The Effect of Maternal Separation on the Stress Response of *A. burtoni*

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> Any situation in which some individuals prevent others from engaging in the process of inquiry is one of violence.
>
> *Paulo Freire*
> *Pedagogy of the Oppressed*

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Preface

Science has a history as an oppressive institution. That being said, I think that science also has the ability to liberate individuals. We must strive to understand how the spaces we create impact one another and to interrogate the ways in which we judge people's ability to control their actions. I hope that at the least this thesis makes one think of how plastic we are to our day-to-day experiences.

List of Abbreviations

11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
ACTH	Adrenocorticotropin hormone
CRH	Corticotropin-releasing hormone
CPP	Conditioned place preference
C_q	Cycle quantification
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HLG	High licking & grooming
HPA	Hypothalamic-pituitary-adrenal
LLG	Low licking & grooming
PCR	Polymerase chain reaction
PVN	Pareventricular nucleus
qPCR	Quantitative polymerase chain reaction
SQ	Starting quantity

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Abstract

The ability of a developing organism to adjust to its early life environment is an important adaptation. That being said, when the adult environment does not match the early environment, these adjustments can be harmful. Exposure to early life stress is known to be correlated with a number of neuropathologies and developmental disorders. Early life stress is able to reprogram the hypothalamic-pituitary-adrenal axis of organisms, leading to an alteration in the stress response. In mammals, poor maternal care is associated with an increase in circulating corticosteroids and anxiety-like behavior in stressful situations. This change in responsiveness is also accompanied by a decrease in inhibitory feedback of the hypothalamic-pituitary-adrenal axis, as measured by decreased glucocorticoid receptors. The present study aimed to model these mammalian studies in the mouthbrooding cichlid species *A. burtoni*. Mouthbrooding females were placed into one of two conditions: maternal care or maternal separation. In the maternal care condition, fry were allowed to stay with their mothers for the first two weeks post-fertilization. In the maternal separation condition, fry were removed from their mothers' buccal cavities shortly after fertilization. Stress-related behavior was measured through boldness and aggression assays while stress response was measured through glucocorticoid receptor expression. Fry separated from their mothers spent more time in the bottom of the novel tank compared to unseparated fry, suggesting an increase in boldness. There was no significant difference between conditions in body mass, aggression, or glucocorticoid receptor expression. Further research is needed in order to assess whether maternal separation in *A. burtoni* is comparable to low maternal care in mammals.

Dedication

And in our hearts
How beautiful the flames that will
flare up in a ring

Chika Sigawa
"Mountain Range"

For Langston.

Chapter 1

Introduction

1.1 A Brief History of Nature *vs* Nurture

A defining feature of living organisms is that they are able to respond to stimuli in their environment. In other words, they behave. Each behavior requires a stimulus, or multiple stimuli, that triggers a chain reaction of internal responses, changing how an organism exists in its environment. In understanding why an animal responds to a stimulus in the way that it does, there are two places to start. One can look to the organism's genotype: was this behavior inherited genetically from its parents? Or one can look to the organism's upbringing: was this behavior learned in response to the environment? Traditionally, these two possibilities have been thought of as separate and exclusive, as in the phrase "nature *vs* nurture"; however, this notion of the two as separate influences has since changed.

The dichotomy of nature and nurture as we know it today has its unfortunate beginnings in the field of eugenics. The phrase was popularized by the father of eugenics, Francis Galton, in the late 19th century in an effort to understand if human "ability" was heritable. He defined nature as "all that a man brings with himself into the world" and nurture as "every influence from without that affects him after his birth" (Galton, 1874). While there was not yet a concept of DNA, both Darwin's theory of evolution and Mendel's inheritence experiments were in circulation. The

interest in nature *vs* nurture remained within developmental psychology until late in the 20th century when behavioral and developmental neurosciences were popularized.

In the early and mid 20th century, the fields of animal behavior and genetics were being revolutionized in ways that would ultimately contribute to the debate of nature and nurture (Krubitzer and Kahn, 2003). In the 1930's a pioneering behavioral scientist by the name of Nikolaas Tinbergen began studying behaviors holistically, as a product of individual experience and evolution. He was interested in creating a scientifically rigorous way by which to observe and comment on behavior. What emerged was the modern field of ethology and a set of four categories to study a behavior through: causation (mechanism), survival value (adaptation), ontogeny, and evolution (Tinbergen, 2005). Tinbergen's four questions were important in examining a single behavior as a product of an individual's experiences and that individual's lineage. That being said, there was still not that much known about molecular biology and its role in behavior.

Abstract concepts of DNA and RNA as a heritable molecule had been proposed by the early 20th century in response to heritability studies (Koltzoff, 1934; Hershey and Chase, 1952), but it wasn't until Francis Crick and James Watson published a study in 1953 on the structure of DNA (notably, the study relied heavily on prior work by Rosalind Franklin) that the field of modern genetics really began (Watson and Crick, 1953). Using information about base pairs and amino acids published by other labs at the time, Crick proposed the central dogma of genetics in 1958 (**?**). This crucial concept states that DNA is translated into RNA, which is then transcribed into amino acids that are linked together to form proteins.

The last big step in getting to our current concept of nature and nurture was the popularization of epigenetics. Epigenetics in short refers to the factors that change the ability of DNA to be transcribed, contributing to changes in gene expression. Much of modern behavioral sciences is aimed at understanding how the environment

influences an organism's epigenome.

Because we now understand gene expression is often altered by the environment, our notion of nature *vs* nurture becomes rather arbitrary. Behaviors can instead be thought of as an intertwining of nature *and* nurture (Sasaki and Kim, 2017; Meaney, 2006). Rather than understanding the ratio of environmental to genetic influence on a behavior, we can instead examine how certain genotypes make an organism more vulnerable to environmental influences or how the environment influences the ways in which the genome is utilized. This research provides a framework for which to examine any biological process, and it is through this lense that this thesis is written.

1.2 The Hypothalamic-Pituitary-Adrenal Axis

1.2.1 Activation of the HPA Axis

If you have made it this far in life, you have at some point felt *stressed*. Stress can be defined as the body's *response to* and *recovery from* a threat that disrupts homeostatis (van Bodegom et al., 2017). An important aspect of the stress response is the production and mobilization of energy. This is made possible through the hypothalamic-pituitary-adrenal (HPA) axis, which functions to produce glucocorticoids. As the name suggests, glucocorticoids play a role in the metabolism of glucose, the body's main source of energy.

The activation of the HPA axis begins with stimulation of the hypothalamus by other brain areas. In the presence of an immediate stressor, brain regions associated with maintaining homeostasis trigger the axis. Take for example the response to a painful stimulus. Pain is sensed by nociceptors in the peripheral nervous system and cause afferent signaling to norepinephrinergic neurons in the hind brain. These hindbrain neurons can in turn stimulate the hypothalamic neurons involved in the HPA axis. It is also possible to activate the HPA axis as an anticipatory response.

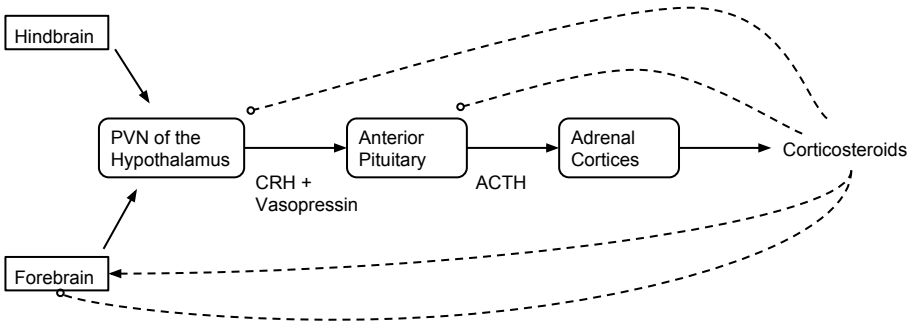

Figure 1.1: Signaling cascade of the HPA axis.
As a response to stress, forebrain or hindbrain projections to the hypothalamus can begin the HPA axis signalling cascade. The paraventricular nucleus (PVN) of the hypothalamus secretes corticotropin-releasing hormone (CRH) and vasopressin into the anterior pituitary. This causes the anterior pituitary to release adrenocorticotropin hormone (ACTH) into the bloodstream. ACTH reaches the adrenal cortices of the adrenal glands, leading to the production of steroid hormones including corticosteroids. While corticosteroids have many targets and regulatory effects, they can also have positive feedback effects (dashed arrowhead) and negative feedback effects (dashed open circle) for the HPA axis itself.

If an animal has been conditioned to associate a given smell with a predator, then in the presence of that smell alone the animal may trigger the HPA axis in anticipation of the danger. This requires polysynaptic signaling from limbic structures involved in learning and fear such as the hippocampus (homologous to telencephalic pallium in teleosts) and amygdala (homologous to the medial pallium in teleots) (Salas et al., 2006). The hippocampus excites the axis through glutamatergic interneurons, whereas it is hypothesized that much of the excitatory amygdalar signaling works through disinhibition In both the immediate and anticipated cases, the activation of neurons within the hypothalamus leads to a stereotyped cascade of signaling.

The hypothalamus is a region of the midbrain known for its role in maintaining allostasis through its involvement in stress, appetite, circadian rhythms, and sexual behavior. In response to a stressor, the paraventricular nucleus (PVN) of the hypothalamus secretes corticotropin-releasing hormone (CRH) and vasopressin, which

bind to receptors in the pituitary gland. The pituitary gland is directly ventral to the hypothalamus and is a main regulator of hormone release. The binding of CRH to CRF_1 receptors in the anterior pituitary leads to the secretion of adrenocorticotropin hormone (ACTH). This excitatory interaction can be potentiated by vasopressin, though vasopressin alone is not enough to produce an effect. ACTH enters the blood stream and travels to the adrenal cortices, which are the dorsal regions of the adrenal glands. In teleosts, the interrenal cells are homologous to the dorsal region as there is not a clear division between their kidneys and their adrenal glands (Pijanowski et al., 2015). ACTH binds to melanocortin 2 receptors, which increases the synthesis of cholesterol. Cholesterol is then transported to the outer mitochondrial matrix where the steroidogenic pathway begins. A major end product of this pathway and thus of the HPA axis is corticosteroids.

1.2.2 Glucocorticoid Receptors

Corticosteroids are steroid hormones that can bind to glucocorticoid receptors (GRs) and mineralocorticoid receptors. The term glucocorticoid refers to corticosteroids that are able to bind to GRs. After being released by the adrenal glands, glucocorticoids travel through the blood stream, pass through the blood-brain-barrier, and freely diffuse into the cytoplasm of neurons. Unbound GRs reside in the cytoplasm as part of a larger protein complex. When a GR is bound to by a glucocortiocoid it goes through a conformational change and sheds the protein complex. It is then transported to the nucleus where it dimerizes with another GR. The homodimer can then interact with other proteins, ultimately leading to the binding of the complex to a glucocorticoid response element (GRE) on the genome. These GREs are often found in the promotor region of their target genes and recruit transcription factors to suppress or enhance transcription of the target gene (Busada and Cidlowski, 2017; Herman et al., 2005). Interestingly, GRs can also localize to the mitochondria, where

they can alter transcriptional elements of mitochondrial DNA. Due to mitochondria's role in steroid and energy production (Lapp et al., 2019), this finding suggests an important long-term regulatory role of glucocorticoids. Ligand binding to GRs can also have nongenomic consequences such as kinase activation, though these pathways are not well understood (Samarasinghe et al., 2011).

In mammals, there are eight known transcriptional isoforms of the GR gene (Saif et al., 2015) and thirteen different post-translational modification sites of the GR protein (Oakley and Cidlowski, 2013). These differences in protein structure alter the cellular function of the GR subtypes (Lu et al., 2007). A genome duplication event happened in the evolution of teleosts, causing them to have two GR paralogues: GR1 and GR2 (**?**). The genetic sequences are highly similar to each other as well as to the GR genes of other species (Greenwood et al., 2003). Both GR1 and GR2 are expressed in corticosteroid responsive brain regions, suggesting that they both maintain signaling functionality.

1.2.3 Regulation of the HPA Axis

Activation of the HPA axis leads to situationally different levels and duration of glucocorticoid release. Inflammatory stressors often lead to prolonged stress responses, as the injury requires sustained energy to repair. Psychological stressors, in contrast, tend to lead to acute responses because there is no tissue repair or immune response required (Herman et al., 2016). Because the stress response is energy intensive, it is important that an organism responds appropriately to threatening situations.

The HPA axis includes negative feedback loops that enact tight regulation. GR's play an inhibitory role in the activation of the HPA axis. They are abundantly expressed within the PVN. Upon activation, they cause endocannabinoid synthesis and release that are able to inhibit glucocorticoid receptors that target CRH neurons. Long term exposure to corticosteroids has also been shown to reduce pituitary

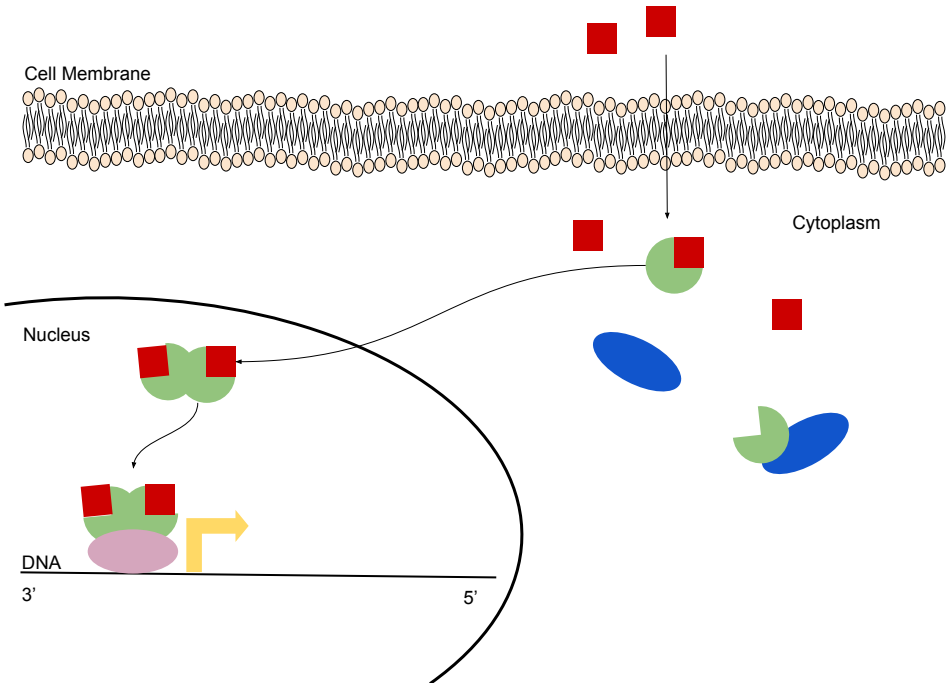

Figure 1.2: Glucocorticoid receptor mechanism of action.
Unbound glucocorticoid receptors (green) reside in the cytoplasm of cells as a part of a larger protein complex (blue). Corticosteroids (red) can freely diffuse through the cell membrane and bind to glucocorticoid receptors. Glucocorticoid receptors then undergo a conformational change, shedding the protein complex, and move into the nucleus. There they form homodimers and bind to glucocorticoid response elements (pink) on the genome. These elements are downstream of promotor regions (yellow) and can alter the transcription of nearby genes.

ACTH release (Keller-Wood, 2015). In addition to regulation within the axis, GRs in the hippocampus and prefrontal cortex are able to inhibit HPA axis activity via GABAergic interneurons.

The HPA axis has the ability to adjust to chronic stress. There is an important distinction to be made by the body between long-term stressors that continuously pose a threat to an organism and long-term stressors that don't actually pose a threat to an organism. Take for example two deer that live in environments coinhabited by humans. The first lives in an area that is frequented by hunters. This deer has to induce a full stress response every time it encounters a human, else the animal will quickly be killed. Now take for example a deer that lives in a zoo. It would surely be a waste of this deer's energy if it were to enter a stressed state every time it encountered a human. While it is adaptive for the former deer to develop a heightened responsiveness to humans, it is adaptive for the latter deer to become desensitized to humans.

A long-term change to the physiology of the HPA axis can occur via epigenetic modification. In other words, the genes encoding proteins necessary for the HPA axis can be made more or less likely to be transcribed as a result of chemical changes to structural elements of the DNA. Epigenetic changes are heritable from parent to daughter cell even though they do not alter the actual DNA sequence, making them an important aspect of early development when cells are rapidly proliferating.

1.3 Stress and Development

1.3.1 Protection Against Early Life Stress

The fact that chronic stress is often unhealthy is quite intuitive. When an organism is forced to expend energy on immediate survival, it must forego less pressing, but very important processes like eating, sleeping, reproducing, and learning. Teleosts and

mammals share a highly-conserved protection mechanism against early-life stress. In both cases, mothers secrete 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) into the prenatal environment. This hormone rapidly deactivates corticosteroids, effectively inhibiting the stress response of embryos (van Bodegom et al., 2017; Faught et al., 2016). As newborns, there is a stress hyporesponsiveness period characterized by a decrease in circulating ACTH and corticosteroids, as well as an overall decrease in responsiveness to stressors (van Bodegom et al., 2017; Barry et al., 1995).

Importantly, both of these defenses can be altered by a highly stressful environment. Repeated maternal exposure to stress decreases 11β-HSD2, increasing prenatal corticosteroid exposure. Additionally, maternal separation is associated with a shortened stress hyporesponsiveness period in mammals (no similar study has been done in fish). These findings suggest that the stress response is plastic to early life experience. The match/mismatch hypothesis argues that this plasticity to the early life environment will be adaptive for the organism if the adult environment matches, but will be maladaptive for the organism if the adult environment is different (Gluckman et al., 2007).

1.3.2 The Effects of Prenatal Stress on Development

Glucocorticoid receptors play an important role in fetal development. While insufficient levels of glucocorticoids are sometimes fatal, causing undeveloped organs, excess circulating glucocorticoids can cause potentially maladaptive developmental reprogramming (Busada and Cidlowski, 2017). The offspring of pregnant mice treated with synthetic glucocorticoids have delayed maturation of neurons and glia as well as delayed vascularization of the brain (Gravanis and Mellon, 2011). Further, prenatal stress exposure is correlated with decreased dendritic spine density in the cingulate gyrus and orbitofrontal cortex (Murmu et al., 2006). These data suggest that prenatal stress alters the physiology and circuitry of the developing brain.

The ability of offspring to learn and form memories is altered by prenatal stress exposure. Compared to controls, offspring of stressed mothers have decreased fear learning in a passive avoidance behavioral paradigm (Sofiabadi et al., 2018). Older rats have impaired spatial memory in the Y-maze and working memory in the radial arm maze when they were exposed to prenatal stress (Vallée et al., 1999). Rats also display a corresponding decrease in CAMKII and CREB mRNA expression in the hippocampus under these conditions (Sun et al., 2017).

Animals exposed to prenatal stress are also more susceptible to addictive behavior, as measured by conditioned place preference (CPP). In this paradigm, animals are conditioned to associate a drug with a given environment and then their preference for that environment over a neutral environment is measured in the absense of the drug. Rats with mothers exposed to stress have increased nicotine CPP as well as increased dopamine D_2 receptor gene expression in nucleus accumbens, a brain region associated with reward and thus addiction (Said et al., 2015). Prenatal stress has also been shown to increase CPP in response to benzodiazepines (Lakehayli et al., 2015), cocaine (Pastor et al., 2018), and morphine (Vey et al., 2016) just to name a few. This implies that changes in stress circuitry impacts other complex neural systems as well. In addition to addictive behavior, prenatal stress is a predictor of psychiatric disease in adults. Rodents exposed to prenatal stress show increased anxiety, depressive, and schizophrenic-like behavior compared to offspring of non-stressed dams (Weinstock, 2017). Taken together, these findings have major implications for understanding underlying mechanisms in neuropathologies.

1.3.3 Maternal Care and Stress in Rats

Stress in the postnatal environment can also be influential to an organism's development. The neuroscientist Michael Meaney has done years of groundbreaking work on how maternal care alters the stress response in rats. Rat mothers exhibit con-

sistent differences in the time spent licking and grooming their young during their first week of life (Meaney et al., 1996). This difference takes place during a critical period of the rats' neural development. As a result, pups reared by high licking and grooming (HLG) mothers and low licking and grooming (LLG) mothers have distinct phenotypes and gene expression profiles (Weaver et al., 2004). Meaney cross-fostered pups from HLG and LLG mothers. As a result, pups born to LLG dams, but reared by HLG dams had a similar phenotype to those born to and reared by HLG dams (Francis et al., 1999). This indicates that the mother's behavior is largely responsible for differences in the pups phenotypes and gene expression.

Meaney's lab examined how circulating stress hormones differed in pups reared by HLG and LLG mothers (Liu et al., 2000). HLG pups had reduced circulating levels of ACTH and corticosterone in response to restraint stress. Additionally, HLG pups appeared to have enhanced regulatory feedback in stressful situations, as they suppressed ACTH to a greater extent after being pre-treated with corticosterone (the murine equivalent of cortisol). HLG pups also developed higher GR expression in the hippocampus as adults, a brain region associated with HPA-axis inhibition. These molecular differences are also associated with distinct behavioral phenotypes between the two groups (Caldji et al., 1998). Rats reared by HLG dams exhibited more exploratory behavior, as measured by an open field paradigm, compared to those reared by LLG dams. Additionally, LLG pups exhibited a longer latency to start eating when placed in a novel environment compared to HLG pups. These findings indicated that maternal care can influence offspring's responses to stress as adults.

In 2004, Meaney's lab published a paper on the epigenetics of the above discoveries (Weaver et al., 2004). The lab found that the epigenetic state of the GR promoter gene was altered by maternal licking and grooming. This difference in methylation state was contingent on the rearing, not the biological, mother. GR receptor gene methylation was decreased and acetylation increased in HLG rats, consistent with earlier

studies. Taken together, the work of Meaney's lab demonstrates that rats reared by LLG dams have a hypersensitive stress response, characterized by increased circulating stress hormones, decreased hippocampal GR receptors, and decreased exploratory behavior.

Table 1.1: Summary of Meaney's work comparing high licking and grooming and low licking and grooming pup phenotypes.

Phenotype	**Pups Reared by HGL Dams**	**Pups Reared by LLG Dams**
Circulating stress hormones	Low	High
Habituation to Corticosterone	High	Low
Hippocampal GR expression	High	Low
Anxiety-like behavior	Low	High
GR gene methylation	Low	High
GR gene acetylation	High	Low

1.3.4 Early Social Environment and Stress in Cichlids

Barbara Taborsky has examined how early social environment influences fish stress response. Most of Taborsky's work is with *Neolamprologus pulcher*, a highly social cichlid species that lives in family units and collectively raises offspring. Immature fish help to keep eggs clean and well-oxygenated while adults defend the eggs against predators and conspecifics (Arnold and Taborsky, 2010).

Much of Taborsky's work has focused on how early life social experience affects social behavior and stress response in adults. Fish were divided into three groups: those raised with adults and immature helpers, those raised with just helpers, and those raised with neither helpers nor adults. In the following studies, fry raised in the presence of just helpers and fry raised in the presence of helpers and adults had the same trends. Taborsky found that *N. pulcher* fry raised in the absence of adults and helpers had decreased social competency, showing energetically costly levels of aggression in territory disputes (Arnold and Taborsky, 2010). Fish raised with adults

had decreased whole-brain GR1 expression and CRH compared to those raised in the presence of adults or helpers (Taborsky et al., 2012).

This was complemented by a similar study in *Astatotilapia burtoni*. Fry raised in social groups (*i.e.*, naturalistic upbringing) had higher whole-brain GR mRNA expression compared to fry raised in pairs (Solomon-Lane and Hofmann, 2018). A follow-up study in *N. pulcher* examining specific regions of the brain showed that fry raised in the presence of adults and helpers had increased in GR1 mRNA expression in the telencephalon (Nyman et al., 2017). This difference in results suggests that changes in GR1 expression are brain region specific. Antagonizing GRs in fry reared without adults and helpers resulted in more appropriate levels of aggression, indicating that the social effects of different rearing environments are mediated in part by GR (Nyman et al., 2017). Lastly, *N. pulcher* fry raised in the absence of adults and helpers displayed more neophobia in behavioral tests, which is indicative of higher stress in new environments (Bannier et al., 2017).

Table 1.2: Summary of Taborsky's work comparing *N. pulcher* fry raised in the presence and absence of adults and adolescents.

Phenotype	**Fry Raised with Older Fish**	**Fry Raised without Older Fish**
Social competency	High	Low
Circulating CRH	Low	High
Whole brain GR1 expression	Low	High
Telencephalic GR1 expression	High	Low
Anxiety-like behavior	Low	High

1.3.5 *Astatotilapia burtoni* as a Model Organism

A. burtoni, also referred to as *Haplochromis burtoni*, is a highly social cichlid species known for its extremely plastic phenotypes related to dominance. There exists two distinct dominance phenotypes in males: those with territory that are reproductively active and those without territory that are reproductively suppressed (Fernald, 1977).

Males with territory are dominant and have bright blue or yellow body coloration with a thick black stripe that passes over their eyes (Border et al., 2019). They have vertical black stripes along their body, a black spot on their gill cover, and a red splotch just caudal of that. Non-territorial males are subordinate and resemble females, with camouflage coloration. The difference in dominance is also correlated with a difference in stress and hormonal regulation (Renn et al., 2008). Dominant males have increased testosterone and gonad size, while subordinate males have increased levels of cortisol and experience faster growth. Males are able two switch between these phenotypes throughout their lives in response to changes in their social interactions.

Female *A. burtoni* also exhibit social dominance, though unlike the males, both dominant and subordinate females are reproductively active. Females take part in a brood care strategy known as mouthbrooding. The female lays eggs and immediately picks them up, storing them in her buccal cavity. Shortly after this, the males fertilize the eggs. The mother keeps the offspring in her mouth for about two weeks as the eggs develop into fry. After the initial release, fry remain close to their mother and return to their mother's mouth in the presence of danger. Brooding mothers voluntarily starve themselves; however, in stressful situations they will eat their offspring. Mouthbrooding helps to protect the developing fry from predators and conspecifics, potentially reducing their exposure to stressful experiences.

1.3.6 Current Investigation

The work of Barbara Taborsky has demonstrated that social fish species are plastic to their early life experience, showing changes in behavior and stress hormone expression as a result of social environment. Taborsky's work, however, is focused on the effect that social environment has on social behavior. There is little known about how maternal separation affects mouthbrooding fish. It is unknown to what extent mouthbrooding influences the neurophysiological development of fry. By comparing

the effects of this offspring care strategy to what is known in mammals, we can better understand the evolution of neuronal plasticity in response to early life experience, especially as it relates to parental care.

A recent thesis by Destine Krenik examined the role that maternal separation in *A. burtoni* had on behavior and neural GR expression (Krenik, 2018). Offspring were either reared with their mothers or were separated as eggs and raised without any adults. The thesis examined anxiety-like behavior and aggression in both conditions, but did not find any significant treatment-based differences. The brains of the fish were extracted and divided into forebrain, midbrain, and hindbrain for GR mRNA analysis. Krenik found significantly lower GR mRNA expression in the hindbrain of fry separated from mothers. The following research is conceptually based on this thesis with three major methodological changes. The first is that the paradigm used to measure anxiety-like behavior in this thesis takes places in a more naturalistic tank to encourage some exploratory behavior. The second change is that this thesis will examine whole-brain GR mRNA expression rather than section the brain into three regions. Lastly, the current research uses three different GR primers, as opposed to Krenik's use of a single primer, in order to quantify more splice variants. The rationale for these changes is to collect different data for the same overarching question in order to build a better understanding as to how maternal separation alters GRs.

I hypothesized that fish separated from their mothers would exhibit more anxiety like behavior and more aggression compared to those brooded by their mothers. This heightened stress response would be accompanied by a decrease in GR1a, GR1b, and GR2 receptor expression.

Chapter 2

Methods

2.1 The Fish

Table 2.1: Brood information

Brood ID	Condition	Number of Fry
0-0	Unseparated	1
Z-1-3	Separated	2
Z-1-7	Unseparated	3
Z-1-9	Unseparated	3
Z-2-1	Unseparated	2
Z-2-4	Separated	3
Z-2-9	Separated	1
Z-3-1	Separated	3
Total	**Unseparated = 4 broods** **Separated = 4 broods**	**Unseparated = 9 fry** **Separated = 9 fry**

The parental generation of the focal juveniles originated from a wild-caught stock of *A. burtoni* collected in 2005 from Lake Tanganyika in East Africa (Renn et al., 2009). Social groups containing males and females of the same generation were kept in 30 gallon aquaria at a temperature of 28°C and a pH of 8.5. Each aquarium's bottom was covered in gravel, and terra cotta pot pieces were placed in the tank to act as shelters and territory markers. Once a female fish began mouthbrooding, she was removed from the aquarium and randomly placed into one of two experimental

conditions.

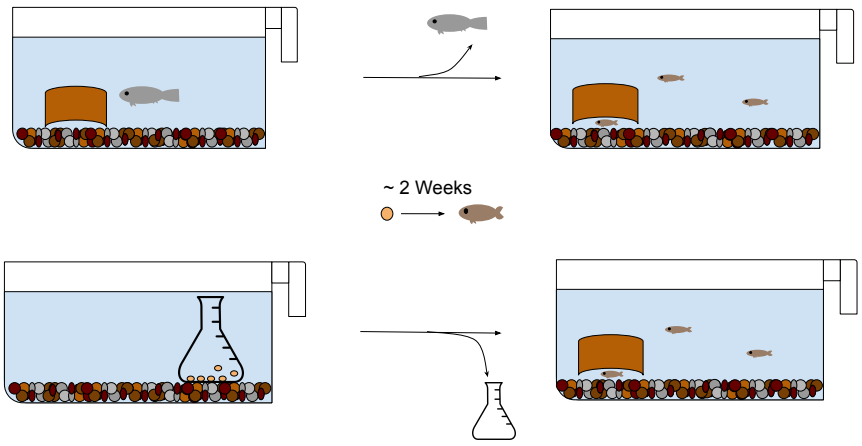

Figure 2.1: Experimental conditions.
Mouthbrooding fish were selected to be in either of two experimental groups. In the first condition (top) mothers were transferred to individual tanks containing gravel and a terra cotta piece. After about two weeks, when fry were able to leave their mothers' mouths, the mothers were moved back into their home aquaria. In the second condition (bottom), eggs were removed from the buccal cavity and placed in a flask within an individual tank. After about two weeks the fry were able to freely swim and the flask was removed.

In the unseparated condition, mothers were removed from their home tank, weighed, and measured. They were then placed individually in small tanks containing gravel and a piece of terra cotta pot. Mothers continued to brood their young until the fry were old enough to regularly leave their mother's mouth (approximately 2 weeks post-fertilization), at which point the mother was removed from the tank to prevent her from eating the fry.

In the separated condition, mothers were weighed and measured and then the eggs were removed from their mouths by gently pulling down the bottom jaw. The eggs were then placed in a 250 mL flask within an new tank containing graveland circulating water. Once the eggs developed into freely moving fry (approximately 2 weeks post-fertilization), they were able to leave the flask and swim around the tank. At this point the flask was removed from the tank and a piece of terra cotta was added.

Figure 2.2: Timeline of experiment.
Two to three days after fertilization of eggs occurred, the broods were placed into one of two conditions: maternal separation or no maternal separation. After approximately two weeks, the fry were old enough to freely swim around their tanks. At this point, depending on the condition, either the mother or the flask was removed from the tank. The fry were allowed to age for about 130 days, at which point they were exposed to the boldness and aggression assays. Immediately after the behavioral testing, the fish were decapitated and their brains were extracted. RNA extraction and qPCR analysis took place after a variable amount of time.

2.2 Behavioral Tests

Behavioral testing took place 130-131 days after the brooding mothers were placed into experimental conditions and consisted of two assays: boldness and social. Both assays were performed on the same day. Directly after each fish took part in the boldness assay, the brood was observed in the social assay. Prior to the start of behavioral testing, the focal brood was moved in its home tank to the testing area. The fish were allowed to acclimate to the change in lighting for 10 minutes. Fish were moved into the appropriate experimental tank (see below) for testing. A video camera was placed approximately two feet from the front side and no humans were present in the room during behavioral recordings. The recorded behavior was scored blind to the experimental condition using the video recordings.

Boldness Assay

Boldness, or willingness to explore novel and open environments, is often used as a

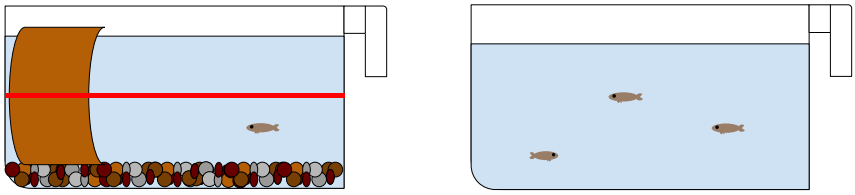

Figure 2.3: Behavioral paradigms.
Two paradigms were used to assess behavioral phenotypes of the fish. In the boldness assay (left) individual fish were placed in a novel tank containing gravel and a terra cotta shelter. After 10 minutes of habituation, time spent in the top half of the tank, in the bottom half of the tank, and behind the shelter was recorded for an additional 10 minutes. In the aggression assay (right) the whole brood was placed into a single, novel tank. As with the other assay, the fish were allowed to adjust to the change in environment for 10 minutes. The number of chases, charges, and bites between fish were recorded for 10 minutes.

measure of stress (Bannier et al., 2017; Francis et al., 1999). Animals that are stressed tend to freeze in place, seek cover, and avoid open spaces. Focal fish were individually placed in a novel experimental tank containing gravel and a terra cotta shelter. Three sides of the tank were covered in white paper to minimize external stimuli. A piece of red tape was horizontally placed on the outside of the tank, dividing it into top and bottom. The fish were allowed to acclimate for 10 minutes in the experimental tank before their behavior was scored for another 10 minutes. Time spent in three distinct areas of the tank were recorded: top half of tank, bottom half of the tank, under the shelter. The fish was considered to be in a given region once its eyes crossed the border.

Aggression Assay

Because *A. burtoni* are highly social fish, it is relevant to assess how maternal separation affects their social behavior. Aggression is known to be tightly correlated with stress, thus measuring aggression within broods is important in analyzing their overall stress response (Gammie and Stevenson, 2006; Averli et al., 2004; Honess and

Marin, 2006; Takahashi et al., 2018). Individual broods were transferred into a novel tank containing only water. Three sides of the tank were covered in white paper to minimize external stimuli. The fish were allowed to acclimate to the new environment for 10 minutes before scoring began. The number of charges, bites, and chases between fish that occurred in 10 minutes was counted by hand. The total sum of aggressive behaviors was then divided by the number of fish in the brood to create a score. Broods of only one fish in them were excluded from this paradigm.

Table 2.2: Aggression Ethogram

Behavior	**Description**
Charge	Fry A increases swim speed in the direction of Fry B. In response, Fry B moves to avoid Fry A.
Chase	Fry A swims towards Fry B. As Fry B swims away from Fry A, Fry A follows the path of Fry B.
Bite	The rostral end of Fry A makes contact with Fry B. Fry B reacts by swimming away from Fry A.

2.3 Gene Expression Assay

Directly following behavioral testing, fish were measured for length and weight and quickly decapitated. The brains were then extracted and placed into 1 mL of RNALater and stored at 4 °C until needed for RNA isolation. RNA from each individual's whole brains was extracted using a Maxwell 16 LEV simplyRNA Tissue kit (Promega AS1270). RNA quality and concentration were confirmed using gel electrophoresis and nanodrop. For each sample, 100 ng of isolated RNA was reverse transcribed into cDNA with an Invitrogen Reverse Transcription kit (Thermo Fisher 18080093).

Previously validated primers for GR1a, GR1b, and GR2 were used for qPCR (Solomon-Lane and Hofmann, 2018). The reference gene rpl32, which is a ribosomal protein coding gene, was used as a reference for GR expression. Each reaction contained cDNA (1 μL), 2x Immuno Mix (10 μL), forward and reverse primers (1

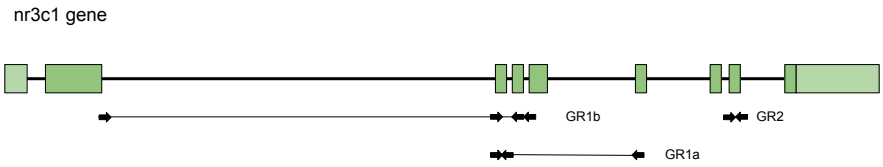

Figure 2.4: Primer design.
Primers sequences were taken from a study by Taborsky *et. al.* The locations of the primers were then found by comparing the sequences to *A. burtoni* transcriptional sequences using the NCBI BLAST database. The GR2 primer is designed to target the homologous duplicated GR receptor (nr3c1) gene. Primers that span introns are denoted by two arrows in the same direction.

μL of 100 nM each), 50x SYBR Green (0.5 μL), and nuclease-free water (6.5 μL). The program used for the qPCR reaction included a hot start (95°C, 5:00 min), 40 cycles that each ended with a fluorescent plate read (94°C, 0:45 min; 60.9°C, 1:00 min; 72°C, 0:30 min), 72°C for 2:00 min, and a melt curve analysis from 65°C to 95°C that measured fluorescence at 0.5°C intervals every five seconds. qPCR was run using a BioRad CFX Connect Real-Time PCR Detection System and the data were analyzed with

The starting quantity (SQ) for each sample was calculated by adjusting the cycle quantification (C_q) value by the efficiency of the primer in order to correct for starting concentration of cDNA. The mean SQ for each sample (samples were run in duplicate) was then divided by the mean SQ of rpl32.

Table 2.3: Genes and corresponding qPCR primer information

Target	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Melting Temperature	Efficiency
gr2	TGC CTC TGT CAC TGC CAC CGT AG	AGT CGT CTG CGT CTG AAG TAA CTG	60.9 °C	100.7%
gr1a	TCA TAA GAT CTG TTT GGT GTG CTC	GTA GTT GTG CTG GCC TTC AAC	60.9 °C	106.9%
gr1b	TGT TGG CTT CTC CGG TTC ATC AC	GTT GTG CTG GCC ATC TGT GTT T	60.9 °C	94.4%
rfl32	TGC TGA TGC CCA ACA TCG GTT	TCT TGG AGG AGA CAT TGT GGG	60.9 °C	99.2 %

Troubleshooting

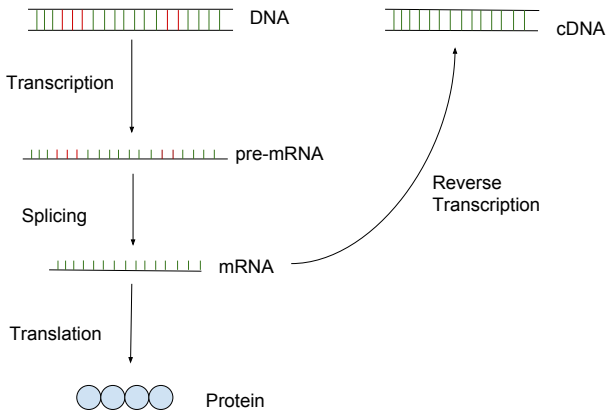

Figure 2.5: The central dogma of molecular biology including reverse transcription.
The central dogma of molecular biology explains how DNA informs protein structure. DNA is transcribed into pre-mRNA. The introns (red), or non-coding regions, of the pre-mRNA are then spliced out, leaving only the exons (green). The spliced mRNA is then translated into a sequence of amino acids that together form a protein. Reverse transcription is the process of making mRNA into a double stranded nucleic acid known as cDNA or rtDNA. Unlike DNA, cDNA does not contain the sequences of introns.

Prior to working with experimental fish, an age-matched brood was used to troubleshoot behavioral testing. Following that, the brood was euthanized using MS-222 to practice brain extractions. Two of the extracted brains were used for gene expression troubleshooting. RNA was extracted from the brains and reverse transcribed into cDNA. All of the primer sets were tested using PCR on a gradient of melting temperature using this cDNA. The most effective melting temperature was then selected for qPCR. The efficiency of individual primers at the selected melting temperature was calculated with qPCR using a two-fold dilution series ranging from 2^0 - 2^{-5} of the cDNA.

Chapter 3

Results

3.1 Size

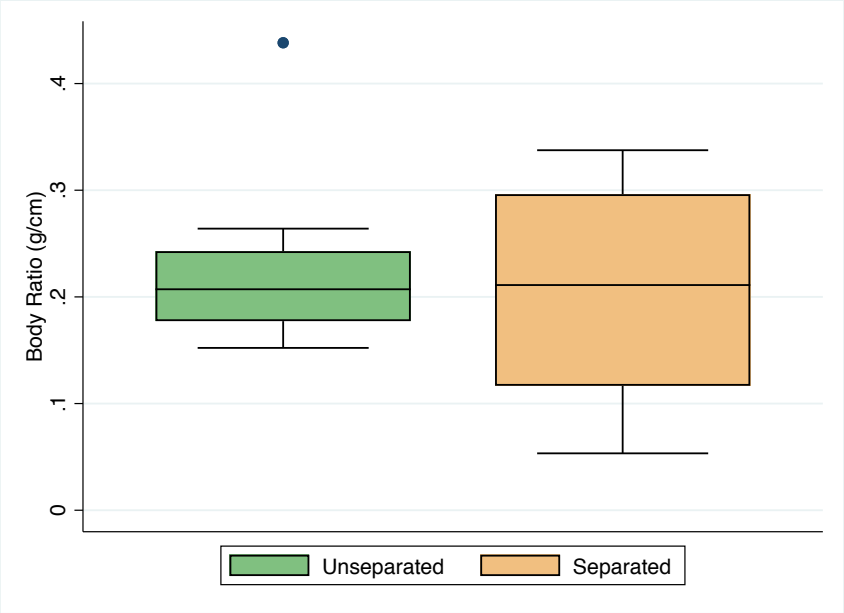

Figure 3.1: Comparison of fry body ratio.
The body ratio of each fry was calculated as the body weight in grams divided by the length in centimeters. There was no statistically significant difference between the size of fry kept with their mothers ($M = 0.23$, $SD = 0.09$) and fry separated from their mothers ($M = 0.20$, $SD = 0.11$), $t = 0.70$, $p = 0.25$.

Because glucocorticoids alter development, each fry's body composition was as-

sessed. Prior to euthanasia each fry's weight and length, from the nose to the beginning of the caudal fin, were measured. Body ratio was calculated to be the weight divided by body length. The conditions were compared using a student's t-test for independent means. There was no significant difference between fry kept with their mothers (M = 0.23 g/cm, SD = 0.09) and fry separated from their mothers (M = 0.20 g/cm, SD = 0.11), $p = 0.25$. There was also no significant difference between the length alone (M separated = 2.31 cm, M unseparated = 2.49 cm) $p = 0.51$ and the weight alone(M separated = 0.52 g, M unseparated = 0.60 g) $p = 0.65$. Broods in which there were only one fish were significantly greater in body ratio ($p < 0.05$), length ($p < 0.05$), and weight ($p < 0.01$) compared to broods with both two or three fry.

3.2 Boldness

The boldness assay is meant to assess the fry's anxiety-like behavior in a novel environment. The experimental tank was divided into three parts: the shelter, where fry could hide behind; the bottom of the tank, where fry were exposed, but could blend in with the gravel; and the top of the tank, where fry were in completely open water. The data were non-normally distributed, so a Wilcoxon rank test was used to analyze them. Fry that were separated from their mothers spent a significantly greater percentage of their time in the the bottom of the tank (M = 43%) compared to those unseparated from their mothers (M = 20%), p < 0.05. There was no significant difference between conditions in the time spent at the top (M separated = 10%, M unseparated = 5%, $p = 0.22$) and behind the shelter (M separated = 45%, M unseparated = 72%, $p = 0.08$)

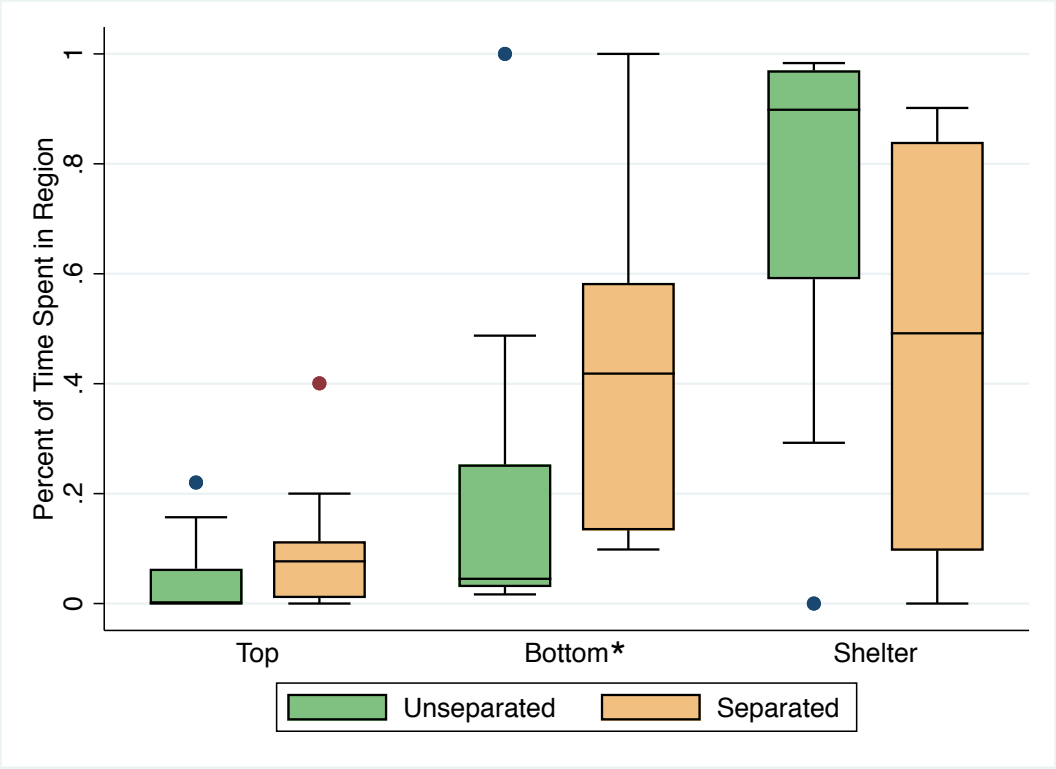

Figure 3.2: Effect of maternal separation on boldness.
Fry were placed individually into a novel tank divided into three parts: top, bottom, and shelter. The percent of time spent in each region over the course of 10 minutes was recorded. Fry separated from their mothers spent significantly more time in the bottom of the tank compared to those that were unseparated ($p < 0.05$). There was no significant difference between groups for time spent in shelter ($p = 0.08$) and in top ($p = 0.22$)

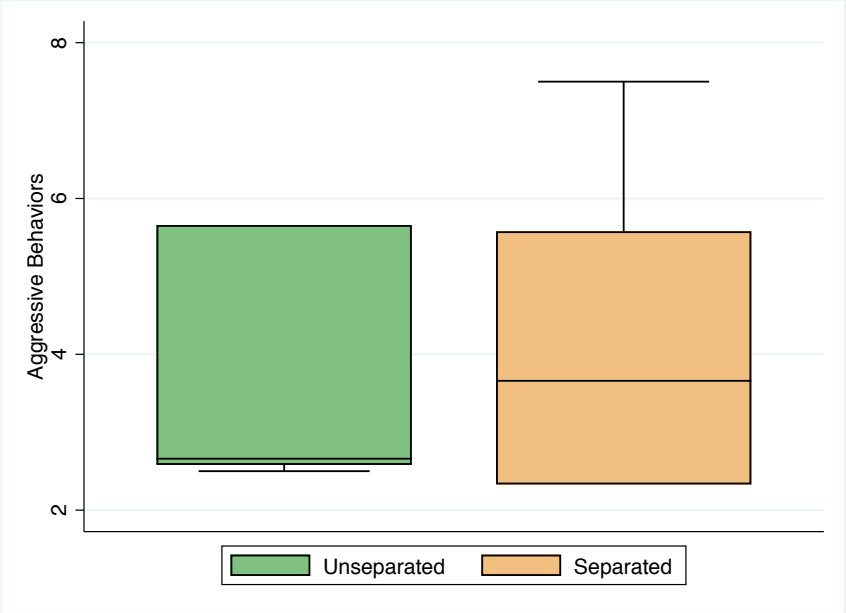

Figure 3.3: Comparison of tank aggression between groups.
Fry aggression was measured as the amount of charges, chases, and bites that occurred within 10 minutes, divided by the number of fry in the tank. Broods in which there was only one fry in the tank were not tested. As measured by a Wilcoxon rank-sum test, there was no difference between fry separated from their mothers (rank sum = 67) and fry kept with their mothers (rank sum = 69), $z = 0.11$, $p = 0.92$.

3.3 Aggression

To measure brood aggression, the total number of charges, chases, and bites were summed. This sum was then divided by the number of fry in the brood to calculate an average aggression score that accounted for brood size. The resulting data were not normally distributed, so to analyze the aggression, a Wilcox Ranked Sum test was used. There was no significant difference in the number of attacks per fish between the two groups (M separated = 4.12, M unseparated = 3.75 , $p = 0.92$).

3.4 Glucocorticoid Receptor Expression

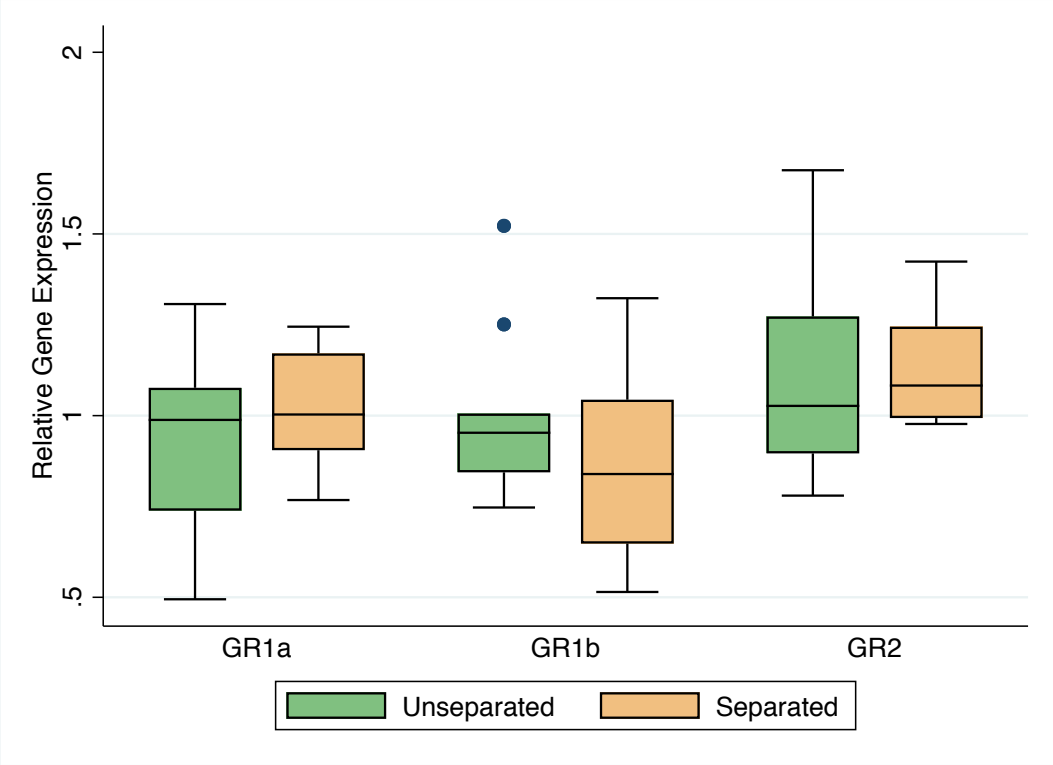

Figure 3.4: GR expression profile for experimental conditions.
Relative expression for GR1a, GR1b, and GR2 were calculated by adjusting the Cq by primer efficiency and then by dividing this starting quantity by starting quantity of the control gene rpl32. There was no significant difference in the expression levels between groups (GR1a $p = 0.38$, GR1b $p = 0.31$, GR2 $p = 0.63$).

GR1a, GR1b, and GR2 expression between conditions were analyzed individually using an independent means t-test. One fry (brood Z-2-9) was excluded from expression analysis due to an absense of rpl32 expression data and outlying data points for the focal genes.

There was no statistically significant gene expression between treatment groups for all three genes of interest (GR1a M separated = 1.02, M unseparated = 0.92, p = 0.38; GR1b M separated = 0.86, M unseparated = 1.00, $p = 0.31$; GR2 M separated = 1.14, M unseparated = 1.12, $p = 0.63$). There was a statistically significant correlation between GR1a and GR2 receptor expression and between GR1b and GR2 receptor expression ($p < 0.05$).

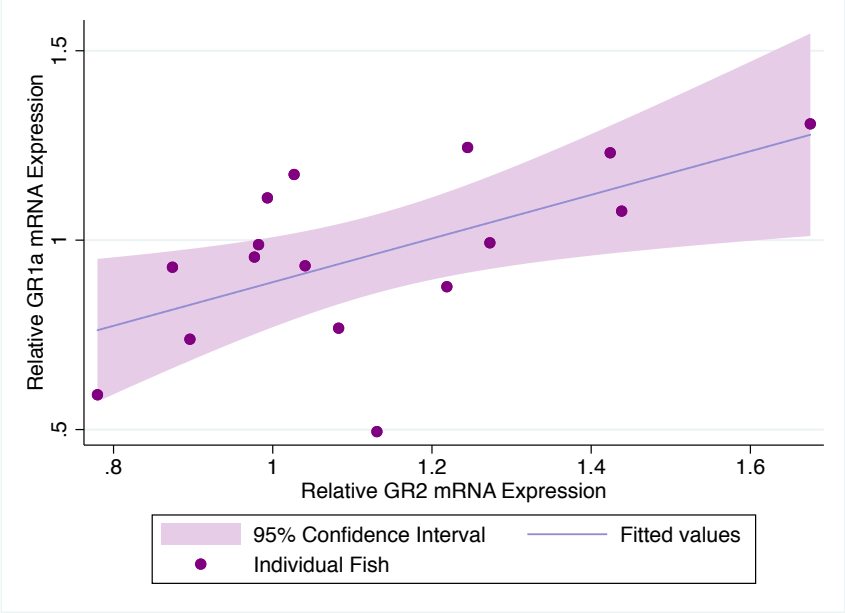

Figure 3.5: Relationship between GR1a and GR2 expression.
GR1a expression was positively correlated with GR2 expression, $p < 0.05$.

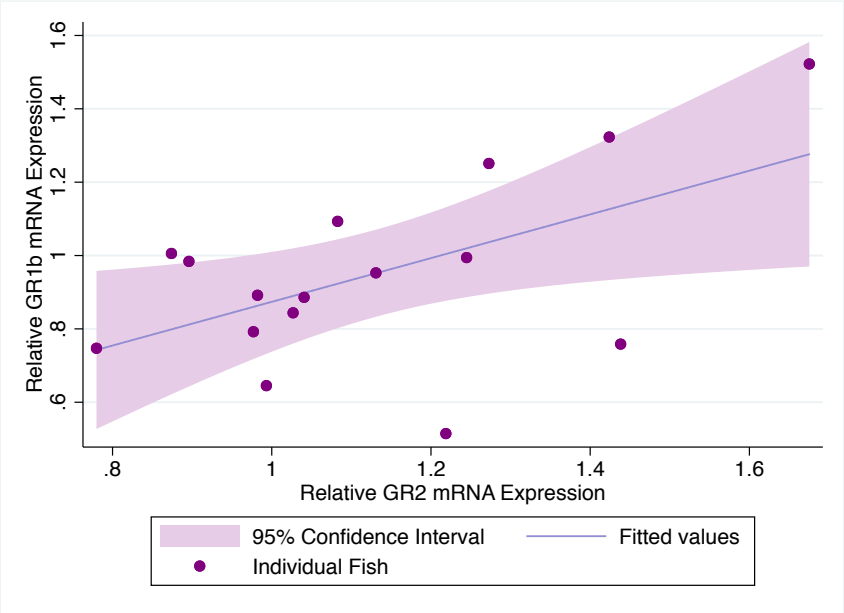

Figure 3.6: Relationship between GR1b and GR2 expression
GR1b expression was positively correlated with GR2 expression, $p < 0.05$

Chapter 4

Discussion

The results of the present study indicate that maternal separation in *A. burtoni* does not have a strong enough effect on the fry's stress response to be detected by the behavioral and GR expression assays used. The finding that fish separated from their mothers spent more time in the bottom of the novel tank is difficult to interpret given that the time spent in the top and behind the shelter were not statistically different. While the top of the tank is completely open and the shelter is unexposed, the bottom of the tank is both exposed to the uncovered side of the tank and offers camouflage amongst the gravel. Because there is a trend towards separated fry spending more time in the top and less time behind the shelter, as compared to unseparated fry, the increased time spent at the bottom of the tank can be interpreted as increased boldness. This is counter to the hypothesis that fry separated from their mothers would exhibit more anxiety-like behavior and thus stay hidden behind the shelter. Boldness has been shown to be influenced by both learned avoidance and by mother's experience (**?**). As a result, the fry in the brooded group could have learned avoidance of open spaces from their mothers.

There was no significant difference in aggression between groups, unlike the findings by Taborsky that *N. pulcher* raised without adults have higher levels of aggression

compared to those raised with adults (Arnold and Taborsky, 2010). It is possible that the fry in the present study were too young to express the distinct social behaviors seen in adults. For the most part, the focal fry had not developed phenotypic markings associated with social status. The most aggressive and mature fry (brood 0-0) could not be included in the aggression analysis because it killed all of its siblings.

Approximately half of the video recordings for the behavioral assays were lost, making additional analysis impossible. It would have been useful to have a count of entries into each of the regions for the boldness assay. In addition to time spent in open spaces as an indicator of reduced stress, the degree of exploration the novel tank is also indicative of reduced stress. In the aggression assay, it would be helpful to have assigned each individual fish an aggression score rather than averaging the whole brood. It is possible that while there was no correlation between average brood aggression and GR expression, there could have been a correlation between individual aggression and expression.

Although there was no difference in GR expression between conditions, there were correlations between GR1a expression and GR2 expression and between GR1b expression and GR2 expression. This implies that there are fish with distinct and consistent GR expression profiles. In the present study, not all variables that can influence GR expression were accounted for. For one, sex is known to influence the degree of HPA-axis regulation that occurs in response to early life stress. Female rats exhibit a larger change in HPA-axis function in response to early life stress compared to males (McCormick et al., 1995). Social dominance is also known to influence GR expression profiles in *A. burtoni*, with dominant males having overall higher levels of GR1a and GR2 expression (Korzan et al., 2014). *A. burtoni* take on dominant and subordinant phenotypes as they mature. While most of the focal fish were too young to establish distinct hierarchies, one brood (0-0) had a male fish with prominent dominance markings and behaviors, suggesting that dominance could have

been established in other broods by 130 days.

Additional methods for measuring stress response regulation can be used in order to gain a better understanding of the ways in which maternal separation is affecting the HPA axis. Like GRs, mineralocorticoid receptors are down regulated in animals exposed to early life stress (Gass et al., 2001). Besides receptor expression, circulating receptor ligands can be used as a measure of HPA-axis activation. As mentioned before, fish with low stress early in life have lower CRF levels due to both decreased HPA axis activation and negative feedback onto the hypothalamus via GR activation (Taborsky et al., 2012). The extent of negative regulation within the HPA axis can be tested by exposing the fry to excess cortisol and measuring their subsequent CRF levels (Liu et al., 2000).

Given that the work of Michael Meaney primarily models maternal care and not maternal separation, it may be worth altering the experimental conditions to better mirror the rat model. One potential measure of maternal care in *A. burtoni* is filial cannibalism. Lab stock mothers exhibit higher instances of cannibalizing their young compared to wild stock mothers. This is likely as a result of the artificial separation of fry and mothers within lab stock leading to a decrease in selective pressure for kin selection (Renn et al., 2009; Lonstein and Gammie, 2002). This measure is of course not useful in a study dependent on observing the phenotypes of fry; however, the distance between fry and mother after initial release is a measure correlated with filial cannibalism that could be used. A preliminary study by the Renn lab found that on average wild stock mothers stay significantly closer in proximity to their fry compared lab stock mothers. This measure of maternal care would be useful if there is a notable variation within a single stock, since to use wild stock as the high maternal care group and lab stock as the low maternal care group would introduce a number of other variables associated with differences in genotype and phenotype that exist between the groups. This experimental paradigm also has the advantage of being a

natural occurring phenotype in contrast to the induction of maternal separation that occurred in the focal experiment.

Another difference between the present study and Meaney's work is that the former examined whole brain GR expression, whereas the latter studied regional GR expression. Because GRs are widely expressed throughout the brain and can have both excitatory and inhibitory feedback roles depending on the brain region, it is possible that any regional changes in expression were muted by changes in the opposite direction within another brain region (Busada and Cidlowski, 2017; Herman et al., 2005). Unfortunately, the telencephalic pallium and medial pallium, regions expected be responsive to early life stress, of *A. burtoni* are much smaller than the homologous structures in rats (hippocampus and amygdala respectively) and thus hard to isolate. Additionally, there is not a clear homologue of the mammalian prefrontal cortex, a region known to be heavily affected by fluctuations in glucocorticoids, in teleosts (Lupien et al., 2009; Yamamoto, 2009).

The question of how maternal separation in *A. burtoni* effects the offspring neurodevelopment is still open to being examined. While there exist a number of untested methods for answering the question, there is also the question of what a teleost model of maternal care can be. Given the large influence that early life stress exposure has on the developing brain and body, it is important to continue research that uncovers the mechanisms behind HPA axis reprogramming.

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