Underlying tendencies of behaviour: Examining stress and anxiety in convict cichlid fish

by

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Abstract

Animal behaviour varies across hierarchical levels made up of behaviours, personality traits, and behavioural types/syndromes. Stress coping style and anxiety are examples of behavioural types, or covariance of personality traits within individuals, and can be influenced by prior stress exposure. A common test used to measure anxiety in rodents is the elevated plus maze, and here an adapted version for use in fish (the submerged plus maze) was validated using the benzodiazepine diazepam. I show that fish spent more time in and entered more open arms of the maze after diazepam exposure than after vehicle exposure, mirroring validations used for the elevated plus maze. The submerged plus maze maintains construct validity for testing anxiety in fish. The effect of developmental stress exposure on adult convict cichlid fish personality traits and a behavioural syndrome was examined. At the individual level, significant effects of early life stress were not seen in adulthood on personality traits, though stress exposure did result in the disruption of the formation of an exploration-boldness syndrome that was present in the unstressed population. These results suggest that an exploration-boldness syndrome is the default syndrome in convict cichlids but may not have provided adaptive benefit for fish in the stressed population due to the level of predation stress in their developmental environment and was therefore not formed. I have demonstrated behavioural plasticity in response to environmental manipulations.

Preface

This thesis is an original work by Brittany Hope. No portions of it have been previously published. Chapter 3 involved a collaboration with Dr. Suzy Renn from Reed College in Portland, Oregan, USA, in which she performed the enzyme immunoassay on the cortisol samples as described in that section.

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Animal Care and Use Committee for Biosciences, Project Name "Social Determination of Sex and Social Behaviour in a Cichlid Fish," AUP 00000055.

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Introduction

Early life environments can influence behavioural phenotypes to be adaptive for the expected later environment. The classification of these changes as either adaptive or maladaptive is contingent upon whether the early and late environments impose similar demands. The mismatch hypothesis explains this context dependency by proposing that concordant juvenile and adult environments result in positive adaptive programming, while discordant juvenile and adult environments result in negative adaptive programming (Nederhof & Schmidt, 2012). This idea suggests that costs and benefits of changes induced by early environments are context-dependent. Individuals exposed to early life stress may therefore be better able to cope with stress in adulthood because the contexts match, as opposed to individuals with no prior exposure to stress (i.e., mismatch). This enhanced ability to cope in "matched" environments may also depend on the severity of the stressor in early life in the manner of an inverted-U function. Stress exposure at either extreme of this curve may result in maladaptive phenotypes, while moderate stress levels may provide advantageous adaptation (Romeo, 2015; Lyons & Parker, 2007). It is possible that the mechanism involved in phenotypic programming has upper and lower bounds that stress levels must remain within for adaptive changes to occur, and exceeding those boundaries results in maladaptation. As such, adaptive phenotypic programming may be both context and severity dependent and therefore it is possible that stress responses can be adaptive depending on the environment present during development. Since stress reactivity can vary between individuals and populations, it is important to study the deeper underlying tendencies of the behaviours associated with it and how they can be manipulated by external stimuli.

Animal behaviour may vary across hierarchical levels of personality traits and behavioural types and syndromes (see Fig. 1.1). This hierarchy likely has three levels, with

individual behaviours at the bottom, leading into personality traits, and ending with behavioural types. A group of behaviours can generate among-individual variance that corresponds to personality traits. Among-individual covariance in those traits corresponds to behavioural types. Behavioural types also have another level, behavioural syndromes, that relies on inter-individual covariance between personality traits within a population.

The second level of the hierarchy is individual variance, or personality traits. Animal personality is a term commonly used to describe consistent, individual differences in behaviour across time or contexts (Réale, Reader, Sol, McDougall, & Dingemanse, 2007) and is divided into different traits, such as boldness, exploration, aggressiveness, activity, and sociability. Although boldness is a frequently used term in animal personality research, a review by Toms et al. (2010) found that not all studies defined boldness the same way, leading to confusion about its true definition and concerns about construct validity. The most common descriptions involve latency to approach novelty, particularly novel objects and areas, and will be the definition applied in this thesis. The underlying construct of the boldness trait can be operationalized in tests, such as the emerge latency test, by the latency of the animal to emerge from an enclosure into a novel arena. Exploration is the pattern of responses to novel situations, such as freezing/hiding behaviours, scototaxis (i.e., preference for dark areas over light areas; Maximino et al., 2010) and thigmotaxis (i.e., travelling close to walls instead of in the centre of an arena; Réale et al., 2007; Champagne, Hoefnagels, de Kloet, & Richardson, 2010). Exploration can be operationalized using tests such as the open field test, plus maze, novel tank diving test, light/dark preference test, and many others. These tests provide outcomes that can then be interpreted as freezing, thigmotaxis, scototaxis, etc. Aggressiveness is agonistic behaviour between individuals and activity refers to the overall activity level of individuals (Réale et al.,

2007). Aggressiveness can be operationalized using tests such as paired aggression tests or mirror aggression tests, in which aggressive behaviours (e.g., biting, scratching, frontal displays, and other attacks) are tallied against an individual perceived as an intruder (whether real or a mirror image). Activity can be operationalized in many of these tests by rate or amount of movement, though it is suggested to measure activity in non-novel and non-risky settings (i.e., home environment) to obtain a baseline level of activity that can then be compared to activity in novel or risky settings. Levels of variance in these traits can be indicative of adaptive strategies.

The third level of the hierarchy is covariance between traits, or behavioural types/syndromes. Multiple personality traits, or sets of behaviours, can be intercorrelated within or between individuals, indicating the presence of a behavioural type or syndrome (Sih, Bell, & Johnson, 2004; Dingemanse et al., 2007). Behavioural types are patterns of covariance within an individual, while behavioural syndromes are patterns of covariance across a population (Bell, 2007; Sih et al., 2004). Inter-individual covariance in personality traits (i.e., behavioural syndromes) can have both developmental (Fischer, Ghalambor, & Hoke, 2016) and genetic origins (Bleakley, Martell, & Brodie, 2006). Relationships between traits may result in sub-optimal behaviour expression in certain situations to prevent one genetically-linked trait from causing detrimental behaviour in the other, therefore favouring optimal relationships between traits over optimal situational responses (Sih et al., 2004). It is also possible that circumstances can influence behavioural traits/syndromes and the timing of that experience may change the effect on the trait/syndrome (Sih et al., 2004). Examples of such trade-offs include the balancing of foraging behaviour with predator-avoidance behaviour. If an organism is under constant predation threat, an optimal response to the threat may be prolonged hiding behaviours. However, if the organism never emerges from hiding, it cannot forage. An optimal relationship

between these behaviours would allow for a balance between foraging and hiding behaviours that best ensures survival of the organism and would vary according to the level of predation risk and food availability at the time the relationship is formed. These traits/syndromes may therefore indicate adaptive strategies. Commonly studied behavioural syndromes include aggression-boldness and exploration-boldness (Sih et al., 2004; Bell & Sih, 2007; Dingemanse et al., 2007; Mazué, Dechaume-Moncharmont, & Godin, 2015). Mazué et al. (2015) found an exploration-boldness syndrome in juvenile convict cichlids, in which bold fish explored more than shy fish. Bell and Sih (2007) elicited an aggression-boldness syndrome in sticklebacks using predation stress, in which bold fish were less aggressive than shy fish. Behavioural types/syndromes can be composed of any combination of correlated personality traits.

Stress coping style refers to the behavioural and physiological responses of an individual in response to a stressor and is frequently described along a continuum between proactive and reactive coping (Koolhaas et al., 1999). Proactive coping style is consistent with active responses (e.g., exploration, aggression, active avoidance), while reactive coping style is consistent with passive responses (e.g., immobility, low aggression; Øverli et al., 2007). Coping style is frequently characterized by associations between the behaviours that make up boldness, exploration, activity, and aggression traits. The common operationalization of coping styles using suites of personality traits or behaviours that span across multiple traits implies that coping style may be a behavioural type (Sih et al., 2004).

Another possible behavioural type is anxiety. Anxiety-like behaviours include thigmotaxis, freezing, and avoidance (e.g., latency to emerge or investigate novelty). Because these behaviours span across multiple personality traits, it may be reasonable to classify anxiety as a behavioural type. Stress coping style and anxiety both encompass boldness and exploration

traits, but should be viewed as different behavioural types because coping style includes aggression while anxiety does not. Anxiety is frequently studied in rodents using an elevated plus maze test, in which preference for closed arms/avoidance of open arms and freezing indicates anxiety and lack of arm preference indicates lack of anxiety (Pellow, Chopin, File & Briley, 1985). Anxiety is also studied using open field test in many species and novel dive tank test in fish, in which increased thigmotaxis and bottom-dwelling indicates anxiety (Stewart et al., 2012).

Previous research has shown that prior stress exposure influences coping style and anxiety by reducing thigmotaxis (Champagne et al., 2010), emerge latency (Brown, Jones, & Braithwaite, 2005), freezing/hiding (Moscicki & Hurd, 2015), and anxiety-like behaviours (D'Aquila, Brain, & Willner, 1994). This shift toward more proactive coping styles as a result of developmental stress exposure has been termed stress resilience (Lyons & Parker, 2007) and supports the mismatch hypothesis. In developmental contexts where stress exposure preferentially triggers behavioural programming that generates proactive coping styles, matching stressful contexts later in life lead to enhanced recovery and survival (Nederhof & Schmidt, 2012).

My primary objective was to investigate the effect of moderate developmental stress on behavioural variation at different levels of the hierarchy using convict cichlid fish (*Amatitlania nigrofasciata*). This objective focused on the effects of stress on coping style and anxiety. To ensure the observed effects would parallel those seen in model species, appropriate model paradigms would need to be applied. In Chapter 2, I conducted a pharmacological validation of an adaptation of the elevated plus maze for use in fish, the "submerged plus maze." Validating an adaptation of such a commonly used anxiety test contributes to a larger arsenal available for

behavioural research and using a non-model species allows for future studies to account for taxonomic spread. In Chapter 3, I examined the effect of juvenile stress exposure on the behavioural hierarchy in adult fish. This experiment employed the emerge latency test, open field test, and the newly-validated submerged plus maze to assess changes in the personality traits boldness and exploration and in the exploration-boldness behavioural syndrome. Studying the way variance/covariance of behaviour is affected by early life stress is an important step in clarifying the impact of different types and durations of stress. This species is also frequently used for studying social behaviour and a noticeable gap exists in the literature surrounding their behavioural responses to stress exposure.

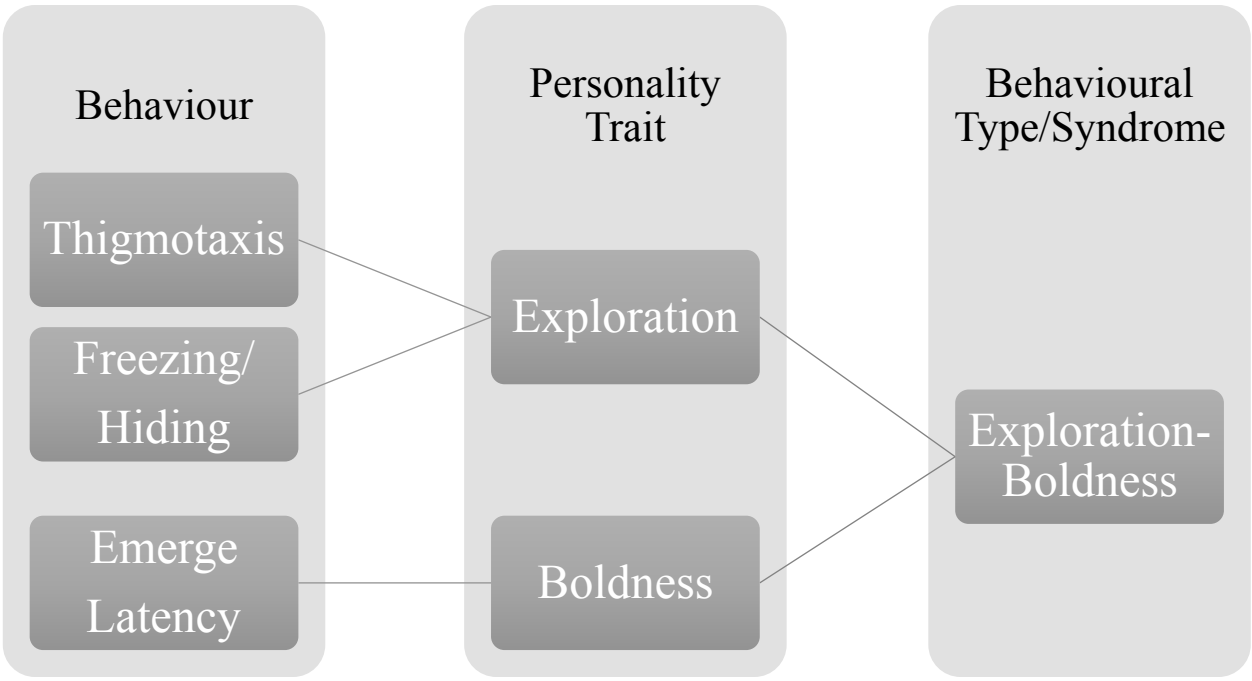

Figure 1.1: Schematic of the hierarchy of behaviour. As defined in this thesis, behaviours make up personality traits. Correlated personality traits then make up behavioural types in individuals and behavioural syndromes in populations.

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Chapter 2: Validation of the submerged plus maze as a measure for piscine anxiety using diazepam

Introduction

Rodents are a commonly used model species for animal behaviour research, but using other species can allow for more economical and efficient studies, as well as provide deeper understanding of the evolution and function of behavioural traits (Taborsky et al., 2015). One important consideration when selecting new species is the validity and specificity of the behavioural assays employed, such that the construct validity of those measures is mirrored between species. The elevated plus maze is a behavioural test used to measure anxiety that is well-documented and validated in rodents. Anxiety is indicated by the tendency of a rodent to avoid the two open arms of the elevated plus maze, which is driven by an aversion to open spaces (Pellow, Chopin, File, & Briley, 1985; Treit, Menard, & Royan, 1993). As the popularity of studying anxiety in fish increases, it is important to develop a set of standard and well-validated assays similar to those used in rodents. Consistency across experimental species is key in ensuring studies can be accurately compared for behavioural effects. Fish already have measures such as the open field test and the light/dark preference test for studying thigmotaxis (i.e., travelling close to walls instead of in the centre) and scototaxis (i.e., preference of dark areas), similarly to rodents. Missing, however, is an adaptation of the elevated plus maze. Sackerman et al. (2010) developed an "aquatic light-dark plus maze" to study anxiety in zebrafish that consists of a plus-shaped apparatus where arms alternate between having white and black walls. In their implementation, the black arms would be analogous to the closed arms of the elevated plus maze and the white arms to the open arms. Unfortunately, since the elevated plus maze is dependent on an aversion to open spaces, their test cannot be considered equivalent

to the elevated plus maze because it tests scototaxic behaviour instead of thigmotaxis. To best ensure an approximation of the elevated plus maze, the non-black arms should be transparent to mimic an open space, such as the apparatus described in this study. The use of transparent arms in the present apparatus allows a closer representation of the elevated plus maze.

New behavioural apparatuses require validation to ensure specificity of the exhibited behaviours to the assumptions of the paradigm (i.e., construct validity). Validation can include both behavioural and pharmacological considerations. Anxiety is frequently characterized by freezing, hiding, defecation, urination, and thigmotaxic behaviours, all of which can be quantified within the plus maze behavioural assay. Pharmacological validations of the plus maze can involve both anxiolytic and anxiogenic substances such as diazepam and yohimbine, respectively. Anxiolytic substances should decrease anxiety-like behaviours and anxiogenic substances should increase them. Sackerman et al. (2010) used the benzodiazepine chlordiazepoxide to validate their plus maze variant, despite known sedative effects in zebrafish at those doses (Bencan, Sledge, & Levin, 2009). Bencan et al. (2009) demonstrated anxiolytic effects on zebrafish thigmotaxis at doses that did not also induce sedation in a novel dive tank test. Both rodents and fish decrease thigmotaxic behaviour in open field tests following diazepam administration (Treit & Fundytus, 1989; Schnörr, Steenbergen, Richardson, & Champagne, 2012). As thigmotaxis appears to be the main response to open spaces in the rodent elevated plus maze, diazepam would be a reasonable choice to use in a pharmacological validation of our "submerged plus maze."

Pharmacological manipulations of behaviour rely on the presence of the appropriate receptors for the drugs involved. Fish possess benzodiazepine-$GABA_A$ receptors that demonstrate similar action and binding as in rodents and humans, although possibly with some

functional differences (Betti, Giannaccini, Gori, Bistocchi, & Lucacchini, 2001; Anzelius, Ekström, Möhler, & Richards, 1995; Nielsen, Braestrup, & Squires, 1978). Since low doses of benzodiazepine site agonists reduce anxiety and high doses induce sedation, it is important to select the lowest dose providing the desired effects. Since diazepam has a greater affinity for the benzodiazepine binding site than chlordiazepoxide and the histidine residue responsible for the anxiolytic effects of diazepam is conserved in zebrafish (Renier et al., 2007), the present study selected diazepam as the anxiolytic substance. Diazepam binds to $GABA_A$ receptors to reduce neuronal inhibition and subsequently reduces anxiety. Because diazepam does not induce any visible behavioural effects at its highest solubility in water (Renier et al., 2007), the solvent dimethyl sulfoxide (DMSO) was used to allow administration by immersion. While rodents are typically administered diazepam by injection, administration by immersion for assessing acute behavioural effects is preferred for fish due to the necessity of anesthetizing the fish prior to injection. Administration by immersion allows for the drug to be administered passively through the skin and gills.

The current study was conducted to provide a validated adaptation of the elevated plus maze for use in fish, termed the "submerged plus maze." Specifically, I used the benzodiazepine diazepam to assess individual differences between drug and vehicle treatment on the behaviour of convict cichlid fish in the submerged plus maze. A within-subject design is possible because rodents do not habituate to the open arms of the elevated plus maze after repeated exposure (Pellow et al., 1985; Treit et al., 1993) and the anxiolytic effect of diazepam does not carry over to drug-free trials (Treit et al., 1993). I hypothesized that diazepam exposure would result in increased time spent in open arms and increased entries into open arms to demonstrate anxiolysis and reduced freezing and hiding behaviours to indicate increased overall activity.

Methods

Subjects and Housing

Subjects consisted of 20 laboratory-bred convict cichlid fish size-matched by mass (m = 1.92 ± 0.5 g). Subjects were housed in 40 L (51 cm × 31 cm × 25.5 cm) aquaria partitioned by transparent dividers into six compartments, at a density of one fish per compartment, allowing identification of fish without physical tags. This housing arrangement also prevents physical harassment commonly seen in communal housing while not subjecting fish to prolonged social isolation from solitary housing. Fish were fed *ad libitum* six days a week with various prepared dried fish foods and aquaria were maintained at 20°C on a 12L:12D light cycle. Fish were tested for behaviour in open field test and submerged plus maze in a within-subjects manner, such that each fish was subjected to each test with and without exposure to diazepam. Inter-trial intervals were set at approximately 48 hours and inter-test intervals were set at approximately 72 hours to allow for drug washout and resting periods. Test order and drug exposure order was randomized and all fish were drug and environment naïve before the onset of the experiment.

Drug Administration

Administration of diazepam by immersion was accomplished by placing a fish in a beaker filled with the drug solution for 3 min and then placing it in a separate holding container (e.g., small plastic aquarium specimen container) for 5 min to allow the drug to take effect (Bencan et al., 2009). The fish was transferred between containers and the test apparatus in a transparent cup with holes and slats in it to allow liquid to pass easily into and out of the cup to reduce handling. Fish were then tested in the selected behaviour test before being returned to their home compartment to await their next trial (see Fig. 2.1). During drug trials, fish were

exposed to a 2.5 mg/L dose of diazepam (dose chosen from pilot dose-response curve, unpublished; Professional Compounding Centers of America) in vehicle (0.5% DMSO in tank water at RT). Control trials were performed using exposure to vehicle (44 µL DMSO in 500 mL tank water at RT) in the dosing step. Note that the dose refers to the concentration of the substance in the water, not the amount bioavailable to the fish. Fish were observed throughout the dosing and holding periods for evidence of sedation (e.g., gross motor effects, postural imbalance). The total duration of each trial (drug exposure to completion) was 15-16 min per fish. Drug solution was made fresh for each fish and holding container water and apparatus water were replaced with fresh oxygenated tank water between each trial.

Submerged Plus Maze

I examined anxiety levels with a submerged plus maze apparatus (adapted from the elevated plus maze; Pellow et al., 1985). The apparatus is shaped as a plus symbol, with four arms alternating between transparent and black plexiglass walls (each arm 12 cm × 4.5 cm × 13 cm), filled with water to a depth of 10 cm (see Fig. 2.2). This arrangement results in two visually closed arms, two visually open arms, and a center area. To quantify travel within the arms, arms are marked every 1.5 cm away from the center, creating eight lines. The center of the maze is also marked with lines 1.5 cm apart, horizontally and vertically, forming a 3 × 3 grid. An acclimation chamber (transparent plastic cylinder, 4.5 cm × 4.5 cm × 13 cm) was placed in the center of the maze. A webcam viewed the apparatus from above to observe all movements and a black curtain occluded the experimenter.

Each fish was placed in the acclimation chamber directly from the holding container for two minutes, after which, the chamber was removed and the fish allowed to swim freely within

the maze. The fish's actions were recorded for five minutes (per Pellow et al., 1985). I scored behaviour according to the number of lines crossed per area (visually closed, visually open, and center) and the amount of time spent in each area (visually closed, visually open, and center). Line crossing was defined as the fish passing the line with its head up to the pectoral fins. Additionally, total number of lines crossed, number of entries into new arms, and number of entries into open arms were quantified.

Open Field Test

I assessed tendency to explore a novel environment with an open field test (Toms, Echevarria, & Jouandot, 2010). Open field tests were conducted in a 20 L tank (40 cm × 25 cm × 21 cm) filled with 10 cm of water with markings to designate location in the tank (fifty 5 cm × 5 cm squares; see Fig. 2.3). An acclimation chamber (opaque plastic cylinder, 5 cm × 5 cm × 13 cm) was placed in the center of the tank. A curtain occluded the experimenter from view while a video camera recorded all trials.

Each fish was placed into the acclimation chamber of the apparatus directly from the holding container and allowed to acclimate for two minutes before the chamber was removed. The movement of the fish was then recorded for five minutes. I scored behaviour using JWatcher (Blumstein, Daniel, & Evans, 2010). Outcome variables included: amount of time spent in each type of square (corner, outer, and inner) and number of squares entered of each type (corner, outer, and inner). Entering a square was defined as the fish crossing the line of that square with its head up to the pectoral fins.

Protocols were approved by the University of Alberta Biological Sciences Animal Policy and Welfare Committee (protocol number 00000055) and adhere to the guidelines of the Canadian Council for Animal Care.

Data Analysis

All statistical analyses were performed using R version 3.1.3 (R Foundation for Statistical Computing, Vienna, Austria). Significant *p*-values were set at 0.05.

I performed repeated measures ANOVA model comparisons of treatment according to the methods in Faraway (2006) by calculating the X^2 from the difference between the model likelihood estimates. Fish identity was classified as a random effect, while all other variables were classified as fixed effects. In the null model, the outcome variable was assessed as a function of fish identity, trial order (drug or vehicle), mass, standard length, and handling time to control for potential confounds. The alternative model was identical to the null model but included treatment to assess the difference between the two models as a function of treatment. These model comparisons were applied separately to each outcome variable. I also performed a Chi-squared test to assess the categorical tendency of fish to enter or not enter an open arm in the plus maze as a function of treatment.

Results

There was a significant effect of diazepam exposure on time spent in closed arms (X^2 (1) = 8.54, p = 0.0035), open arms (X^2 (1) = 4.02, p = 0.045), and the centre area (X^2 (1) = 6.02, p = 0.014; all n = 20; Fig. 2.4). After exposure to diazepam, fish spent more time in open arms and the centre area and less time in the closed arms. There was also a significant effect of diazepam

exposure on lines crossed in closed arms (X^2 (1) = 6.50, p = 0.011; Fig. 2.5) and total lines crossed (X^2 (1) = 6.50, p = 0.011; Fig. 2.6), where fish crossed more lines after diazepam treatment than after vehicle. Mean lines crossed in open arms (X^2 (1) = 2.16, p = 0.14) and the centre area (X^2 (1) = 0.90, p = 0.34) did not differ between diazepam and vehicle trials (Fig. 2.5).

Fish entered more open arms (X^2 (1) = 6.64, p < 0.001) and more new arms (X^2 (1) = 4.63, p = 0.031) after diazepam exposure than after vehicle (Fig. 2.7). The percentage of open arm entries out of new arm entries was also significantly increased by diazepam exposure (X^2 (1) = 6.17, p = 0.013; Fig. 2.7). Fish were more likely to enter an open arm after drug exposure than after vehicle (X^2 (1) = 3.91, p = 0.048; Table 2.2).

There was no significant effect of drug exposure on open field outcome variables (see Table 2.3). There were no significant relationships between any outcome variables and mass, standard length, or handling time.

Discussion

In this study, I demonstrated an anxiolytic effect of diazepam on convict cichlid behaviour in a submerged plus maze apparatus. Fish exposed to a 2.5 mg/L dose of diazepam spent significantly less time in closed arms of the apparatus, crossed more total lines, and performed more entries into new arms than fish exposed to vehicle (tank water and DMSO). This dose did not elicit behavioural changes in the open field test.

The lack of anxiolytic effect induced by this dose of diazepam in the open field test is likely a result of a non-optimal dose rather than diazepam failing to produce anxiolysis in the open field test. Diazepam has previously been used to elicit an anxiolytic effect in an open field test in larval zebrafish (Schnörr et al., 2012), indicating test performance is sensitive to the drug

albeit not at this concentration. Since the dose used was determined by the optimal dose in the submerged plus maze, it is likely that the two tests are differentially sensitive to the drug and therefore have different optimal doses for anxiolytic behaviour. This difference is further complicated by the observation that half of the fish exhibited postural imbalances in both drug and vehicle trials, suggesting an effect of DMSO on behaviour or buoyancy. The postural change elicited by DMSO in the open field test was not evident in the submerged plus maze. Future studies should examine the postural and behavioural effects of this popularly used solvent.

Plus maze experiments generate outcomes that can be split into behaviours indicative of activity and behaviours indicative of anxiety. Outcomes such as total arm entries or exploration levels (lines crossed) are frequently used to operationalize activity, while outcomes such as time spent in open arms and entries into open arms frequently operationalize anxiety. Activity levels are important to consider when experimental substances, such as benzodiazepines, may have sedative effects and for establishing a level of exploration. Outcomes relating to the open arms are important when considering anxiety because aversion to open spaces is a fear-inducing stimulus (Treit & Fundytus, 1989).

Like rodents, convict cichlid fish display a preference for the closed arms of the plus maze over the open arms. While this preference is pervasive regardless of drug exposure, aversion to the open arms is significantly reduced after diazepam administration. Time spent in open arms and entries into open arms were increased in fish by diazepam administration, as observed in pharmacological validations of the elevated plus maze in rats (Pellow et al., 1985; Treit et al., 1993). This reduction in open arm aversion is indicative of a reduction in anxiety caused by the drug. Since pharmacological intervention by a substance with known anxiolytic

effects induces anxiolysis in the submerged plus maze, the submerged plus maze is validated in this study as a measure of anxiety in fish.

Although plus maze literature typically treats activity levels as a separate consideration to anxiety, activity was influenced by diazepam exposure. Fish entered more arms, crossed more total lines, and crossed more lines in closed arms after exposure to diazepam than after exposure to vehicle only. This increase in activity suggests that diazepam exposure at this dose does not result in sedation. It is also important to note that the anxiolytic and activity effects seen here are strong enough to overcome the impact of sedation observed in four fish that displayed abnormal postural balance. This overall increase in exploration indicates that the effect of diazepam extends past anxiety-like behaviours to also influence activity.

The anxiolytic effect of diazepam has been shown in both rodents and zebrafish in various behaviour tests, such as the novel tank diving test, open field test, light-dark preference test, and elevated plus maze. Diazepam exposure tends to reduce a variety of anxiety-like behaviours, such as thigmotaxis (Schnörr et al., 2012), bottom-dwelling (Bencan et al., 2009), and scototaxis (Maximino, da Silva, Gouveia, & Herculano, 2011), while chlordiazepoxide, another benzodiazepine compound, seems to only have anxiolytic effects that are specific to scototaxic behaviour (Maximino et al., 2011; Sackerman et al., 2010). In a previous study by Sackerman et al. (2010), chlordiazepoxide was used to validate an "aquatic light-dark plus maze" as a model of anxiety in zebrafish. Their finding that chlordiazepoxide increased the amount of time spent in white arms and the percentage of entries into white arms parallels those found in studies of elevated plus maze with rodents and the present study. However, the test they designed is more indicative of scototaxis than thigmotaxis, and Treit et al. (1993) demonstrated that the main driver behind rodent behaviour (i.e., aversion to open arms) in the elevated plus may be

thigmotaxis. Thigmotaxis is attenuated by diazepam exposure in the novel tank diving test (Bencan et al., 2009), elevated plus maze (Treit et al., 1993), open field test (Treit & Fundytus, 1989), and now the submerged plus maze. In contrast, chlordiazepoxide does not attenuate thigmotaxis in the novel tank diving test (Bencan et al., 2009; Sackerman et al., 2010), so it's possible that chlordiazepoxide effects are exclusive to scototaxic anxiety-like behaviours and not thigmotaxic. The anxiolytic effect of 0.05% DMSO exposure observed by Sackerman et al., (2010) may also be specific to scototaxis, since a similar effect is not apparent with thigmotaxis (Sackerman et al., 2010; Bencan et al., 2009). The present study also used a lower percentage of 0.008% DMSO in the dosing beaker, possibly at a dose too low to produce anxiolysis. Although both thigmotaxis and scototaxis are anxious behaviours, they are qualitatively different and therefore may be differentially sensitive to various anxiolytic substances. Because the retina is rich in GABA receptors, it is possible that diazepam can alter visual field sensitivity due to hyperpolarization. However, it is unlikely that this diazepam exposure impaired the ability of fish to distinguish between the transparent and black arms because the dose and duration was much lower than the high doses shown to affect vision (Steenbergen, Richardson, & Champagne, 2011; Elder, 1992).

In conclusion, this study provides a new, validated measure of anxiety for use in fish that provides a close representation of the commonly used elevated plus maze. The sensitivity of this test to an anxiolytic substance, diazepam, provides a framework for behavioural and pharmacological assessments of anxiety that expands the current repertoire of model organisms and behavioural assays. Future studies would benefit from incorporating this measure into existing anxiety batteries.

Outcome Measure	Drug Mean ± SEM	Vehicle Mean ± SEM	X^2 (1)	*p*
Closed time	295.0 ± 1.3	298.9 ± 0.4	8.54	0.004
Center time	3.5 ± 1.0	1.0 ± 0.4	6.02	0.014
Open time	1.6 ± 0.7	0.1 ± 0.1	4.02	0.045
Closed lines	59.4 ± 13.1	24.4 ± 6.0	6.50	0.011
Center lines	2.5 ± 0.7	1.7 ± 0.6	0.90	0.34
Open lines	2.1 ± 0.9	0.6 ± 0.6	2.16	0.14
Total lines	64.0 ± 13.6	27.2 ± 6.5	6.50	0.011
Arm entries	2.0 ± 0.2	1.4 ± 0.2	4.63	0.031
Open entries	0.35 ± 0.11	0.05 ± 0.05	6.64	0.001

Table 2.1: Model comparisons of plus maze outcome variables as a function of drug exposure.

	Drug	Vehicle
Entered an open arm	7	1
Did not enter an open arm	13	19

Table 2.2: Frequency table depicting the number of fish that either entered or did not enter an open arm as a function of drug or vehicle trial. Fish were more likely to enter an open arm after drug exposure than after vehicle ($p = 0.048$).

Outcome Measure	Drug Mean ± SEM	Vehicle Mean ± SEM	X^2 (1)	*p*
Outer time	78.8 ± 13.6	52.1 ± 9.6	3.17	0.08
Corner time	218.1 ± 13.8	240.2 ± 12.2	1.79	0.18
Inner time	3.0 ± 0.6	7.6 ± 3.8	1.65	0.20
Outer squares	126.8 ± 15.2	106.6 ± 17.6	1.20	0.27
Corner squares	25.2 ± 3.1	21.9 ± 3.6	0.83	0.36
Inner squares	10.2 ± 1.9	15.6 ± 3.0	2.92	0.09
Total squares	162.1 ± 19.9	144.0 ± 23.3	0.52	0.47

Table 2.3: Model comparisons of open field test outcome variables as a function of drug exposure.

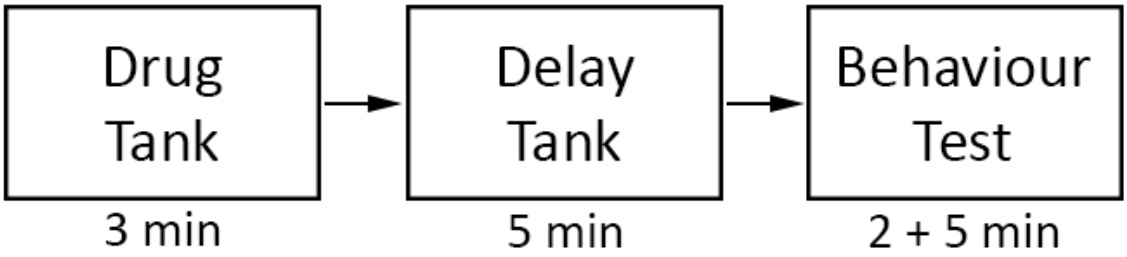

Figure 2.1: Anxiolytic administration procedure. Fish was first placed into a tank containing the drug treatment (diazepam + vehicle or vehicle only) for 3 min. Next, the fish was moved to a delay tank (tank water) for 5 min to allow the drug to take effect. Finally, the fish was moved to the appropriate behaviour test, where it acclimated for 2 min before the 5 min testing period.

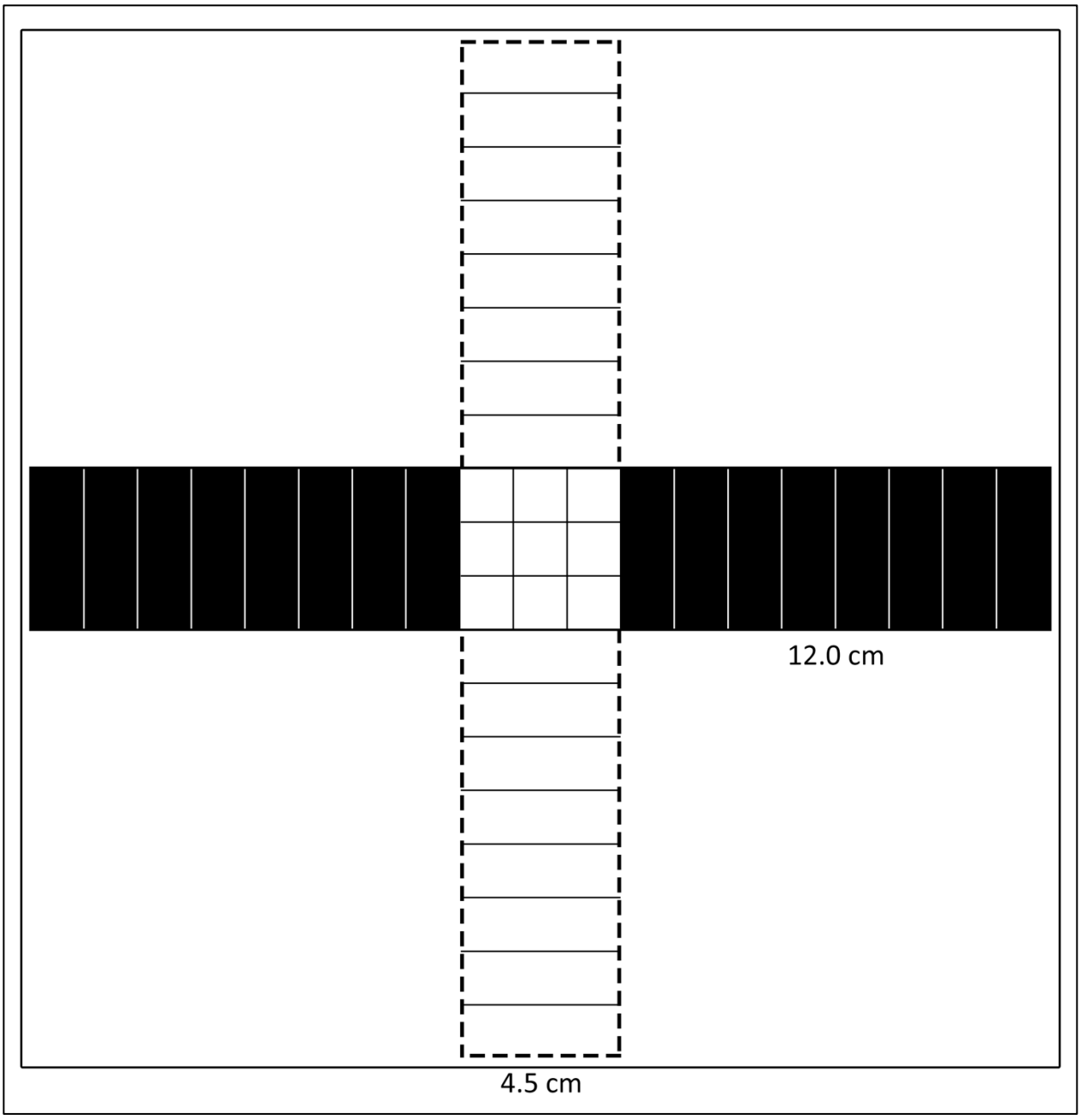

Figure 2.2: Submerged plus maze apparatus. The apparatus is shaped as a plus symbol with alternating black (black fill) and transparent (white fill, dashed lines) arms. Arms are marked to quantify travel within the maze. Fish were placed in an acclimation chamber in the centre area for two minutes before they were released to explore the maze for five minutes.

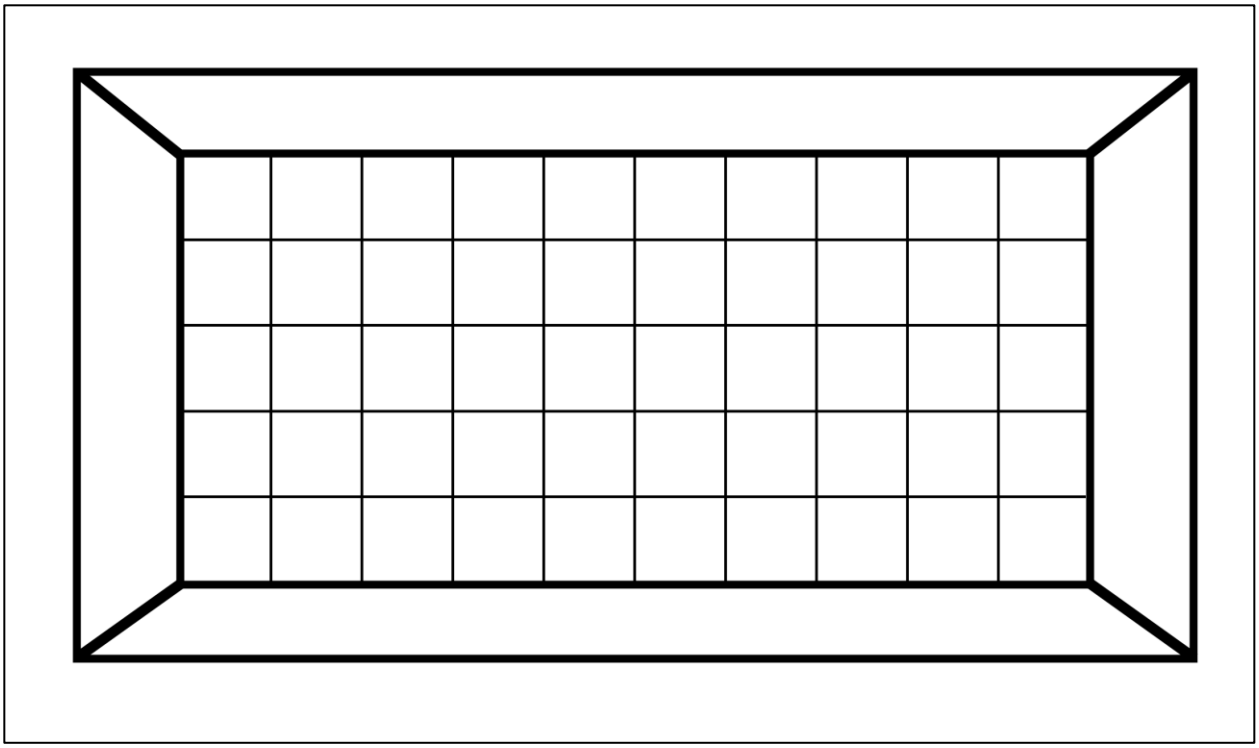

Figure 2.3: Open field apparatus. A grid of 10 squares by 5 squares designates location (corner, outer, and inner squares) within the open field apparatus. Fish were placed in an acclimation chamber in the centre area for two minutes before they were released to explore freely for five minutes.

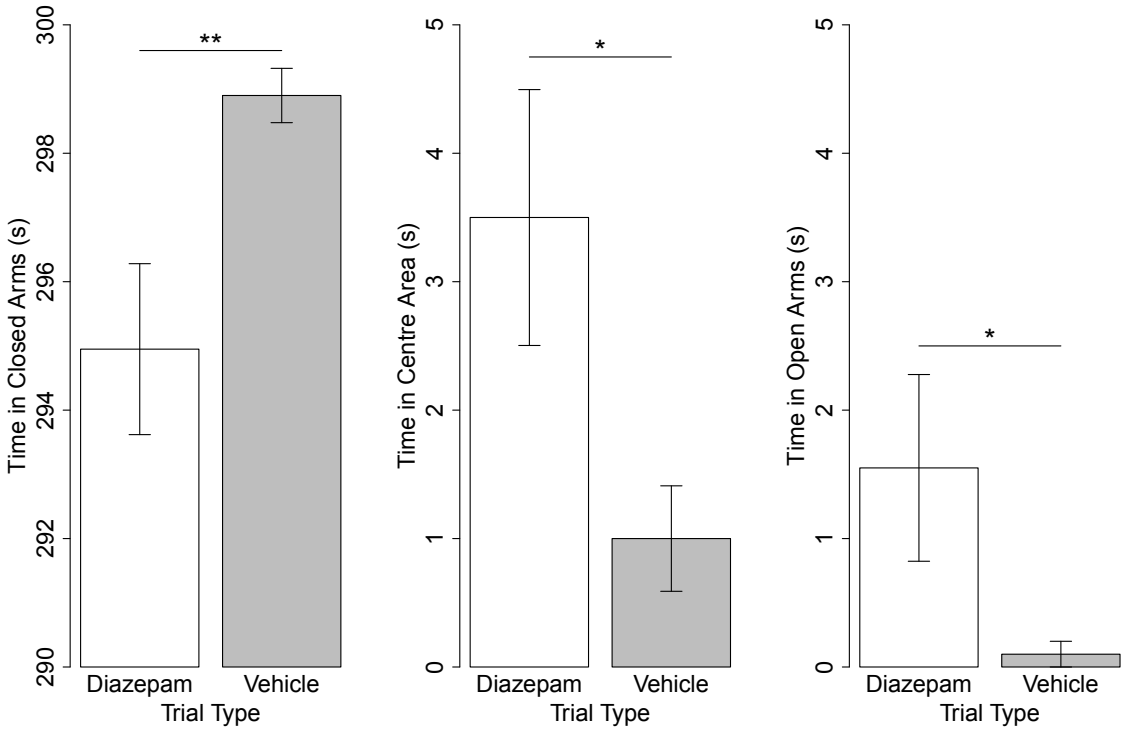

Figure 2.4: Barplots of time spent in closed arms, the centre area, and open arms as a function of drug exposure. After diazepam exposure, fish spent less time in closed arms ($p = 0.0035$) and more time in the centre area ($p = 0.014$) and in open arms ($p = 0.044$) as opposed to after vehicle exposure (* $p < 0.05$; ** $p < 0.001$)

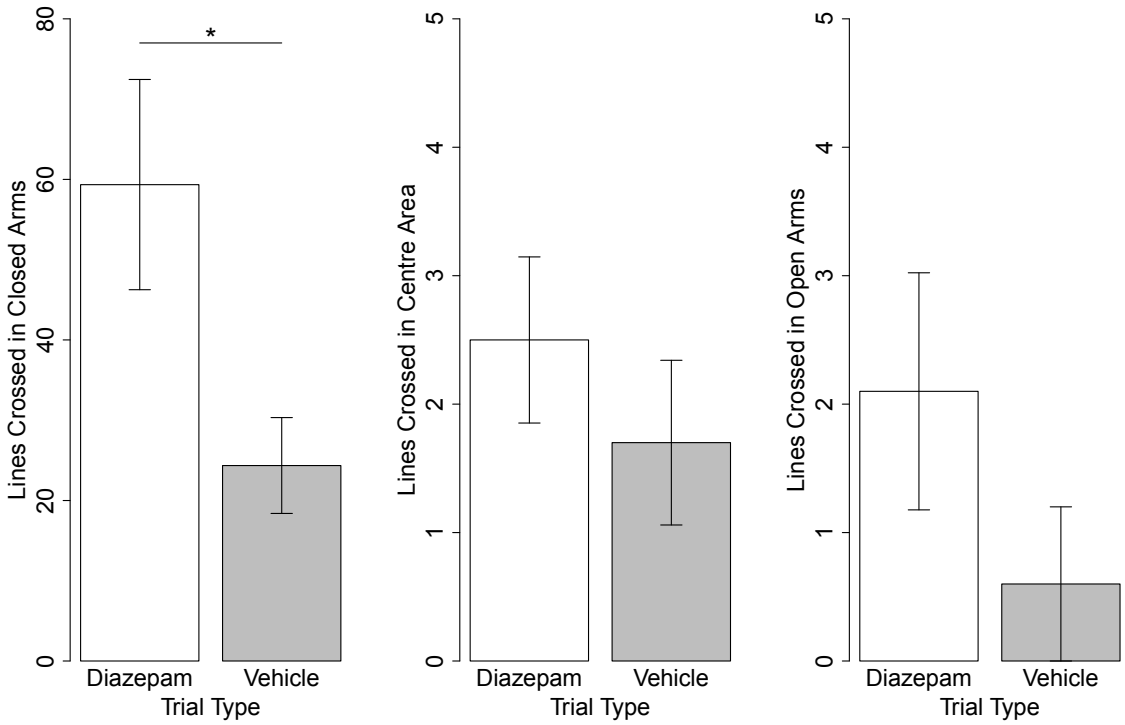

Figure 2.5: Barplots of lines crossed in closed arms, the centre area, and open arms as a function of drug exposure. Fish crossed more lines in closed arms ($p = 0.011$) after diazepam exposure than after vehicle. Lines crossed in the centre area ($p = 0.34$) and in open arms ($p = 0.14$) did not differ between diazepam and vehicle trials (* $p < 0.05$).

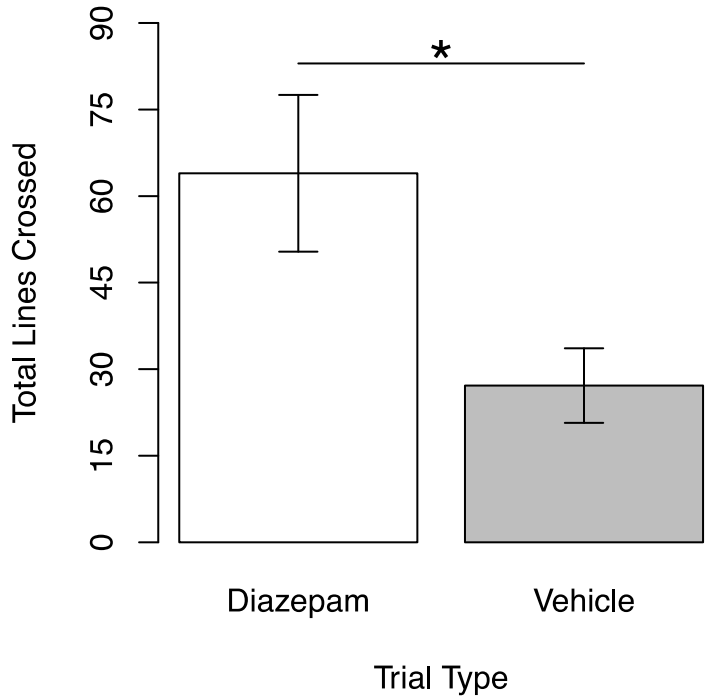

Figure 2.6: Barplot of total number of lines crossed as a function of drug exposure. Fish crossed more total lines after diazepam exposure than after vehicle ($p = 0.011$; * $p < 0.05$).

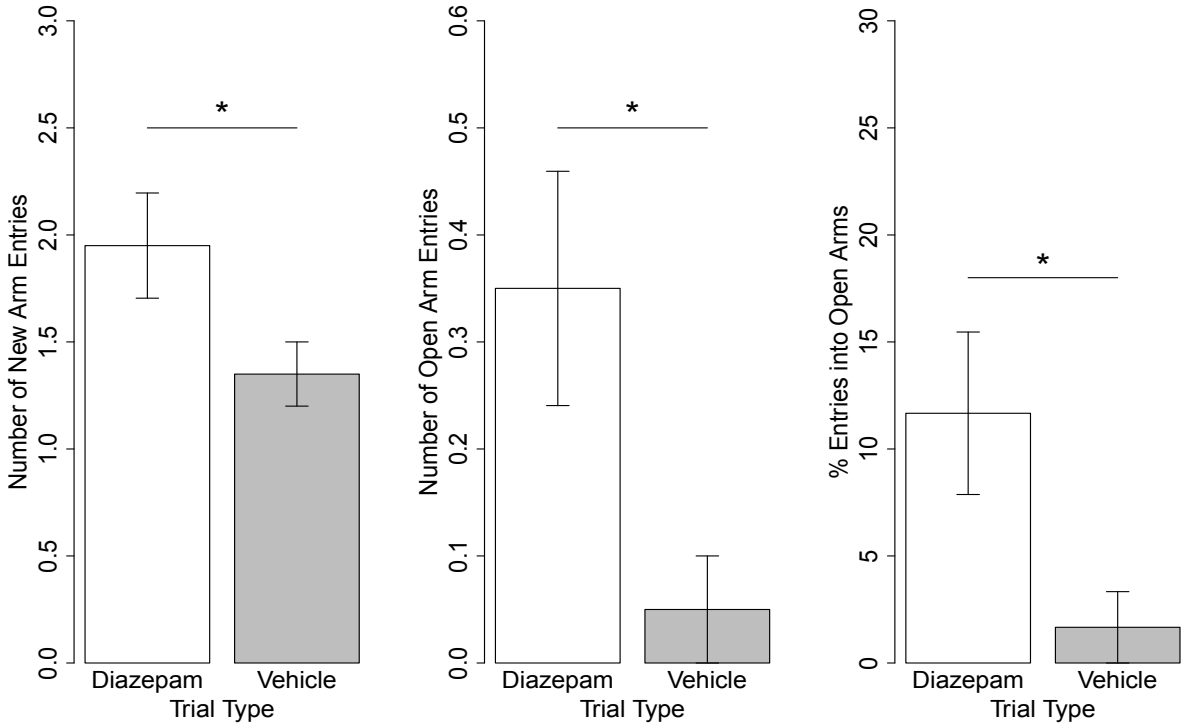

Figure 2.7: Barplots of total entries into new arms, entries into open arms, and percent entries into open arms as a function of drug exposure. After diazepam exposure, fish entered more new arms ($p = 0.031$) and more open arms ($p < 0.01$). The percent of entries into open arms was also increased by diazepam exposure ($p = 0.013$). Note that since the percent of entries into open arms is a ratio of the previous entry measures, is not an independent representation (* $p < 0.05$).

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Chapter 3: Effect of juvenile stress exposure on individual personality and population-level behavioural syndromes

Introduction

Animal personality refers to behavioural variation within individuals (Réale, Reader, Sol, McDougall, & Dingemanse, 2007). Some commonly examined personality traits are boldness, exploration, and aggressiveness. Boldness is the latency to approach novel objects or environments, exploration addresses patterns of movement in a novel environment, and aggression is behavioural conflict between individuals (Toms, Echevarria, & Jouandot, 2010; Réale et al., 2007).

While personality traits are constructed by intra-individual variation, intra-individual covariation indicates the presence of a behavioural type and inter-individual covariance signifies a behavioural syndrome (Sih, Bell, & Johnson, 2004; Dingemanse et al., 2007; Bell, 2007). These traits/syndromes may be adaptive because they can be influenced by environmental conditions (Sih et al., 2004). Some commonly studied behavioural syndromes are aggression-boldness and exploration-boldness (Sih et al., 2004; Bell & Sih, 2007; Dingemanse et al., 2007; Mazué, Dechaume-Moncharmont, & Godin, 2015). Juvenile convict cichlids display an exploration-boldness syndrome in which boldness and exploration are positively correlated (Mazué et al., 2015). Alternately, predation stress elicits an aggression-boldness syndrome in sticklebacks, producing bold fish that are less aggressive than shy fish.

The patterns of behavioural and physiological responses to a stressor are called stress coping style (Koolhaas et al., 1999). Coping style can be described on a continuum between proactive (e.g., active responses and low cortisol reactivity) and reactive (e.g., passive responses and high cortisol reactivity; Øverli et al., 2007). Coping style is similar to anxiety because they

are both operationalized by boldness, exploration, and activity personality traits, although coping style also includes aggression and stress hormone levels (e.g., cortisol). These traits can be measured using behavioural tests such as emerge latency test, open field test, and plus maze and are typically sensitive to stress exposure treatments (Stewart et al., 2011; Toms et al., 2010).

Personality traits and behavioural syndromes can be influenced by predation pressure and other stressors (Bell & Sih, 2007; Brown, Burgess, & Braithwaite, 2007). Previous studies in fish have found that stress exposure reduces freezing/hiding behaviours (Moscicki & Hurd, 2015), thigmotaxis (Champagne, Hoefnagels, de Kloet, & Richardson, 2010), and emerge latency (Brown, Jones, & Braithwaite, 2005). Stress-naïve rodents also tend to prefer closed arms, while stressed rodents showed no arm preference, suggesting that stress exposure influences anxiety-like behaviour (D'Aquila, Brain, & Willner, 1994). Bell and Sih (2007) exposed sticklebacks to predation and uncovered an aggression-boldness behavioural syndrome previously absent in the same population. Juvenile stress exposure has been shown to elicit stress resilience effects, whereby organisms exposed to moderate stress as juveniles demonstrate more proactive coping styles as adults (Lyons & Parker, 2007). This shift toward proactive coping is characterized by increased boldness and exploration accompanied by decreased cortisol reactivity (Koolhaas et al., 1999; Øverli et al., 2007).

Here I examine the effects of juvenile stress exposure on adult convict cichlid (*Amatitlania nigrofasciata*) personality and behavioural syndromes. To explore differences in personality and behavioural syndromes between stressed and unstressed fish, an ecologically relevant simulated predation attack was used: chasing with a dip net. If juvenile stress exposure leads to adaptive adult phenotypes, stressed individuals should display more bold and

exploratory behaviours with lower cortisol levels than controls and stressed populations should display a stronger exploration-boldness behavioural syndrome compared to controls.

Methods

Subjects and Housing

Subjects consisted of 94 laboratory-bred convict cichlid fish from five different broods. Each brood was randomly split in half into two separate tanks, creating five treatment tanks (n = 42 total) and five brood-matched control tanks (n = 52 total). Fish in treatment tanks were subjected to stress by being chased with a dip net for two minutes per day, starting the day they became free-swimming (i.e., approximately 6 days post-fertilization), for 14 days. Fish in control tanks were not stressed by net chasing at any stage of life (beyond that required to administer tests in adulthood). Subjects were housed in 40 L (51 cm × 31 cm × 25.5 cm) mixed-sex communal aquaria with brood mates corresponding to treatment. Fish were fed *ad libitum* six days a week with various prepared dried fish foods and aquaria were maintained at 20°C on a 12L:12D light cycle. I injected a unique identifier consisting of one or two elastomer tags (Visible Implant Elastomer, Northwest Marine Technology Inc., Shaw Island, WA, USA) in various colours under the scales at one of four possible locations in all fish. This identification method is used extensively in fish because of its brief procedure and recovery durations with no adverse behavioural effects. Nine months after hatching, fish were tested for behaviour in emerge latency test, open field test, and submerged plus maze on consecutive days, and then a cortisol sample was taken six days later, following the same sequence for all fish.

Behavioural Tests

Emerge Latency Test

I tested boldness with an emerge latency test (Toms et al., 2010). The emerge latency apparatus consisted of a black plexiglass enclosure (20 cm × 20.5 cm × 26 cm) with a steel base (25 cm × 25.5 cm) against one interior wall of a 40 L tank (51 cm × 31 cm × 25.5 cm) filled with 1 cm of aquarium sand and 11 cm of water. The experimenter opened a door (19.5 cm × 25 cm) in the front of the enclosure by pulling a string while occluded from view by a curtain (see Fig. 3.1). A webcam recorded all trials.

Each fish was placed into the plexiglass enclosure of the apparatus and allowed to acclimate for two minutes before the door was lifted. The latency to vacate the enclosure was recorded for up to 300 seconds. After completion of the test, fish were identified by their elastomer tag under ultraviolet light.

Open Field Test

I assessed tendency to explore a novel environment with an open field test (Toms et al., 2010). Open field tests were conducted in a 20 L tank (40 cm × 25 cm × 21 cm) filled with 11 cm of water with markings to designate location in the tank (fifty 5 cm × 5 cm squares). An acclimation chamber (opaque plastic cylinder, 5 cm × 5 cm × 13 cm) was placed in the center of the tank. A curtain occluded the experimenter from view while a video camera recorded all trials.

Each fish was placed into the acclimation chamber of the apparatus and allowed to acclimate for two minutes before the chamber was removed. The movement of the fish was then recorded for five minutes. After completion of the open field test, fish were identified by their elastomer tag. I scored behaviour using JWatcher (Blumstein, Daniel, & Evans, 2010). Outcome variables included: amount of time spent in each type of square (corner, outer, and inner) and

number of squares entered of each type (corner, outer, and inner). Entering a square was defined as the fish crossing the line of that square with its head up to the pectoral fins. The outcome variables were compiled in a principal component analysis (PCA) to summarize the behavioural outcomes of the open field test. To prevent issues associated with collinearity due to time spent in each area summing to 300 seconds, I excluded the amount of time spent in corner squares from the PCA.

Submerged Plus Maze

I examined anxiety levels with a submerged plus maze apparatus (adapted from the elevated plus maze, see Chapter 2; Pellow, Chopin, File, & Briley, 1985). The apparatus is shaped as a plus symbol, with four arms alternating between transparent and black walls (each arm 12 cm × 4.5 cm × 13 cm), filled with water to a depth of 10 cm. This arrangement results in two visually closed arms, two visually open arms, and a center area. To quantify travel within the arms, arms are marked every 1.5 cm away from the center, creating eight lines. The center of the maze is also marked with lines 1.5 cm apart, horizontally and vertically, forming a 3 × 3 grid. An acclimation chamber (transparent plastic cylinder, 4.5 cm × 4.5 cm × 13 cm) was placed in the center of the maze. A webcam viewed the apparatus from above to observe all movements and a black curtain occluded the experimenter.

Each fish was placed in the acclimation chamber for two minutes, after which, the chamber was removed and the fish allowed to swim freely within the maze. The fish's actions were recorded for five minutes. Upon trial completion, fish was weighed (in grams), measured for standard length (in centimeters), and identified by their elastomer tag. I scored behaviour according to the number of lines crossed per area (visually closed, visually open, and center) and

the amount of time spent in each area (visually closed, visually open, and center). Line crossing was defined as the fish passing the line with its head up to the pectoral fins. The outcome measures above were compiled into a PCA to summarize the behavioural outcomes of the plus maze. To prevent collinearity issues due to time spent in each area summing to 300 seconds, I excluded the amount of time spent in closed arms from the PCA.

Cortisol Sampling

I collected water-borne cortisol samples for stress hormone analysis by placing a fish in a sterilized 250 mL beaker filled with 150 mL of distilled water. The experimenter was occluded from view by white barriers positioned around the beaker to form a four-walled testing area. The beakers and nets were sterilized with 100% ethanol and rinsed with distilled water between each fish.

A trial consisted of removing a fish from its communal holding tank using a sterilized dip net and placing the fish in the beaker. The fish remained in the beaker for 30 minutes to allow for ample cortisol collection (Archard, Earley, Hanninen, & Braithwaite , 2012), upon which the contents of the beaker, fish included, were poured into another sterilized beaker through a second sterilized dip net to allow collection of the fish. The fish was then weighed, measured to standard length, and identified by its elastomer tag. Water samples were transferred to a freezer-safe container and frozen until hormone extraction.

Cortisol Extraction and Analysis

I extracted steroid hormones from thawed water samples using a solid phase extraction method with C18 columns (Bond Elut 200 mg 3 mL; Agilent Technologies, Santa Clara, CA,

USA), a 20-port vacuum manifold (VM20, Sigma-Aldrich), and a vacuum (Earley et al., 2006; Kidd, Kidd, & Hofmann, 2010; Sebire, Katsiadaki, & Scott, 2007). Columns were primed with 2 × 2 mL of 100% methanol followed by 2 × 2 mL of distilled water before drawing the samples through under vacuum pressure. After the sample had completely passed through the column, 2 mL of distilled water was used to purge salts. Columns were then sealed with Parafilm and frozen at -20°C until elution. Using 3 mL of 100% ethanol, hormones were eluted from the columns into glass vials and processed by drying under a nitrogen flow before being stored at -20°C. For the enzyme immunoassay (EIA), an experimenter suspended the pellets in 300 µL of EIA buffer (Enzo cat#ADI-901-071) to be assayed in 100 µL duplicates. Adhering to manufacturer's instructions, cortisol levels were calculated using a linear fit of serial dilutions of supplied standards. Coefficients of variation of cortisol level duplicates did not exceed 20%, except for eight individuals which were removed from the analysis.

Protocols were approved by the University of Alberta Biological Sciences Animal Policy and Welfare Committee (protocol number 00000055) and adhere to the guidelines of the Canadian Council for Animal Care.

Data Analysis

All statistical analyses were performed using R version 3.1.3 (R Foundation for Statistical Computing, Vienna, Austria). Significant *p*-values were set at 0.05. Four fish with incomplete data were excluded from analyses.

I collapsed the outcome measures of open field and plus maze behaviours into composite behavioural scores using principal component analysis separately for each test (the *princomp()*

function was used). Before performing the principal component analyses, I removed one time-related variable from each test to remove collinearity caused by the three variables summing to 300 seconds. I determined collinearity in time-related outcome measures using Kendall correlations between time spent in inner, outer, and corner squares of the open field test and between time spent in open, closed, and center areas of the plus maze. I then compared the tau values of each pairwise correlation and subsequently removed the outcome variable with the two strongest tau values from the principal component analysis for that test (time spent in corner squares in open field and closed arms in plus maze). Principal component analysis allowed me to reduce the number of variables from six outcome measures to two principal components for each test.

Kendall's tau was used for all tests of association between pairs of variables for its robustness and ability to detect both linear and non-linear effects. I performed pairwise correlations using Kendall's tau to examine relationships between weight and the five behavioural variables (emerge latency, two open field principal components, and two plus maze components).

To assess the effect of treatment on individual behaviour, I performed repeated measures ANOVA model comparisons of treatment according to the methods in Faraway (2006) by calculating the X^2 from the difference between the model likelihood estimates. Brood was classified as a crossed random effect, while all other variables were classified as fixed effects. In the null model, the behavioural variable was assessed as a function of brood, mass, and handling time to control for potential confounds. The alternative model was identical to the null model but included treatment to assess the difference between the two models as a function of stress exposure. These model comparisons were applied separately to each behavioural variable. I also

used this method to assess the effect of treatment on cortisol levels with brood, coefficient of variance, and handling time included in both models.

I examined the relationship between cortisol and the five behavioural variables using type II Wald chi-squared tests and the *lmer()* function. The behavioural variable served as the dependent variable and cortisol was set as a covariate with treatment and brood as categorical factors (fixed and random effects, respectively).

To investigate the presence of behavioural syndromes, I performed pairwise Kendall correlations between variables for each treatment. The distributions of the resulting *p*-values were compared to the uniform distribution predicted by the null hypothesis using one-sample Kolmogorov-Smirnov tests, as in Seaver and Hurd (2017).

Results

There was no significant difference between the stress and control treatment groups in body mass ($M_{stressed}$ = 0.50 g, $M_{control}$ = 0.48 g; $t(87.1)$ = 0.14, p = 0.89) or body length ($M_{stressed}$ = 2.25 cm, $M_{control}$ = 2.20 cm; $t(81.0)$ = 0.39, p = 0.69).

Principal component analysis of behaviour in the open field test revealed two components accounting for the majority of the variability (89%, see Table 3.1), which were used for subsequent analyses and discussion. The first principal component (PC1) of the open field test accounted for 54% of the variance and is characterized by inactivity in corner squares, which can be interpreted as freezing and hiding behaviour (Table 3.1). Fish with high open field PC1 scores spent less time in inner and outer squares and entered fewer squares of any kind, therefore displaying more freezing and hiding behaviours. The second principal component (PC2) accounted for 35% of the variance and was sensitive to the time and movements in inner versus

outer squares, suggesting negative thigmotaxis (Table 3.1). Fish with high open field PC2 scores spent more time in inner squares and entered more inner squares, entering fewer outer and corner squares, therefore demonstrating less thigmotaxis. Principal component analysis of behaviour in the plus maze revealed two components also accounting for the majority of the variability (72%, see Table 3.1). The first principal component (PC1) of the plus maze accounted for 45% of the variance and, like PC1 of open field, is characterized by inactivity and time spent in closed arms, also describing freezing and hiding behaviour (Table 3.1). Fish with high plus maze PC1 scores spent less time in the center and open arms and crossed fewer lines of any kind, therefore displaying more freezing and hiding behaviours. The second principal component (PC2) accounted for 27% of the variance and was sensitive to the time and movements in closed arms and center area versus open arms, describing movement tendencies (Table 3.1). Fish with high plus maze PC2 scores crossed more lines in the center and closed arms and spent more time in closed arms, spending less time and crossing fewer lines in open arms. Plus maze PC2 can be characterized by movement and presence in closed and center areas opposing movement and presence in open arms.

Heavier fish had lower open field PC2 scores than lighter fish (Kendall correlation: $n = 90$, $\tau = -0.25$, $p << 0.01$) and higher plus maze PC2 scores than lighter fish (Kendall correlation: $n = 90$, $\tau = 0.23$, $p = 0.002$). For example, heavier fish exhibited more thigmotaxis in the open field test and preferentially moved and lingered in closed and center areas of the plus maze test. The remaining variables were not correlated with weight (Kendall correlation: $n = 90$, Emerge latency: $\tau = -0.007$, $p = 0.93$; open field PC1: $\tau = -0.1$, $p = 0.17$; plus maze PC1: $\tau = -0.01$, $p = 0.89$).

Open field PC1 scores were correlated with plus maze PC1 scores (Kendall correlation: n=90, $\tau = 0.16$, $p = 0.017$; Table 3.2), meaning that fish who exhibited fewer freezing and hiding behaviours in the open field test also exhibited fewer freezing and hiding behaviours in plus maze. Open field PC2 scores were not correlated with plus maze PC2 scores (Kendall correlation: n = 90, $\tau = -0.002$, $p = 0.98$), indicating that movement patterns in open field and plus maze are not related (see Table 3.2 for all pairwise correlations).

There was no significant difference in any of the behavioural variables between stress and control treatment groups. The difference in open field PC1 scores between fish in the stressed treatment and fish in the control treatment approached significance (X^2 (1) = 3.32, p = 0.068, Fig. 3.1), such that stressed fish exhibited fewer freezing and hiding behaviours in the open field test. Stressed fish produced more cortisol than controls (X^2 (1) = 5.29, $p = 0.021$, Fig 3.2). The remaining variables did not differ significantly by treatment (Emerge latency: X^2 (1) = 1.28, $p = 0.26$; open field PC2: X^2 (1) = 0.01, $p = 0.91$; plus maze PC1: X^2 (1) = 0.004, $p = 0.95$; plus maze PC2: X^2 (1) = 0.32, $p = 0.57$).

Cortisol was not correlated with any of the behavioural variables (Emerge latency: X^2 (1) = 0.52, $p = 0.47$; open field PC1: X^2 (1) = 0.68, $p = 0.41$, open field PC2: X^2 (1) = 0.22, $p = 0.64$; plus maze PC1: X^2 (1) = 0.14, $p = 0.71$; plus maze PC2: X^2 (1) = 0.45, $p = 0.50$). Despite this, this analysis did demonstrate an effect of treatment on open field PC1 (X^2 (1) = 4.25, $p = 0.04$).

To test for the presence of a behavioural syndrome, I examined the pattern of relationships between our five behavioural variables in each treatment. Table 3.3 lists the Kendall's tau for these relationships for control and treatment groups, respectively. I compared the distributions of the *p*-values from these pairwise correlations against the null hypothesis, which predicts that *p*-values will be uniformly distributed. In the stressed treatment, the *p*-values

for these relationships did not differ significantly from uniform (Kolmogorov-Smirnov: D = 0.22, p = 0.64; Fig. 3.3), showing no evidence of a behavioural syndrome. The control group showed positively skewed p-values with a significant bias towards small p-values (Kolmogorov-Smirnov: D = 0.54, p = 0.003; Fig. 3.3), demonstrating significant patterns of covariance between behavioural traits. This revealed the presence of a behavioural syndrome within the control, but not the stressed, population. To assess the full range of personality variation within these populations, data was not standardized by brood, though the trend remained upon examination of standardized scores.

Discussion

I predicted that juvenile stress exposure would result in increased boldness and exploratory behaviours in stressed individuals. This prediction was incongruent with the finding that behaviour was not influenced by stress treatment. I also hypothesized that juvenile stress exposure would result in a stronger exploration-boldness behavioural syndrome, but found no evidence of an exploration-boldness behavioural syndrome in the stressed population.

Juvenile stress exposure did not have a significant effect on personality traits related to stress coping style. The near-significant difference in freezing and hiding behaviours between stressed and control individuals indicates that stress exposure may have some influence on adult behaviour. The tendency toward fewer freezing and hiding behaviours in stressed individuals is in agreement with significant results of previous studies (Moscicki & Hurd, 2015; Champagne et al., 2010). It is possible that the degree of stress during development was too mild and was not intense enough to cause visible effects at the individual level. The exact amount of stress required to permanently alter individual personality may exceed what I administered.

Alternatively, greater statistical power may be required to detect these effects, necessitating a larger sample size.

This level of stress exposure may not have altered scores on simple personality traits but did alter cortisol; fish in the stressed treatment had significantly higher cortisol levels than control fish. Since juvenile stress exposure should result in more proactive coping styles, cortisol levels should be similarly altered, following the profile of lower cortisol release in response to stress (Koolhaas et al., 1999; Øverli et al., 2007). The cortisol reactivity of fish in the stressed treatment does not suggest a shift toward proactive coping but instead toward reactive coping. This result must be interpreted with caution, however, because it does not compare baseline cortisol levels to stress responsive cortisol levels within individuals. Archard et al. (2012) found no significant effect of predation history on near-baseline cortisol concentrations obtained using a similar procedure, although they did find an effect of predation on cortisol release rates after exposure to the open field test (i.e., stress responsive levels). Additionally, an important consideration when examining water-borne cortisol levels is the potential for the procedure to induce a stress response. While the water-borne collection method is preferable due to its relative non-invasiveness, it is vulnerable to the handling and confinement stress it produces (Wong, Dykstra, Campbell, & Earley, 2008). Wong et al. (2008) found that the first exposure to the collection beaker generates a stress response in convict cichlids that habituates after 3-4 presentations, indicating that a habituation protocol may be required to obtain baseline cortisol levels using this method. It is possible that the change in cortisol levels from baseline to stress responsive is smaller in the stress-experienced fish than in the controls, albeit with a higher baseline. Alternatively, the degree of stress during development may not have been at a level

which results in decreased cortisol levels and instead resulted in increased levels (i.e., maladaptive phenotype).

While this degree of stress exposure may not have elicited significant behavioural effects at the individual level, it did alter population-level covariance. The presence of significant covariance between behaviours seen in the control population shows a deeper underlying personality structure that was absent in the stressed population. Mazué et al. (2015) found a similar exploration-boldness syndrome in juvenile convict cichlids in which boldness and exploration were positively correlated. This contrasts with Bell and Sih's (2007) finding that predation stress exposes a behavioural syndrome absent in control fish, while our stress treatment seems to have diminished the relationships that contribute to the behavioural syndrome present in the controls. It is possible that the differing developmental timing of stress exposure in these studies had contrasting influences on the resulting behavioural syndrome (Sih et al., 2004), such that juvenile stress exposure may eliminate a syndrome, while adult stress exposure may produce a syndrome. Additionally, Bell and Sih (2007) examined an aggression-boldness syndrome, while I examined an exploration-boldness syndrome. This difference in the examined behaviours may explain these apparently opposing effects of stress on personality syndrome. Aggression-boldness syndromes may be elicited by stress, while exploration-boldness syndromes may be naturally occurring, as seen in other species (Mazué et al., 2015; van Oers, Drent, de Goede, & van Noordwijk , 2004; Wilson & Godin, 2009). It does not seem that sticklebacks are naturally constrained to produce an aggression-boldness syndrome, but under certain circumstances, the syndrome may provide survival advantage, leading to the development of the syndrome. The similarity between juvenile (Mazué et al., 2015) and adult convict cichlids indicates continuity of an exploration-boldness syndrome throughout life. The exploration-boldness syndrome

demonstrated by the stress-naïve population suggests natural constraints may be pushing toward the development of the syndrome, but when faced with a challenging experience early in life, those constraints may no longer be present or exert the same influence, leading to the absence of the syndrome seen in the stress-experienced population. It is possible that the stress exposure could have disrupted the creation or maintenance of this default syndrome. While this stress exposure may have impaired the development of this syndrome, other syndromes may have resulted. It is possible that an aggression-boldness syndrome or another syndrome was produced in this population. Stress may therefore have different effects on different types of behavioural syndromes.

In conclusion, I was unable to cause lifelong changes in personality traits in individuals subjected to transient predator stress during early life. Despite this, transient predator stress did result in population-level changes in behavioural covariance. The exploration-boldness syndrome present in the unstressed population was absent in the population exposed to stress.

Open Field		
Behaviours	Component 1	Component 2
% Variance Explained	54	35
Time in outer squares	-0.51	0.01
Outer squares entered	-0.48	-0.44
Time in inner squares	-0.39	0.55
Inner squares entered	-0.44	0.46
Corner squares entered	-0.40	-0.54
Plus Maze		
Behaviours	Component 1	Component 2
% Variance Explained	45	27
Lines crossed in closed arms	-0.47	0.37
Time in open arms	-0.54	-0.45
Lines crossed in open arms	-0.56	-0.41
Time in center	-0.26	0.39
Lines crossed in center	-0.34	0.58

Table 3.1: Principal components 1 and 2 from the principal component analyses for open field and plus maze outcomes. Time spent in corner squares (open field) and time spent in closed arms (plus maze) were excluded due to collinearity for their respective principal component analyses.

		Open Field PC1	Open Field PC2	Plus Maze PC1	Plus Maze PC2	Cortisol
Emerge Latency	tau	0.195	0.167	0.144	-0.021	0.001
	p	0.008	0.024	0.051	0.77	0.99
Open Field PC1	tau		0.146	0.158	-0.083	0.023
	p		0.041	0.027	0.25	0.76
Open Field PC2	tau			0.008	-0.002	-0.04
	p			0.91	0.98	0.60
Plus Maze PC1	tau				-0.137	-0.09
	p				0.056	0.24
Plus Maze PC2	tau					-0.069
	p					0.37

Table 3.2: Pairwise correlations between behaviour and cortisol variables are shown with the Kendall's tau and corresponding *p*-values.

Stressed				
	Open Field PC1	Open Field PC2	Plus Maze PC1	Plus Maze PC2
Boldness	0.006	-0.072	0.113	0.157
Open Field PC1		0.161	0.236	-0.063
Open Field PC2			-0.117	0.074
Plus Maze PC1				-0.007

Unstressed				
	Open Field PC1	Open Field PC2	Plus Maze PC1	Plus Maze PC2
Boldness	0.352	0.358	0.142	-0.177
Open Field PC1		0.148	0.135	-0.094
Open Field PC2			0.077	-0.067
Plus Maze PC1				-0.231

Table 3.3: Relationships between behavioural variables in stressed and unstressed populations. Kendall's tau values are shown for the relationships between the five behavioural variables for the stressed (top) and unstressed (bottom) populations. Figure 3.3 displays the pattern of *p*-values for these relationships to depict patterns of covariance.

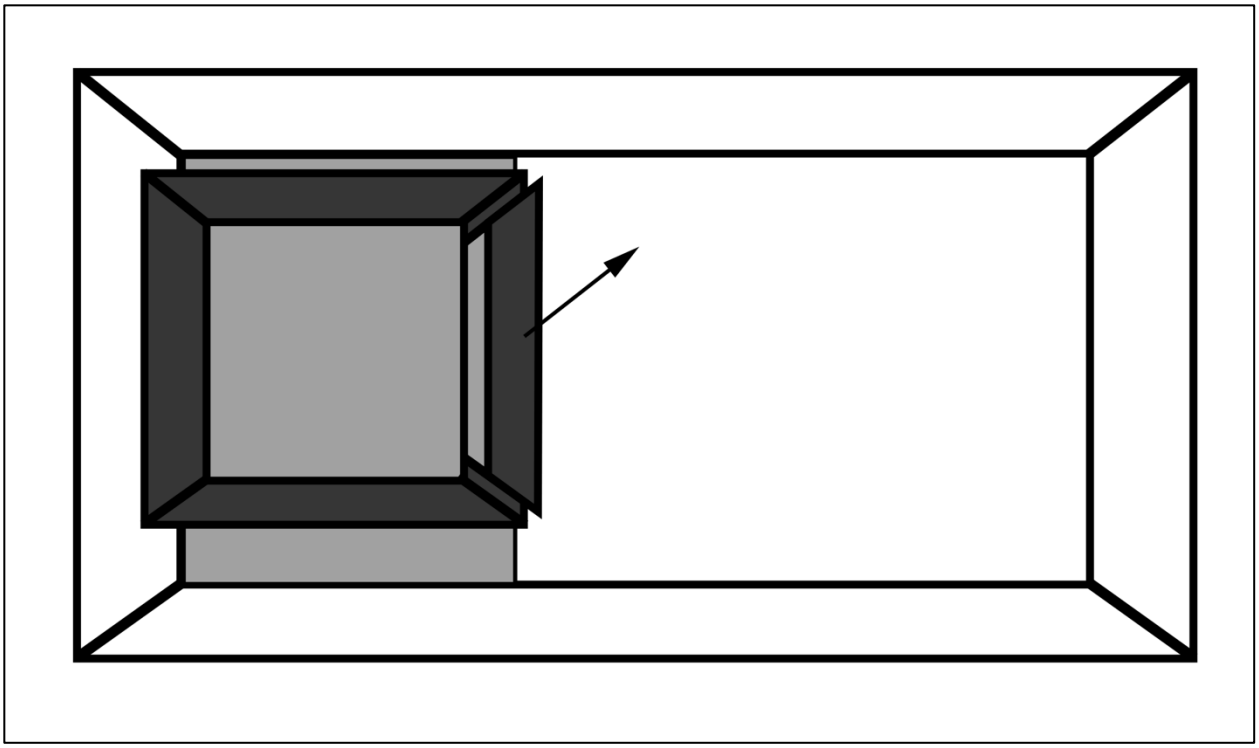

Figure 3.1: Emerge latency apparatus. An enclosure sits atop a steel base with a raised door facing into the open area of the tank. Fish were placed in the enclosure with the door closed for two minutes before the door was raised by a string. Latency to emerge from the enclosure was recorded for up to five minutes.

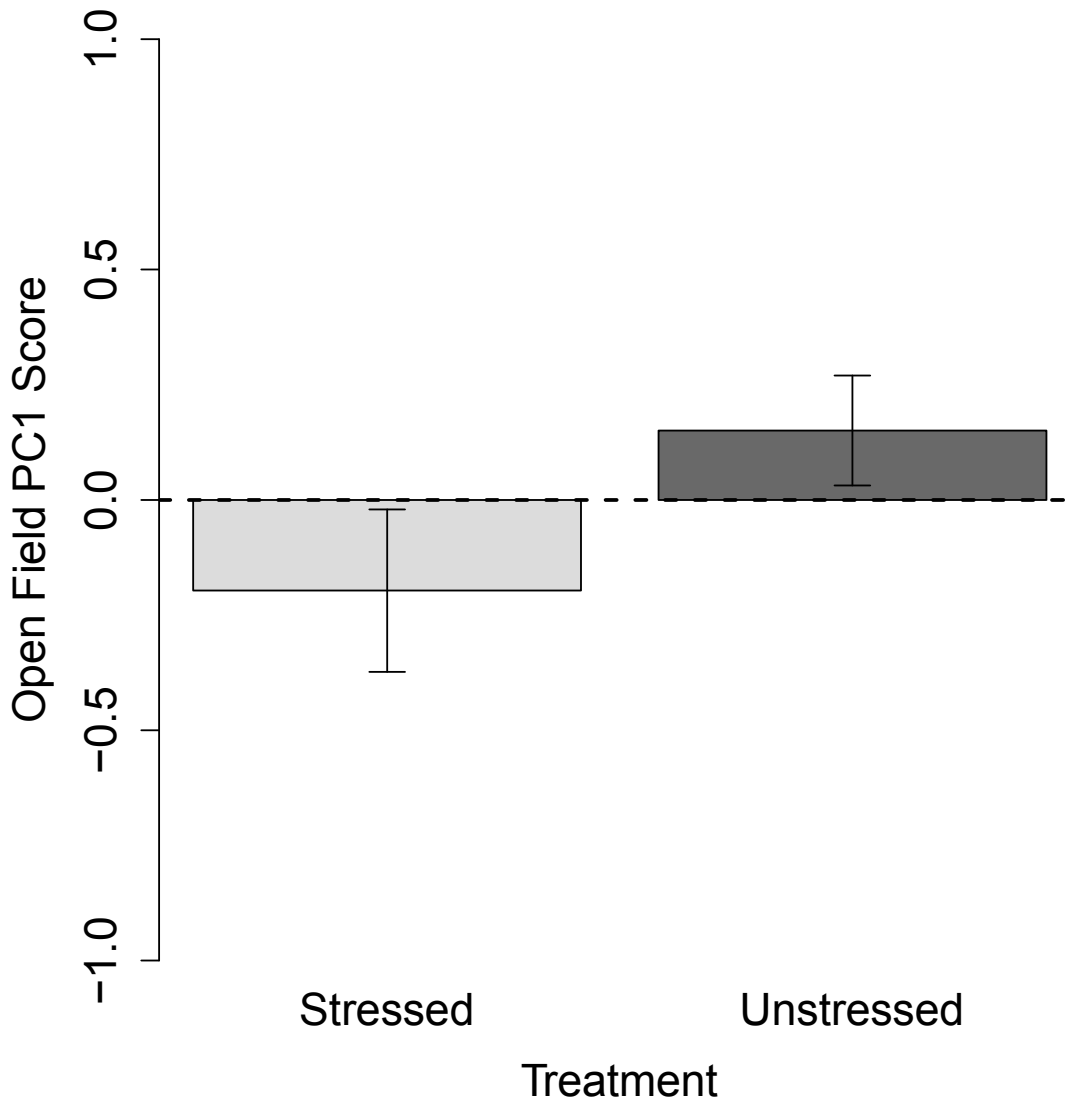

Figure 3.2: Open field PC1 scores by stress exposure. The tendency of stressed fish to exhibit fewer freezing and hiding behaviours (i.e., lower open field PC1 scores) than unstressed individuals approached significance in a model comparison including treatment and covariates ($p = 0.068$).

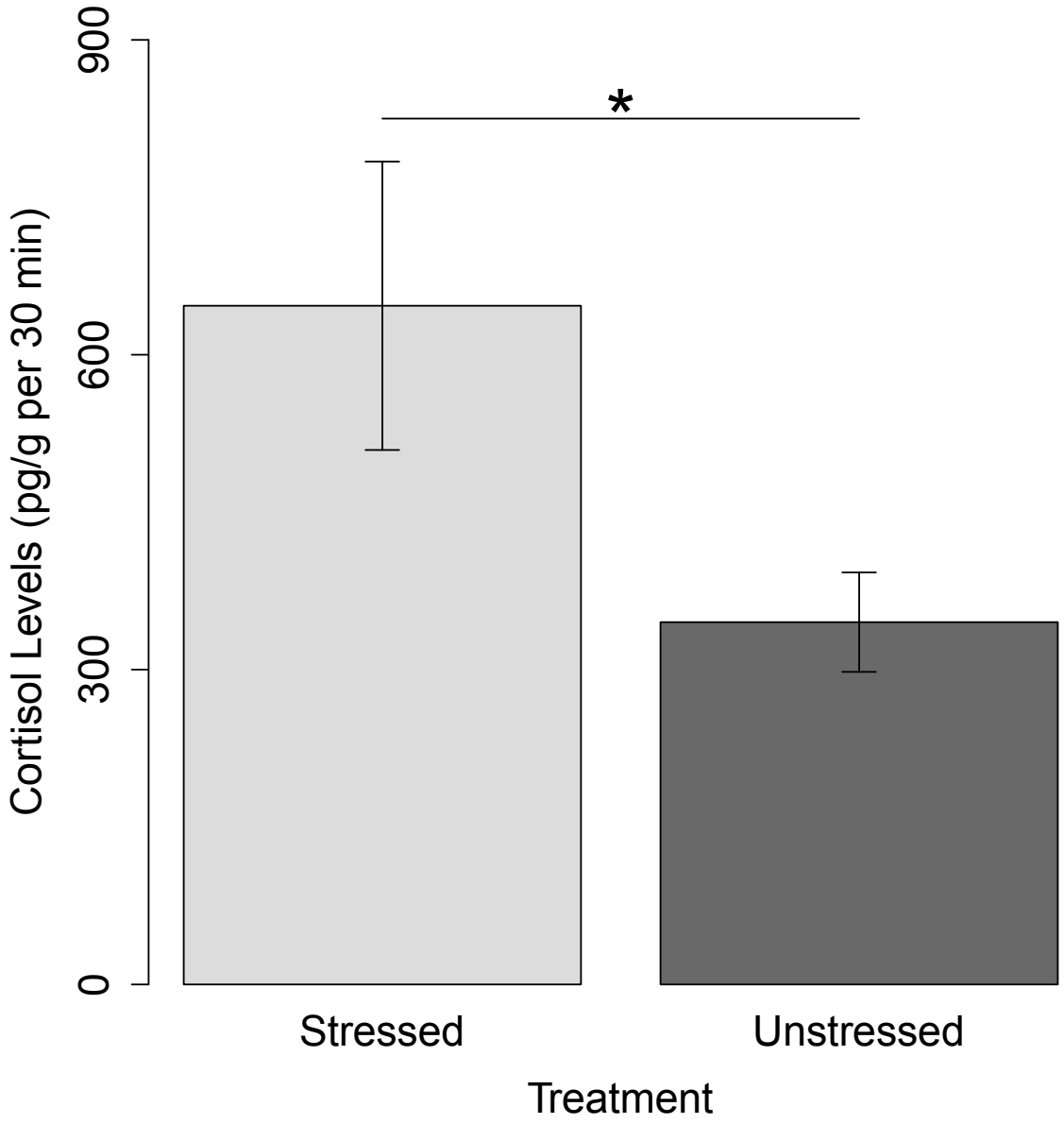

Figure 3.3: Cortisol levels by stress exposure. A model comparison including treatment and covariates showed that fish in the stressed treatment produced higher levels of cortisol than unstressed fish ($p = 0.04$; * $p < 0.05$).

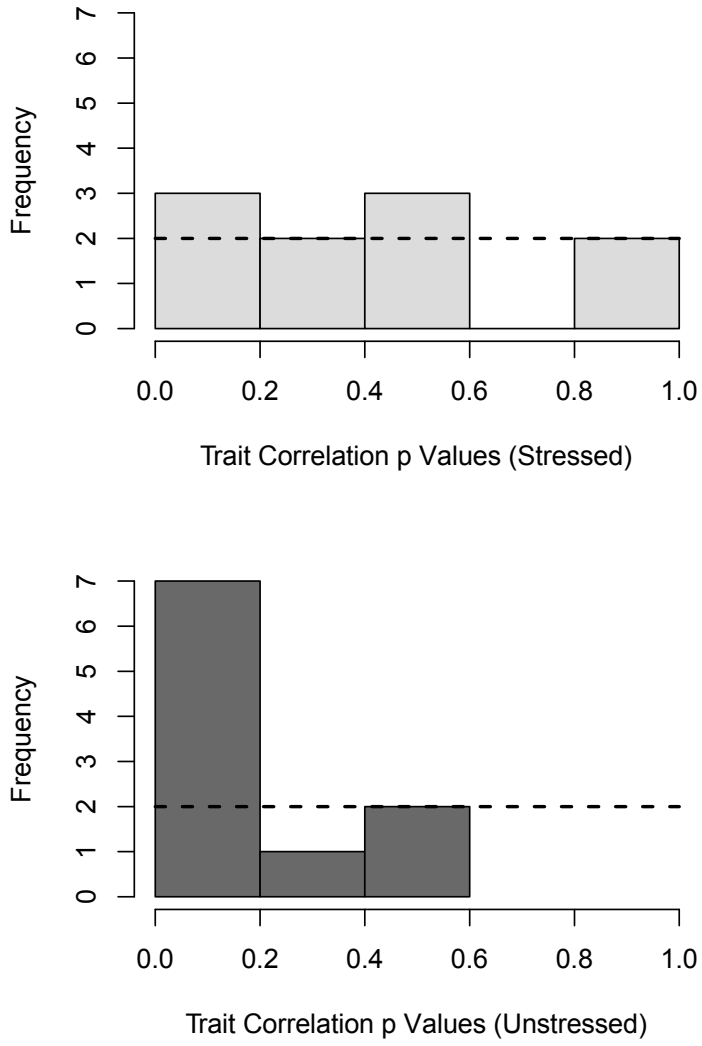

Figure 3.4: Distributions of *p*-values by treatment population. Behavioural covariance in the stressed treatment resulted in *p*-values that did not differ significantly from uniform (top; Kolmogorov-Smirnov: $D = 0.22$, $p = 0.64$). The unstressed population demonstrated patterns of behavioural covariance with a significant bias towards small *p*-values (bottom; Kolmogorov-Smirnov: $D = 0.54$, $p = 0.003$). This revealed the presence of a behavioural syndrome within the unstressed, but not the stressed, population.

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Chapter 4: Discussion

The aim of this thesis was to investigate changes elicited by developmental stress on the behavioural hierarchy in adult convict cichlid fish. To test the effect of early life stress on stress coping style and anxiety, I validated a new behavioural assay for studying anxiety and incorporated it in a battery of measures associated with anxiety. In Chapter 2, I used diazepam to validate the submerged plus maze as a test of anxiety in fish. Drug exposure resulted in more time spent in and entries into open arms than did vehicle exposure. In Chapter 3, I then examined the effect of developmental stress exposure on adult personality and behavioural syndromes. Early life stress resulted in the absence of the exploration-boldness syndrome that was present in the control population. Taken together, these chapters demonstrate an ability to measure anxiety-like behaviour in non-model species.

When behavioural assays are adapted for use in a new species with different physical constraints (i.e., living in water vs. on land), they should be validated to ensure construct validity. Validations ensure the behaviours are paralleled between species and the results may be accurately compared. By using an adapted version of the elevated plus maze, the submerged plus maze, I was able to assess anxiety-like behaviours. While Chapter 2 used the raw outcome measures to validate the submerged plus maze, Chapter 3 used a PCA of the outcome measures to examine the effect of stress exposure. Ultimately, the test remained the same and therefore the behaviours observed remain the same. Using PCA on the outcome measures allowed me to extract the largest sources of variation, reduce the complexity of the dataset, and examine deeper underlying tendencies. This allowed me to focus on the higher levels of the behavioural hierarchy. Using the raw outcome measures also allowed me to closely mirror existing

validations of elevated plus maze (Treit, Menard, & Royan, 1993; Pellow, Chopin, File & Briley, 1985).

Anxiety levels should be prone to modification by pharmacological and environmental interventions (Stewart et al., 2012; Champagne, Hoefnagels, de Kloet, & Richardson, 2010). In both Chapter 2 and 3, I demonstrate the plasticity of anxiety to external factors. Chapter 2 demonstrates the effectiveness of an anti-anxiety drug, diazepam, in changing a major anxiety-like behaviour, thigmotaxis. At an individual level, diazepam increased time in open arms in the submerged plus maze, indicating a modulatory effect on behaviour by an pharmacological substance. Chapter 3 demonstrates a population-level effect of stress exposure on behavioural covariance in three tests of anxiety. The stress-experienced population did not display the default exploration-boldness syndrome seen in unstressed controls. This indicates a shift from one set of behavioural associations that are typically expressed under "normal" evolutionary contexts to another set that may be more adaptive for stressful contexts. The mismatch hypothesis supports this phenotypic shift due to early life programming (Nederhof & Schmidt, 2012).

My initial hypothesis about the effects of developmental stress on the hierarchy of behaviour was not supported. I believed that if a syndrome already existed, organisms would simply alter their behaviour along the spectrum of that syndrome, and if no syndrome existed, one that provides survival value would appear. Given this belief, I did not anticipate predation pressure preventing the formation of a naturally occurring syndrome. Though unexpected, perhaps it is reasonable to conclude that something about the correlation structure of the exploration-boldness syndrome must not be conducive to success in an environment prone to predation attacks (Sih, Bell, & Johnson, 2004). This highlights the adaptability of behavioural covariance and behavioural plasticity. If a certain interaction of behaviours does not provide

survival benefit, that set of interactions should not be formed. Likewise, if another set of interactions will increase survival rate, those associations should be formed, assuming natural selection is unhindered. Unfortunately, it is unlikely that natural selection can induce change without constraints because there are a finite number of instructions that can be coded into DNA. These limitations result in the development of patterns of constrained behaviours specific to a given context. This effect may be a result of pleiotropy, but that would imply movement along the association/syndrome, a conclusion not evident in this thesis. Therefore, it is more likely that the relationship between traits is plastic and optimal for the given context. Behavioural plasticity could be advantageous when it occurs in response to pressures from the given environment.

Since the developmental environment of the stressed fish obviously influenced behaviour at some level, it is difficult to reconcile the finding that simple personality scores were not affected. There must be some driving factor behind the difference in behavioural syndromes between the treatment groups. A population-level effect is usually due to the summation of individual-level effects, suggesting some sort of presently undetected trend in individual personality and behavioural types. I believe that the trend toward significance in freezing and hiding behaviours between treatments in open field indicates that there may be a real effect given a larger sample, thereby increasing power. It is also possible that the sensitivity and specificity are not strong enough to detect these effects. Numerous other studies demonstrate an effect of stress exposure on individual personality, in which prior stress exposure increases activity level (Moscicki & Hurd, 2016), increases boldness (Brown, Burgess, & Braithwaite, 2007), and decreases anxiety (D'Aquila, Brain, & Willner, 1994). The deviation of the present study from this pattern of observations might lead to closer inspection of potential confounds, such as genetics or parental care. Controlling for confounds is important, though lack of such

consideration does not necessarily negate the observed effects. It is possible that the effects may become stronger upon controlling for confounds, as did the effect of treatment on freezing/hiding behaviours when I controlled for the genetic effect of brood (unreported results, Chapter 3). I believe that the environment during development has the ability to influence adult personality but I was unable to detect those effects in this study. It is also important to note that significant effects were detected at the individual level in Chapter 2, which used a within-subjects experimental design. While within-subjects designs make it easier to uncover differences in individual variance, they are not always possible.

This thesis aimed to assess the effects of an anxiolytic compound and developmental stress exposure on anxiety responses in multiple environments. The compound reduced individual anxiety responses, while stress exposure altered the default behavioural profile of anxiety responses at a population level. In conclusion, my thesis demonstrated significant effects of diazepam and developmental stress exposure on adult convict cichlid behaviour, though at varying levels (individual vs. population level effects).

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