish were measured (35 days), the first 12 days were compared to the last 12 days of the experiment.

Day	Time	Position	Cohesion	Coordination	Aggression
1	Beginning	-0.4	0.3	-0.8	0.1
2	Beginning	0.2	0.1	-0.5	-0.1
3	Beginning	0	0.9	-1	0.1
4	Beginning	0	1	-0.8	-0.3
5	Beginning	0.2	1.1	-1	-0.1
6	Beginning	0	0.7	-0.7	-0.2
7	Beginning	0.4	0.7	-1	0
8	Beginning	0.4	0.9	-0.9	0
9	Beginning	0.6	0.3	-0.5	-0.1
10	Middle	0.6	0.4	-0.5	0
11	Middle	0.6	0.6	-1.2	-0.2
12	Middle	0.4	0.7	0.3	0
13	Middle	0.4	1.1	0.5	-0.1
14	Middle	0.6	1.4	0.1	-0.5
15	Middle	-0.2	2	0.5	0
16	Middle	0.6	1.4	-0.3	-0.1
17	Middle	1	1.4	0	0.1
18	End	0.8	1.1	-0.6	-0.1
19	End	0.8	1.6	0.5	-0.1
20	End	0.8	1	0.5	0
21	End	0.8	1.9	0.7	-0.5
22	End	0.4	1.4	0.3	0.2
23	End	0.2	1.4	-0.2	-0.2
24	End	0.6	1.3	0.5	-0.1
25	End	0.4	1.6	0.4	-0.1
26	End	0.8	1.4	0.7	-0.2

Table 5-2: Zebrafish average difference scores for the feeding assay during Experiment 1, where ‘Beginning’ relates to days 1 through 9, ‘Middle’ represents days 10 to 17 and ‘End’ represents days 18 to 26.

Results

Facial Recognition Experiment 1

Behavior during the first nine days of the experiment During the beginning of the experiment, there was no difference in the position of fish when presented with either a caretaker associated with feeding or a novel object ($t_{1,16} = 0.77$, $p = 0.45$). This test accounted for data when fish stayed in the feeding area before and after the facial presentation of a caretaker. When excluding fish that did not move, and only accounting for fish that moved into or away from the feeding area, there also was no significant difference when fish were presented with either caretaker ($t_{1,16} = 0.99$, $p = 0.33$).

When examining group behavior, the fish were more cohesive ($t_{1,16} = 6.30$, $p < 0.0001$) and less coordinated ($t_{1,16} = -6.40$, $p < 0.0001$) during feeding compared to during the presentation of a novel object, but there were no differences in fish aggression ($t_{1,16} = -1.34$, $p = 0.19$).

Behavior during the last nine days of the experiment During the final nine days of experiment 1, fish moved into, or stayed in, the area associated with food when presented with the caretaker associated with feeding, but moved away from the food area when presented with the caretaker associated with novel object presentation ($t_{1,16} = 3.34$, $p < 0.005$; Figure 5-4). These results were also seen again when excluding data of fish staying in the food area before and after the facial presentation of a caretaker ($t_{1,16} = 3.96$, $p < 0.005$; Figure 5-5). Thus, by the end of experiment 1, the zebrafish appeared to be recognizing the role performed by the different caretakers.

Fish were also seen to be more cohesive during trials associated with feeding than when being presented with a novel object ($t_{1,16} = 12.04$, $p < 0.001$; Figure 5-6). Fish were also less aggressive during feeding, compared to when they were presented with a novel object ($t_{1,16} = -3.21$, $p = 0.0054$; Figure 5-7). There was no difference in fish coordination ($t_{1,16} = 1.68$, $p = 0.11$).

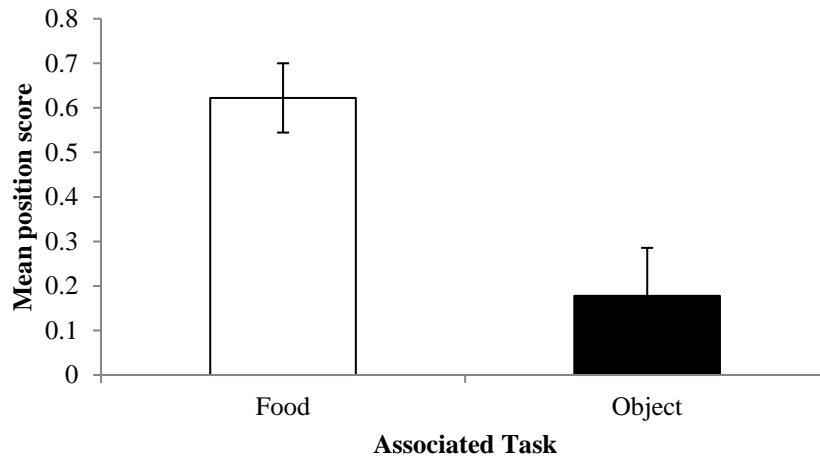


Figure 5-4: Mean position score (\pm s.e) for fish that were exposed to either a caretaker associated with food or with the presentation of a novel object. This position score includes fish that stayed in the feeding area both before and after the presentation of the caretakers.

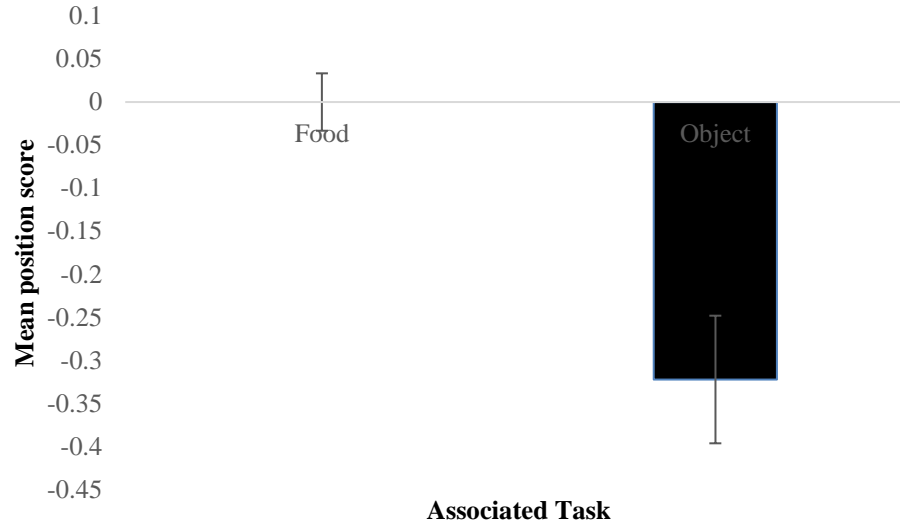


Figure 5-5: Mean position score (\pm s.e) for fish that were exposed to a caretaker associated with food or exposed to a caretaker associated with the presentation of a novel object. This position score excludes fish that stayed in the feeding area both before and after the presentation of either caretaker.

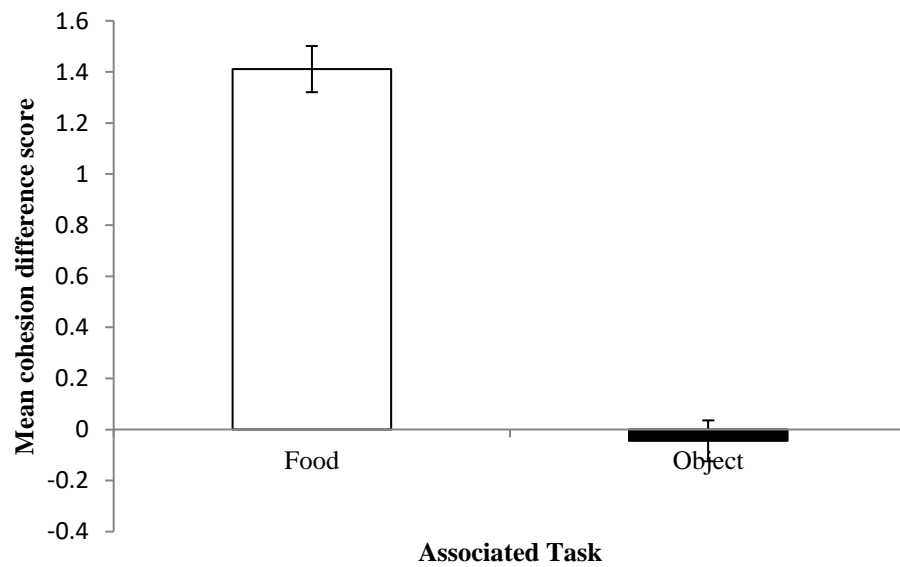


Figure 5-6: Mean cohesion difference score (\pm s.e) of fish before and during feeding, and before and during novel object presentation.

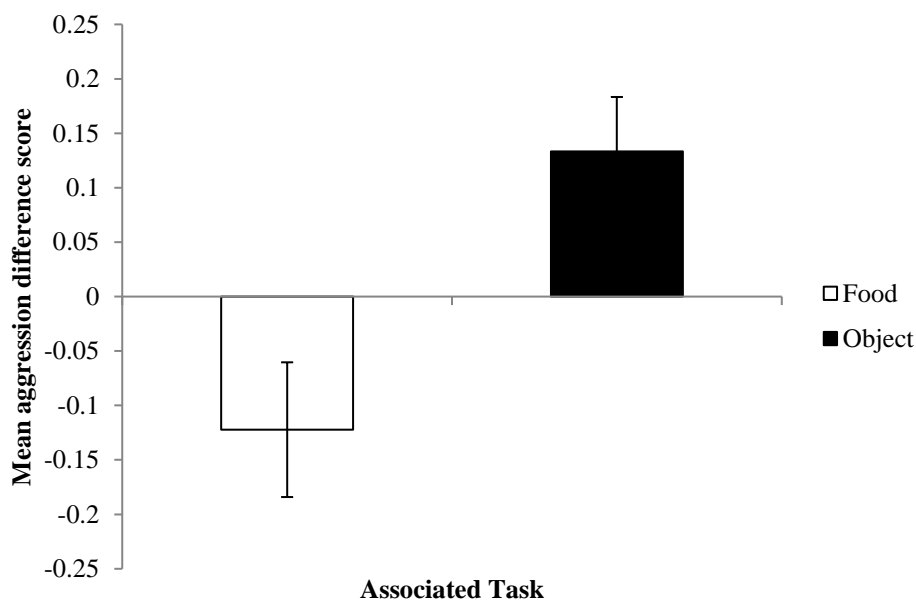


Figure 5-7: Mean aggression difference score (\pm s.e) of fish before and during feeding, and before and during novel object presentation.

Facial Recognition Experiment 2

Behavior during the first 12 days of the experiment During the beginning of the experiment, there was no difference in the position score when fish were presented with a caretaker associated with feeding compared to the net-chasing caretaker ($t_{1,22} = -0.039$, $p = 0.96$). When excluding fish that stayed in the food area before and after facial presentation, there was still no significant effect ($t_{1,22} = 0.096$, $p = 0.92$). Similarly, when comparing the number of fish in the feeding area, there were no differences regardless of which caretaker was present ($t_{1,22} = 1.39$, $p = 0.17$). With regard to group social behavior, there were no differences in cohesion ($t_{1,22} = -0.06$, $p = 0.94$), coordination ($t_{1,22} = 0.51$, $p = 0.61$), or aggressive behavior ($t_{1,22} = 0.66$, $p = 0.51$) when fish were presented with either caretaker.

Behavior during the last 12 days of the experiment At the end of the experiment, however, a higher number of individual fish moved into the area associated with feeding when presented with the feeding caretaker, but fish moved away from the feeding area when presented with the net-chasing caretaker ($t_{1,22} = 3.71$, $p < 0.005$; Figure 5-8). This result is also reflected by the position of the majority of fish following the caretakers presenting their faces ($t_{1,22} = 3.76$, $p < 0.005$; Figure 5-9). These results were consistent when excluding fish that stayed in the feeding area before and after facial presentation ($t_{1,22} = 4.11$, $p < 0.005$; Figure 5-10). Lastly, when examining group behavior, there were no differences in cohesion ($t_{1,22} = -4.30 \times 10^{-17}$, $p > 0.99$), coordination ($t_{1,22} = -0.05$, $p = 0.95$), or aggressive behavior ($t_{1,22} = 1.54$, $p > 0.99$) when fish were presented with either caretaker.

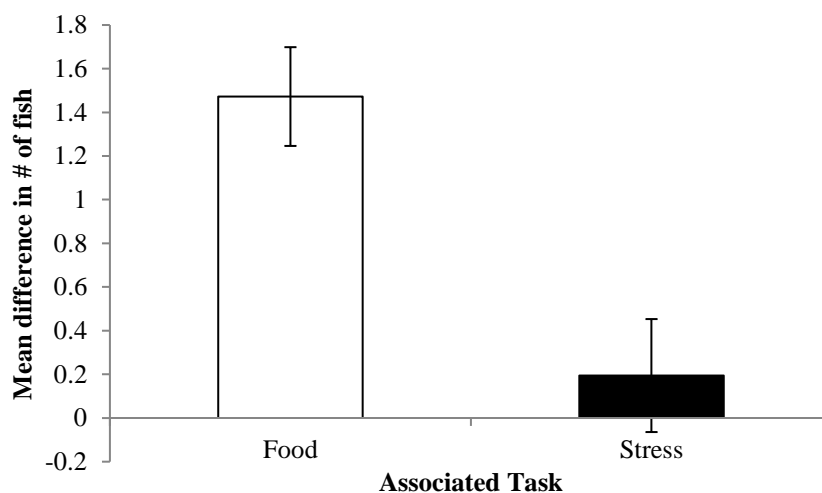


Figure 5-8: Mean difference between the number of fish in the feeding area (\pm s.e) after being exposed to a caretaker associated with food, or a caretaker associated with a stressor.

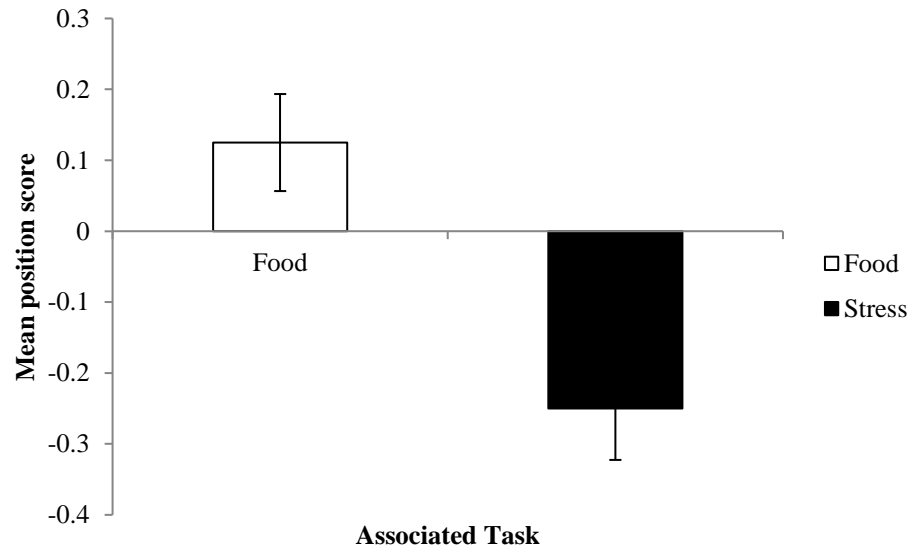


Figure 5-9: Mean position score (\pm s.e) for fish that were exposed to a caretaker associated with food, or a caretaker associated with a stressor. This position score includes fish that stayed in the feeding area both before and after the presentation of either caretaker.

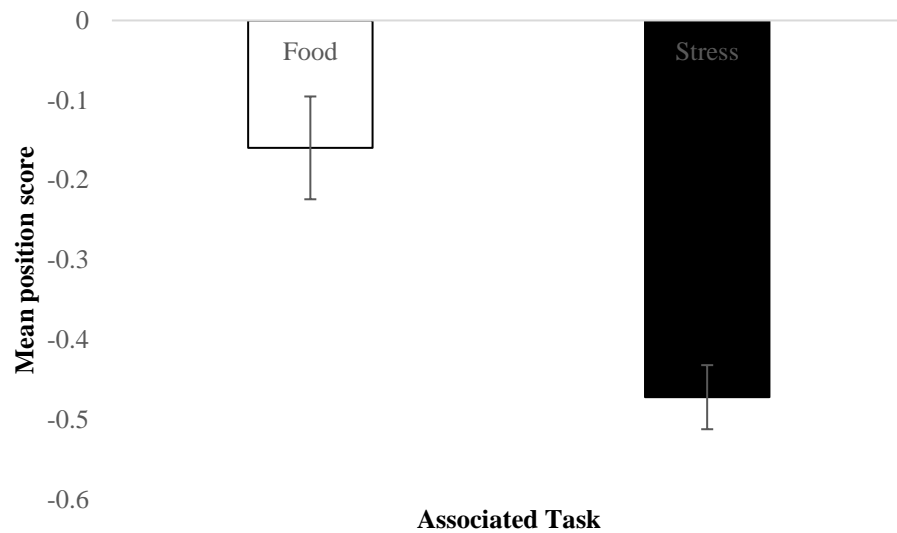


Figure 5-10: Mean position score (\pm s.e) for fish that were exposed to a caretaker associated with food, or a caretaker associated with a stressor. This position score excludes fish that stayed in the feeding area both before and after the presentation of either caretaker.

Discussion

Both experiments demonstrate that zebrafish can recognize individual human caretakers associated with different basic husbandry tasks. In the first experiment, fish moved into the area associated with food after the presentation of the caretaker associated with feeding but moved away from the food area after the presentation of the caretaker associated with novel object presentation. Similarly, in the second experiment fish moved into the feeding area after the presentation of the caretaker associated with feeding, and moved out of the feeding area after the presentation of the caretaker associated with net-chasing. These results were further corroborated when examining the actual number of fish within the feeding area, both before and after caretaker presentation. Importantly, these effects are only found at the end of the experiments, after the fish have been given time to learn the experimental protocol and the features of the two caregivers.

Signaled Predictability

In both experiments, zebrafish understood the significance of the human caretaker cue; when tested, zebrafish moved to the correct location within their home tank after seeing individual caretakers associated with their respective tasks. Other species have also successfully identified the meaning of signaled cues in prior studies. Greiveldinger and colleagues (2007), for example, examined feeding and anxiety-related behaviors between groups of lambs exposed to a predictable or unpredictable sudden loud noise. It was found that lambs from the signaled predictable treatment displayed fewer startle responses, a lower heart rate, and consumed more food than lambs from the unpredictable group.

Similarly, Rimpley and Buchanan-Smith (2013) also identified beneficial effects of incorporating a signaled predictability routine, but in zoo animals. Here, researchers knocked before entering capuchin monkey enclosures for the predictable treatment before performing feeding, cleaning, and other standard husbandry duties. In contrast, for the unpredictable treatment, keepers entered without any prior signal. Knocking before entering was found to alleviate stress-related behaviors, and capuchins within the predictable treatment displayed fewer self-destructive behaviors compared to animals in the unpredictable treatment.

Although adding predictability has been shown to deliver numerous benefits for captive mammals, this phenomenon has only recently been investigated in other taxa (Bassett and Buchannan-Smith 2007, Galhardo *et al.* 2011). Galhardo and colleagues (2011) examined cichlid fish (*Oreochromis mossambicus*) behavior and stress physiology before and after exposure to a predictable signal prior to a positive (feeding) or negative (confinement for 30 minutes) event. The results showed that fish given a predictable signal before the confinement stressor excreted less cortisol than fish in the unpredictable treatment. Again, but this time in fish, we see how incorporating signaled predictability could be beneficial for captive animals, with decreased cortisol circulation helping animals to avoid approaching a physiological state of distress (Bassett and Buchannan-Smith 2007, Oliveira and Galhardo 2009, Hau *et al.* 2016).

A cue card, a simple knock, and a light being switched on are all cost-effective and straightforward cues that were understood by captive animals, and therefore, were able to be utilized to decrease stress exposure within a captive environment (Greiveldinger *et al.* 2007, Galhardo *et al.* 2011, Rimpley and Buchanan-Smith 2013). However, due to the frequency to which captive animals interact with their caretakers, the work within this chapter aimed to examine whether these individuals themselves could be a recognized cue for upcoming husbandry events. And it was found that zebrafish were able to recognize individual human faces because of their different responses to both caretakers dependent on their respective roles.

Facial Recognition

Newport and colleagues (2016) also examined facial recognition with a species of fish, but in a more restricted way. Archerfish (*Toxotes chatareus*) were conditioned with a food reward to spit a jet of water at a particular two-dimensional image of a human face, and it was found that fish were able to learn key features associated with a specific picture. In a continuation of their experiment, and after the removal of the food reward, researchers tested whether fish could discriminate the learned human face when paired with a total of 44 other novel human faces. Again, the archerfish were able to spit a jet of water at the face image they were trained to recognize.

More recently, human facial recognition has been identified in pigs as well. Wondrak and colleagues (2018) exposed pigs to an apparatus displaying two images: a human's face and the back of the same individual's head. Pigs were divided into two treatment groups where the first was trained to use their snout to press the face image, and the second was trained to press the back-of-head image to receive a food reward. After examining 10 pairs of novel images, it was found that both groups were successfully able to identify the correct image corresponding to their training. Both groups were then exposed to additional pairs of novel images and did not receive a food reward for correct responses. Again, it was found that both groups were successful at identifying the correct image. Overall, both of these studies highlight how different species can successfully carry out human facial recognition. The studies I report within this chapter further contribute to this work by showing how zebrafish could consistently recognize and discriminate between real, three-dimensional human beings, and also, remember specific tasks associated with different faces.

The ability to associate an aversive event with a human face has also been detected in wild crows. In Marzluff and colleagues (2010) masked researchers captured and banded crows

within the field; these individuals were assigned to the “threatening” treatment group. Whereas, masked individuals that did not partake in this process were assigned to the neutral treatment. It was found that crows were significantly more aggressive when exposed to individuals from the threatening treatment group compared to the neutral group. Thus, facial recognition and learning to associate specific facial features with certain experiences have been observed in a species of bird, and as my results demonstrate, in fish as well.

Facial recognition is believed to be a sophisticated form of object recognition (Mandeville *et al.* 2003, Hoffman *et al.* 2007, Marzluff *et al.* 2010, Jüttner *et al.* 2016, Newport *et al.* 2016). To be capable of facial recognition, fish need to be able to perceive different color combinations, e.g., hair or eye color and differentiate between simple and complex shapes, e.g., different facial bone structures. Also, fish would need to be capable of associative learning and episodic memory by being able to recall specific facts about an event, i.e., which caretaker performed what task, and be able to make future decisions regarding this information, i.e., where within the fish tank to move to prepare for or escape from an impending interaction.

Zebrafish Cognition

Previous work has demonstrated visual discrimination learning in zebrafish, with fish being able to distinguish between simple objects (objects with a single shape, such as a cube) of different colors and shapes. Oliveira and colleagues (2015), for example, tested zebrafish object recognition memory through a one-trial recognition test. Fish were first exposed to two small LEGO™ blocks that were identical in color, size, and shape. During the test phase fish were again placed within the same arena containing two LEGO™ blocks. However, one block was swapped out for another of a different color.

It was determined that fish were able to perceive and discriminate between objects, by fish spending more time around the novel block compared to the familiar (Oliveira *et al.* 2015). Furthermore, fish were also able to discriminate between objects of different shapes (i.e., round vs. rectangular), and different colors and shapes. Thus, zebrafish are capable of displaying cognitive abilities that underlie perception, learning, and memory (Ebbesson and Braithwaite 2012, Oliveira *et al.* 2015, Patton and Braithwaite 2015). May and colleagues (2016) also examined object recognition memory in zebrafish, but with objects that were more complex.

Fish were exposed to two identical LEGO™ figures which had a spherical head, a cubed body, two square legs, and a helmet or visor partially shielding the face. Fish were first placed into an arena containing the two identical figures and allowed to investigate them for a period of time. During the test phase fish were placed in the same arena, but one of the figures was exchanged for a distinctly different one. In this study, unlike that of Oliveira and colleagues (2015), the zebrafish spent more time around the familiar figure compared to the novel. Here, zebrafish were again able to discriminate between objects, but for some reason moved towards and spent more time around the familiar object. It is not clear why the fish approached a novel object in one study (Oliveira *et al.* 2015), and a familiar object in the other study (May *et al.* 2016), but both studies highlight how zebrafish are capable of recognizing and remembering objects of different shapes and colors.

In a follow-up study, whether zebrafish were capable of episodic memory (encoding what, where and when regarding a previous event) was tested using a one-trial recognition test (Hamilton *et al.* 2016). A neutral framework was used to minimize confounding effects of motivation, or responses potentially being driven by appetitive or aversive stimuli (Oliveira *et al.* 2015, May *et al.* 2016, Hamilton *et al.* 2016). Fish were exposed to two environmental contexts before testing where two objects were arranged in specific locations, within a yellow arena and an opposite arrangement within a blue arena. Fish were allowed to acclimate to these two objects

and their arrangement within each arena. Later fish were tested either in the yellow or the blue arena with one of the objects being in the correct original arrangement, whereas the other object was in an incorrect arrangement by reflecting the other arena (Hamilton *et al.* 2016). Here, zebrafish were able to recall their past experience during testing and discriminate between the object in the correct vs. incorrect location, by spending more time around the latter. Hamilton and colleagues (2016) suggest that their experiment demonstrates zebrafish are capable of distinguishing between complex objects as well as are capable of a cognitively complex ability, i.e., episodic memory.

Another type of learning and memory that zebrafish are capable of, and that is also likely directly relevant to the facial recognition experiments within this chapter, is associative learning. Associative learning involves exposing an animal to the pairing of a neutral cue with a positive or negative stimulus, and in the end the animal learns to apply the positive or negative nature (valence) of the stimulus to the cue itself (Colwill *et al.* 2005, Galhardo *et al.* 2011, Aoki *et al.* 2015). Associative learning is relevant to the two experiments that I conducted because zebrafish needed to learn that the facial presentation of the caretaker (the neutral cue) was associated with either a positive, i.e., feeding or novel object presentation in the first experiment, or a negative stimulus, i.e., net-chasing, in the second experiment.

Furthermore, associative learning of positive or negative stimuli has previously been identified in zebrafish. Colwill and colleagues (2005) used a T maze arrangement to demonstrate that zebrafish could associate a color or a pattern, i.e., horizontal versus vertical lines, cue with a food reward. Similarly, Aoki and colleagues (2015) also found evidence that zebrafish were capable of associatively learning a negative stimulus. Here, fish had to associate a change in color with the onset of an electric shock. Fish were either exposed to a non-shock or shock trial, and if fish that were exposed to the latter did not reach the shock-free goal area within 15 seconds, a shock was delivered. It was determined that fish in the electric shock treatment made a higher

percentage of correct choices in identifying the non-shock goal area and had a lower latency to reach this area, compared to the non-shock treatment.

Group Social Behavior

In both experiments reported in this chapter, I examined whether group social behavior was affected by the positive or negative roles associated with individual human caretakers. Cohesion, coordination, and aggression are all examples of these behaviors (Špinka 2012, Graham *et al.* 2018a, Graham *et al.* 2018b). Examining group rather than individual behavior has been argued to provide a better picture of whether adding predictability into the captive zebrafish environment improves their overall welfare (Graham *et al.* 2018b). High cohesive and coordinated behavior, coupled with low aggression has been perceived to be indicative of positive emotional reactions to stimuli, specifically when animals perceive themselves to be safe and not threatened (Špinka 2012, Graham *et al.* 2018a).

Recently, group social behavior has been examined in zebrafish to determine if fish valued the opportunity to explore a novel area within their home tank, as a potential form of enrichment (Graham *et al.* 2018a). Enrichment is considered to be a valuable tool for the welfare of captive animals for a variety of reasons, such as promoting cognitive stimulation which is beneficial for emotional and mental well-being (Morgan and Tromberg 2007, Graham *et al.* 2018a, Graham *et al.* 2018b). Furthermore, it is well known that novelty is considered to be cognitively stimulating and enriching (Morgan and Tromberg 2007, Graham *et al.* 2018a and b). Graham and colleagues (2018a) found that cohesion and coordination behavior increased, while aggressive behavior decreased, the day after a divider was removed and fish were able to access a novel area within their home tank.

For the first facial recognition experiment, I also examined the potential positive effects of novelty, as well as feeding, but I aimed to examine which task generated a greater positive response from zebrafish. I predicted that fish would perceive feeding more rewarding than the presentation of a novel object, due to the former being a strong and innate motivational driving force for survival. It was also predicted that these results would be present during the last rather than the first nine days of the experiment due to the fish needing time to learn the experimental paradigm.

During the first nine days of experiment 1, the fish were more cohesive and less coordinated while feeding compared to when presented with a novel object, but there was no difference in fish aggression. It seems that fish did find food more rewarding due to the cohesion results. However, the results for coordination and aggression do not fully support this per Graham and colleagues (2018a). The data from the last nine days of my experiment, however, better fit the idea that food was a more rewarding stimulus. At the end of experiment 1, zebrafish were more cohesive during feeding than during the presentation of a novel object. Fish were also found to be less aggressive while feeding; however, there was no difference in coordination. It is possible that due to the way food was presented in a floating circular ring, that this made it physically difficult for fish to feed in a coordinated manner. Fish also likely became less aggressive during feeding because the fish learned to use the food ring arrangement and cooperate more, during these trials where a consistent stimulus was delivered. In contrast, decreased interest for the novel object trials could be due to a different object being presented daily in attempts to make every interaction interesting, or novel. But how to interpret the novel object presentation data is challenging because not all objects would necessarily be perceived as “equal,” and zebrafish may have perceived some objects to be more interesting or more threatening than others.

To try to avoid these complications, experiment 2 used a clearly rewarding task, i.e., feeding, with a recognized negative task, i.e., chasing with a dip-net. Given the clear differences between these two types of stimuli, I expected to find differences in social behavior. However, I found no significant effects for cohesion, coordination, or aggression during either the first or the last 12 days of the experiment. It is not clear why differences in social behavior were not detected. Despite facing a threatening stimulus, differences in defense-related behaviors were still predicted to be identified, e.g., increased cohesion during net-chasing might, therefore, indicate shoaling behavior which is performed for better protection during a threat (Magurran 1990, Seghers and Magurran 1995, Swaney *et al.* 2015).

Future work is needed to try and resolve why measures that appear to work well for assessing social behavior in other contexts have been difficult to untangle in the current experiments. This could be resolved by incorporating some physiological measures such as cortisol production immediately following the facial presentation of the caretaker associated with a stressor and compare this against the caretaker associated with a positive task, such as feeding. Alternatively, a behavioral measure that has consistently been shown to indicate anxiety levels in zebrafish could be used, e.g., staying close to the tank-bottom and avoiding the rest of the water column until fish are comfortable to explore (Wu *et al.* 2010, Depasquale *et al.* 2016, Graham *et al.* 2018b).

Overall Conclusion

The experiments described in this chapter demonstrate that zebrafish are capable of recognizing the faces of individual human caretakers associated with different kinds of tasks. Together, these results suggest there would be merit in research investigating signaled predictability in the form of facial recognition to allow zebrafish to prepare behaviorally and

physiologically for impending husbandry tasks. This could, therefore, alleviate stress in their captive environment for this increasingly used species.

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Education

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B. Sc (Hons.) Towson University, USA, 2010 – 2014
Biology and Interdisciplinary Studies (Animal Behavior)

College Teaching Experience

- 2017** Teaching Assistant at Penn State University
Courses: Animal Welfare (WFS 461) and Wildlife Behavior (WFS 460)
- 2015** Teaching Assistant at Penn State University
Course: General Fishery Science (WFS 410)

Publications

Ahlbeck Bergendahl, I., **Miller, S.**, Depasquale, C., Giralico, L., & Braithwaite, V. A. (2017).
Becoming a better swimmer: Structural complexity enhances agility in a captive-reared fish.
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Posters and Presentations

- 2017** Controlled exposure to stress in early life: effects on adult anti-predator behavior and exploration in domesticated and wild guppies (*Poecilia reticulata*). Presentation for the Animal Behavior Society Conference Poster Symposium, at the University of Toronto-Scarborough Canada.
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Grants and Awards

- 2016** Smithsonian Tropical Research Institute (STRI) Fellowship
- 2016** American Society of Ichthyologists and Herpetologists Edward C. Raney Fund Award
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