Comparative Maternal Phenotypes in a Mouthbrooding Cichlid

A Thesis

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&

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List of Abbreviation

ELISA	Enzyme-linked immunosorbent Assay
GSI	Gonad-somatic index
DOM	Dominant phenotype
SUB	Submissive phenotype
WS	Wild stock
LS	Lab stock
B2	Two days into the brooding cycle (treatment group)
B14	Fourteen days into the brooding cycle (treatment group)
R2	Two days after releasing fry (treatment group)
R14	Fourteen days after releasing fry (treatment group)

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Abstract

Maternal care behavior is plastically adjusted based on external and internal cues. Differences in maternal phenotypes can be observed between laboratory raised animals and their wild-raised conspecifics, eliciting the question of how environmental stimuli processing and internal physiological signals integrate to produce adequate maternal behavior. These inextricably linked networks have often been studied in isolation, but a thorough investigation of maternal phenotypes requires an investigation of the interplay between internal and external sources that affect reproductive mechanisms. Using an integrated comparative approach, this study examines behavioral, physiological, and hormonal contrasts between lab-raised and wild-raised stocks of the brooding cichlid fish *Astatotilapia burtoni*, which demonstrate divergent patterns of maternal investment. Brooders were placed into treatment groups at various time points throughout the brooding cycle and behavioral, morphological, and hormonal data was collected and compared between stocks and brooding stages. Results indicate significant differences in rates of filial cannibalism and weight regulation between the two stocks, revealing a phenotype contrast between good and inept brooders. These findings contribute to the limited compendium on the neural mechanisms which influence maternal care behaviors.

I dedicate this research to my mom for having a superior maternal phenotype.

Background

Introduction

"It is not the strongest of the species that survive, nor the most intelligent, but the one most responsive to change."
-Charles Darwin

Animal behavior is a complex neural process that requires the brain to sense and integrate internal and external stimuli in order to produce an appropriate behavioral response to fluctuating demands in the environment. Yet often there are contradictory internal and external pressures that demand vastly different behaviors, such as when an animal must balance its own metabolic demands while simultaneously allocating limited energy resources to raising its offspring. These competing behaviors are resolved through a complex cross-talk between brain systems that must adjust the animal's internal state to motivate it towards performing the most appropriate behavior. The transition from one behavioral drive to another is crucial, as the evolutionary fitness of an organism depends on its ability to navigate and respond in the most effective way to events in the environment. When changes in the environment occur, a demand is placed on the animal to adjust its behavioral output by plastically recalibrating its physiology based on the salient input. The underlying mechanisms of how the brain determines the most appropriate behavior based on neural stimuli integration is highly complex, and understanding the networks underlying behavioral plasticity in response to the environment will inevitably lead to a better understanding of the functionality of this enigmatic organ.

Why Study Cichlids?

The cichlid fish *Astatotilapia burtoni* is a superb model in which to study behavioral plasticity and the transition from different behavioral states in response to environmental fluctuations (Fernald & Hirata, 1977). Native to Lake Tanganyika in South Africa, *A. burtoni* belongs to a large and diverse family of cichlid species that has experienced rapid diversification across the Great Lakes of Africa, resulting in divergence of phenotypes from a large family of closely related species. (Barlow, 2000). Such a wide range of divergent phenotypes from closely-related species provides opportunity to compare the mechanisms of behavioral plasticity that have evolved to produce unique adaptations to the environment. In particular, *A. burtoni* demonstrate a high amount of behavioral plasticity from socially-mediated environmental cues, making this species a well-suited model organism for socially-mediated behavioral phenotypes (Renn *et al*. 2009).

Male *A. burtoni* live within in social hierarchy systems, and display behavioral and color-polymorphism phenotypes that correspond to distinct motivational states (Korzan & Fernald, 2006, Figure 1.1). In both natural and laboratory settings, approximately 20% of *A. burtoni* males will assume a dominant male phenotype, in which the male displays bright coloration accompanied by an increase in the growth hormone gonadotropin that drives an increase in body size and gonad development (Davis & Fernald, 1990, Figure 1.1), and estradiol, testosterone, and cortisol hormone titers, which are involved in promoting aggressive behaviors (Alcazar *et al*. 2016). This advance up the social hierarchy is accompanied by dark pigmentation bands across the body and face, and bright coloration, making it apparent as to the individual's ranking of

the social hierarchy and motivational state (Hofmann, 2003). Approximately 80% of males assume the non-territorial phenotype and do not exhibit bright coloration or body bars, and instead remain austere in coloration, invest most of their time to food seeking, and do not attempt courtship behaviors with females (Fernald, 1977).

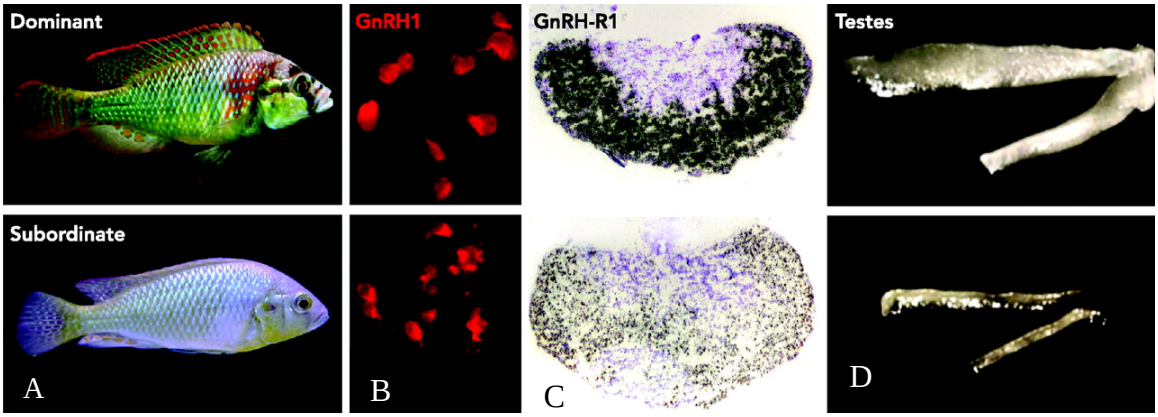

Figure 1.1: Male *A. burtoni* phenotype contrasts.
Demonstrated here are the distinct phenotype differences between dominant (top) and non-dominant (bottom) male A. burtoni. (A) show the color polymorphisms distinct to each phenotype; (B) shows enlarged neurons in dominant males in the preoptic area of the brain that produce gonadotropin signaling molecules (immunohistochemical staining); (C) shows an increased amount of gonadotropin molecules produced within the reproductive axis (in-situ hybridization); (D) compares morphological differences in testes development between the two phenotypes. (Maruska & Fernald, 2011).

Male *A. burtoni* behavioral and morphological plasticity has attracted substantial investigation into the hormonal regulation of male social dominance hierarchies, but the bases by which females regulate the social phenotype plasticity are less well understood (Renn, 2012). Female *A. burtoni* were once considered to display no social dominance hierarchies, and alternate only from a gravid, brooding, or non-gravid phenotype, with some displays of maternal aggression when defending their fry (Fernald, 1977).

However, Renn *et al*. (2012) showed that when females are housed in tanks with only female conspecifics, a few females will display a male-typical dominant phenotype characterized by increased aggression towards non-aggressive females, expression of eye-pigment bars, and increased levels of testosterone and estradiol, but in contrast to males, do not experience an increase in ovary growth compared to non-dominant females (Figure 2). Additionally, in natural settings, female A. burtoni exhibit even greater variance in phenotype expression as they cycle from the gravid to non-gravid phenotype, expressing distinct motivational states that influence either maternal aggression, maternal protection, mate-seeking, or egg development. Each of these unique female phenotypes are characterized by distinct morphological, hormonal, and behavioral traits that have been understudied, yet offer a valuable opportunity to explore mechanisms by which behavioral phenotypes are altered based on integrated cues from the environment, and the internal state of the organism.

Figure 1.2: Aggressive Female Morphology
Morphological differences between females displaying the non-dominant phenotype (right) and females displaying vertical eye bars also observed in the dominant male phenotype (left) (Renn *et al.* 2012).

The Mouthbrooding Reproductive Cycle

Reproduction is a powerful motivator in any animal's life, and in many species, it requires extreme physiological and behavioral adjustments in order to produce successful offspring. Female *A. burtoni* reproduce by mouthbrooding, which is a reproductive method that has fascinated biologists for decades due to its robust behavioral transitions of different maternal care phenotypes (Barlow, 2000). After spawning with a male, female *A. burtoni* gather the fertilized eggs into their buccal cavity (i.e. mouths) until the eggs have fully developed into free-swimming fry, taking anywhere from 12-28 days (Figure 3). This method of reproduction requires that the female voluntarily starve herself, involving significant alterations in the release of steroid hormones of brooders throughout the brooding cycle to suppress hunger and decrease the need for food intake (Grone *et al*. 2012). During the early brooding stage, *A. burtoni* enter an early-stage brooding phenotype, in which the brooder shifts from motivation towards mate-seeking behaviors, to a drive towards hiding behaviors and energy conversation, allowing them to prepare for fasting. Late-stage brooders undergo another round of behavioral plasticity which is characterized by maternal aggression and protection behaviors in preparation of the release of their fry (Oliveira et al. 1998). Additionally, late-stage brooders display body pigmentations seen in both males and females engaging in dominant and/or aggressive behaviors. It is currently unknown which signaling factors contribute to this phenotype switch to late-stage brooding, but may involve feedback about the developmental progress of the fry (Specker & Kishida, 2000).

After the brooder has released her free-swimming fry, a third phenotype emerges, characterized by increased maternal care and maternal aggression behaviors, and by

resumption of motivation to seek food and development of gonads for future reproductive potential. Maternal care behaviors exhibited by the female may include: hovering over her fry, taking her fry back into her mouth if a threat is sensed in the environment or if fry are pecking at their mother's mouth to seek shelter (colloquially referred to as 'bus-stopping') (Renn *et al.* 2009). The female may also display aggressive maternal behaviors, such as the expression of body, eye, and face pigment bars, and physical aggression towards conspecifics (Renn *et al.* 2009). The final phenotype expressed by brooders occurs several days to weeks after the release of the fry, which is characterized by extinction of maternal care behavior, and eventual return to mate-seeking behavior once again. The obvious behavioral and morphological phenotype switches throughout the brood cycle allows for accurate identification of the distinct brood stage of individual brooders, which opens up an opportunity to evaluate characteristics of the entire brooding cycle, further advocating the role of this model organism in the investigation of maternal phenotypes.

Mouthbrooding represents the importance of behavioral plasticity in transitioning between motivational states that encourage energy investment either towards somatic growth, or future reproductive potential. As such, if at any time during the brooding cycle female *A. burtoni* sense elevated threats of predation, or a decrease in food availability, she may deem the environment unfavorable for potential offspring and cannibalize her fry (referred from here on out as 'filial cannibalism'), allocating her energy into ensuring future reproductive potential (Manica, 2002). As such, rates of filial cannibalism can thus be represented as reproductive success or failure in when the environment is

controlled, providing a behavioral determinant of distinct successful or inept maternal phenotypes.

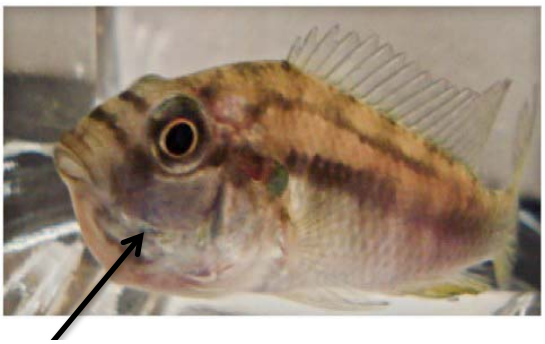

Figure 1.3: Morphology of Mouthbrooding
A mouthbrooding *A. burtoni* with an expanded buccal cavity filled with eggs.

Maternal Hormones

The high energy cost of the brooding process involves a complex neuroendocrine system that co-regulates maternal care and feeding behaviors (O'Rourke & Renn, 2015). The release of steroid sex hormones facilitate motivational and morphological adjustments in response to reproduction in vertebrates, inducing metabolic changes, territorial aggression, mate seeking and maternal care behaviors (Bender, *et al.* 2008; Grattan & Kokay, 2008). Of particular interest to the present study is the interaction and mediation of the estrogen estradiol, and the androgen testosterone. During development, both estradiol and testosterone are involved in sex determination, with higher amount of testosterone promoting masculine behavior and morphology, and estradiol promoting contrasting feminine morphology (Elbrecht & Smith, 1992). Testosterone is converted to estradiol by the enzyme aromatase, and inhibiting aromatase during development results in a permanent male phenotype, even if genetically, the animal was female (in chickens: Elbrecht & Smith, 1992). Additionally, Huffman *et al.* (2013) showed that inhibiting

aromatase in a population of male *A. burtoni* decreased aggressive behaviors, but had no effect on reproductive behaviors. In addition to morphological effects, testosterone is also well-known for mediating aggressive behaviors in many animal species, (Mehta & Beer, 2010), and estradiol in mediating maternal care behaviors (Ribeiro *et al.*2012). The highly contrasting morphological and behavioral effects induced by either estradiol or testosterone, and the function of aromatase in converting testosterone into estradiol, indicates that these two sex hormones are critical in mediating switches from maternal care behavior, to maternal aggression (Huffman *et al.* 2013).

Testosterone and Estradiol in Mediating Maternal Behaviors

In addition to promoting a masculine phenotype during development, testosterone has also been identified as an important mediator for maternal aggression and courtship behaviors in females (in rats: Rosenblatt *et al.* 1994; in songbirds: Cain & Ketterson, 2013; Wittingham & Schwabl, 2002). In female cichlids, the highest levels of testosterone are present just before spawning, then drop significantly once the female becomes gravid (Renn *et al.* 2012). Testosterone titers during the early brood stage are low, and increase slightly after females release their fry with the biggest spike occurring several days to weeks after the end of the brooding cycle (Renn, 2009). Also, it has been observed that the quality of maternal care decreases and likelihood of filial cannibalism increases past a certain elevated threshold of testosterone, suggesting that testosterone titers must be finely regulated to produce appropriate maternal behaviors (Dantzer, *et al.* 2011).

Estradiol is implemented in maternal care, but also plays a significant role in aggression as demonstrated by elevated estradiol levels in male and female *A. burtoni*

that are expressing the dominant phenotype (O'Connell *et. al.* 2013). However it is not clear whether this increase in estradiol promotes aggressive territorial behavior via the same pathways by which female *A. burtoni* produce aggressive maternal care behaviors for the purpose of offspring protection. Female *A. burtoni*, estradiol levels peak just before spawning, then sharply drop once the female becomes gravid followed by a gradual increase post-release; a similar pattern is seen for testosterone (Martin *et al.* 2004). In paternal cichlid species in which males partake in mouthbrooding, testosterone and estradiol levels sharply decrease after the first day of mouthbrooding, experience a minor, insignificant increase several days before releasing, and finally increase sharply to levels at or above the initial pre-spawning amounts (Specker & Kishida, 2000). Integrating physiological, behavioral, and hormonal trends throughout the brooding cycle allows for opportunity to examine how the mechanisms of integration of internal and external stimuli utilized during brooding correspond to differential maternal phenotypes.

Divergent Maternal Phenotypes

The exorbitant cost of the reproductive process reduces the female's capacity for future reproductive potential by reducing her energy intake and hence growth rate during the brooding period. As such, successful mouthbrooding is a highly costly reproductive behavior that requires an adequate maternal fitness phenotype capable of balancing metabolic demands with environmental threats. When environmental conditions are not favorable for either survival of the parent or offspring (presence of predators, food and mate availability), brooders may cannibalize their eggs, trading immediate reproductive success for future viability - a characteristic of their life history strategy (Manica, 2002).

A unique opportunity to examine the physiological basis of 'good' or 'bad' maternal phenotypes in *A. burtoni* has come about in the observation of divergent maternal phenotypes between lab-raised and wild-raised stocks. An original stock of *A. burtoni* was obtained from Lake Tanganyika during the 1970s and has since spawned dozens of generations in laboratory settings, in which natural environmental conditions were not perfectly mirrored and artificial selection has absolutely occurred. In these artificial laboratory settings, eggs are commonly stripped from a female's brood and raised by artificial incubation, depriving fry of a natural upbringing and displacing mothers from the selective pressures of the full brood cycle (Renn *et al.* 2009). Recently, a new wild stock (WS) of *A. burtoni* was collected from Lake Tangankya and demonstrates largely different behavioral phenotypes than their equivalent lab stock (LS) (Renn, *et al*. 2009). Specifically, WS females exhibit higher rates of maternal care behaviors and achieve higher rates of successfully producing offspring, while LS have a higher tendency for filial cannibalization and experience greater body mass loss 14 days into brooding, (Renn, *et al*. 2009) suggesting that the gene expression profiles of these different stocks has changed through genetic accommodation within the past ~40 years. It has not yet been investigated, however, how circulating hormone titers and body mass fluctuates throughout the entire brooding cycle between LS and WS to contribute towards motivation to engage in or inhibit filial cannibalism behaviors. Furthermore, while animal behavior studies have documented body mass differences between wild and captive stocks (Atlantic Bluefin tuna: Pousis, *et al.* 2012), and specifically differences in reproductive potential between stocks (Black tiger prawn: Brady *et al.* 2013), few studies

have integrated hormonal, morphological, and behavioral measurements to explain maternal phenotype plasticity throughout the entire brooding cycle.

Currently, the networks which influence somatic growth motivation and reproductive behavior have been explored as isolated behaviors, yet little research has examined the networks which integrate both drives to produce behaviors (O'Rourke & Renn, 2015). Thus, the aim of this study is to investigate the interplay between hormonal, behavioral, and physiological mechanisms that promote a successful maternal phenotype. These phenotypic changes are crucial adaptations during the brooding cycle which ultimately determine the success of the offspring. Animals that incur a high cost for reproduction, such as brooding cichlids, require mechanisms of maternal care behaviors that are coordinated by a complex neuroendocrine circuit which has not been fully explored. By comparing hormone levels with observable morphological and behavioral characteristics between different stocks, it is possible to better understand the plasticity between the complex neural networks which influence maternal care.

Research Question

How do estradiol titers, rates of filial cannibalism, and morphology of brooding LS and WS fluctuate throughout the brooding cycle to promote distinct maternal phenotypes?

Hypothesis

It was hypothesized that differences in maternal phenotypes and reproductive success between lab-raised and wild-raised *A. burtoni* would be reflected by differences in rates of filial cannibalism, body morphology, and estradiol titers throughout the brooding cycle. Specifically, LS were hypothesized to have lower rates of filial cannibalism, greater preservation of body mass during brooding, and higher estradiol titers, which are all factors that contribute to more successful reproductive outcomes.

Methodology

Samples

The brooding cycle was split into four distinct brood stages which made up 4 treatment groups. Brooding females were characterized by their expanded buccal cavities and specific brood stages were identified by examining the developmental stage of the developing fry in the buccal cavity (Figure 2.1). The B2 group consisted of females who had been mouthbrooding for two days and exhibited behaviors characteristic to the early brood stage, including: hiding from conspecifics, abstaining from eating, and lacking vertical body pigment bars. During this stage, females are likely undergoing extreme metabolic adjustments as they begin fasting and reducing their expenditure of energy. The B14 group consisted of females who were 2 weeks into the broody cycle and close to releasing their fry. This stage was identified by expression of body, face, and eye pigment bars, hiding, and immobility. As mentioned, it is thought that expression of these bars is characteristic to threat signaling behavior and thus this brood stage is thought to be undergoing internal changes to express aggressive, protective maternal behaviors. Since the brood stage length is variable, it was most important that data was collected by animals displaying appropriate behavioral cues in line with their selected treatment group. As such, samples from this group included animals within 13 – 15 days of the brooding cycle that were displaying the appropriate maternal phenotype. The R2 group consisted of females who had released their fry from their mouths two days previously, regardless of whether the brooder had retaken her fry back into her mouth. During this

brood stage, the female is undergoing metabolic plasticity as she starts increasing her food intake, and becomes highly aggressive and protective of her fry. Maternal behavior during this stage is highly variant and there are several phenotypes which the females may rapidly cycle though. The aggressive maternal phenotype includes females who display dark and constant pigment bars, and bite conspecifics that approach her fry. The protective maternal phenotype may be identified by females who hover over their fry and will take their fry back into their mouths when approached by a conspecific.

The R14 group consisted of females who were stripped of their fry 2 days post release, and had spent 12 additional days alone with a stimulus male. During this stage, the female does not have any maternal care behaviors and is instead investing a high amount of energy into gonad development for future reproductive potential.

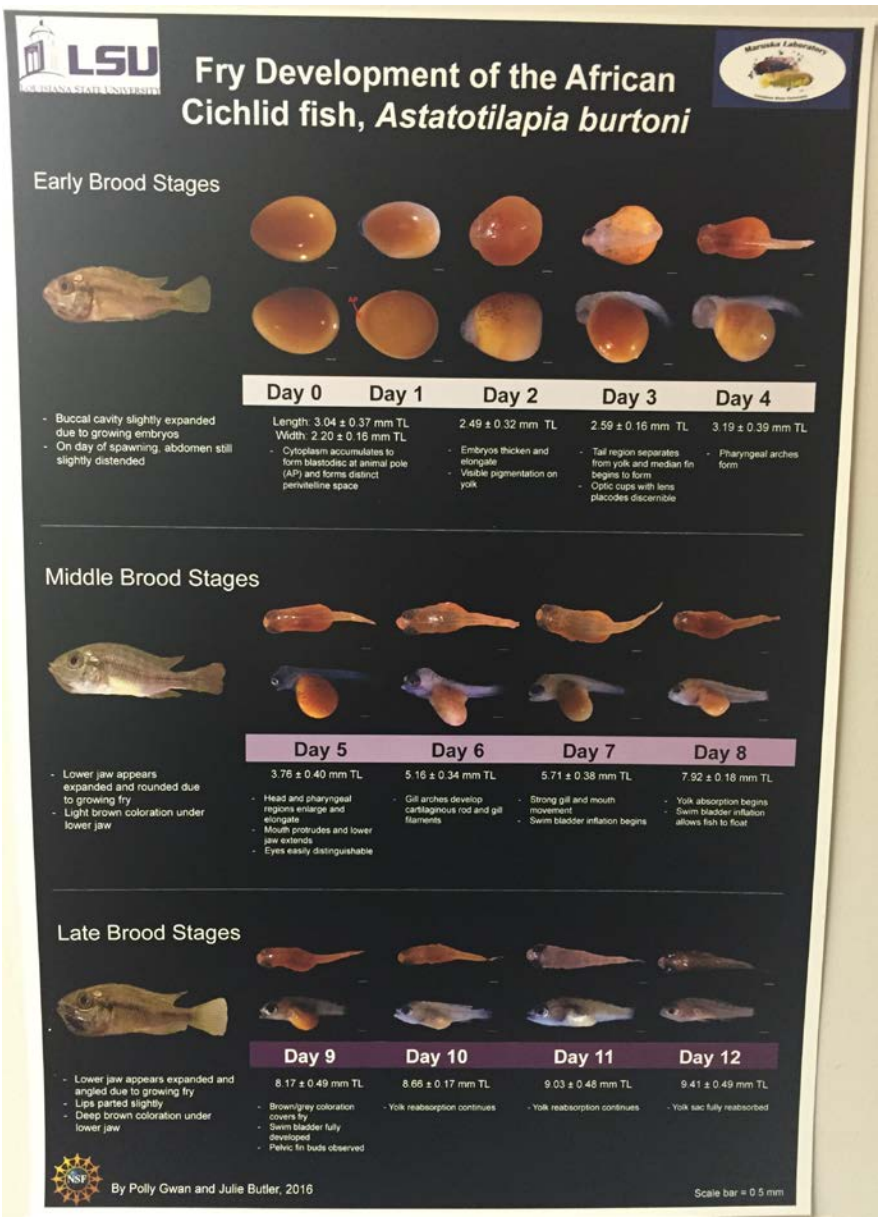

Figure 2.1: Fry Developmental Stages of *A. burtoni.*

Brood stages were identified by the developmental stage of the egg according to these images provided by Gwan & Buffer (2016).

Experimental Set Up

The tanks used for the experiment were standard 5 gallon zebrafish aquaculture systems with terra cotta pot shelters and gravel. A medium-sized (~3g $\pm$ 1g) stimulus male fish was kept behind a cylindrical mesh barrier so that visual & olfaction cues were sensed by the female, but the female could not be attacked by the male. The stimulus male phenotype was varied across tanks, and often consisted of the male cycling between dominant and subordinate phenotypes several times during the length of the female's brood cycle. The male stimulus fish was fed daily with standard cichlid pellets explicitly within his confines so that the female had visual and olfactory cues regarding the availability of food, but did not have access to it. Once females released their fry, they were fed a diet of standard fish flakes. Tanks were kept on a 12/12 day/night cycle with a half an hour dawn and dusk settings. The salinity and pH levels of tanks were matched to the average levels of Lake Tanganyika.

Behavioral Observations

Behavioral observations were made within one hour of dissection. Brooders were examined for 5 minutes each, and the absence or presence or specific behaviors were checked off using an original *A. burtoni* ethogram (Table 2.1).

Active Behavior	Post Spawn (B2, B14)	Post Release (R2, R14)
Swimming	Y / N	Y / N
Taking fry into mouth	N/A	Y / N
Hovering over fry	N/A	Y / N
Attacking stimulus fish	Y / N	Y / N
Eating	N/A	Y / N
Inactive Behavior		
Hiding	Y / N	Y / N
Floating on one side	Y / N	N/A
Ignoring "bus-stopping" fry	N/A	
Physical Appearance		
Absence/presence of pigment bars	Y / N	Y / N

Table 2.1. Brooding *A. burtoni* ethogram.
Behavioral observations were taken before dissection for each brooder, and the presence or absence of the behaviors specified on this ethogram were noted.

Dissection

Dissections were made between 1600-1800 hours. Brooders were removed from their tanks and anesthetized in MS-222 for approximately one minute, or until the animal displayed balance disproportions and immobility. Body length measurements were taken from the most rostral region of the head to the most caudal end of the body, excluding the tail, and body mass weight measurements were recorded. Blood was drawn by clipping a one centimeter section of the caudal region of the body, allowing adequate exposure of blood vessels to be drained into a micro capillary tube and immediately placed in a dry tube on ice. The animals were sacrificed via cervical dislocation within five minutes after the initial retrieval from the source tank. Brain tissue for future gene expression analysis was obtained by anatomizing the jaw of the animal and making a dorsal cut along the scalp to remove the dorsal skin and skull. The optic nerves were severed and the brain and rostral end of the spinal cord was removed and placed in a tube of 500 ml RNALater. The time of animal retrieval to collection of the brain took no longer than 10 minutes. Blood was centrifuged at 10,000 x g for 10 minutes at 10 ° C, and the extracted serum was kept at -80 ° C for 3 months.

Enzyme-Linked Immunosorbent Assay

Fluctuations of estradiol plasma titers throughout the brood cycle were measured using a 7-standard dilution control series enzyme-linked immunosorbent assay (ELISA), Roche). Serum was diluted with molecular grade water to a 1:42 dilution range with 6 microliters of serum and 194 microliters of solvent. Due to the size variation of brooders and food intake restrictions, samples that did not have 6 ul of serum instead used a 1:66

and 1:99 ratio with 3 ul and 4 ul of blood, respectively. A separate dilution series was set up to ensure that the assay was sensitive enough to detect these smaller amounts. The assay was scanned with a spectrophotometer (Nanodrop 1000) with an optical density reading of 405 nm.

Statistics

The contrasting rates of filial cannibalism were analyzed between stocks using a Fisher's exact t-test. A two-way ANOVA and Tukey's post hoc test was performed to test for average brood sizes between stocks and within treatment groups. Body condition contrasts between stocks and within treatment groups were analyzed using a two-way ANOVA and a Tukey's post hoc. Estradiol fluctuations were calculated using a two-way ANOVA and a Tukey's post hoc to test the effects of stock and treatment group on estradiol titers. A Pearson's linear correlation analysis was performed to measure the correlation between estradiol titers and gonado-somatic index growth. All statistics were performed in R (R-project 2016), and the corresponding R-Scripts for each analysis can be found in Appendix A.

Results

Note: Mean values proceed standard deviations within parentheses in text, and standard error of the mean is depicted in figures.

Rates of Filial Cannibalism Are Significantly Higher in LS Compared to WS

Collectively, 72 brooders were placed into treatment tanks, out of which 32 were LS, and 40 were WS. A total of 15 brooders cannibalized their eggs before releasing, of which 10 were LS, and 5 were WS (Table 2). A Fisher's t-test showed that WS were significantly more likely to cannibalize than LS ($p < 0.007$).

Stock	# of Broods Cannibalized	# of Broods not Cannibalized	% Cannibalized
LS	13	18	42%
WS	5	35	12%

Table 3.1. Rates of filial cannibalism by stocks.
The numerical values of the number of broods cannibalized by each stock. LS are significantly more likely to cannibalize their entire brood than WS (Fisher's t-test $p < 0.007$).

LS Engage in Partial Filial Cannibalism

Partial filial cannibalism, in which only some offspring are eaten by the parent, is a common phenomenon in teleost fish (Manica, 2002). As such, it is likely that brood sizes may have started out larger during the early brooding stages for each individual, and some fry may have been cannibalized during the later brooding stages. A two-way ANOVA testing for the effects of stock and brood stage on brood size revealed that there was no overall difference in the mean brood size at the time of sacrifice between stocks ($p = 0.36$, LS mean = 18.05 ± 12.07 fry, WS mean = 15.23 ± 6.79 fry), however for LS, B14 samples had a significantly smaller brood size than B2 samples ($p = 0.001$, LS B2 mean = 33.67 ± 14.05, LS B14 mean = 12.50 ± 9.80), but WS did not follow this trend ($p = 0.99$, WS B2 mean = 18.40 ± 5.32, WS B14 mean = 17.50 ± 9.09, Appendix A: Script 1, Figure 3.1). Interestingly, LS B2 brooders were significantly more likely to have larger broods than WS B2 brooders ($p = 0.05$).

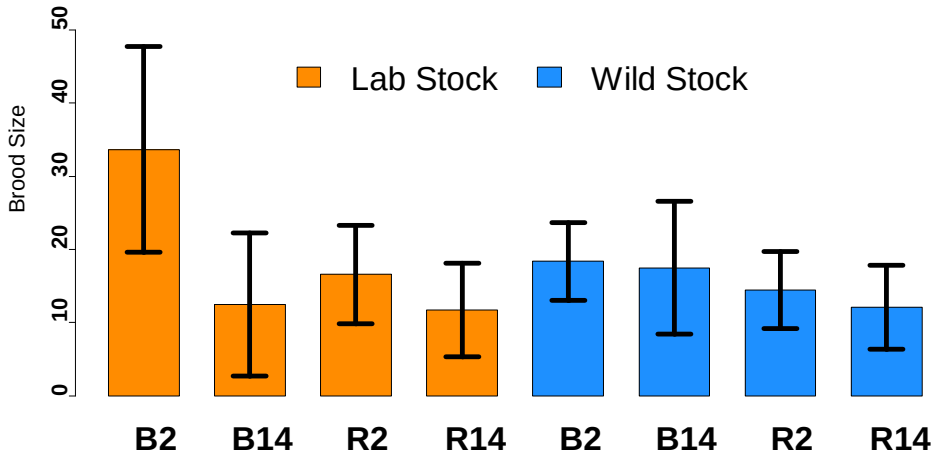

Figure 3.1: Brood Size fluctuation throughout the brooding cycle

A two-way ANOVA was performed to test for the effects of stock and brood stage on brood size. The y-axis represents the average number of eggs from broods within a treatment group, which is displayed on the x-axis. On average, B2 LS females start the brooding cycle with significantly more eggs than WS ($p = 0.05$), though the B14 LS average brood size is significantly reduced and not significantly different than the mean WS B14 broods ($p = 0.15$). There was no significant difference in the overall brood size across all treatment groups between LS and WS ($p = 0.36$).

LS Lose Significantly More Body Mass during Mouthbrooding than WS

Weight and length measurements at the beginning of the brood cycle were not completely standardized due to limited numbers of available subjects. Residual mass was therefore selected as a measurement to test for the effects of mouthbrooding on body condition, as it provides a measure of body condition that is independent of body length, effectively eliminating variance in length as a confounding variable in the analyses of body condition. Residual mass was calculated using a linear regression model to derive the equation of a curvilinear line, representing the amount of body mass gain (g) per unit of length (mm) gain across all subjects in the experiment. The equation of this line was then used to calculate the predicted mass for each individual, which was subtracted from actual mass to obtain residual mass. A two-way ANOVA was performed to determine significant differences in residual mass between stocks and within brooding treatments. Results show that there was no significant difference in residual mass fluctuation when comparing combined treatment groups within stocks ($p = 0.26$; WS mean = 0.04 ± 0.24 g, LS mean = -0.05 ± 0.30, Appendix A: Script 2), however there was a significant difference in residual body mass fluctuation between stocks and within brood stages ($p = 0.047$, LS B2 mean = 0.16 ± 0.20 g vs WS B2 mean 0.07 ± 0.19 g, LS B14 mean = -0.22 ± 0.21 g vs WS B14 mean 0.03 ± 0.35 g, LS R2 mean = 0.09 ± 0.20 g vs WS R2 mean = -0.09 ± 0.12 g, LS R14 mean = 0.32 ± 0.31 vs WS R14 mean 0.05 ± 0.24 g;, Appendix A: Script 2, Figure 3.2), indicating greater fluctuation of body mass across the brood cycle in LS compared to WS A Tukey's post-hoc test revealed that within LS, residual mass for B14 brooders was significantly less than R14 brooders ($p = 0.016$, Appendix A: Script 2).

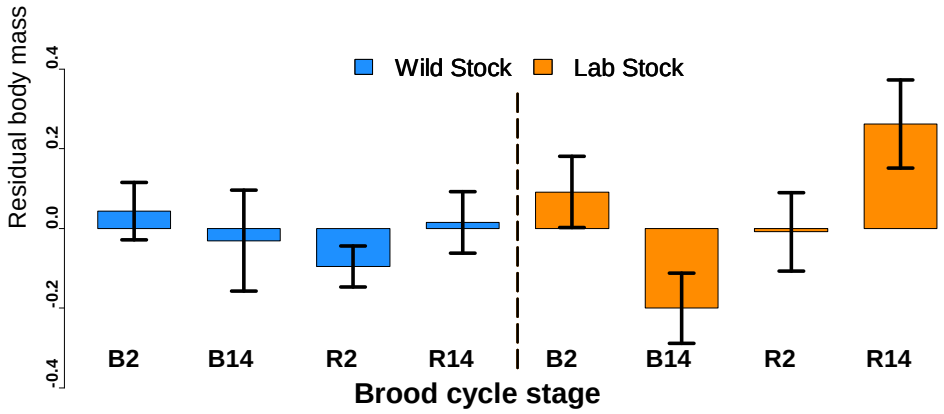

Figure 3.2: Residual body mass fluctuation throughout the brooding cycle.
A two-way ANOVA revealed a significant difference in body mass between B14 and R14 LS brooders ($p = 0.016$). When treatment groups were combined across stocks, there was no significant difference in body mass fluctuation between LS or WS ($p = 0.26$).

Estradiol Increases after Fry Release for Both Stocks

Blood serum levels of estradiol were measured from a collective total of 32 samples, out of which 13 were LS, and 19 samples were from WS. A preliminary two-way ANOVA showed excessive heteroscedasticity within residuals; therefore estradiol titers were log-transformed to control for this skewness, and to preserve the validity of the data. There was no significant difference in log-transformed estradiol titers between WS and LS throughout the brooding cycle ($p = 0.29$; mean WS = 12113.89 $\pm$ 10908.21 pg/mL, mean LS = 9525.33 $\pm$ 8587.43 pg/mL, Appendix A: Script 3). However, there was an interaction between estradiol concentrations and treatment group ($p = 0.003$; mean B2 = 4227.07 $\pm$ 1995.86 pg/mL, mean B14 = 5395.18 $\pm$ 2538.95 pg/mL, mean R2 = 11324.30 $\pm$ 7168.98 pg/mL, mean R14 = 17041.43 $\pm$ 12706.88 pg/mL; Appendix A: Script 3; Figure 3.3). For both stocks, estradiol levels sharply increased after release of

fry, resulting in a significant difference between R14 and both B2 and B14 (Tukey's HSD p = 0.006 and 0.016), and an insignificant difference between R2 and B2 (Tukey's HSD p = 0.094; Appendix A: Script 3; Figure 3.3).

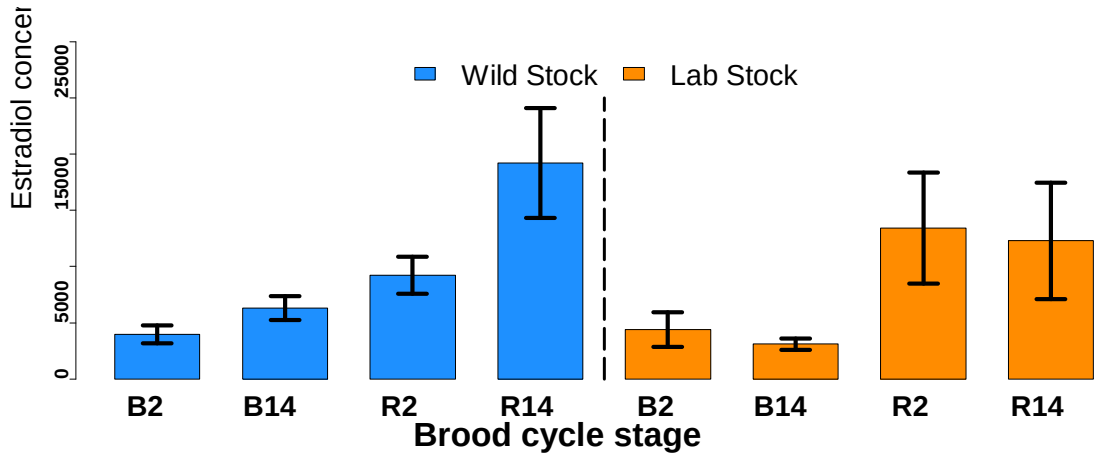

Figure 3.3: Estradiol blood titers throughout the brooding cycle.
A two-way ANOVA between estradiol titers, stock, and treatment group show that estradiol titers increase significantly in R14 individuals relative to both B2 (p = 0.006) and B14 individuals (p = 0.016), and tend to increase in R2 individuals relative to B2 individuals (p = 0.094).

Estradiol is Not Correlated with GSI

Gonado- somatic index, which is (gonad mass/body mass) * 100, was used as a representative measurement of the amount of energy allocated towards reproductive viability. To determine whether estradiol is correlated with gonad growth, a linear correlation analysis between GSI and estradiol levels was selected as an appropriate correlational test. Since both estradiol concentrations (Figure 3.3) and GSI (Figure 3.4) were significantly higher in brooders compared to non-brooders (estradiol: Welch two-sample t-test $p < 0.001$; GSI: Welch two-sample t-test $p < 0.001$) correlations were analyzed both within lumped brooding classes (B2 and B14), and within lumped non-brooding classes (R2 and R14) to eliminate brooding class as a confounding variable.

Estradiol titers were log-transformed to address excessive variability among treatment groups. Results revealed no significant correlation between GSI and estradiol in lumped R2+R14 non-brooders (Pearson's r = 0.347, p = 0.146; Appendix A: Script 4a, Figure 3.5), or lumped B2+B14 brooders (Pearson's r = 0.203, p = 0.526; Appendix A: Script 4b, Figure 3.5).

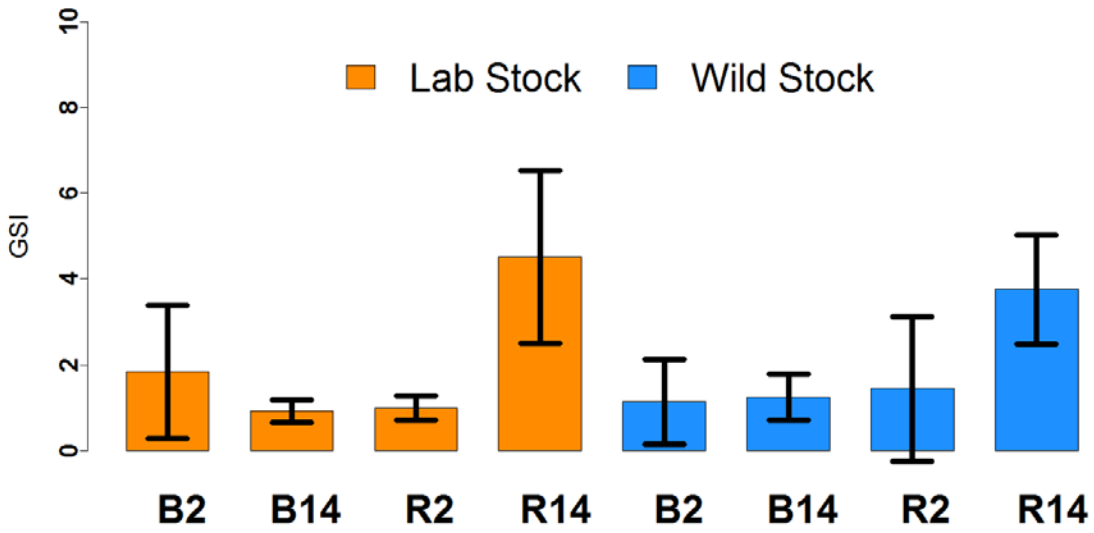

Figure 3.4. GSI fluctuations throughout the brooding cycle.
A Welch two sample t-test showed a significant increase in GSI in nonbrooders compared to brooders ($p < 0.001$). A two-way ANOVA between GSI, stock, and treatment group showed that there was no significant difference in GSI between stocks ($p > 0.733$), however for both stocks, GSI in R14 nonbrooders was significantl higher than in B2 brooders ($p < 0.001$), B14 brooders ($p < 0.001$), and R2 brooders ($p < 0.001$, Appendix A: Script 5).

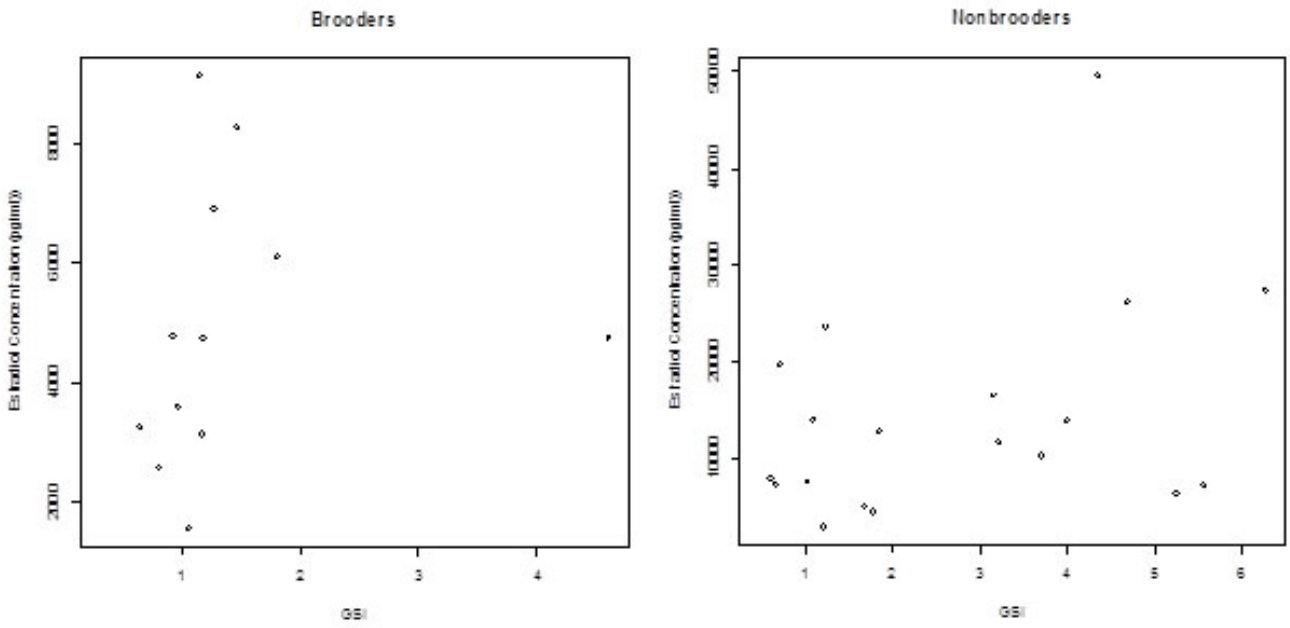

Figure 3.5. Correlation between GSI and estradiol in brooders and nonbrooders. (Stocks Combined). The y-axis represents estradiol blood titers at the time of sacrifice. The x-axis represents GSI, which is (gonad mass/body mass) 100. A Pearson's correlation showed no significant relationship between GSI and estradiol for either brooders brooders ($r = 0.203$, $p = 0.526$) or for non-brooders ($r = 0.347$, $p = 0.146$)

Discussion

Plasticity of reproductive behaviors is an essential criterion for the survival of many species. In many cases, the reproductive process is dynamic and adequate parental care requires successfully integrating internal and external cues to motivate the parent towards care behaviors at the appropriate time in the reproductive cycle, which ultimately determines the survival outcome of the offspring. In the environment, natural threats to a parent such as predation, food, and mate availability, puts selective pressure on the parent to adjust its reproductive behavioral output in a way that is most suited to handle the current environmental threat. When an animal is bred in artificial conditions and does not have selective environmental pressures, then behaviors which may be counterproductive and hinder its survival in the wild will not be as influential in determining its reproductive success, and as a result, these inadequate behaviors will be inherited by the next generation of offspring. As a result, laboratory-raised animals may produce drastically different behaviors to the same stimulus as their wild-raised conspecifics, and may be inferior in their reproductive success rates. The purpose of this experiment was to establish evidence for the development of robust maternal phenotype differences between *A. burtoni* stocks as a result of differential environments, and to explore the mechanisms by which these varying maternal phenotypes are produced.

Behavioral Variance

One of the major aims of this experiment was to determine whether there was variance in maternal success rates among stocks. It was previously observed that LS *A. burtoni* exhibit a higher rate of filial cannibalism than their WS conspecifics, (Renn *et al.* 2009) thus filial cannibalism behavior was selected as a measurement of failed maternal outcomes. The significantly higher rates of filial cannibalism in LS compared to WS support the Renn *et al.* (2009) findings that LS brooders, who have been subjected to natural selection for the past 5 decades, have developed a maternal phenotype characterized by poorer rates of offspring outcomes, and higher rates of complete filial cannibalism.

Unexpectedly, it was shown that LS begin the brooding cycle with significantly more eggs than WS, and likely undergo partial filial cannibalism, in which brooders will eat a few of their eggs while also sparing some of the brood. This finding suggests that the increased rates of filial cannibalism within LS may not necessarily be due to an inferior maternal phenotype, and may in fact be adaptive. The increased fecundity (i.e. ability to produce abundant offspring), as well as the higher rates of both partial and full filial cannibalism, suggests an alternative life-history strategy has developed within LS. Increased fecundity provides brooding LS an opportunity to offset the cost-to-benefit ratio of the taxing brooding cycle by providing a food reservoir to LS, while also increasing the chances of the survival of some of the brood – a hypothesis first put forward by Rohwer (1978). This strategy of using offspring as a source of food to increase current and future reproductive viability is a common strategy in many species

of teleost fish (Rohwer, 1978), but the majority of findings concern paternal-caring fish, and until now this reproductive strategy had not yet been identified in *A. burtoni* females.

It is worth mentioning that WS were obtained from the northern end of Lake Tanganyika, while LS were sourced from the southern end of the lake. Thus, it is possible that aspects of this life-history strategy may have developed prior to the artificial selection imposed by the laboratory settings. More than likely, however, the artificial upbringing of previous LS generations exacerbated or influenced this maternal strategy. Furthermore, it has been shown that tropical male scissortail sergeants, who participate in paternal nest guarding, will invest a higher amount of paternal care towards a brood if the previous brood was unsuccessful (Manica, 2004). Therefore, it is possible that increased fecundity of LS may be an adaptive response to the generations of egg-stripping and incompletion of the brood cycle brought on by an upbringing in an artificial laboratory setting, though this hypothesis requires additional evidence. Additionally, filial cannibalism is thought to be driven by the presence of a small, and therefore undesirable mate, (Wong *et al.* 2016) and opportunities for future investigation into the cannibalism rates between LS and WS should examine the effect of mate selection, and whether the presence of either a small or large stimulus male influences the female's motivation to cannibalize.

Metabolic Regulation

This study hypothesized that the differences in maternal phenotypes in mouthbrooders likely involves differences in metabolic regulation, as feeding and reproduction brain networks experience significant cross-talk (O'Rourke & Renn, 2015). It was shown here that WS and LS do not differ in their initial starting weights at the

beginning of the brood cycle, but after brooding for 2 weeks, LS show a decrease in residual mass, followed by a significant increase 2 weeks after releasing their fry, while WS are able to stabilize their body mass throughout the entire brooding cycle without a significant gain or loss in body mass (Figure 3.2). The data here supports the hypothesis that WS have a superior ability to regulate body mass throughout the brooding cycle, whereas LS are unable to prevent significant body mass reduction during the reproductive cycle. Additionally, this study shows a trend in the irregular patterns of weight loss/gain in LS, showing that not only do LS rapidly lose weight while mouthbrooding, but that the body mass is also rapidly gained after the release of the fry. This is in contrast to WS, who do not experience significant weight loss during brooding, or significant weight gain after releasing their fry. Further support for superior metabolic regulation in LS compared to WS comes from a comparative analysis by Renn *et al.* (2009), that examined the percentage of body mass lost between LS *A. burtoni,* and the first generation of WS *A. burtoni* that were the ancestors of the fish used in the present experiment. It was shown that brooding LS lost a similar amount of weight as nonbrooding LS that were artificially starved, while there was no significant difference in the amount of weight lost between starved WS and brooding WS. Therefore, it is likely that the observed differences between lab and wild stock filial cannibalism may be due in part to differences in metabolic regulation; LS are more likely to cannibalize, because they are literally in more danger of somatic harm from starvation than WS. Furthermore, the increase in initial brood size in LS may also be a strategy developed to counteract the deficiencies in metabolic stability throughout the LS reproductive cycle.

The processes by which mouthbrooders are able to suppress hunger drives and reduce energy expenditure are plentiful and have been the topic of many prior investigations (in cichlids: Grone *et al.* 2012). Previously in the lab in which the present study was conducted, it was demonstrated that the apoptotic gut turnover rate in *A. burtoni* was significantly reduced in WS brooders compared to starved, or control WS groups (Bacheller, data not shown). This data, taken with the results from the present experiment, suggest that WS *A. burtoni* are able to adapt to the costly mouthbrooding process by allocating limited energy away from cell turnover within the gut. Alternative methods of energy conservation that have recently been explored include the alteration of circulating orexigenic signaling molecules. Specifically, Kalpana (2017) showed that neurons that release the appetite-stimulating hormone neuropeptide Y (NPY), are reduced in a mouthbrooding cichlid species compared to starved or controlled groups, while Grone *et al.* (2012) showed an increase in NPY receptor mRNA expression in day 14 brooders, however neither of these studies examined stock differences. Though it is not clear how NPY signaling in the brain of mouthbrooders influences plasticity in feeding behaviors, it is likely that NPY signaling and receptor expression, along with a plethora of other anorexic and orexigenic signaling molecules, is involved in the regulation of feeding behavior during the brooding cycle. The brain tissue samples derived from the fish in the present experiment will allow for a future comparative investigation into the differential expression levels of various signaling hormones and their corresponding receptors involved in feeding behaviors.

Influence of Estradiol in Maternal Care

Estradiol is a major contributor to reproductive behavior and maternal care, and abolishment of estradiol-producing neurons has been shown to decrease maternal care behavior (in mice: Rosenblatt, 1994). Thus it was hypothesized that estradiol titers would significantly vary between LS and WS as a characteristic of differential maternal phenotypes between stocks. This hypothesis was not confirmed, as there was no significant difference in estradiol concentrations between stocks at any stage during the brood cycle. However contrasting trends in estradiol concentrations are apparent between both stocks. Specifically, WS start the brooding cycle with low amounts of estradiol, and concentrations steadily increase as the brood cycle progresses (Figure 3.3). Contrarily, LS experience a slight decrease in average estradiol titers between B2 and B14 time points, followed by a drastic increase in estradiol at the R2 time point, and have no pronounced change between the R2 and R14 time points. It was also shown that estradiol concentrations post-release increase in LS quicker than they rise in post-release WS. The rapid rise in estradiol post-release in LS raised the question of whether estradiol was involved in mediating maternal aggression since estradiol titers are also elevated in DOM female and male *A. burtoni* (female: Renn *et al.* 2009; male: Alcazar *et al*. 2016). Additionally, if estradiol is related to an increase in aggression, then the premature increase in estradiol in R2 LS may be a driving force which influences the increased cannibalism rates seen in LS.

Estradiol has been shown to peak in females just before spawning, decrease during brooding, and increase after releasing fry (Baroiller *et al.* 2014; Martin *et al.* 2004) which suggests that estradiol influences gonad development, which is on hold

during the brood cycle. However no prior experiment from the time of this writing had examined the correlation between estradiol and GSI at the reproductive time points that were selected for the present experiment. The present experiment showed that estradiol increased significantly during the late-release stage when *A. burtoni* were supposedly experiencing a transition from a behavioral state characterized by motivation to brood, to a motivation towards mate-seeking behaviors and energy investment towards egg development. The increase in estradiol post-release for both stocks suggested that estradiol was involved in driving egg development in preparation for the next reproductive cycle. Increased estradiol has been identified as a mediating factor which increases ovarian development (Baroiller *et al.* 2014) and vitellogenisis (yolk formation) by allocating energy from somatic growth into reproductive development (Davis *et al.* 2008) however the lack of a correlation between estradiol and GSI in the present experiment contradicts this explanation. It is worth mentioning that GSI tends to be higher in B2 LS than B2 WS, and in B14 LS compared to B14 WS. When considering the evidence towards greater brood sizes in B2 LS, it can be concluded that LS invest greater amounts of energy into egg development as part of their alternative reproductive strategy.

To resolve the effect of estradiol on maternal aggression and gonad development, a second experiment was performed which involved exposing DOM and SUB non-brooding females to exogenous estradiol, and comparing levels of aggressive behaviors, residual mass gain, and GSI development at 2 days, and 14 days into the estradiol treatment. DOM females showed a significant increase in GSI, body mass, and body length 14 days into treatment, however at day 2 of treatment, GSI in DOM females was

not significantly greater than GSI in SUB females, refuting the hypothesis that an increase in GSI drives aggressive behavior, as increased GSI should be apparent in DOM females at day 2 (O'Rourke, data not shown). The increase in GSI at day 14 for DOM females was more than likely the result of increased food access to DOM, and decreased food intake for SUB females. Furthermore, if estradiol was involved in mediating maternal care behaviors, then estradiol titers should be highest in R2 brooders, with a decrease in R14 brooders when maternal care behaviors are abolished. However the exact opposite trend was observed in this study, leaving the definitive role of estradiol in the brooding cycle inconclusive. Though estradiol was selected as a hormone candidate in the present study, the interacting effects of other sex steroids, primarily progesterone, testotsterone, and prolactin, most definitely interact with each other in complex and ill-understood ways throughout the brooding cycle, and an integration of other hormonal titers not limited to solely estradiol is needed to fully understand the complex endocrinology behind the brooding process.

Conclusion

An evaluation of behavioral, morphological, genetic, and hormonal data revealed key differences in divergent maternal phenotypes across *A. burtoni* stocks. First, differential patterns and rates of filial cannibalism suggest that LS have developed alternative life-history reproductive strategies as a result of the artificial selection within a laboratory setting. Secondly, LS were shown to have a lack of metabolic stability throughout the brooding cycle compared to WS, suggesting that the higher rates of filial cannibalism in LS may in fact be an adaptive strategy to compensate for this reduction in weight stability during the brooding cycle. Third, estradiol titers were not significantly

different between LS and WS brooders throughout the brooding cycle, but a contrasting pattern of estradiol fluctuation existed between stocks, suggesting that estradiol may be indirectly involved in producing contrasting maternal phenotypes. Finally, estradiol is likely not a contributor to egg development during the brooding cycle, as there was no correlation between GSI and estradiol for either WS or LS. The robust difference between rates of filial cannibalism between stocks demonstrates the validity of *A. burtoni* as a model organism for investigating the molecular mechanisms which influence maternal care.

Evolution has shaped an incredibly diverse scope of organisms and behaviors that are uniquely suitable for the environment of a given species. Reproduction is a behavior that serves the same purpose for every species (creation of offspring and continuation of the species), yet the diverse mechanisms by which organisms are equipped to handle reproduction varies. This diversity has important implications for the inspiration of medical engineering, as advances in medicine often result from studying the differential ways natural selection has shaped biological functions using highly conserved biological compounds. The mechanisms by which *A. burtoni* brooders regulate metabolism, suppress food intake, and achieve such pronounced behavioral plasticity is indeed a window into the biological underpinnings of a plethora of human diseases, such as nutritional and metabolic diseases, wasting disorders such as anorexia, and hormonal regulation of human fertility. Understanding how diverse organisms achieve fluctuations in physiological and behavioral states is essential for the future application of these mechanisms towards advancing human health and wellbeing.

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Appendix A: R-Scripts

Highlighted numbers indicate values referenced in Results section

Script 1 – *two-way ANOVA between treatment, stock, & brood size*

```
> m<-aov(brood_size~treat*stock);summary(m)
              Df Sum Sq Mean Sq F value  Pr(>F)
treat          3 1036.6   345.5   6.058 0.00164 **
stock          1   50.5    50.5   0.885 0.35223
treat:stock    3  625.2   208.4   3.654 0.02008 *
Residuals     41 2338.5    57.0
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
23 observations deleted due to missingness
> TukeyHSD(m)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = brood_size ~ treat * stock)

$treat
              diff       lwr       upr     p adj
B2-B14   10.19841270   1.558529 18.838297 0.0150670
R14-B14  -3.35714286 -11.146031  4.431745 0.6586410
R2-B14   -0.04945055  -7.838338  7.739437 0.9999982
R14-B2  -13.55555556 -22.324507 -4.786604 0.0009387
R2-B2   -10.24786325 -19.016815 -1.478912 0.0163565
R2-R14    3.30769231  -4.624123 11.239507 0.6815039

$stock
         diff       lwr       upr     p adj
WS-LS -2.07145 -6.543366 2.400466 0.3550196

$`treat:stock`
                    diff       lwr         upr     p adj
B2:LS-B14:LS   22.0000000   6.437258 37.56274215 0.0012633
R14:LS-B14:LS  -0.7500000 -16.312742 14.81274215 0.9999999
R2:LS-B14:LS    4.1000000 -10.499146 18.69914621 0.9847524
B14:WS-B14:LS   5.0000000  -8.020724 18.02072426 0.9193684
B2:WS-B14:LS    5.9000000  -8.699146 20.49914621 0.8973103
```

R14:WS-B14:LS -0.3888889 -13.095815 12.31803687 1.0000000
R2:WS-B14:LS 2.0000000 -11.020724 15.02072426 0.9996488
R14:LS-B2:LS -22.7500000 -39.798130 -5.70187013 0.0026988
R2:LS-B2:LS -17.9000000 -34.073276 -1.72672393 0.0209717
B14:WS-B2:LS -17.0000000 -31.764114 -2.23588645 0.0142928
B2:WS-B2:LS -16.1000000 -32.273276 0.07327607 0.0517927
R14:WS-B2:LS -22.3888889 -36.877013 -7.90076441 0.0003447
R2:WS-B2:LS -20.0000000 -34.764114 -5.23588645 0.0022281
R2:LS-R14:LS 4.8500000 -11.323276 21.02327607 0.9778843
B14:WS-R14:LS 5.7500000 -9.014114 20.51411355 0.9137176
B2:WS-R14:LS 6.6500000 -9.523276 22.82327607 0.8888179
R14:WS-R14:LS 0.3611111 -14.127013 14.84923559 1.0000000
R2:WS-R14:LS 2.7500000 -12.014114 17.51411355 0.9987664
B14:WS-R2:LS 0.9000000 -12.844642 14.64464171 0.9999989
B2:WS-R2:LS 1.8000000 -13.448311 17.04831091 0.9999396
R14:WS-R2:LS -4.4888889 -17.936635 8.95885730 0.9603599
R2:WS-R2:LS -2.1000000 -15.844642 11.64464171 0.9996609
B2:WS-B14:WS 0.9000000 -12.844642 14.64464171 0.9999989
R14:WS-B14:WS -5.3888889 -17.104096 6.32631780 0.8191915
R2:WS-B14:WS -3.0000000 -15.054848 9.05484823 0.9925059
R14:WS-B2:WS -6.2888889 -19.736635 7.15885730 0.8066691
R2:WS-B2:WS -3.9000000 -17.644642 9.84464171 0.9838243
R2:WS-R14:WS 2.3888889 -9.326318 14.10409558 0.9978045

Script 2 – *two-way ANOVA between stock, treatment, & residual mass*

> m<-aov(resid_mass~treat*stock);summary(m)

Df Sum Sq Mean Sq F value Pr(>F)

treat 3 0.4120 0.13734 2.335 0.0853 .

stock 1 0.0766 0.07663 1.303 0.2592

treat:stock 3 0.5001 0.16671 2.835 0.0477 *

Residuals 49 2.8817 0.05881

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

>TukeyHSD(m)

Tukey multiple comparisons of means

95% family-wise confidence level

Fit: aov(formula = resid_mass ~ treat * stock)

$treat

diff lwr upr p adj

B2-B14 0.18326365 -0.07045432 0.43698163 0.2327352
R14-B14 0.20327916 -0.02948257 0.43604088 0.1067899
R2-B14 0.06258019 -0.18118415 0.30634454 0.9031720
R14-B2 0.02001550 -0.22315064 0.26318165 0.9962471
R2-B2 -0.12068346 -0.37440143 0.13303451 0.5892220
R2-R14 -0.14069897 -0.37346069 0.09206276 0.3839237
$stock
diff lwr upr p adj
WS-LS -0.07474115 -0.2073356 0.0578533 0.2628267
$`treat:stock`
diff lwr upr p adj
B2:LS-B14:LS 0.37072664 -0.09410539 0.83555868 0.2096262
R14:LS-B14:LS 0.52761364 0.06278161 0.99244568 0.0160082
R2:LS-B14:LS 0.29869203 -0.14450792 0.74189199 0.4089672
B14:WS-B14:LS 0.23593160 -0.17864400 0.65050719 0.6221288
B2:WS-B14:LS 0.28047818 -0.14659998 0.70755635 0.4423824
R14:WS-B14:LS 0.25913203 -0.12469039 0.64295445 0.4066942
R2:WS-B14:LS 0.12142791 -0.29314769 0.53600351 0.9818536
R14:LS-B2:LS 0.15688700 -0.32861423 0.64238823 0.9686351
R2:LS-B2:LS -0.07203461 -0.53686665 0.39279742 0.9996574
B14:WS-B2:LS -0.13479505 -0.57241994 0.30282984 0.9759095
B2:WS-B2:LS -0.09024846 -0.53973525 0.35923833 0.9981589
R14:WS-B2:LS -0.11159461 -0.52020478 0.29701556 0.9878818
R2:WS-B2:LS -0.24929873 -0.68692362 0.18832616 0.6209504
R2:LS-R14:LS -0.22892161 -0.69375365 0.23591042 0.7715737
B14:WS-R14:LS -0.29168205 -0.72930694 0.14594284 0.4232726
B2:WS-R14:LS -0.24713546 -0.69662225 0.20235133 0.6615556
R14:WS-R14:LS -0.26848161 -0.67709178 0.14012856 0.4417455
R2:WS-R14:LS -0.40618573 -0.84381062 0.03143916 0.0867264
B14:WS-R2:LS -0.06276044 -0.47733603 0.35181516 0.9997065
B2:WS-R2:LS -0.01821385 -0.44529201 0.40886431 1.0000000
R14:WS-R2:LS -0.03956000 -0.42338242 0.34426242 0.9999780
R2:WS-R2:LS -0.17726412 -0.59183972 0.23731147 0.8732287
B2:WS-B14:WS 0.04454659 -0.35274736 0.44184054 0.9999609

R14:WS-B14:WS 0.02320044 -0.32717989 0.37358077 0.9999990
R2:WS-B14:WS -0.11450369 -0.49832611 0.26931873 0.9798627
R14:WS-B2:WS -0.02134615 -0.38643409 0.34374179 0.9999996
R2:WS-B2:WS -0.15905027 -0.55634422 0.23824368 0.9063956
R2:WS-R14:WS -0.13770412 -0.48808445 0.21267621 0.9142835

Script 3 - *2 -way ANOVA between stock, treatment, & log-transformed estradiol titers*

```
>m<-aov(log(est)~treat*stock);summary(m)
            Df Sum Sq Mean Sq F value  Pr(>F)
treat        3  8.014  2.6712   6.254 0.00273 **
stock        1  0.496  0.4958   1.161 0.29199
treat:stock  3  0.655  0.2185   0.512 0.67817
Residuals   24 10.251  0.4271
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

>TukeyHSD(m)
  Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = log(est) ~ treat * stock)

$treat
             diff       lwr       upr     p adj
B2-B14  -0.2587463 -1.3144084 0.7969159 0.9050986
R14-B14  1.0160084  0.1585649 1.8734519 0.0160143
R2-B14   0.6543435 -0.2787389 1.5874258 0.2407170
R14-B2   1.2747547  0.3150951 2.2344143 0.0062784
R2-B2    0.9130898 -0.1147136 1.9408931 0.0942414
R2-R14  -0.3616649 -1.1845662 0.4612364 0.6252018

$stock
          diff       lwr       upr     p adj
WS-LS 0.2464039 -0.2391004 0.7319082 0.3053164

$`treat:stock`
                    diff       lwr       upr     p adj
```

```
B2:LS-B14:LS   0.19661473 -1.77929235 2.1725218 0.9999725
R14:LS-B14:LS  1.19825592 -0.67625412 3.0727660 0.4326021
R2:LS-B14:LS   1.18536528 -0.68914477 3.0598753 0.4458186
B14:WS-B14:LS  0.65919435 -1.15175440 2.4701431 0.9224332
B2:WS-B14:LS   0.23534499 -1.92915276 2.3998427 0.9999498
R14:WS-B14:LS  1.63116434 -0.08002139 3.3423501 0.0694820
R2:WS-B14:LS   1.06502791 -0.80948213 2.9395380 0.5751365
R14:LS-B2:LS   1.00164119 -0.65152128 2.6548037 0.4980750
R2:LS-B2:LS    0.98875054 -0.66441192 2.6419130 0.5138167
B14:WS-B2:LS   0.46257962 -1.11814604 2.0433053 0.9746572
B2:WS-B2:LS    0.03873026 -1.93717682 2.0146373 1.0000000
R14:WS-B2:LS   1.43454960 -0.03082231 2.8999215 0.0580511
R2:WS-B2:LS    0.86841318 -0.78474929 2.5215756 0.6631087
R2:LS-R14:LS  -0.01289064 -1.54342168 1.5176404 1.0000000
B14:WS-R14:LS -0.53906156 -1.99105080 0.9129277 0.9148493
B2:WS-R14:LS  -0.96291093 -2.83742097 0.9115991 0.6864623
R14:WS-R14:LS  0.43290842 -0.89257035 1.7583872 0.9546599
R2:WS-R14:LS  -0.13322801 -1.66375905 1.3973030 0.9999890
B14:WS-R2:LS  -0.52617092 -1.97816016 0.9258183 0.9240727
B2:WS-R2:LS   -0.95002028 -2.82453033 0.9244898 0.7001069
R14:WS-R2:LS   0.44579906 -0.87967970 1.7712778 0.9473638
R2:WS-R2:LS   -0.12033737 -1.65086841 1.4101937 0.9999945
B2:WS-B14:WS  -0.42384936 -2.23479811 1.3870994 0.9929438
R14:WS-B14:WS  0.97196998 -0.26198359 2.2059236 0.2010589
R2:WS-B14:WS   0.40583355 -1.04615568 1.8578228 0.9803266
R14:WS-B2:WS   1.39581934 -0.31536638 3.1070051 0.1702101
R2:WS-B2:WS    0.82968292 -1.04482713 2.7041930 0.8172841
R2:WS-R14:WS  -0.56613643 -1.89161519 0.7593423 0.8418427
```

Script 4 - Pearson's correlation between estradiol titers & GSI (nonbrooders, stocks combined)

4a Correlation in non-brooders (R2 and R14, stocks combined):

```
> cor.test(log(est),GSI)

        Pearson's product-moment correlation

data:  log(est) and GSI
t = 1.5242, df = 17, p-value = 0.1458
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
```

-0.1275604 0.6919685
sample estimates:
cor 0.3467386

4b Correlation in brooders (B2 and B14, stocks combined):

> cor.test(log(est),GSI)

Pearson's product-moment correlation

data: log(est) and GSI
t = 0.6566, df = 10, p-value = 0.5263
alternative hypothesis: true correlation is not equal to 0
95 percent confidence interval:
-0.4195533 0.6959963
sample estimates:
cor 0.2032991

Script 5 – *two-way ANOVA between GSI, stock, & treatment group*

---

m<-aov(GSI~stock*treat);summary(m)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
stock	1	0.17	0.173	0.117	0.734
treat	3	86.98	28.994	19.683	1.85e-08 ***
stock:treat	3	4.19	1.396	0.948	0.425
Residuals	48	70.71	1.473		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> TukeyHSD(m)

Tukey multiple comparisons of means
95% family-wise confidence level

Fit: aov(formula = GSI ~ stock * treat)

$stock

	diff	lwr	upr	p adj
WS-LS	0.1136967	-0.5540039	0.7813974	0.733565

$treat

	diff	lwr	upr	p adj
B2-B14	0.3214319	-0.9492682	1.592132	0.9067036
R14-B14	2.8806157	1.6985335	4.062698	0.0000003
R2-B14	0.1461207	-1.0747285	1.366970	0.9886760
R14-B2	2.5591838	1.3256830	3.792685	0.0000078
R2-B2	-0.1753112	-1.4460113	1.095389	0.9828821
R2-R14	-2.7344950	-3.9165771	-1.552413	0.0000009

$`stock:treat`

	diff	lwr	upr	p adj
WS:B14-LS:B14	0.32258251	-1.7541140	2.3992790	0.9996489
LS:B2-LS:B14	0.91461958	-1.4138220	3.2430611	0.9140427
WS:B2-LS:B14	0.21604598	-1.9232785	2.3553705	0.9999807
LS:R14-LS:B14	3.60679118	1.2783496	5.9352327	0.0002784
WS:R14-LS:B14	2.83785200	0.8862910	4.7894130	0.0007454
LS:R2-LS:B14	0.07410868	-2.1459732	2.2941906	1.0000000
WS:R2-LS:B14	0.52271222	-1.5539843	2.5994087	0.9924748
LS:B2-WS:B14	0.59203706	-1.6001182	2.7841923	0.9885663
WS:B2-WS:B14	-0.10653654	-2.0966656	1.8835925	0.9999998
LS:R14-WS:B14	3.28420867	1.0920534	5.4763639	0.0004735
WS:R14-WS:B14	2.51526948	0.7285141	4.3020248	0.0011952
LS:R2-WS:B14	-0.24847383	-2.3251703	1.8282227	0.9999388
WS:R2-WS:B14	0.20012970	-1.7225176	2.1227770	0.9999762
WS:B2-LS:B2	-0.69857360	-2.9501476	1.5530004	0.9747473
LS:R14-LS:B2	2.69217160	0.2601937	5.1241495	0.0205357
WS:R14-LS:B2	1.92323242	-0.1507653	3.9972301	0.0871782
LS:R2-LS:B2	-0.84051090	-3.1689525	1.4879307	0.9434267
WS:R2-LS:B2	-0.39190736	-2.5840626	1.8002479	0.9991187
LS:R14-WS:B2	3.39074520	1.1391712	5.6423192	0.0004367
WS:R14-WS:B2	2.62180602	0.7626300	4.4809821	0.0011658
LS:R2-WS:B2	-0.14193730	-2.2812618	1.9973872	0.9999989

WS:R2-WS:B2 0.30666624 -1.6834628 2.2967953 0.9996670
WS:R14-LS:R14 -0.76893918 -2.8429369 1.3050585 0.9352512
LS:R2-LS:R14 -3.53268250 -5.8611241 -1.2042409 0.0003885
WS:R2-LS:R14 -3.08407896 -5.2762342 -0.8919237 0.0012056
LS:R2-WS:R14 -2.76374332 -4.7153043 -0.8121824 0.0010974
WS:R2-WS:R14 -2.31513978 -4.1018951 -0.5283844 0.0036178
WS:R2-LS:R2 0.44860354 -1.6280929 2.5253000 0.9970554