Maternal Effect on *Astatotilapia Burtoni* Cichlid Fish

Anxiety-Mediated Behavior and “Personality”

A Thesis

Presented to

The Division of Mathematics and Natural Sciences

Reed College

In Partial Fulfillment

of the Requirements for the Degree

Bachelor of Arts

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May 2012
Approved for the Division

(Biology)

Suzy C.P. Renn
Acknowledgments

Suzy, thank you for making me do six impossible things before breakfast. You’ve been my motivating factor and understood when all the pieces felt like they were crumbling to bits. You always talked about how your students got to go off and do “all the cool things,” but you are the backbone behind that academic inspiration.

Tim, no set of classes ever made me happier. Thank you for helping me bridge the gap between biology and psychology, and for nurturing my excitement for comparative cognition research. Lavinia, thank you for your support and providing us all with more information about the abilities of our feathered friends.

Every biology professor I’ve ever had at Reed: thank you for drilling in me a work ethos only thought possible in robots, for allowing me to express creativity in independent projects, and letting me know I can be a scientist without having to give up the artist inside. Thank you for expanding my mind from the macro world to the one that underlies it, and empirically showing me that everything is connected. Thank you Ben and Joanna at CUS and the Bio stockroom for all your additional support. Chris and Katrina, I am so lucky to have you as lab partners and friends.

To my friends, who have always been stronger to me than blood: you have given me the most precious gifts of laughter and love without the need for reason. Nick Herbst, for having gone through every emotion humanly possible with me, for all the mutual breakdowns, for the best ideas, for the worst ideas, for that alter ego no one will ever let you forget, and for those sporadic wiggly dances at 2 a.m. during the writing of this thesis. Thank you for being my brother and best friend. To Rex, for sharing early environments, and supporting his sister in her crazy endeavors. To Tyler Ink Broeker, for never being anything but sweet. And to Isabela, for always reminding me what is real.

To everybody and everything Reed: you have given me the lowest lows and highest highs. You have taught me about biology, life, and myself. You have proven to me that anything is possible and that science is magic. You have done nothing but make me a stronger person. Thank you for being my four-year fairy land.
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Abstract

Behavioral syndrome and personality are relatively new terms used in the fields of behavioral ecology and evolutionary biology. However, many fields can gain from the practice of researching suites of correlated behaviors across situations and contexts. Limiting a study to just one behavioral variable or situation limits the view on potential restrictions of any given behavior. For instance, a behavior that seems advantageous in one situation or context may be non-optimal in another. A popular behavioral syndrome to test for is boldness, as most populations of animals can be classified on a shy-bold continuum. Boldness has been used as a measure for risk-taking behavior, fearfulness, emotionality, and anxiety. Anxiety-mediated behavior is well understood as a maternal effect in rat populations, and the mechanism behind it has been extensively studied. Being a fundamental social experience in the early development of many animals, the level of maternal care an individual receives can impact later life behaviors. Fish raised with and without mothers were used in this study in order to investigate the evolution of anxiety-mediated behavior and obtain a biological understanding of the bold behavioral syndrome. In order to accomplish these aims I conducted rigorous behavioral observations in one exploratory and one feeding assay at two stages in development (juvenile and adult). This study was the first to provide comparable data between anxiety-mediated behavior of fish and rats, and provides evidence to support the evolutionary conservation of anxiety-mediated behavior influenced by maternal care. It also has implications for the conservation of its mechanism, and provides revisions to basic nomenclature in personality research.
To Betty Brownlie

May you rest in the sunshine
knowing you gave me all the maternal care
I needed to be who I am today.
Introduction

“No one supposes that all the individuals of the same species are cast in the very same mould. These individual differences are highly important for us, as they afford materials for natural selection to accumulate, in the same manner as man can accumulate in any given direction individual differences in his domesticated productions.

These individual differences generally affect what naturalists consider unimportant parts; but I could show by a long catalogue of facts, that parts which must be called important, whether viewed under a physiological or classificatory point of view, sometimes vary in the individuals of the same species. I am convinced that the most experienced naturalist would be surprised at the number of the cases of variability, even in important parts of structure, which he could collect on good authority, as I have collected, during a course of years.”

Origin of Species
Charles Darwin

1.1 Focus

Continuing Darwin’s argument of individual variation, many scientists have debated and examined the use of individual and group “personality” or “behavioral syndromes” (see section 1.2). In some studies, nomenclature from this scientific literature has overlapped with topics concerning maternal effects, anxiety-mediated behaviors, underlying neural systems, and physiology. This thesis was motivated by this interconnectedness of animal behavior and the realized importance of having a biological understanding of cognitive and psychological functions.

Behavioral syndrome literature ranges across many sample species, but is biased in favor of mammals. A contributing factor to this fact is the anthropocentric belief that animals phylogenetically close to humans are most able to provide comparable insight into our behavioral deficiencies (see section 1.5). However, for evolutionary, developmental, and practical concerns, we must wonder at the consistency of maternal
effects throughout the animal kingdom. Practically, similarities between fish and other animal classes may allow fish to be used as model organisms in the field. Developmentally, comparative studies directed at the effect of early experiences in fish may better inform how to promote more energetically favorable behavior throughout the ontogeny of an individual or population. Evolutionarily, this area of research may find the maternal effect on anxiety-mediated behavior to have either a more recent or distant origination for vertebrates.

This thesis compares the anxiety-mediated behavior of the Lake Tanganyikan cichlid (pronounced sick-lid) fish, *Astatotilapia burtoni*, raised with and without mothers in a laboratory setting. Although I do not examine data from a species other than *A. burtoni*, research was conducted to ensure that tests between rats and fish were comparable (Gosling 2001, Caldji *et al.* 1998, Taborsky and Kotrschal 2010, Arnold and Taborsky 2010, Brown *et al.* 2007) in order to discuss the potential mechanism and behavioral significance of anxiety in fish. The results will be interpreted based on the existing understanding of maternal effects, and will take the contentious issue of behavioral syndromes into consideration.

### 1.2 Behavioral syndrome, personality, et cetera

#### 1.2.1 Personality defined

In 1998, behavioral ecologists called out to evolutionary biologists. The common misconception that deviation in a given behavior was non-adaptive accompanied the notion that seemingly successful behavior was the adaptive mean (Wilson 1998). However, the need to adopt a “set of expectations about adaptive individual difference within single populations that can be applied to all traits” (Wilson 1998, Dall *et al.* 2004) was answered by debates, studies, and the emerging field of animal personality.

Personality has been through many incarnations and definitions, changing when referred to by different, yet related, fields of study. Personality psychologists have defined it “as those characteristics of individuals that describe and account for consistent
patterns of feeling, thinking, and behaving” (Pervin & John 1997); though vague, the mention of consistency, as well as incorporation of a cognitive and behavioral pathway satisfied many. Sih and Bell gave an ecological and evolutionary perspective on personality, and coined the term “behavioral syndrome”, which grew to be synonymous with “personality” (Bell 2007). Theirs is the idea that both species and populations exhibit “suites of correlated behaviors across situations,” situations being distinct from contexts (Sih et al. 2004). A situation, or “given set of conditions at one point in time,” may include various levels along an environmental gradient (e.g. presence versus absence of a predator) or different sets of conditions across time (e.g. breeding versus non-breeding season). The context of any given behavior has a specific functional category; this includes feeding, mating, parental care, or antipredator behavior (Sih et al. 2004). A behavioral syndrome can transpire across different situations in either the same or different contexts. An example of different situations in the same context is latency to feed (context) in the presence and absence of a predator (situations). An example of different situations with different contexts would be latency to feed (context) in the presence of a predator (situation) and aggression to conspecifics (context) in the absence of a predator (situation). However, a rigorous study of behavioral syndrome would include at least two different context-specific behaviors. In this way, tests can compare both similar (e.g. latency to feed versus latency to feed; aggression versus aggression) and different contexts (e.g. latency to feed versus aggression to conspecifics) across different situations (e.g. in the presence and absence of a predator).

This thesis will compare *A. burtoni* “bold” behavioral syndrome (see section 1.2.2) across different temporal situations (as juveniles and adults) in two contexts (feeding and exploration). By measuring two contexts I will be able to compare feeding and exploration behaviors of fish in different sets of conditions across time. I will also be able to compare various context-specific behaviors in fish raised with high maternal care (HMC) to fish raised with low maternal care (LMC). In this way I will be using a developmental approach to examine the extent that maternal care affects the population’s overall behavioral syndrome.
1.2.2 The “shyness-boldness continuum”

While it would be infinitely more organized if populations separated into distinct groups, behavioral syndromes, like most systems in nature, function on a continuum. Thus, while it may not be possible to define a population as absolutely shy or bold, it is possible to place them on a continuum between the two. In this way, a study can conclude that one population is “more shy” or “more bold” than another.

Many of the methods used to study bold behavior often overlap with what other fields use for “anxiety-mediated behavior”. For example, the open-field test, or its aquatic counterpart that I will refer to as the “open-tank test”, has long been used in fields like neurobiology to measure emotionality, behavioral fearfulness, or anxiety-mediated behavior (Walsh 1976, Caldji et al. 1998). Meanwhile, studies less concerned with the mechanism of this behavior deem open-field/tank tests a study of boldness, exploration, and/or fear (Gosling 2001, Brown et al. 2007). In behavioral ecologist literature, “the terms shy and bold refer to the propensity to take risks, especially in novel situations. Shy individuals react to novelty by retreating, reducing activity levels and becoming more vigilant (similar to symptoms of mild stress), whereas bold individuals are more likely to approach novel objects, increase activity levels and exploratory behaviour” (Brown et al. 2007). Since this thesis examines the behavior of fish in different situations, while taking theories of both personality and underlying neural mechanism into consideration, the terms “bold/shy” and “anxiety-mediated” are relative and will be used interchangeably.

1.2.3 The core of the problem: nomenclature

Despite having several designations and re-definitions, behavioral syndromes remain a debated topic among animal behaviorists. The schism spans from scientists like Sih and Bell (2004, 2007), who coined the phrase, to more conservative views like Neff and Sherman (2004), who remain unconvinced that further study of behavioral syndrome will bring any novel discoveries to Biology.

The initial problem with this debate is unfortunately common within many scientific communities: nomenclature. Colloquial phrases or words are utilized for scientific nomenclature, but are subsequently loaded with entirely new depths of
meaning. Too late and too often it becomes apparent that new, not commonly used phrases could cause less confusion. However, when this decision is made, the audience for new research is further limited to an even more select group of ivory tower scientists. Additionally, the change in title may detract from original meanings, cause further confusion, or have no effect on the viewers’ reaction at all. This is how the term behavioral syndrome was created, and how “personality” came back into the behaviorists’ vocabulary.

I am not the first to notice the seemingly trifle lack of appropriate scientific nomenclature; in his review of animal personality, Gosling (2001) paints temperament as a frequently used scapegoat for animal researchers who seek to “avoid using the word personality” due to its casual nomenclature and anthropomorphic associations. Furthermore, terms were created to counter the plethora of studies without an agreed and standard nomenclature. Many studies have either the same label to refer to different constructs (e.g. the “jingle fallacy”) or different names to refer to the same construct (e.g. “jangle fallacy”). For example, the term “shyness” is frequently associated with constructs like “emotionality, curiosity, or assertiveness” (jingle fallacy). Many studies also use one of the following terms exclusively, failing to acknowledge synonymous vocabulary created in previous literature (jangle fallacy): “exploratory, curious, novel seeking, risk prone, or bold” (Gosling 2001). In order to avoid these fallacies, relate my findings to relevant mechanistic literature, and help further unite the body of work surrounding personality in fish, the aforementioned terms “anxiety-mediated behavior” (Caldji et al. 1998), “bold” and “shy” (Sih et al. 2004, Bell and Stamps 2004, Brown et al. 2007) will be consistently used in this thesis.

1.2.4 Contributions of personality: a debate

Behavioral syndromes have the potential to explain non-optimal behaviors that carry over situations (e.g. inappropriately high activity in the presence of a predator) and how individual variation is maintained within a species or population (Sih et al. 2004, Bell 2007). With these claims, Sih aimed to convince behaviorists to study correlated traits “together, as a package, rather than as isolated units”. Neff and Sherman (2004)
challenged Sih’s maladaptive behavioral syndromes with examples of adaptive behavioral plasticity. However, the two are actually complementary (Sih et al. 2004).

While syndromes can explain suboptimal behavior in an isolated context, they do not necessarily result in maladaptation (Sih et al. 2004). Neff and Sherman used the Bluegill Sunfish to display how adaptive behavioral plasticity explains the altered paternal defense in response to the experimental changes in paternity (e.g. on average, the father would defend fry less if they were not his biological offspring, and defend them more if they were biologically his). However, individual differences in response to reduced paternity showed that, while some males completely abandoned their nests, others defended the mixed brood. Rather then disputing that reduced paternity reduces defense (which is a behavioral plasticity question), Sih (2004) asked about consistency in other contexts; do aggressive fathers remain aggressive when the fry are biologically theirs, when predators are present, or towards other males during courtship? Aggressive correlations across contexts would explain the variation in what would be expected to be low care in cases for low paternity. Perhaps the tradeoff for being particularly aggressive in other contexts (e.g. towards other males during courtship) ensures him more opportunity to mate continuously, and outweighs the cost of spending energy defending non-biological kin. Essentially, while adaptive behavioral plasticity definitely exists in populations, it is limited.

More obvious benefits of personality studies are their contributions to ultimate questions in ecological and evolutionary research. Ecologically, behavioral syndromes give insight into the tradeoffs that may limit a species or population’s ability to adapt to limiting environmental factors, and since behavioral correlations across contexts (e.g. reproductive, predator-prey, and dispersal behaviors) can reflect birth, death, and dispersal rates, behavioral syndromes can be incorporated into ecological analysis (Sih et al. 2004). By treating behavioral syndromes as other phenotypic traits, personalities of different species can be compared and can help determine the evolutionary path of certain adaptive and maladaptive behaviors. For example, gene sequences already associated with personality traits like anxiety can be examined across a range of animals, and the time that the trait was introduced to the genome can be estimated (Gosling 2001). Evolutionary models can also be made with behavioral syndromes, and “in any such
situation, we would expect correlations between those behavioural traits that involve risks that might prevent individuals from reaping the returns from reproductive investments” (Wolf et al. 2007). Additionally, comparative studies between species may help to understand the development of personality along with connections between personality and health (*see section 1.2.5*).

### 1.2.5 The future of personality research

The last section highlights the contributions of behavioral syndrome’s ultimate questions (e.g. adaptation and phylogeny), which describe its initial draw to behavioral research. While these questions have yet to be fully and rigorously explored, modern researchers see the value in asking proximate questions as well. Harkening back to Nikolaas Tinbergen’s “four questions” (1963), both ultimate and proximate (e.g. mechanism and ontogeny) aspects of behavior are necessary to fully comprehend a given phenotype. Recent attention is especially directed at developmental research, (Bell 2007, Trillmich and Hudson 2011) but has been slow to gain momentum. Initially, supporters of personality did not want to weaken their concept by admitting that behavioral stability, once thought to be required for behavioral syndromes (Bell and Stamps 2004), may actually change throughout an individual’s ontogeny.

Many still agree that personality traits should remain relatively stable over time and across a variety of different contexts, but that does not inhibit traits from being “susceptible to change through life experiences” (Brown *et al.* 2007, Wilson 1998). Even Bell (2007) admits that, since behavioral variation is most likely maintained via (1) the variation and frequency of life history strategies in a population and (2) the variation in state (e.g. health, body size), an individual’s personality may indeed change throughout its life. This does not necessarily mean the population’s behavioral syndrome will also change, but it does open the doorway to more questions about the stability of personality throughout ontogeny and research with a developmental approach. If personality does change, researchers would like to know at which stage, and under what circumstances, these changes occur (Trillmich and Hudson 2011); just because state (Bell 2007) is one possible determining factor, there may be many more.
Recent studies have already begun questioning the influence of genetics, heritability, and early development in animal personality. This research is particularly interested in the heritability and development of boldness. In one study, lab-reared tropical poeciliid (*Brachyraphis episcopi*) fry descended from fish caught in either high or low predation areas, were examined at baseline boldness, which would be proof of inheritance, or at the very least early environmental influence (Brown *et al.* 2007). Inheritance and early environmental influence could not be discerned because the eggs were not forcibly separated from their mother, and fry may have had access to social inference. The extent that boldness was learned was tested by comparing baseline boldness data to data compiled from the same open-tank test after two weeks of continuous chasing with a net (e.g., predator proxy). Fry descended from high predation areas displayed a shorter latency to emerge than fry descended from low predation areas, and all fry that underwent the predation condition displayed a shorter latency to emerge regardless of ancestry. Findings may indicate some level of personality inheritance and some ability to alter boldness traits in high-pressure conditions.

Questions generated from the developmental approach have inspired other proximate questions concerning mechanism. Trillmich and Hudson (2011) ask how individual behavioral phenotypes establish in development. Brown (2007) asks about the mechanism that governs the population differences mentioned in the above study (Brown *et al.* 2007). Could it be related to the same mechanism that governs physiological stress? Is the mechanism governing a personality in one species similar to the mechanism governing the same personality in another? In certain psychopharmacology research, this question has valuable and applicable uses. For example, one study is using their findings on the mechanism for risky personality, “novelty seeking” behavior in rats to hypothesize responses to serotonergic drugs in high-risk personality, “sensation seeking” humans (Verheij *et al.* 2009). In their experiment, they found that risky rats given a serotonergic injection became less risky, which was the desired result. However, they also discovered a potential danger; less risky rats given the same injection became more risky, providing evidence to delay the therapeutic distribution of this treatment.
1.2.6 A word of caution

Personality can be a useful tool when defining clusters of behaviors, correlating with other traits, or as a starting point to deeper exploration; however, pitfalls do exist. For example, using personality as a means to explain a behavior does not advance the search for an underlying mechanism, which may be present on a neurological, physiological, and/or developmental level. At best, invoking personality at the conclusion of an experiment infers a grouping of “aggressive” or “bold” individuals. At worst, this “nominalist fallacy” acts to prevent the search, and parallels the use of “anthropomorphism” (Gosling 2001, Wynne 2007) or “insight” (Kacelnik 2009) in cognitive research.

Personality needs to be incorporated into the question of research to provide informative results into the mechanism of behavior. In this experiment, I predicted that the lack of maternal care in an early social environment would increase levels of stress in individuals, and that this would correlate with a significantly “less bold” (or “shy”) behavioral syndrome than individuals raised with mothers. Because each juvenile fish was tagged, I can also identify individual personality through adulthood. In this way, personality can be correlated with other traits and empirically tested.

1.3 Maternal effects in mammals

1.3.1 Rats

The study of maternal effects was greatly influenced by the discovery that rat phenotypes can vary as a result of external rather than genetic influence. Typically, traits are determined via the sequence of nucleotides passed from one generation to the next. The mechanism behind epigenetic inheritance is “above” or “over” genetics: processes like DNA methylation are able to cause a heritable change in gene expression without changing the DNA sequence of an organism (Russell 2006). Cross-fostering experiments
have demonstrated this kind of epigenetic inheritance in rats (Francis et al. 1999, Liu et al. 1997, Caldji et al. 1998).

While pups bred from mothers who exhibit high levels of maternal care, like licking/grooming and arched-back nursing (LG-ABN), are reported to become mothers who exhibit high levels of LG-ABN, pups bred from mothers with low levels of LB-ABN generally become mothers with low levels of LG-ABN (Francis et al. 1999). However, this behavioral trait is not strictly genetic, but more malleable. In the cross-fostering experiments mentioned above, even pups bred from low LG-ABN (“bad”) mothers raised with high LG-ABN (“good”) mothers became “good” mothers, and pups bred from “good” mothers raised with “bad” mothers became “bad” mothers (Sapolsky 2004, Francis et al. 1999, Weaver et al. 2004). Additionally, pups raised with high LG-ABN mothers exhibited lower levels of stress as adults, while the reverse was true for pups raised with low LG-ABN mothers (Sapolsky 2004, Champagne et al. 2003, Francis et al. 1999, Liu et al. 1997, Caldji et al. 1998).

The mechanism for stress reduction takes place in the hippocampus; following a real or perceived threat (stressor), the hypothalamic-pituitary-adrenal (HPA) axis releases glucocorticoids (GC) like corticosterone or pituitary adrenocorticotropic hormone (ACTH) in rats (Conrad 2008, Liu et al. 1997). These steroid hormones are comparable to cortisol, also associated with stress levels, in humans (Conrad 2008). When raised with high LG-ABN mothers, offspring express more hippocampal GC receptor (GR) messenger RNA. After GC has promoted sufficient stress-mediated behavior in response to a stressor, GR mRNA returns in a negative feedback loop to the HPA axis; here, GR mRNA inhibits the synthesis of hypothalamic releasing factors for ACTH, like hypothalamic corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) (Liu et al. 1997). As a result, offspring raised with high LG-ABN mothers produce a lower level of ACTH and corticosterone in response to stressors, and are more able to terminate the stress response once the stressor is no longer a threat. A timely termination of stress response is critical because the “racing heart, high blood pressure, and maximized blood flow” necessary for escaping a stressor can wreck havoc on a body over time (Conrad 2008). On a behavioral level, these offspring have reduced fearfulness and respond better to novelty (Caldji et al. 1998). This stress-mediated behavior, like
maternal care, is an epigenetically inherited trait, and is determined based on the maternal care provided in the first 10 days after birth (Francis et al. 1999, Liu et al. 1997, Meaney and Aitken 1985).

If maternal care is not administered in the “critical period” of a species, GR mRNA is down-regulated, the synthesis of hypothalamic releasing factors for ACTH in the HPA axis is not inhibited, and termination of stress responses to a stressor is unlikely (Liu et al. 1997, Caldji et al. 1998). As a result, rats generally become behaviorally fearful, overly hesitant in novel situations, and low LG-ABN mothers (Francis et al. 1999, Caldji et al. 1998).

1.4 Fish brains and behavior

1.4.1 The benefits of studying fish

Comparative studies between distantly related animals, like rats and fish, may provide insight into unknown neural mechanisms in fish. They may also help to identify the evolutionary origin or ecological consequence of certain behaviors, and may broaden the potential fish have as models in personality or psychological research (Bell and Stamps 2004, Brown et al. 2007, Grosenick et al. 2007, Gomez-Laplaza 2011). Whereas much of the mechanism behind stress-mediated behavior has been studied in rats, relatively little can be said about the mechanism behind the same behavior in fish. Collecting behavioral fish data similar to that already collected in rats provides scope for a comparative look into the mechanism mediating stress in fish.

While fish are only evolutionarily vital in their phylogenetic distance from mammals, they are valuable to ecological research in many aspects. Fish have relatively quick reproductive and developmental timeframes, and are highly sensitive to environmental and social variables. Therefore they make excellent model organisms for matching specific cognitive abilities with ecological (selecting force) factors, or rather, specific regions of the brain (Bshary et al. 2002). Hence, fish are practical organisms for generation-long research and developmental questions. Even when studies do not test
many species simultaneously, such as in this thesis, the results from fish behavioral studies alone can be used as a ‘null-hypothesis’, or viewed comparatively, in mammalian research (Bshary et al. 2002).

From quantity discrimination to complex social recognition, fish have already become a model system in a range of cognitive and behavioral studies. It has recently been demonstrated that angelfish “count” using a similar technique as many mammals and avian species (Gomez-Laplaza 2011). This technique, called Weber’s law, involves discriminating quantities according to ratio. The experiment confirmed that angelfish preferred to shoal with larger groups and hence could differentiate shoal size up to a 2:1 ratio. For example, given the choice between twelve or three fish (4:1 ratio), focal fish chose to shoal with the former. Even though discrimination below a 2:1 ratio was not achieved, this limitation is common among many animals without language for exact numbers. Even human tribes, like the Munduruku, who lack words for numbers above five, quantify and discriminate large number sets via ratios (Pica et al. 2004). Following Weber’s Law, as ratios decreased below 2:1, and quantities became more similar, Muduruku success decreased.

Also once thought to be a uniquely human trait, transitive inference (TI) has been demonstrated in primates, rats, birds, and recently in fish (Rapp et al. 1996, Gillian 1981, Davis 1992, Bond et al. 2003, Grosenick et al. 2007). Using logic to infer that if “A > B and B > C, then A > C,” fish can infer social rank without any acoustic or olfactory cues. After watching pair wise fights between male individuals A, B, C, D, and E, and being given the option between A and E or B and D, focal fish consistently preferred to locate themselves near E or D, the lower ranking males (Grosenick et al. 2007). Fry receiving maternal care may be exposed to a form of TI from their mother as well as their siblings (Brown et al. 2007). Hence, it is important to keep TI in mind when designing an experiment and speaking about inheritance versus learned behavior.
1.4.2 Brain mechanism of fish

Recent developments in the Taborsky Lab (2010) signify that early social environments don’t just influence mammalian behavior; fish behavior can be altered as well. However, the mechanism for which this, and maternal effects, may operate is relatively unknown. A major factor contributing to the mystery behind the mechanism of stress-mediated behavior in fish is that fish employ the lateral pallium in lieu of a hippocampus (Spence et al. 2011, Vargas et al. 2009).

The fish pallium has been found to have structures similar to the mammalian hippocampus, amygdala, and isocortex; these are the lateral pallium (which is crucial for spatial learning), the medial pallium (which mediates avoidance learning), and dorsal pallium (which mediates short-term memory processes) respectively (Vargas 2009). However, the homology of the fish and mammalian brain structures have been debated due to the different way in which the fish telencephalon develops (via eversion). However, this has not inhibited comparative studies from proposing homologies. Evidence providing that GR receptors are most abundant in the fish and rat forebrain supports the theory that “expression and function of GR in the CNS arose early in vertebrate evolution and may be conserved” (Teitsma et al. 1998, Yao et al. 2008, Vargas 2009).

This thesis will use behavioral data in a comparative approach to contribute to research providing evidence for typically-hippocampal functions being affected by lack of maternal care in fish. Significant differences between HMC and LMC fish will support that the lateral pallium of LMC fish lack negative feedback loops of GR mRNA similar to that of the hippocampus of mammals raised without maternal care. Insignificant differences could mean that fish up-regulate GR mRNA into negative feedback loops regardless of maternal care, thus enabling all fish to appropriately react to stressors.
1.4.3 Influence of early environments

Factors of early environmental influences usually fall under the category of variability or social interaction. Maternal care is a social factor of early environment influences, but many other factors support and lead to the question of parental importance. For example, habitat landmark variability, food regime variability, conspecifics behavior, and sibling inference have been shown to contribute and shape fish behavior.

Enrichment and restoration research discovered that variability in an early rearing environment promotes flexibility in cod behaviors that will better ensure survival in the wild (Braithwite and Salavanes 2005). By installing variable spatial and foraging cues, like kelp and cobble, fry experience typical obstacles and structures present in their natural habit. Additionally, food regimes, in which the distribution and availability of pellets is variable, mimic encounters with wild prey. Fry raised in variable visual environments with a variable food regime were faster to consume live prey, recover from a stressful experience, explore a novel environment, and exhibited “bolder, more curiosity driven behavior” (Braithwite and Salavanes 2005).

Variable food regimes during early development can also affect long-term cognitive abilities in fish. Kotrschal and Taborsky (2010) distributed food rations to juvenile cichlid fish Simochromis pleurospilus at constant or variable (from high to low or from low to high) amounts. Fish with variable diets (especially those that switched from high to low amounts) significantly outperformed fish with non-variable diets in a simple associative learning task. Tested one year later, adults preformed similarly to juveniles, demonstrating that exposure to variability in early environment can affect adult behaviors in cichlid fish.

The mechanism behind the differences in these responses can be seen on eco-, nuero-, and physiological levels. Artificially variable environments cultivate a need for behavioral flexibility in fry, which consistently requires adaptation to change. In wild populations, this same requirement exists with natural selection providing the incentive for individuals to adapt quickly. However, since artificially variable environments simulate natural stochasticity, individuals respond as they would in a natural setting (Braithwite and Salavanes 2005). Mechanistically, it has been speculated that
environmental variability “evokes repeated neural stimulations resulting in [increased synaptic development, enhanced brain development, and] faster and better learning” (Braithwite and Salavanes 2005, Kotrschal and Taborsky 2010). The continual problem-solving tasks that comprise variable environments give fish the occasion to increase synaptic development while becoming conditioned to respond more quickly to foraging or predator-avoidance opportunity when it is present.

Early social environment can also influence fry behavior through adulthood. In the cooperatively breeding cichlid *Neolamprologus pulcher*, the presence of adults in early ontogeny allowed fry to develop energetically favorable social cues as adults (Arnold and Taborsky 2010). In a social performance test, fish were given the role of “shelter-owners” or “invaders” in a test tank. Appropriate territory-defending behavior included restrained aggression (threat displays), while newcomers quickly submitted to territory-owners. Fish raised without adults exhibited costly open aggression behavior as defenders, and required more time to submit as invaders. Whether attributed to the fact that adults expose fry to continuous water-borne hormones, enable fry to increase interactions with siblings by decreasing the need for vigilance, or allow fry to eavesdrop social (including acoustic and olfactory) cues directly, parents and adult brood-helpers influence the acquisition of appropriate and cost-effective offensive and defensive behaviors in critical development stages.

### 1.5 The human connection

Research into the maternal effect of anxiety-mediated behavior or “boldness” in non-human animals can ultimately relate to studies on child abuse or neglect, which can be applied in clinical psychology, behavioral therapy, and the pharmaceutical process. Because maternal effects are epigenetic, and not genetic in nature, there is potential for behavior reversal with the appropriate manipulation (Weaver *et al.* 2006). Through the use of environmental enrichment (Bredy *et al.* 2003), neonatal handling (Bredy *et al.* 2004), and intracerebroventricular infusion of histone deacetylase inhibitor trichostatin A (TSA) or methionine (Weaver *et al.* 2006), rat research has already begun to explore
methods of altering deleterious effects that lack of maternal care has on later-life cognitive ability and anxiety-mediated behavior. Finding the effects of maternal neglect on rat pups may be synonymous in many ways to the effects maternal neglect has on human children. Similarly, finding methods to alter rat anxiety-mediated behavior throughout ontogeny may help to do the same for abused human children, teenagers, and adults. If fish process maternal effects, like anxiety-mediated behavior, with the same mechanism as fish, they could also be used as a sample species in this area of research.

1.6 This study

While rats have been extensively used to study the behavioral differences (Caldji et al. 1998) and mechanistic underpinning of maternal effects (Francis et al. 1999, Liu et al. 2000, Champagne et al. 2003, Sapolsky 2004, Weaver et al. 2004), fish studies are just beginning to explore the effects of early social environments on later-life behavior (Arnold and Taborsky 2010). This project seeks to contribute to this growing body of work and is one of the first to make its data comparable to rats. My objective is twofold: I aim to (1) investigate the evolution of maternal effect mechanism using a behavioral and comparative approach and (2) obtain a biological understanding of behavioral syndromes using a developmental approach.

In order to facilitate my aims, I have provided an apt methodology for quantitatively measuring anxiety-mediated (“bold”) behavior in fish, examined the behavioral difference between fish raised with and without mothers in different stages of development, and investigated the existence and stability of a behavioral syndrome. The cichlid fish, Astatotilapia burtoni, offers an adequate system for a comparative study to laboratory rats. They are uni-parental, providing only maternal care, and are phylogenetically distant.

The “feed test” and “open-field test” (Caldji et al. 1998) frequently used to measure anxiety-mediated behaviors in rats has been adapted to quantify fish behavior. Arena diameters were scaled down appropriately and made aquatic. I have constructed an ethogram for each context (feeding or exploration) that encompasses a range of context-
specific behaviors, and use it to perform focal observations of individuals from each brood. I then draw comparisons between social (LMC and HMC) and temporal situations (juvenile and adult), and use these to draw conclusions about maternal effects in fish, comparability to rats, and the development of behavioral syndromes.

In a sample population with a high degree of genetic similarity, behavioral changes between LMC and HMC can be assumed to be epigenetic and developmental by nature. If these results are significant, they will provide evidence for evolutionally conserved anxiety-mediated behavior and implications for an evolutionarily conserved underlying neural mechanism. If the behaviors of LMC and HMC are not significantly different, results will provide evidence for a more recent evolutionary origin. Additionally, if bold behaviors are conserved across situations and contexts, this study will provide evidence for behavioral syndromes. It will also expose the effect socio-temporal situations can have on an individual’s behavioral syndrome and stability.
Materials and Methods

2.1 Study species

*Astatotilapia burtoni* is a maternal mouth-brooding cichlid fish endemic to Lake Tanganyika in East Africa. They are generalists, and like most rock-dwelling cichlids, are opportunistic feeders (Lang *et al.* 2006, Carleton *et al.* 2008). In previous evolutionary and neuroethological studies, *A. burtoni* has been proven to be a viable model organism partially due to the fact that it is a fitting representative of one of the potential founding cichlid lineages (Lang *et al.* 2006). *A. burtoni* is an ideal model organism in this study because the species has been said to have the highest level of parental care known in fishes (Barlow 2000). The individuals used in this thesis were either the F1 or F2 generation from fish caught in 2005 by Susan Renn from the same geographical area of Lake Tanganyika.

2.2 Fish care

Laboratory conditions mimicked the cichlids’ natural habitat as much as possible. All fish were kept on a 12:12 light:dark cycle, with water pH and salinity kept high (pH 8.6, salinity at 630 – 650 µS/cm). All tanks were kept on a self-regulating system with constant aeration, and water was filtered once a day from 10:15 to 10:30 h. If not in testing phase (two consecutive days as juveniles and adults), fish were fed commercial dry food (TetraMin) once a day. Each brood (8-10 fish) had separate housing aquariums, and except for the bubbling methyl blue beakers, each aquarium contained equal amounts of gravel and terracotta potshards as per the laboratory aquarium standard. As this study utilized a non-invasive measure of anxiety in fish, no animals were sacrificed in the creation of thesis. This research is in compliance with publication 86-23, “Principles of Animal Care”, revised in 1983 of the National Institutes of Health and the Renn Laboratory animal care protocol approved by the Reed Animal Care Committee.
2.3 Development of ethograms

Since this thesis aims to have comparable data to previous anxiety-mediated behavior research, I obtained fundamental response variables from existing rodent and fish literature (Caldji et al. 1998, Bell and Stamps 2004, Brown et al. 2007, Lantova et al. 2011). Since researchers of similar behaviors have been criticized for not “using a standard set of characteristics translated into species-typical behaviors” (Gosling 2001), I have attempted to use the behavioral labels prior research has already defined. These response variables were tested in preliminary behavioral assays with unrelated fish (Renn Lab, Roetker 2011) in order to ensure relevant and quantifiable behaviors in A. burtoni. “Extra” fish, from the same broods as those employed for this study, were also tested in preliminary assays in order to troubleshoot experimental procedure and develop an appropriate recording method. Additionally, while raising experimental fry, qualitative evidence of certain behavioral differences (e.g. vertical movement) between conditions were observed and incorporated in testing.

Anxiety-mediated behaviors were quantified by conducting continuous, focal samples for both the open-tank and feed test. Continuous recording was chosen because it “gives true frequencies, latencies, and durations of behavior” (Martin and Bateson 1993, Morrison et al. 2006). All focal behavioral observations were conducted using JWatcher, a Java-based event recorder developed specifically for animal behavior research (http://www.jwatcher.ucla.edu). JWatcher applies the same ethogram to all observations, and creates focal observation data files using unique key codes mediated by the ethogram file. Each keystroke is electronically timed, and may be made mutually exclusive with any other behaviors (See Appendix A: JWatcher). The ethograms for the feed test (Table 2.2) contained behaviors recorded exclusively as states (i.e. behaviors with durations), while the open-tank test (Table 2.1) recorded two behaviors (areas explored and moved areas) as events (i.e. behaviors counted as singular points in time) as well.
2.3.1 Open-tank test

All behaviors were measured in response to a novel environment, and relate to an exploratory context. Exploration is the most comparable variable to rats in the feed test (Caldji et al. 1998), and is measured by inner area behavior, which is mutually exclusive with outer area behavior. Likewise, swimming is mutually exclusive with immobility. However, start box behavior, also being in the outer area, is only mutually exclusive with swimming, as activity in the outer area is disregarded in analysis (See Appendix A: JWatcher). Events (areas explored and moved area) were often counted manually in subsequent video observations, but were included in the JWatcher ethogram for consistency. In preliminary observations the open-tank test rarely, if ever, observed aggression. Hence, aggression was not included in the open-tank test ethogram.

Table 2.1: Open-tank test complete ethogram.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner area</td>
<td>Entire body outside red tape</td>
<td>State</td>
</tr>
<tr>
<td>Outer area</td>
<td>Any part of body inside red tape</td>
<td>State</td>
</tr>
<tr>
<td>Swim</td>
<td>At least one full body length from previous position</td>
<td>State</td>
</tr>
<tr>
<td>Immobile</td>
<td>A pause in swimming behavior</td>
<td>State</td>
</tr>
<tr>
<td>Start box</td>
<td>In crack behind or inside of start box</td>
<td>State</td>
</tr>
<tr>
<td>Areas explored</td>
<td>Total number of areas entered out of 12 total areas</td>
<td>Event</td>
</tr>
<tr>
<td>Moved area</td>
<td>Total number of times crossed green tape</td>
<td>Event</td>
</tr>
</tbody>
</table>

2.3.2 Feed test

While all behaviors in this assay were tested in the context of feeding, three behaviors (below 3 cm, between 3-5 cm, and above 5cm) associated specifically with vertical movement. Lines were drawn directly on feed test tank at appropriate levels. Latency and duration eating are the most comparable variables to rats in the feed test (Caldji et al. 1998).
and are measured by a single key. Latency to eat is measured \textit{in situ} by the time of the first feed keystroke, while JWatcher computes the total duration.

Table 2.2: Feed test complete ethogram.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed</td>
<td>Approach food flake and take into mouth</td>
<td>State</td>
</tr>
<tr>
<td>Swim</td>
<td>At least one full body length from previous position</td>
<td>State</td>
</tr>
<tr>
<td>Immobile</td>
<td>A pause in active (swimming or aggressive) behavior</td>
<td>State</td>
</tr>
<tr>
<td>Aggressive</td>
<td>Accelerated swim or bite towards reflection</td>
<td>State</td>
</tr>
<tr>
<td>Below 3 cm</td>
<td>Entire body under 3 cm line</td>
<td>State</td>
</tr>
<tr>
<td>Between 3-5 cm</td>
<td>Entire body above 3 line but below 5 cm line</td>
<td>State</td>
</tr>
<tr>
<td>Above 5 cm</td>
<td>Entire body above 5 cm line</td>
<td>State</td>
</tr>
</tbody>
</table>

### 2.4 Experimental procedure

Adult fish were kept in mixed gender social groups until they spawned. Pregnant, mouth-brooding females were either immediately stripped of eggs or relocated to individual housing (10 l; 26.5 cm × 18 cm × 17.5 cm) for approximately four weeks before being returned to original tank. Brood size was restricted to 8-10 fry; if mothers produced less than 8 fry, they were dismissed from experimental testing, labeled “extra HMC” or “extra LMC”, and used for preliminary trials. Likewise, if broods contained more than 10 fry, extra fry were randomly selected and equally dismissed. Contrary to optimal design, all mouth-brooding females (with HMC eggs) were relocated before all LMC condition eggs were harvested (see Appendix C: Troubleshooting and Confounds).

Eggs stripped from mothers were relocated into large, well-aerated beakers with approximately 1-2 mL methyl blue to prevent fungal infections during metamorphose to fry. Eggs kept with their mothers did not require methyl blue because pregnant \textit{A. burtoni} continuously roll eggs inside their mouths to inhibit the growth of fungi on offspring.
Since the eggs in bubbling methyl blue received the benefit of fungus elimination (one function of *A. burtoni* maternal care), but were not raised with mothers to benefit from any other form of maternal care, this condition received only low levels of maternal care (LMC). However, eggs raised with mothers received natural fungicide via mouth-brooding, and had access to transitive inference from their mothers’ behaviors and hormones. Generally speaking, these fry were exposed to higher levels of maternal care (HMC).

As soon as LMC fry absorbed their egg yolk, and began to ingest food, they were transferred to larger containers (10 l; 26.5 cm × 18 cm × 17.5 cm), out of sight of HMC tanks. Approximately 2.5 weeks after mothers released HMC fry, mothers were relocated back to their original tanks. Measures were taken to ensure the developmental ages were similar in both conditions. For instance, the age count for HMC fry did not begin until they were released from mothers’ mouths and free swimming. Likewise, the age count for LMC did not begin until they had, like the HMC fry, lost their yolk sac and were free swimming. So day 1 for LMC was the day they were transferred to 10 l tanks.

Both LMC and HMC conditions had the same amount of time (approximately 31 days) free swimming in 10 l tanks before being transferred to 20 l tanks in the testing room. After four weeks of habituation, approximately on day 60, juvenile subjects of both conditions underwent the first series of anxiety-mediated behavior assays (open-tank test and feed test). Individual fish were tested on the same day as siblings. At the end of this series all subjects were tagged for individual identification on either the right or left side with pink, orange, green, yellow, or blue visual implant elastomer (VIE) tags (See Appendix B: VIE Tagging Fry). Fry were then transferred back to each brood’s respective 20 l tanks.

On approximately day 90, adult subjects of both conditions underwent the same series of anxiety-mediated behavior assays, followed by individual identification via the VIE tags. Despite several factors restricting the ability to control for standardized testing timeframes for each brood (see Appendix C: Troubleshooting and Confounds), most tests were conducted between 14:00 and 19:00 h. On day 126, fish were individually re-identified, photographed, and weighed for body condition. From photographs, the standard length (SL, to 0.01 mm) was measured using ImageJ image analysis software.
Standard length was measured from the tip of the mouth to the base of the tail (Figure 2.1). Weight was measured (to 0.01 g) using a digital scale. If VIE tags were particularly hard to see, a handheld black light was used to aid in individual identification. Occasionally, when fish completely discharge VIE tags, they were given the identifier “NT” (no tag). In any step of this process fish were not deprived water for more than 1 min.

### 2.4.1 Open-tank test

The test tank measured 74 cm × 30 cm (75 l; Figure 2.2). Water depth (10 cm), start box dimensions (19 cm high × 8 cm wide × 10 cm long), observation technique, and non-threatening, novel arena furnishings from a fish perspective were based on previous open-tank test designs (Brown et al. 2007). The start box was constructed from aquarium grade black Plexiglas with a matte surface so that fish could not see out of a closed start box or be distracted by their own reflections. Observations utilized a small slit cut in the black plastic wrapped around the test tank; in this manner, latency to emerge could be recorded in situ without influencing the fishes’ behavior. The tank was furnished with gravel, four upturned terracotta potshards, and four artificial aquarium plants. The “twelve equal-sized areas” to measure activity in an unfamiliar environment also originated in previous fish personality research (Bell and Stamps 2004). These areas were differentiated by thin green tape adhered directly to the top of the test tank, and covered every territory in the tank. However, neurobiology research on rat exploration requires the “entire body of the animal [to be] away from the immediate vicinity of the wall (>10 cm) enclosing the open field” (Caldji et al. 1998). Since this project aims to obtain comparable data to rat studies, the dimensions of the open field were converted to those of the test tank using Equations 2.1 and 2.2. Designated “outer areas” were discriminated by thin red tape in a similar fashion as the green tape.

\[
\frac{\text{rat outer area} \times \text{tank length}}{\text{rat arena diameter}} = \text{tank outer area along tank length} \quad (2.1)
\]

\[
\frac{\text{rat outer area} \times \text{tank width}}{\text{rat arena diameter}} = \text{tank outer area along tank width} \quad (2.2)
\]
Prior to testing, all fish from the intended test brood were captured and gently placed in individual holding beakers. Using beakers to transfer fish into the start box decreased anxiety due to net chasing and handling immediately before an individual’s trial (Brown et al. 2007). Each fish was given a 2 min habituation period in the start box before its sliding door was manually raised. Sessions were recorded for 5 min on an aerial video camera, after which each fish was placed back into his/her individual container and left without access to food for 2 days. Latency to emerge, measured with a stopwatch, was the only response recorded in situ because more accurate activity and location-oriented movement could be provided by the camera’s aerial perspective. Additionally, video recordings were renamed and scrambled by a colleague. This enabled me to conduct the JWWatcher focal continuous sampling blind. Contrary to optimal design, housing tanks were not randomized, and in situ observation was not blindly conducted (see Appendix C: Troubleshooting and Confounds).

2.4.2 Feed test

This test was adapted from novelty-induced suppression of appetitive behavior tests in rats (Caldji et al. 1998); therefore, results will be comparable. Since rat results found there to be no difference between testing in a novel or familiar environment, a novel tank was used to obtain greater experimental control and unobstructed observations. Unlike the open-tank test, potshards and plants were not used in this arena because the experimental design sought to limit distraction and exploration behavior. However, preliminary qualitative data prescribed the inclusion of gravel; without it, reflections were more evident, fish engaged in higher levels of aggression, and spent less time exhibiting feeding behavior.

The test tank measured 26.5 cm × 18 cm (10 l; Figure 2.3), and like the open-tank test, water depth was kept consistently at 10 cm. Fresh water and equal amounts of fresh food were supplied prior to each fish’s test session. Food flakes were required to be small and added directly before fish entered arena to prevent food from dropping below surface level. 80% of food was required to be at surface level at the start of all individual test
sessions. At the end of each fish’s 10 min *in situ* continuous, focal recording in JWatcher, fish were caught and returned to their individual beakers.

Figure 2.1: Line drawn from tip of snout to base of tail measures fish standard length (mm) in ImageJ.

Figure 2.2: A digital replication of the open-tank test (74 cm × 30 cm, 75 l). Red lines indicate outer areas (4.62 cm wide at the width, 1.88 cm wide at length), and green lines define 12 exploration areas (18.5 cm × 10 cm). Latency to emerge from start box (SB; 19 cm × 8 cm × 10 cm) was observed from slit made in black plastic on exterior tank. Four potshards (orange triangles) and four aquarium plants (digital replica of plant) were interchangeably dispersed in arena. Water depth was manually kept at 10 cm.
2.5 Data analysis and software

For each focal recording file, JWatcher calculated many basic statistics for each behavior. Of these, I chose to present all state durations in order to include an individual’s behavior repertoire of the entire 5 or 10 min session (i.e. includes the first and last behavior). For open-tank test analysis, I mainly used counts (i.e. occurrence) and the total time of occurrences (reported in milliseconds). For feed test analysis, I used the total time of occurrences and proportion of time spent in a given state. JWatcher files were exported into R using the .cd.res file type, then organized in Excel, and analyzed in Jmp. Tables and figures were created in Microsoft Excel and Jmp.

Figure 2.3: A digital replication of the feed test tank (V cm × V cm, 10 l). Dark green line measures 3 cm from the bottom of the tank. Light green line measures 5 cm from the bottom of the tank. Water was kept manually at 10 cm, and 80% of food (red, orange, and yellow dots) remained at surface of water at beginning of feed test.
One-way analysis of variance (ANOVA) tests were performed to examine differences between fish tested at different times, and fish raised with high and low levels of maternal care. Analysis of co-variance (ANCOVA) tests were preformed to examine correlations between specific behaviors (e.g. latency to emerge and exploration) and to obtain residuals from morphological measures (e.g. weight and standard length). They were also initially used to determine boldness behavior correlations for behavioral syndromes. However, principle component analysis (PCA) was more efficient at determining correlations between boldness behaviors.

2.5.1 Principal component analysis

PCA is frequently used to break multi-dimensional data into fewer dimensions. Previously explained as “a variable reduction procedure” (O’Rourke et al. 2005), PCA was particularly suited for exploring behavioral syndromes because it tests for correlations, or redundancy, in a large number of variables. Redundancy among variables allows the statistician to then “reduce the observed variables into artificial variables” (O’Rourke et al. 2005). Using the first PCA conducted in this thesis as an example (Figure 3.10), I will explain how PCA analyzed the data, and how to interpret it.

Eight behaviors from both behavioral assays (feed and exploration tests) were ranked into eight categories called “principle components” (PCs). Data points from each observed variable were “optimally-weighted” (O’Rourke et al. 2005), or computed along a regression line with each of the other variables. One variable’s regression line would be the baseline (0) while every other behavior’s observed data point would be the residual along that line. PCA repeats this for every behavior.

This thesis limited analysis to the first four principal components (PC1, PC2, PC3, PC4), because together, they explained 80.4% of behavioral correlation (Table 3.2). PC1 is unique in that it accounts for the largest percentage of variance in total behavioral correlation. In other words, PC1 is most redundant among all other behaviors, and is correlated with more of the total behaviors than the other PCs. PC2 is unique as well. It also correlates with all behaviors, or the total “syndrome” very well, but is uncorrelated with PC1. In fact, all PCs are statistically independent of any other PC so that each PC
gives focus to only a few behaviors that can be given weight independently of other behavioral influences.

The positive or negative nature of the behaviors within a PC is also informative. If all numbers in a PC are negative except for two, then the behavior being characterized most in that PC are those two behaviors. Likewise, the closer a behavior is to 1.0, the more significantly correlated it is to all other behaviors, and makes it prominent within the PC.

PCs obtained from PCA were used in two-way ANOVAs to identify population-wide behavioral syndromes. The same PCs were also used between HMC and LMC broods to test for differences in behavioral correlations (e.g. behavioral syndrome).
Results

In total, 258 focal observations recording the behavior of 136 fishes in the feed test tank were conducted. Though a total of 269 videos recorded the behavior of 136 fishes in the open-tank test, most of the raw video data was unsalvageable (see Appendix C: Troubleshooting and Confounds). However, the in situ behavioral observation, “latency to emerge”, was recoded in 269 tests for 136 fishes, and was used in analysis. Additionally, a total of 18 focal observations recording the behavior of 2 broods with different levels of maternal care were conducted using undamaged open-tank test video. Each individual who survived to adulthood was tested twice in each test over a period of 39 days. Exceptions to this were the fish in HMC brood 1; they were included in the open-tank tests, but not the feed tests as adults. As a result, 6 HMC and 8 LMC broods were used in both tests as juveniles and in the open-tank test as adults; 5 HMC and 8 LMC broods were used in the feed test as adults. Both the feed and open-tank tests for HMC and LMC juveniles acquired 58 and 78 individual observations, respectively. The feed test for HMC and LMC adults acquired 49 and 73 individual observations, and the open-tank test for adults acquired 59 and 74 individual recordings. Of the open-tank test individual recordings, 10 HMC and 8 LMC sessions were observed and analyzed.

3.1 Testing timeframe

Deviations from a systematic testing timeframe may have been a confounding factor (see Appendix C: Troubleshooting and confounds). However, testing all fry at approximately the same age standardized test measures. Additionally, major behaviors (e.g. latency to emerge) were graphed by brood testing date to discern if broods tested early in the year differed from broods tested later (Figure 3.1 and Figure 3.2). There were no trends apparent in either developmental stage, nor were there any significant differences between adult individual broods (ANOVA; F ratio = 1.5810, p = 0.1001) or dates
(ANOVA; F ratio = 1.3940, p = 0.1843). However, when significant differences were detected among juvenile dates tested (F ratio = 4.0839, p = <0.0001), post-hoc Tukey’s honestly significant difference (HSD) tests were performed to identify apparent differences (Table 3.1). Tukey’s HSD tests ensured that there were no specifically significant differences in behavior dependent on testing day. Major behaviors in the feed test, like latency to swim, latency to eat, and time eating experienced similar results.

### 3.2 Maternal effects

#### 3.2.1 Feed test

Fry raised with high levels of maternal care demonstrated a shorter latency to begin eating (Figure 3.3(a); F ratio = 3.9500, p = 0.0489) and spent more time eating in a novel environment (Figure 3.4(a); F ratio = 33.1377, p < 0.0001) than did fish raised with low levels of maternal care. The same was true for latency to feed (Figure 3.3(b); F ratio = 9.7089, p = 0.0023) and time spent eating as adults (Figure 3.4(b); F ratio = 50.2055, p < 0.0001). However, because adults had a longer latency to begin eating (F ratio = 9.6987, p = 0.0005) and spent less time eating (F ratio = 33.2991, p < 0.0001), data from the two developmental stages were not combined, but presented separately.

Vertical behavior in the feed test was also analyzed between fish raised with and without maternal care. There was no significant difference between developmental stages in behaviors below 3 cm (ANOVA; F ratio = 0.0128, p = 0.9100), between 3-5 cm (ANOVA; F ratio = 0.1203, p = 0.7290), and above 5 cm (ANOVA; F ratio = 0.5184, p = 0.4722), so vertical behavior data for juvenile and adult fish were combined (Figure 3.5). While all fish showed a preference for areas below 3 cm, LMC fish were more likely to behave between 3-5 cm (ANOVA; F ratio = 6.3526, p = 0.0124) or above 5 cm (ANOVA; F ratio = 10.5757, p = 0.00013) when compared to HMC fish. Not surprisingly, LMC fish spent significantly less time in areas below 3 cm (ANOVA; F ratio = 9.4764, p = 0.0023).
3.2.2. Open-tank test

A small sample of the study’s adult *A. burtoni* population (n = 18) was used to examine “exploration” as defined by Michael Meaney (Caldji et al. 1998). Although there was no statistically significant maternal effect on exploration (F ratio = 0.9266, p = 0.3501), adults raised with mothers generally spent more time exploring the inner area of the open-tank test than adults raised without mothers (Figure 3.6). This result was the most significant variable tested between the two broods. However, the other behaviors tested mirrored these results with corresponding trends. HMC fish spent less time near the start box (Figure 3.7(a); F ratio = 0.2338, p = 0.6353), traveled to a greater quantity of areas (Figure 3.7(b); F ratio = 0.1698, p = 0.6857), and moved more frequently into different areas (Figure 3.7(c); F ratio = 0.1338, p = 0.7193).

The entire *A. burtoni* population was used to explore “latency to emerge” behavior (Figure 3.8(a)) which has previously been used to measure rodent (Lantova et al. 2011) and fish (Brown et al. 2007) anxiety. Since juveniles and adults did not have significantly different responses (F ratio = 0.0691, p = 0.7929), the developmental stage data was combined to provide 269 observations for 59 HMC and 78 LMC fish. No significant differences were found (ANOVA; F ratio = 1.1186, p = 0.2912), but HMC fish tended to have longer latencies to emerge. The same generalization was found when comparing focal broods HMC2 and LMC4 (Figure 3.8(b); F ratio = 0.1435, p = 0.7070).

An interesting correlation between latency to emerge and exploration behavior in focal broods was also found (ANCOVA; Figure 3.9; F = 24.7966, p = 0.0001). It is not surprising that exploration increased as the latency to enter exploration field decreased. However, there is another repeated trend, though not statistically significant; when compared to brood LMC4, brood HMC2 consistently took longer to emerge yet also explored for a longer duration of time (Figure 3.6 and Figure 3.9).
3.3 Behavioral syndromes

3.3.1 “Consistency in different situations over contexts”

Most (33.9%) of the variation in boldness behavior was characterized by latency to swim, latency to feed, and vertical (below and above 3 cm) behavior (PC1; Table 3.2). It is unsurprising that below and above 3 cm were inversely correlated with each other. By ethogram definition (Table 2.2), when fish that spent more time above 3 cm, spent less time below 3 cm. But it is more interesting that the three remaining behaviors are positively correlated. In this respect, PC1 separates fish that are slower to begin eating and swimming from those that are quicker. The positive correlation between latencies and duration below 3 cm indicates that slower fish generally spend more time on the bottom of the tank. These correlations being the most prominent, PC1 clearly defines vertical speed.

Much (23.8%) of the variation in boldness behavior was characterized by duration of aggression, swimming activity, and time spent eating (PC2; Table 3.2). These results are unique because the correlations within PC2 are independent of the correlations within PC1. Swimming and aggression appear to be positively correlated, composing a single measure of activity. However, activity does not appear to be energetically favorable when seen correlated with all other variables; active fish eat for a shorter amount of time, are slower to begin eating, and slow to begin swimming. Interestingly, active fish also spend more time above 3 cm and have a shorter latency to emerge from start box. Summarizing these correlations, PC2 describes inefficient activity.

Variables characterizing PC1 and PC2 contributed most to the overall correlation of boldness behaviors, as their arrows extend furthest from the center of the 2D loading plot (Figure 3.10). Because vertical speed and inefficient activity most explained all fishes’ syndromes, temporal situations were plotted on PC1 and PC2 axes (figure 3.11). Adults and juveniles displayed inverse behavioral syndromes that were significantly different from each other (ANCOVA; F ratio = 9.0904, p = 0.0251). Juveniles utilized a strategy that increased vertical speed (PC1) by increasing inefficient activity (PC2).
However, as adults, the same fish increased *vertical speed* (PC1) by decreasing *inefficient activity* (PC2).

In order to better identify the variation between behavioral strategies, another PCA was performed for juveniles and adults separately (Table 3.3, Table 3.4, Figure 3.12). The main difference between juveniles and adults in PC1 was that juvenile fish with high vertical speed swam for a longer period of time, while adults with high vertical speed swam for a shorter duration. While juveniles and adults both had the same type of correlations producing inefficient activity, the main difference in PC2 was that adults had stronger correlations between these behaviors (Table 3.4) while juveniles’ were very weak (Table 3.3). Correlations for adults and juveniles were visualized as before (Figure 3.12).

### 3.3.2 Maternal effect on behavioral syndrome

Fish raised with high levels of maternal care did not demonstrate different levels of vertical speed (PC1) when compared to fish raised with low levels of maternal care (Figure 3.13). However, the clustering effect along the x-axis (PC2; Figure 3.13) signified that HMC and LMC fish might differ in another way. The clustering effect proved significant, and evidence was given that LMC fish produced higher levels of inefficient activity in comparison to HMC fish (ANOVA; Figure 3.14).

### 3.4 Morphological measures

Rearing conditions did not affect fish weight (F ratio = 0.2833, p = 0.5955) or standard length (F ratio = 0.0033, p = 0.9544). From the regression that determined weight (g) and standard length (mm) were correlated (Figure 3.15; F ratio = 737.3266, P <0.0001), residuals were produced. These residuals provided a measure of “body condition” which supplied more information than weight or size alone. Positive residuals indicate a “good” body condition which is heavier (g) than long (mm). Fishes with negative residuals have “bad” body conditions, and for their length, weigh less than they should. Even though
LMC fish had better body conditions (Figure 3.16), the difference was not significant (F ratio = 1.5900, P = 0.2097). While most fish were measured on day 126, the fish measured on day 136 did not differ in body condition either (Figure 3.17; F ratio = 0.0383, p = 0.8452).
3.5 Figures and tables

Figure 3.1: Mean ± SE juvenile latency to emerge behavior. The highest fish latencies were recorded on 12/13/2011 (HMC5), but this may be independent of time (see Table 3.1). Each bar represents one brood. Red bars indicate fry raised with high levels of maternal care; blue bars indicate fry raised in methyl blue. Broods are presented in order of date tested. Juvenile tests began with brood HMC1 in December and ended with brood LMC14 in February.

Figure 3.2: Mean ± SE latency to emerge behavior for adult broods. No broods (F ratio = 1.5810, p = 0.1001) or dates (F ratio, p = 0.1843) were significantly different from each other. Each bar represents one brood. Red bars indicate fish raised with high levels of maternal care; blue bars indicate fish raised in methyl blue. Broods are presented in order of date tested. Adult tests began with brood HMC1 in December and ended with brood LMC14 in March.
Table 3.1: Brood HMC5 tested in December (12/13/2011) had the highest least square mean, but it was not different from broods tested in January (1/21/2012) and February (2/5/2012). Tukey’s HSD Test shows that levels not connected by same letter are significantly different. Levels represent the date of juvenile open-tank test for all broods.

<table>
<thead>
<tr>
<th>Level</th>
<th>Least Sq Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>12/13/11</td>
<td>A 359.75000</td>
</tr>
<tr>
<td>2/5/12</td>
<td>A B 210.50000</td>
</tr>
<tr>
<td>1/21/12</td>
<td>A B 198.00000</td>
</tr>
<tr>
<td>2/9/12</td>
<td>B 178.20000</td>
</tr>
<tr>
<td>12/23/11</td>
<td>B 162.00000</td>
</tr>
<tr>
<td>12/21/11</td>
<td>B 155.40000</td>
</tr>
<tr>
<td>12/28/11</td>
<td>B 144.20000</td>
</tr>
<tr>
<td>2/6/12</td>
<td>B 140.10000</td>
</tr>
<tr>
<td>1/12/12</td>
<td>B 125.40000</td>
</tr>
<tr>
<td>2/10/12</td>
<td>B 115.70000</td>
</tr>
<tr>
<td>12/27/11</td>
<td>B 105.70000</td>
</tr>
<tr>
<td>12/3/11</td>
<td>B 82.60000</td>
</tr>
<tr>
<td>1/29/12</td>
<td>B 62.88889</td>
</tr>
</tbody>
</table>

Figure 3.3: Mean ± SE latency to begin eating in a novel environment for HMC and LMC (a) juvenile and (b) adult *A. burtoni*. HMC fish have a significantly shorter latency to feed as juveniles (p < 0.05) or adults (p < 0.0001). Sample sizes (number of individuals) are shown within bars.
Figure 3.4: Mean ± SE duration eating in a novel environment for adult HMC and LMC (a) juvenile and (b) adult *A. burtoni*. HMC fish spend a significantly longer time eating as juveniles (*p* < 0.0001) or adults (*p* < 0.0001). Sample sizes (number of individuals) are shown within bars.

Figure 3.5: Time budgets of vertical behavior for 49 HMC and 78 LMC fish. All fish spent a majority of their time in the bottom 3 cm of the feed test tank, but fish raised without mothers spent 7% more time above 3 cm (“between” *F* ratio = 6.3526, *p* = 0.0124; “above” *F* ratio = 10.5757, *p* = 0.00013). Black regions indicate proportion of time fish spent below 3 cm; grey regions indicate proportion of time spent above 3 cm. Adult and juvenile data were combined, nearly doubling the number of individual observations and sample size (*n* = 249).
Figure 3.6: Mean ± SE duration exploring a novel environment for adult broods HMC2 and LMC4. There is no statistical maternal effect (F ratio = 0.9266, p = 0.3501), but HMC fish generally spent a longer time exploring the inner area of the open-tank test than LMC fish. Sample sizes (number of individuals) are shown within bars.

Figure 3.7: Mean ± SE duration exploring a novel environment for adult broods HMC2 and LMC4. There is no statistical significance of any variable, but trends show that (a) HMC2 fish spent a shorter duration of time in or behind the start box (F ratio = 0.2338, p = 0.6353), (b) HMC2 fish generally explored more areas than LMC4 fish (F ratio = 0.1698, p = 0.6857), and (c) HMC2 fish switched areas more frequently than LMC4 fish (F ratio = 0.1338, p = 0.7193). Sample sizes (number of individuals) are shown within bars.
Figure 3.8: Mean ± SE latency to emerge behavior for juvenile and adult fish from (a) all broods and (b) focal broods HMC2 and LMC4. There is no significant maternal effect between all (F ratio = 1.1186, p = 0.2912) or focal (F ratio = 0.1435, p = 0.7070) broods, but there is a trend in both showing that HMC broods generally take longer to emerge from start box. Sample sizes (number of individual observations) are shown within bars.
Figure 3.9: Latency to emerge is significantly correlated with time spent exploring the inner areas of the open-tank test ($F = 24.7966$, $p = 0.0001$). Fish that take longer to emerge explore less, and fish that have shorter latencies explore more. Because exploration data points for all fish were not available, only adult data from broods HMC2 and LMC4 were used in regression analysis and ANCOVA ($n = 18$). There is a trend for HMC2 to explore more and have a longer latency to emerge than LMC4 ($F$ ratio = 8.7290, $p = 0.2113$).
Figure 3.10: PCA 2D loading plot for behaviors measured in foraging and exploratory contexts for all juvenile and adult fish. Arrows of variables that contribute most to PCs extend further out while those contributing least stay close to the center. Variables that neighbor each other generally have positive correlations while those that oppose generally have negative correlations. PC1 variables left of 0.0 positively correlate with each other, while variables right of 0.0 also positively correlate.

Table 3.2: PCA loadings for all behaviors measured in feed and open-tank tests. Behaviors for which loadings were >0.6 (absolute value; in bold in the table) were used for the interpretation of the components. Percents of variation contributed are shown under corresponding PC.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency to swim (secs)</td>
<td>0.68952</td>
<td>0.16091</td>
<td>0.52252</td>
<td>-0.13349</td>
</tr>
<tr>
<td>Latency to feed (secs)</td>
<td>0.66286</td>
<td>0.53067</td>
<td>0.19532</td>
<td>-0.04282</td>
</tr>
<tr>
<td>Time eating (secs)</td>
<td>-0.50821</td>
<td>-0.62055</td>
<td>-0.02861</td>
<td>0.13513</td>
</tr>
<tr>
<td>Swimming (secs)</td>
<td>0.12754</td>
<td>0.63752</td>
<td>-0.18412</td>
<td>0.39108</td>
</tr>
<tr>
<td>Aggression (secs)</td>
<td>-0.14196</td>
<td>0.63844</td>
<td>-0.54883</td>
<td>0.03573</td>
</tr>
<tr>
<td>Below 3cm</td>
<td>0.82168</td>
<td>-0.40393</td>
<td>-0.36940</td>
<td>-0.10674</td>
</tr>
<tr>
<td>Above 3cm</td>
<td>-0.82685</td>
<td>0.40147</td>
<td>0.36369</td>
<td>0.09842</td>
</tr>
<tr>
<td>Latency to emerge (secs)</td>
<td>0.38078</td>
<td>-0.27546</td>
<td>0.11953</td>
<td>0.82300</td>
</tr>
</tbody>
</table>
Figure 3.11: Different temporal situations plotted over PC1 and PC2. Components do not correlate with each other, but together aim to show stability of vertical speed (PC1) and inefficient activity (PC2) over lifetime. Different situations have inverse behaviors, as juveniles (gray) and adults (black) have significantly different PC regressions (ANCOVA; F ratio = 9.0904, p = 0.0251). Principle components characterize the strongest correlating boldness behaviors, and PC1 and PC2 explain 57.7% of boldness behavior variation.
Table 3.3: PCA loadings for behaviors measured in juvenile feed and open-tank tests. Behaviors for which loadings were > 0.6 (absolute value; bold in the table) were used for the interpretation of the components. Percents of variation contributed are shown under corresponding PC.

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>43.7%</td>
<td>20.8%</td>
<td>13.7%</td>
<td>9.93%</td>
</tr>
<tr>
<td>Latency to swim (secs)</td>
<td>0.82437</td>
<td>0.29247</td>
<td>0.19192</td>
<td>0.03146</td>
</tr>
<tr>
<td>Latency to feed (secs)</td>
<td>0.81352</td>
<td>0.39354</td>
<td>-0.03890</td>
<td>0.12018</td>
</tr>
<tr>
<td>Time eating (secs)</td>
<td>-0.63088</td>
<td>-0.46172</td>
<td>0.15834</td>
<td>0.09605</td>
</tr>
<tr>
<td>Swimming (secs)</td>
<td>0.89104</td>
<td>0.21246</td>
<td>0.12741</td>
<td>0.01413</td>
</tr>
<tr>
<td>Aggression (secs)</td>
<td>-0.15836</td>
<td>0.23458</td>
<td><strong>-0.81420</strong></td>
<td>0.49252</td>
</tr>
<tr>
<td>Below 3cm</td>
<td><strong>0.64340</strong></td>
<td><strong>-0.67201</strong></td>
<td>-0.30620</td>
<td>-0.15102</td>
</tr>
<tr>
<td>Above 3cm</td>
<td><strong>-0.66456</strong></td>
<td><strong>0.66090</strong></td>
<td><strong>0.28839</strong></td>
<td><strong>0.14513</strong></td>
</tr>
<tr>
<td>Latency to emerge (secs)</td>
<td>0.28196</td>
<td>-0.47218</td>
<td>0.42389</td>
<td><strong>0.69485</strong></td>
</tr>
</tbody>
</table>

Table 3.4: PCA loadings for behaviors measured in adult feed and open-tank tests. Behaviors for which loadings were > 0.6 (absolute value; bold in the table) were used for the interpretation of the components. Percents of variation contributed are shown under corresponding PC.

<table>
<thead>
<tr>
<th></th>
<th>PC1</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>42.7%</td>
<td>19.9%</td>
<td>11.3%</td>
<td>10.8%</td>
</tr>
<tr>
<td>Latency to swim (secs)</td>
<td><strong>0.68716</strong></td>
<td>0.11449</td>
<td>0.47506</td>
<td>-0.25539</td>
</tr>
<tr>
<td>Latency to feed (secs)</td>
<td>0.55641</td>
<td><strong>0.61008</strong></td>
<td>0.11334</td>
<td>0.00554</td>
</tr>
<tr>
<td>Time eating (secs)</td>
<td>-0.39940</td>
<td><strong>-0.73725</strong></td>
<td>0.06663</td>
<td>-0.03772</td>
</tr>
<tr>
<td>Swimming (secs)</td>
<td><strong>-0.85794</strong></td>
<td>0.01410</td>
<td>-0.23296</td>
<td>0.17530</td>
</tr>
<tr>
<td>Aggression (secs)</td>
<td>-0.32323</td>
<td><strong>0.72867</strong></td>
<td>-0.38234</td>
<td>0.09378</td>
</tr>
<tr>
<td>Below 3cm</td>
<td><strong>0.85044</strong></td>
<td>-0.25100</td>
<td>-0.45430</td>
<td>-0.02429</td>
</tr>
<tr>
<td>Above 3cm</td>
<td><strong>-0.84991</strong></td>
<td>0.24739</td>
<td>0.45832</td>
<td>0.01776</td>
</tr>
<tr>
<td>Latency to emerge (secs)</td>
<td>0.43035</td>
<td>-0.09584</td>
<td>0.20807</td>
<td><strong>0.86860</strong></td>
</tr>
</tbody>
</table>
Figure 3.12: PCA 2D loading plots for behaviors measured in foraging and exploratory contexts for (a) juvenile and (b) adult fish. The major difference in juvenile and adult PC1 was swimming; (a) it clumps together/positively correlates or (b) is far from/negatively correlates with latency behaviors. The major difference in juvenile and adult PC2 was the stronger negative correlation between activity (swimming and aggression) and time eating in adults. Notice, the aggression arrow for juveniles is very short (a) and symbolic of a weak correlation with other variables. Variables that contribute the most to PCs extend further out with their arrows while those contributing least stay close to the center. PC1 components are circled in black.
Figure 3.13: The relationship between PC1 and PC2 shown for different levels of maternal care. HMC (red) and LMC (blue) do not have significantly different PC correlations (F ratio = 0.5280, p = 0.2681). This indicates that they do not have a particular tradeoff between vertical speed (PC1) and inefficient activity (PC2). Maternal conditions do not have differences in their vertical speed (PC1). However, there is a noticeable clustering effect along the x-axis (HMC: left, LMC: right).
Figure 3.14: Mean ± SE differences in second principle component for juvenile and adult fish from broods with different levels of maternal care. There is a significant maternal effect on PC2, which explains 23.8% of variation in all boldness behaviors (F ratio = 59.0831, p < 0.0001). Accordingly, LMC fish demonstrate more inefficient activity (PC2) than HMC fish. Sample sizes (number of individual observations) are shown within bars.
Figure 3.15: Fish weight (g) and standard length (mm) measured at from day 126 to day 135 was significantly correlated (F ratio = 737.3266, P <0.0001) for HMC (red) fish and LMC (blue) fish.
Figure 3.16: Mean ± SE adult fish body condition calculated from weight (g) and standard length (mm) was not significantly different (F ratio = 1.5900, P = 0.2097).

Figure 3.17: Mean ± SE body condition calculated from weight (g) and standard length (mm) was not significantly different between fish measured at day 126 and day 136 (F ratio = 0.0383, p = 0.8452).
Discussion

4.1 Behavioral syndromes

Behavioral syndromes, or “suites of correlated behaviors reflecting consistency in behavior across (two or more) situations,” are often used to “explain non-optimal behaviors that carry over situations” and are able to separate “behavioral types” within a species or population (Sih et al. 2004). Variables in this thesis correlated in interesting ways (Figure 3.10, Table 3.2). The principal component explaining (33.9% of) the boldness syndrome was vertical speed; fish who are quick to swim, eat, and behave in the upper vertical portions of the tank were distinguished from fish who are slow to eat, swim, and stay in the lower portions of the tank. The second component explaining (23.8% of) the boldness syndrome separated fish with inefficient activity from fish with efficient activity. Inefficiently active fish exhibited excessive swimming and aggression with no feeding payoff (slower to begin eating, ate for a shorter duration, longer to start swimming). When considering an individual’s fitness, increased activity in congruence with decreased gain (less food) appears energetically costly and non-optimal. This component could be given the “artificial variable” (O’Rourke et al. 2005) anxiety. Less anxious fish ate more than they swam, suggesting a higher energetic payoff.

“Boldness” was previously defined as the propensity to “approach novel objects, increase activity levels and exploratory behavior” (Brown et al. 2007). According to this thesis, boldness was (57.7%) explained by vertical speed and inefficient activity. However, this appears evolutionarily contradictory.

4.1.1 Ecology and evolution

While it would be evolutionarily beneficial to be “vertically speedy”, it would be deleterious to a fish’s overall fitness to be “anxious”. However, within this syndrome, bold fish are both vertical speedy and anxious. These results succeed in explaining non-optimal behavioral variation in a population, but nomenclature does not allow for
distinction within the syndrome. Distinction between components of behavioral syndromes like this one becomes necessary to provide accurate information in evolutionary and ecological models.

For instance, the bold component anxiety appears to be heavily affected by changes in early development (Figure 3.14) while the bold component vertical speed does not (Figure 3.13). This suggests that the two components explaining the overarching behavioral syndrome may have different mechanisms. If different mechanisms govern different parts of boldness, only one component (e.g. anxiety) may be evolutionarily sensitive and vary over time. I propose that in order to functionally separate the two components, slight revisions to the nomenclature should be made.

### 4.1.2 Revisions to nomenclature

The term “behavioral syndrome” is too broad. Using the behavioral syndrome in this thesis as an example, the two main components of boldness evolutionarily contradict each other (fish fastest to feed waste the most energy). This explains Sih’s “non-optimal” behavior within a population well, but does not allow for distinction of components. For instance, the component describing anxiety was affected by early development (Figure 3.14), where vertical speed was not (3.13). In order to reflect this difference, I would label anxiety as a developmentally-sensitive bold syndrome, and vertical speed as a stable bold syndrome. In this way, fish demonstrate two bold behavioral syndromes instead of one. Fish could be categorized as having a stable “vertically fast” or “vertically slow” behavioral type. Separately, fish could be categorized as having a malleable “anxious” or “calm” behavioral type. As a result, these syndromes affect fitness in different ways, each syndrome could be plugged into evolutionary models independently or at different frequencies in order to reflect differences between populations’ “birth, death, and dispersal rates” (Sih et al. 2004).

Another factor that influences behavioral syndrome stability is time. Some behavioral syndromes have previously been shown to correlate over the entire lifetime (Bell and Stamps 2004). Other behavioral syndromes, such as those in this thesis, have demonstrated the ability to transform over time (Figure 3.3, Figure 3.4, Figure 3.11). Just as correlating behaviors that change due to developmental influence (e.g. maternal care)
have been labeled \textit{developmentally-sensitive} syndromes, correlating behaviors that change over time (e.g. age or “state” (Bell 2007)) can be referred to as \textit{temporally-sensitive} syndromes.

A second critique is on the term “situation”. It has been defined as “a given set of conditions at one point in time [involving] different levels along an environmental gradient or different sets of conditions across time” (Sih \textit{et al.} 2004). I propose to specify situations as either “environmental” or “temporal”. However, this thesis has demonstrated that this even these terms may be too broad.

The temporal situation utilized in this thesis was developmental stage (e.g. age). I made the mistake behavioral syndrome pioneers made initially (Sih \textit{et al.} 2004), and assumed that personality remained consistent over developmental stages. However, fish did not produce consistently correlated behaviors throughout their lifetimes (Figure 3.11). This may have been because fish were sensitive to “state” (Bell 2007) or “life experiences” (Brown 2007, Wilson 1998). A specific life experience that may have contributed to differences in boldness over lifetime may have been the tagging procedure, which took place after juvenile and before adult testing.

To specify temporal situations further, situations could be deconstructed for species in which time or age would influence consistency in their behavioral patterns. Large species slow to reproduce (e.g. ungulates) would utilize “K temporal situations”. These species may have consistent behavioral correlations across larger spans of time, like “breeding and non-breeding season” (Sih \textit{et al.} 2004). Small species quick to reproduce (e.g. rats and fish) would utilize “r temporal situations”. Due to their rapid growth rate, small species’ “state, health, or body size” (Bell 2007) during breeding season one month could be dramatically different during non-breeding season the next month. Hence, these species would have smaller timeframes for consistent behavioral correlations because of their expedited growth rate. Additionally, with these terms defined, it can be made explicit that temporally-sensitive behavioral syndromes should not test for correlated behaviors across temporal situations.
4.1.3 Boldness influenced by maternal care

Within the sample population of *A. burtoni* used for this study, the only difference in experimental rearing was maternal care. HMC fish were allowed the entirety of their maternal mouth-brooding span plus an additional few weeks free-swimming with their mother, enabling transitive inference. LMC fish were stripped from their mother while a majority of their egg yolks remained, were raised in methyl blue, and not allowed observation of HMC broods or adult fish. By doing this, I was able to use a developmental approach to personality, and test whether a specific change in early social environment (e.g. maternal care) would change the behavioral syndrome of the resulting juveniles and adults.

The results indicated that maternal care influences context-specific (feeding and exploration) behaviors in fish. HMC fish are significantly more likely to begin swimming quicker, begin eating quicker, and eat for a longer period of time (Figure 3.3, Figure 3.4). There are also trends indicating that HMC fish may also explore for a longer period of time, travel to a greater amount of areas, and move more frequently in a novel environment (Figure 3.6, Figure 3.7).

Independently, these results do not constitute a boldness behavioral syndrome, which should be judged by correlations between behaviors. Hence, variables were checked for differences in correlations (PCA; Figure 3.13). Although maternal conditions did not have distinct life history strategies along the primary component (vertical speed, 33.9% of boldness variation), they did have significant differences between the secondary component (anxiety, 23.8% of boldness variation).

Using nomenclature provided (*see section 4.1.2*), this indicated that the vertical speed syndrome was not affected by developmental differences in maternal care, but that anxiety syndrome was (Figure 3.13). According to a specific suite of correlated behaviors (PC2, Table 3.2), LMC fish were more anxious that HMC fish (Figure 3.14).
4.2 Maternal effects

Known to impact anxiety-mediated behavior, maternal effects can also regulate glucocorticoid receptors (GR) in rat populations. The correlations between this behavior and hormonal mechanism have been extensively studied and reported in mammals (Liu et al. 1997, Caldji et al. 1998, Francis et al. 1999, Conrad 2008), but remains relatively unexplored in fish. This is partially due to debates on fish brain homology in function and research applicability.

With the data collected in this thesis, populations of fishes and rats can be compared. This information can then be used to access the evolutionary origin of maternal effect on anxiety-mediated behavior. Rats used for maternal effect studies were often tested “no earlier than 100 days of age” (Caldji et al. 1998), and were therefore adults before testing. Fish in my thesis were tested at both the juvenile and adult developmental stage. Despite age, data reflected that of Michael Meaney: fish raised with high levels of maternal care “showed a shorter latency to begin eating and spent more time eating in a novel environment” (Caldji et al. 1998) than did fish raised with low levels of maternal care (Figure 3.3 and Figure 3.4). While both results were significant and similar to rat data statistically, visual representations of latency and time eating behaviors were also similar. In both rats and fish, disparities between animals raised with and without maternal care were more drastic in the time eating behavior (Figure 3.4) than in the latency to feed behavior (Figure 3.3).

Rat research relies heavily on another test of anxiety-mediated behavior, the open-field test. This thesis used the same parameters for “exploration” as previous rat research. Since time spent in the areas 10 cm closest to the walls of open-field arenas were not categorized as exploration behavior (Caldji et al. 1998), restricted zones were converted to tank measurements, labeled “outer” areas (Table 2.1), and applied to the open-tank test in red tape (Figure 2.2). Any time spent in the outer areas was omitted, and only time spent within the central arena was considered exploration.

While only one sample brood of each maternal condition was used to compare this response variable, trends indicated that adult HMC fish spent a longer time to explore (Figure 3.6). When correlated with latency to emerge, an interesting trade-off was seen.
between latency to emerge and exploration; HMC fish tended to take longer to emerge, but explored the center arena for longer (Figure 3.9). LMC fish tended to take a shorter time to emerge, but spent a shorter time in the center arena. Although LMC fish appeared to emerge sooner, they spent more of their time in the outer areas of the arena. By Meaney’s definition, LMC behavior would not be considered exploration, but rather, an attempt to escape (Caldji et al. 1998). If the same trade-off in latency and exploration behavior were assumed for the entire A. burtoni sample population, the trend showing longer latencies for HMC fish (Figure 3.8) would imply that all HMC fish spent longer exploring as well. These results would further support the similarities between rat and fish anxiety-mediated behavior in response to maternal effects.

Additionally, the trade-off in latency and exploration behavior could provide evidence for an argument about the viability of “latency to emerge” as an appropriate response variable of anxiety and boldness (Brown et al. 2007). No only did this behavior negatively correspond to exploration, but it explained the least amount of variation in every PCA identifying the principal components of boldness behavior (Table 3.2, Table 3.3, Table 3.4).

4.2.1 Mechanism of maternal effects

When compared to the data previously collected on rats, the data collected in this thesis supports homology between fish and rat maternally influenced anxiety on a behavioral scale. However, because the mechanism of anxiety-mediated behavior is known in rats, my results may be able to support data from other studies that make the claim for homologous mechanism.

For example, it is known that rat and fish forebrains have the most abundant concentration of GR mRNA (Teitsma et al. 1998, Yao et al. 2008, Vargas 2009), but the fish previously tested have had no other behavioral condition in which to compare their baseline amount of forebrain GR mRNA. Chris Galvin (Renn Lab, 2012) has been conducting an experiment in congruence with my behavioral study in order to compare HMC and LMC fish GR in the liver. Since liver GR has previously corresponded with pallium GR, quantifiably more GR in HMC fish liver would indicate an up-regulation in HMC fish pallium GR.
Not only have both studies employed wild-stock, biologically related parental fish to spawn fry, but Galvin and I have split approximately 50% of the broods from each maternal condition. As Galvin often needed only 5 fish per brood, he utilized the excess from broods containing 13 to 15 fish. Fish not required in experimental brood of either study became part of the “extra” fish broods used for open-tank or feed test preliminary trials.

Since our sample populations are intrinsically related, a significantly higher up-regulation in Galvin’s HMC fish livers would act as a very strong defense for my behaviorally comparative approach, and vice versa. Combined, these results would have significant impact on existing literature. Despite the divergent methods of brain development, results would provide significant evidence for the homology of fish lateral pallium to that of the mammalian hippocampus. Results would also support the argument that CNS expression and function have been evolutionary conserved. Practically speaking, fish could become a model organism for maternal effect studies or more frequently used in neurobiology. Because of their environmental sensitivity, and ability to absorb from and release hormones into their surrounding water source, they may also be desirable in psychopharmacology research.

4.2.2. Evolution of maternal effects

In an evolutionary context, this thesis has provided evidence to indicate that anxiety-mediated behavior, especially context-specific behaviors, like time eating and latency to feed, may have originated very early in vertebrate evolution and have been conserved. Without Galvin’s HMC and LMC GR liver data at hand, I can at least infer that these behavioral studies have reflected something about the mechanism of this behavior as Meaney’s rats have in the past (Caldji et al. 1998). My near homologous results allow me to speculate that fish raised with maternal care up-regulate GR mRNA in feedback loops into the HPA axis, while LMC fish do not. Mechanistic evidence for this argument would support that not only the behavior from maternal effects is conserved, but also that the function of the HPA axis and mechanism of maternal effect also has very distant phylogenetic origins.
4.3 “Anxiety”

The two aims of this thesis are connected by anxiety. The overarching bold behavioral syndrome diverged into its two components (vertical speed and anxiety), making new sub-syndromes. Anxiety proved to be unique by being the only syndrome affected by maternal care. Further, LMC fish proved to exhibit more anxiety than HMC fish (Figure 3.14). These personality results support the results compiled for the mechanism of maternal effects (Figure 3.3 and Figure 3.4). Hence, the mechanism for anxious personality and anxiety-mediated behavior may be similar. This implies that vertical speed personality may have another mechanism entirely. Because vertical speed is not developmentally-sensitive, it may be much more difficult to influence. For instance, where anxiety may be altered by environmental enrichment (Bredy et al. 2003), neonatal handling (Bredy et al. 2004), or drug injection (Weaver et al. 2006), vertically fast fish may always be different from vertically slow fish.

Although no wild population could be “without mothers”, this thesis provides evidence for the existence of developmentally-sensitive behavioral syndromes in that an animal’s early environment can change its life history strategy and maybe even the mechanism which underlies it.
Appendix A: JWWatcher

A.1 Focal master files

A.1.1 Open-tank test

Name: mez_OTT.fmf
Format: Focal Master File 1.0
Updated: Tue Mar 06 00:50:23 PST 2012

------------------------------
Behavior.name.1=inner area
Behavior.description.1=moved into inner square
Behavior.name.2=outer area
Behavior.description.2=moved into outer square
Behavior.name.a=aggression
Behavior.description.a=bitting at tank walls or objects
Behavior.name.b=start box
Behavior.description.b=inside or behind start box
Behavior.name.i=immobile
Behavior.description.i=immobile
Behavior.name.n=new green area
Behavior.description.n=new green area
Behavior.name.o=out of sight
Behavior.description.o=cannot see fish
Behavior.name.s=swim
Behavior.description.s=swim movement
Behavior.name.u=crosses green
Behavior.description.u=# times moved to diff area
DurationMilliseconds=300000
CountUp=true
SoundOn=true
Question.1=File Name (Chris's Code)
Question.2=Date
Question.3=
Question.4=
Question.5=
Question.6=
Notes=
Supplementary=Anxiety Meditated Behavior-- open field/tank test
A.1.2 Feed test

------------------------------------------------------
Name: feed test.fmf
Format: Focal Master File 1.0
Updated: Sat Dec 17 17:08:11 PST 2011
------------------------------------------------------
Behavior.name.2=between 3-5cm
Behavior.description.2=when in middle-tank
Behavior.name.a=aggression
Behavior.description.a=bites tank or reflection
Behavior.name.b=bottom 3 cm
Behavior.description.b=when in bottom of tank
Behavior.name.f=feed
Behavior.description.f=
Behavior.name.i=immobile
Behavior.description.i=
Behavior.name.o=out of sight
Behavior.description.o=
Behavior.name.s=swim movement
Behavior.description.s=
Behavior.name.t=above 5cm
Behavior.description.t=when in the top of the tank
DurationMilliseconds=600000
CountUp=true
SoundOn=true
Question.1=brood #
Question.2=C or E
Question.3=fish #
Question.4=
Question.5=
Question.6=
Notes=
Supplementary=
A.2 Focal analysis master files

A.2.1 Open-tank test

Name: mez_OTT.faf
Format: Focal Analysis Master File 1.0
Updated: Sun Apr 15 18:02:52 PDT 2012

FocalMasterFile=/Applications/JWatcher_V1.0/mez_anxiety_OTT/mez_OTT.fmf

TimeBinDuration=0.0
EndWithLastCompleteBin=true

ScoreFromBeginning=true
ScoreFromBehavior=false
ScoreFromFirstBehavior=false
ScoreFromOffset=false

Offset=0.0
BehaviorToScoreFrom=

OutOfSightCode=o

Report.StateNaturalInterval.Occurrence=false
Report.StateNaturalInterval.TotalTime=false
Report.StateNaturalInterval.Average=false
Report.StateNaturalInterval.StandardDeviation=false
Report.StateNaturalInterval.ProportionOfTime=false
Report.StateNaturalInterval.ProportionOfTimeInSight=false
Report.StateNaturalInterval.ConditionalProportionOfTime=false

Report.StateNaturalDuration.Occurrence=false
Report.StateNaturalDuration.TotalTime=false
Report.StateNaturalDuration.Average=false
Report.StateNaturalDuration.StandardDeviation=false
Report.StateNaturalDuration.ProportionOfTime=false
Report.StateNaturalDuration.ProportionOfTimeInSight=false
Report.StateNaturalDuration.ConditionalProportionOfTime=false
Report.StateAllInterval.Occurrence=false
Report.StateAllInterval.TotalTime=false
Report.StateAllInterval.Average=false
Report.StateAllInterval.StandardDeviation=false
Report.StateAllInterval.ProportionOfTime=false
Report.StateAllInterval.ProportionOfTimeInSight=false
Report.StateAllInterval.ConditionalProportionOfTime=false

Report.StateAllInterval.Occurrence=true
Report.StateAllInterval.TotalTime=true
Report.StateAllInterval.Average=false
Report.StateAllInterval.StandardDeviation=false
Report.StateAllInterval.ProportionOfTime=false
Report.StateAllInterval.ProportionOfTimeInSight=true
Report.StateAllInterval.ConditionalProportionOfTime=false

Report.EventNaturalInterval.EventCount=false
Report.EventNaturalInterval.Occurrence=false
Report.EventNaturalInterval.Average=false
Report.EventNaturalInterval.StandardDeviation=false
Report.EventNaturalInterval.ConditionalNatRate=false
Report.EventNaturalInterval.ConditionalNatIntervalOccurance=false
Report.EventNaturalInterval.ConditionalNatIntervalAverage=false
Report.EventNaturalInterval.ConditionalAllEventCount=false
Report.EventNaturalInterval.ConditionalAllRate=false
Report.EventNaturalInterval.ConditionalAllIntervalOccurance=false
Report.EventNaturalInterval.ConditionalAllIntervalAverage=false
Report.EventNaturalInterval.ConditionalAllIntervalStandardDeviation=false

AllCodesMutuallyExclusive=false

Behavior.isModified.o=false
Behavior.isSubtracted.o=false
Behavior.isIgnored.o=false
Behavior.isEventAnalyzed.o=false
Behavior.switchesOff.o=a,i,s,u,2,1,n,b

Behavior.isModified.n=false
Behavior.isSubtracted.n=false
Behavior.isIgnored.n=false
Behavior.isEventAnalyzed.n=false
Behavior.switchesOff.n=o

Behavior.isModified.i=false
Behavior.isSubtracted.i=false
Behavior.isIgnored.i=false
Behavior.isEventAnalyzed.i=false
Behavior.switchesOff.i=a,o,s

Behavior.isModified.b=false
Behavior.isSubtracted.b=false
Behavior.isIgnored.b=false
Behavior.isEventAnalyzed.b=false
Behavior.switchesOff.b=1,o,s

Behavior.isModified.a=false
Behavior.isSubtracted.a=false
Behavior.isIgnored.a=false
Behavior.isEventAnalyzed.a=false
Behavior.switchesOff.a=i,o,s

Behavior.isModified.2=false
Behavior.isSubtracted.2=false
Behavior.isIgnored.2=false
Behavior.isEventAnalyzed.2=false
Behavior.switchesOff.2=1,o

Behavior.isModified.1=false
Behavior.isSubtracted.1=false
Behavior.isIgnored.1=false
Behavior.isEventAnalyzed.1=false
Behavior.switchesOff.1=2,o,b

Behavior.isModified.u=false
Behavior.isSubtracted.u=false
Behavior.isIgnored.u=false
Behavior.isEventAnalyzed.u=false
Behavior.switchesOff.u=o

Behavior.isModified.s=false
Behavior.isSubtracted.s=false
Behavior.isIgnored.s=false
Behavior.isEventAnalyzed.s=false
Behavior.switchesOff.s=o,a,i,b
A.2.2 Feed test

Name: feed test.faf
Format: Focal Analysis Master File 1.0
Updated: Wed Feb 15 10:12:37 PST 2012

FocalMasterFile=/Applications/JWatcher_V1.0/mez_anxiety_feed test/feed test.fmf

TimeBinDuration=0.0
EndWithLastCompleteBin=true

ScoreFromBeginning=true
ScoreFromBehavior=false
ScoreFromFirstBehavior=false
ScoreFromOffset=false

Offset=0.0
BehaviorToScoreFrom=

OutOfSightCode=o

Report.StateNaturalInterval.Occurrence=false
Report.StateNaturalInterval.TotalTime=false
Report.StateNaturalInterval.Average=false
Report.StateNaturalInterval.StandardDeviation=false
Report.StateNaturalInterval.ProportionOfTime=false
Report.StateNaturalInterval.ProportionOfTimeInSight=false
Report.StateNaturalInterval.ConditionalProportionOfTime=false

Report.StateNaturalDuration.Occurrence=false
Report.StateNaturalDuration.TotalTime=false
Report.StateNaturalDuration.Average=false
Report.StateNaturalDuration.StandardDeviation=false
Report.StateNaturalDuration.ProportionOfTime=false
Report.StateNaturalDuration.ProportionOfTimeInSight=false
Report.StateNaturalDuration.ConditionalProportionOfTime=false

Report.StateAllInterval.Occurrence=false
Report.StateAllInterval.TotalTime=true
Report.StateAllInterval.Average=false
Report.StateAllInterval.StandardDeviation=false
Report.StateAllInterval.ProportionOfTime=false
Report.StateAllInterval.ProportionOfTimeInSight=false
Report.StateAllInterval.ConditionalProportionOfTime=false
Report.StateAllDuration.Occurrence=true
Report.StateAllDuration.TotalTime=true
Report.StateAllDuration.Average=true
Report.StateAllDuration.StandardDeviation=false
Report.StateAllDuration.ProportionOfTime=true
Report.StateAllDuration.ProportionOfTimeInSight=false
Report.StateAllDuration.ConditionalProportionOfTime=false
Report.EventNaturalInterval.EventCount=false
Report.EventNaturalInterval.Occurrence=false
Report.EventNaturalInterval.Average=false
Report.EventNaturalInterval.StandardDeviation=false
Report.EventNaturalInterval.ConditionalNatRate=false
Report.EventNaturalInterval.ConditionalNatIntervalOccurance=false
Report.EventNaturalInterval.ConditionalNatIntervalAverage=false
Report.EventNaturalInterval.ConditionalAllEventCount=false
Report.EventNaturalInterval.ConditionalAllRate=false
Report.EventNaturalInterval.ConditionalAllIntervalOccurance=false
Report.EventNaturalInterval.ConditionalAllIntervalAverage=false
Report.EventNaturalInterval.ConditionalAllIntervalStandardDeviation=false
AllCodesMutuallyExclusive=false
Behavior.isModified.b=false
Behavior.isSubtracted.b=false
Behavior.isIgnored.b=false
Behavior.isEventAnalyzed.b=false
Behavior.switchesOff.b=2,t,o

Behavior.isModified.a=false
Behavior.isSubtracted.a=false
Behavior.isIgnored.a=false
Behavior.isEventAnalyzed.a=false
Behavior.switchesOff.a=f,i,o,s

Behavior.isModified.i=false
Behavior.isSubtracted.i=false
Behavior.isIgnored.i=false
Behavior.isEventAnalyzed.i=false
Behavior.switchesOff.i=a,f,o,s

Behavior.isModified.t=false
Behavior.isSubtracted.t=false
Behavior.isIgnored.t=false
Behavior.isEventAnalyzed.t=false
Behavior.switchesOff.t=2,b,o

Behavior.isModified.2=false
Behavior.isSubtracted.2=false
Behavior.isIgnored.2=false
Behavior.isEventAnalyzed.2=false
Behavior.switchesOff.2=b,t,o

Behavior.isModified.s=false
Behavior.isSubtracted.s=false
Behavior.isIgnored.s=false
Behavior.isEventAnalyzed.s=false
Behavior.switchesOff.s=a,f,i,o

Behavior.isModified.f=false
Behavior.isSubtracted.f=false
Behavior.isIgnored.f=false
Behavior.isEventAnalyzed.f=false
Behavior.switchesOff.f=a,i,o,s

Behavior.isModified.o=false
Behavior.isSubtracted.o=false
Behavior.isIgnored.o=false
Behavior.isEventAnalyzed.o=false
Behavior.switchesOff.o=a,f,i,s,2,b,t
A.3 Example data files

A.3.1 Open-tank test

FirstLineOfData=25
Name: bingo.dat
Format: Focal Data File 1.0
Updated: Sun Apr 15 17:13:32 PDT 2012

FocalMasterFile=/Applications/JWatcher_V1.0/mez_anxiety_OTT/mez_OTT.fmf

# Observation started: Sun Apr 15 17:04:30 PDT 2012
StartTime=1334534670144
# Observation stopped: Sun Apr 15 17:09:30 PDT 2012
StopTime=1334534970144

Answer.1=bingo
Answer.2=4/15/2012
Answer.3=
Answer.4=
Answer.5=
Answer.6=

#BEGIN DATA
0, b
0, i
60501, s
61270, 1
62409, i
151055, s
152260, 2
152340, 1
157657, s
159129, i
159745, s
163587, i
164826, s
165093, i
169748, s
172420, i
179833, s
184279, i
190449, s
191880, i
198363, s
199241, i
208007, s
208205, i
209077, s
212991, i
216560, s
218060, 2
220149, s
221289, i
222550, 1
222560, s
228394, i
234092, s
235084, i
236541, s
245983, s
250473, s
251798, i
259440, s
259819, l
261011, 2
263987, 1
266660, i
267347, s
268148, i
273886, s
276111, i
277295, s
280278, 2
281187, 1
282163, i
283001, s
288872, i
290946, s
291739, 2
292641, i
294315, s
296092, i
296501, s
297412, i
299685, s
300000, EOF
A.3.2 Feed test

FirstLineOfData=25

Name: feed test 2_E6_fish 7.dat
Format: Focal Data File 1.0
Updated: Tue Feb 28 15:33:36 PST 2012

FocalMasterFile=/Applications/JWatcher_V1.0/mez_anxiety_feed test/feed test.fmf

# Observation started: Tue Feb 28 15:22:13 PST 2012
StartTime=133047133656
# Observation stopped: Tue Feb 28 15:32:13 PST 2012
StopTime=133047193656

Answer.1=6 (brood)
Answer.2=E (LMC)
Answer.3=7 (fish#)
Answer.4=
Answer.5=
Answer.6=

#BEGIN DATA
0, i 0, b 40342, s
42030, s 44414, s 44711, a 45031, a
45374, s 46495, s 48464, 2 50424, t
51800, i 52992, s 53352, a 54557, 2
55553, b 57765, i 98048, s 101465, 2
102553, s 103722, t 104922, s 105602, 2
105986, b 106794, s 107618, 2 108275, s
108723, t 113124, 2 113652, s 113916, b
117704, 2 119480, i 122203, a 122969, b
123714, 2 124762, t 127130, i 132403, b
134828, s 135101, 2 135516, s 135812, b
137245, s 140850, s 146999, s 151001, s
153601, s 155369, s 159407, i 165828, s
167621, s 168781, 2 169541, t 170600, s
171048, 2 171325, b 171999, s 172342, 2
173222, s 173616, b 174694, s 175930, 2
176591, s 176727, t 177417, a 177743, a
178106, b 179873, s 181264, a 182281, a
182619, s 184217, a 184545, 2 185145, t
187129, s 187458, 2 188146, s 188450, b
191267, i 193284, s 194931, a 197117, 2
198055, i 209457, s 210633, b 211865, s
212291, s 212473, a 218987, 2 219572, t
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<td>599443</td>
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<td>600000</td>
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Appendix B: VIE tagging fry

Visual implant elastomer (VIE) tags were used in this thesis to identify and track individual fish throughout ontogeny. The youngest fish I tagged was on day 56 and the oldest was on day 62. However, others have tagged fish as young as 2 weeks old (Parker 2009). Since tagging protocol and procedure for elastomer preparation has been outlined before (Parker 2009), I will provide methodology I found to be most effective when tagging small fish in this thesis.

Recommendations when using VIE tags:

1. Upon elastomer preparation, or when retrieving pre-made elastomer from the refrigerator, put syringes on ice. Taking this precaution allows additional syringes to stay cool and remain functional for a longer period of time.

2. Ensure each color elastomer is still functional by gently squeezing the injecting syringes. Needle should be facing up and away from body or peers. Ideally, the elastomer should be fluid, and pressure needed to eject liquid elastomer should be minimal. More pressure needed to apply to syringe indicates that the elastomer is older, or has not been refrigerated properly. Hardened elastomer makes tagging small fish difficult and may result in increased fatalities.

3. Put individual fish on a paper towel. Situate towel so that fish head faces away from the experimenter. With less dominant hand, brace towel on either side of fish body, gently bracing the widest sides of the fish. This precaution prevents excessive movement during injection. If tags require a particular side of the fish to be tagged (left or right), fish merely need to be flipped horizontally, head still facing away from experimenter. This also ensures injection is made beneath scales.

4. Syringe tip should be clean of excess elastomer prior to injection. To minimize the likelihood of lost tags, ensure needle penetrates second layer of skin. However, take care to prevent needle tip from contacting area surrounding brain
or organs. If only faint tag has been injected, but needle tip is very close in
proximity to brain or organs, withdrawal needle, and attempt injection from
further back along the spine.

5. Take note or sketch specific location and side where each individual fish was
tagged. This will make later identification of lost or hard-to-see tags much easier
(see step 7).

6. Immediately following injection, fish should be replaced in water. Fish should
ideally not spend more than 1 minute outside water source. If tagging is taking too
long, place focal fish in individual container of water, tag other fish, and revisit
original subject.

7. If VIE tag is very difficult to see upon later identification, use a handheld
backlight in near to complete darkness to look for tag in body. If tag does not
illuminate even in presence of backlight in darkness, the tag may have fallen out.
Recall notes on individual fish, and double check tag existence before marking
“tag lost” or re-tagging.
Appendix C: Troubleshooting and confounds

C.1 Date tested timeframes:

The process of identifying and retrieving eggs from pregnant *A. burtoni* females has a high learning curve. By the time I was very good at identifying a brooding female, I had collected many mothers, and few broods of LMC eggs. Additionally, the correct concentration of methyl blue required research, purchase, and trial time. In order to foster a surviving brood of eggs alone in bubbling beakers, only a few drops of methyl blue are needed to get a deep robin’s egg color. Even with the correct concentration and consistent aeration, many broods of eggs died without maternal care.

Due to their higher survival rates and the fact that they were collected first, HMC fry began development earlier in the year than LMC fry. Ages were, of course, accounted for in testing. All fry and adult fish were tested at approximately day 60 and 90, respectively. However, the timeframe of dates tested for HMC fish (December 3, 2011 - February 1, 2012) and LMC fish (January 12, 2012 - March 11, 2012) differed by approximately one month. If I were to re-design this thesis protocol, I would take this into account. Not only would I begin by collecting eggs, but I would also collect HMC and LMC broods in alternating sequence. For instance, after collecting one LMC brood, I would collect a HMC brood, and so on.

C.2 Time tested timeframes:

Since broods were tested near, if not on, day 60 and day 90, and experimental broods were close in age, multiple broods were often tested on the same day. Hence, brood test times often spilled into timeframes that I would have liked to avoid. Logistically, video camera battery life and storage capacity were also limiting factors in time efficiency, as several hours were spent waiting for camera battery to charge and downloading videos.
As a result, the earliest behavioral observation I recorded was at 11:30 h and the latest was at 20:30 h. However, a majority of tests were conducted between 14:00 and 19:00 h.

In order to account for variable ambient light levels during testing, additional lights were installed in the test room. Using this method, the focal fish in the test tank and remaining fish in individual beakers were exposed to equal amounts of light. In order to control for light sources at closer proximities, additional lights were used for every test, regardless of time of day.

**C.3 Lost video data:**

Even though a majority of the open-tank test data was lost, I had the foresight to record at least one behavior *in situ*. Additionally, footage of one HMC adult brood remained, along with footage from a LMC adult brood that was tested only 11 days proceeding. Hence, a sample of each condition’s population was analyzed fully. As a result of this learning experience I will endeavor to always use multiple backup hard drives and take advantage of collective backup servers.

**C.4 Blind testing:**

For note-keeping and logistical purposes, I did not have a colleague re-label the fish from different maternal conditions, nor did I have a colleague move the tanks. If I could redesign this thesis I would include this in the experimental setup so that I could conduct all anxiety-mediated behavioral observations blind.

Attempts to justify this were made by restraining open-tank test observations to just one response variable *in situ* (i.e. latency to emerge). All other variables were to be analyzed from scrambled and relabeled video recordings.
References


