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**Expression of heat shock proteins and glucocorticoid receptors
in response to metabolic and reproductive stressors in the
African cichlid fish, *Astatotilapia burtoni***

by

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ABSTRACT

Organisms constantly experience stress in their natural lives, and several mechanisms exist to respond and adapt to these stressors among various species. In particular, there are multiple types of stress responses, which includes both endocrine and cellular changes. The liver plays an important role in regulating the stress response, along with the expression of heat shock proteins (HSPs) and glucocorticoid receptors (GRs) in the body. Little is known, however, about how the expression of HSPs and GRs may change with an animal's reproductive and metabolic state. In this study, molecular approaches were used to examine how the expression of heat shock proteins and glucocorticoid receptors differ in response to metabolic stressors, such as mouthbrooding, in the female cichlid *Astatotilapia burtoni*. Female *A. burtoni* undergo an extreme form of parental care known as mouthbrooding in which they hold their developing young in their mouths for up to two weeks, during which they undergo forced starvation. To examine stress induced by these reproductive processes, cichlids at various stages of the reproductive cycle that also differ in metabolic needs (mouthbrooding and gravid, as well as fed and starved) were collected. Quantification of heat shock proteins (*hsp70*) and glucocorticoid receptors (*gr1* and *gr2a*) in liver samples measured by qPCR revealed that mouthbrooding females had higher expression levels, particularly when compared to gravid females. In addition, mouthbrooders had a positive correlation between the expression of heat shock proteins and glucocorticoid receptors. These results indicate that although part of their natural lifestyle, mouthbrooding induces stress that seems to differ from the stress induced by starvation alone and caring for developing young in the mouth may provide added demands on female physiology. Thus, not only are heat shock proteins and glucocorticoid receptors involved in the general stress response, but they are also affected by reproduction and metabolic condition. This study provides a better understanding of how animals cope with the costs and challenges of reproduction and changing energetic needs, which is important for all living organisms.

INTRODUCTION

Organisms are regularly exposed to various stressors, and how they respond to stress helps them to adapt and survive under constantly changing conditions. Many mechanisms evolved to assist organisms in responding to environmental, physiological, and metabolic stressors and to overcome them (Barton, 2002; Wingfield *et al.*, 1998). Stress is typically defined as an organism's physiological, biological, and psychological response to a 'stressor,' which can result from environmental, external, or internal challenges or demands. The stress response can be categorized into primary and secondary responses. The primary stress response includes endocrine changes, such as the release of catecholamines and corticosteroids, while the secondary stress response involves physiological adaptations such as metabolic, cellular, and hematological changes (Barton, 2002). While these stress responses are common across vertebrates, less is known about the specific mechanisms and molecules that are involved to counteract these stressors to maintain homeostasis.

One important organ crucial in the regulation of both the primary and secondary stress response is the liver. Gluconeogenesis, or glucose synthesis, primarily occurs in the liver and provides glucose to other tissues during periods of stress (Aluru and Vijayan, 2009). This process is activated by stress-induced plasma cortisol, which then binds to glucocorticoid receptors (GRs) expressed in the liver and other tissues to exert physiological changes (Hernández-Pérez *et al.*, 2019). In addition, exposure to stress can result in changes in the expression of numerous genes in the liver. A transcriptome analysis of the liver in the estuarine tapertail anchovy (*Coilia nasus*) revealed that nearly 3,000 genes were upregulated in response to loading stress (Du *et al.*, 2014), suggesting that stressors can cause dramatic changes in liver function.

Heat shock proteins (HSPs) are a group of highly conserved cellular proteins synthesized in response to a diverse range of stressors (Basu *et al.*, 2002). They are found in virtually all living organisms, including fishes, and may help animals survive periods of stress. Three major families of heat

shock proteins are described and categorized based on molecular weight: HSP70 (68-73 kDa), HSP90 (85-90 kDa), and low molecular weight HSPs (16-47 kDa) (Demeke and Tassew, 2016). Each class of heat shock protein varies in localization and function. HSP70 and HSP90 are localized in the liver (Mohanty *et al.*, 2018) and act as molecular chaperones that are involved in protein homeostasis (Genest *et al.*, 2019). Specifically, heat shock proteins regulate the folding of proteins and control misfolded or denatured proteins. Heat shock proteins are implicated in regulating a response to various stressors among fish species. For example, in the doctor fish, *Garra rufa*, the expression of multiple heat shock proteins was increased in the muscle of fish living in elevated temperatures as compared to those living in normal rivers (Oksala *et al.*, 2014). Furthermore, exposure to heavy metals increases HSP expression in the liver of the common carp, *Cyprinus carpio* (Jiang *et al.*, 2015). While some studies also examined the role of heat shock proteins in response to food deprivation (Antonopoulou *et al.*, 2013), there is still a gap in our knowledge of the role HSPs play in the context of stress associated with starvation and reproductive condition.

Another key aspect of the stress response is glucocorticoid receptors, which are a group of nuclear receptors that bind glucocorticoids (GCs) such as cortisol and then act as transcription factors to regulate other gene expression pathways. While three types of GRs are described in most fish species (GR1, GR2, and MR [mineralocorticoid receptor]), GR1 and GR2 are largely involved during periods of stress (Barr and Dokas, 1999; Teles *et al.*, 2013). In addition, the GR subtypes have a lower affinity to cortisol than MR. In the cytoplasm of target cells, GRs associate with HSPs to form multiprotein complexes (Filipović *et al.*, 2007). In particular, *hsp70* and *hsp90* assist in the folding of the binding domain of GRs (Basu *et al.*, 2003), and *hsp70* plays an important role in regulating the proteome by controlling unfolded and denatured proteins (Juhász *et al.*, 2014). While GCs are implicated in several aspects of reproductive physiology, little is known about the exact role that these hormones and GRs

have on liver function during reproductive processes and metabolic trade-offs associated with reproduction and parental care (Whirledge and Cidlowski, 2017).

Reproduction is a costly, yet fundamental, process that contributes to stress in living organisms (Ruhland *et al.*, 2016; Saweck *et al.*, 2019). Parental care, defined as behaviors performed by one or both parents that contribute to offspring fitness, often follows reproduction and is a major energy investment (Smith and Wootton, 1995). While it increases offspring survival, providing parental care to offspring can have detrimental effects on the parent, including loss of body weight and less successful reproductive attempts in the future (Balshine-Earn, 1995; Zięba *et al.*, 2018). Maternal mouthbrooding is one of the most intensive forms of parental care and exists in ~53 genera of fish species. Mouthbrooders will hold their developing young in their mouths, providing care at the cost of their own health because they do not feed during this time, which can last from days to weeks depending on the species. Therefore, it is important for organisms to adopt ways to endure the challenges that come with reproduction and parental care. Heat shock proteins and glucocorticoid signaling, which are involved in the stress response, may aid organisms in adapting to reproductive and parental care related stressors.

The African cichlid, *Astatotilapia burtoni*, is an ideal model organism to study the expression of heat shock proteins and glucocorticoid receptors in response to reproductive and metabolic stressors because females undergo different reproductive states that may contribute to stress in their natural lifestyles. Female *A. burtoni* cycle through three primary stages of reproduction: gravid, brooding, and recovering (Figure 1). Gravid (ripe with eggs) females are sexually receptive and prepare for spawning with dominant males. Following spawning, females care for the developing embryos inside their mouths for approximately two weeks, a process known as mouthbrooding. Typically, *A. burtoni* females do not eat for the entire period of mouthbrooding. Therefore, this forced starvation period may be a major metabolic stressor in female cichlids. Following the brood period and fry release, females undergo a recovery phase when they resume eating and begin investing in egg growth for the next spawning cycle.

The liver functions as an energy reservoir for energetically costly processes, such as reproduction, which includes production of vitellogenin protein needed for yolk accumulation in developing oocytes (vitellogenesis). Thus, it is important to examine the role of the liver in response to the stress that comes with reproduction. Because little is known about the role of heat shock proteins and glucocorticoid signaling in these contexts in cichlid fishes, this study provides insights on the cellular response to metabolic stressors and how they allow animals to adapt and survive stressful periods in their lives.

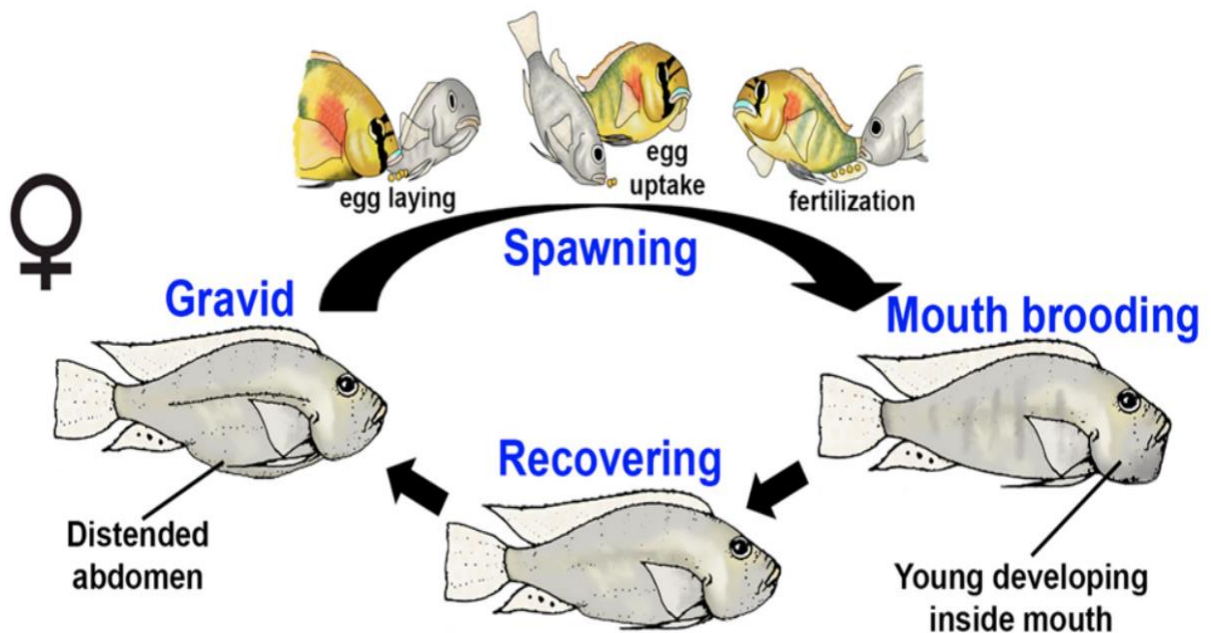


Figure 1. Overview of the female reproductive cycle in the African cichlid fish, *Astatotilapia burtoni*. Gravid females have a distended abdomen holding ripe eggs and are ready to be courted by dominant males. Dominant males actively court gravid and ovulated females. Following spawning, females undergo mouthbrooding, a process in which the developing embryos are held in the buccal cavity of the female for ~2 weeks, and the female does not eat. Fully developed young are released, and the female undergoes a recovering stage until she is ready to reproduce again. Adapted from Maruska & Fernald (2018).

The goal of this study was to examine how the expression of heat shock protein and glucocorticoid receptor genes in the liver responds to the stress caused by mouthbrooding-induced starvation in the female cichlid fish *A. burtoni*. Specifically, I test the hypothesis that mouthbrooding

stimulates the expression of heat shock proteins and glucocorticoid receptors to help overcome the metabolic challenges of reproduction, parental care, and starvation. To determine the effect of reproduction on *hsp* and *gr* gene expression, liver samples from mouthbrooding, gravid, fed, and starved *A. burtoni* females were obtained. Mouthbrooding females were cichlids that kept the developing embryos inside their mouths for the duration of the experiment, fed females were brooding females whose eggs were removed from the mouth and returned to a daily feeding schedule, and starved females were brooding cichlids whose eggs were removed and then deprived of food for the duration of the experiment. Quantitative PCR was performed to compare mRNA levels of two major families of heat shock proteins, *hsp70* and *hsp90*. In addition, expression levels of two glucocorticoid receptors (*gr1* and *gr2a*) were measured to further examine their relationship with heat shock proteins in the stress response. These results provide support that heat shock proteins and glucocorticoid receptors play an important role in regulating stress caused by forced starvation and overcoming the significant energy investment required for reproduction in *A. burtoni*.

MATERIALS & METHODS

Experimental Animals

African cichlid fish, *Astatotilapia burtoni*, were derived from a wild-caught population from Lake Tanganyika in East Africa and raised in a laboratory environment that resembles their natural habitat (approximately 28°C and pH 8.0). The fish tanks contain gravel at the bottom with halved terra-cotta pots that serve as territory shelters and is maintained on a 12-hour light/12-hour dark cycle. Fish in community tanks were fed cichlid flakes daily and supplemented with brine shrimp weekly. All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University, Baton Rouge, LA and were performed in accordance with the guidelines set by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, 2011.

To examine how liver HSP expression may differ across the female reproductive cycle, fish were collected from the following groups: mouthbrooding, gravid, fed, and starved. These fish were all collected as part of separate studies, but I used the collected livers for my analyses of *hsp* and *gr* expression. Females in the mouthbrooding condition were kept with brood intact inside their mouths. Females in the fed condition had their brood removed from their mouths 1 day after spawning and were then fed cichlid flakes every morning for 12 days (the same time as a normal brood period). Females in the starved condition had their brood removed from their mouths 1 day after spawning and were then not fed for 12 days (the same time as a normal brood period). Gravid females were selected based on the presence of a distended abdomen and gravidity was later confirmed after fish collection. For collections, fish were netted from their aquaria, anesthetized and immobilized in ice cold cichlid system water, and then sacrificed with rapid cervical transection. Fish were measured for standard length (SL) and weighed for body mass (BM) and gonad mass (GM), which were used to calculate the gonadosomatic index (GSI) as a measure of reproductive investment [$GSI = (GM / BM) * 100$]. Livers were also weighed, collected, and immediately frozen at -80°C until RNA extractions. Liver mass and body mass were also used to calculate the hepatosomatic index (HSI) as an indicator of energy reserves in fishes [$(\text{liver mass} / \text{body mass}) * 100$]. In addition, Fulton's condition factor was calculated using the formula $K = [BM / (SL^3)] * 100$.

Tissue Collection & mRNA Isolation

Total RNA from liver tissue samples obtained from prior experiments and stored at -80°C was isolated using the standard protocol from Qiagen RNeasy Plus Mini Kit, which includes a step to remove genomic DNA, according to the manufacturer's instructions. RNA was eluted with 100 μL of RNase-free water, concentrations and purity were measured with a Nanodrop spectrophotometer, and the RNA was stored at -80°C until cDNA synthesis.

cDNA Synthesis

Equivalent amounts of RNA (4.7 ng/ μ L) from each liver were put into the cDNA synthesis reaction using the qScript SuperMix (Quantabio). A T100 BioRad Thermocycler was used with the following protocol: 25°C for 5 minutes, 42°C for 60 minutes, 85°C for 5 minutes, and held at 4°C. Dilutions of the 20 μ L synthesized cDNA solutions were made using 17 μ L of the cDNA solution and 34 μ L of nuclease-free water and stored at -20°C until qPCR.

qPCR

To quantify expression levels of two heat shock protein genes (*hsp70* and *hsp90*) and glucocorticoid receptor genes (*gr1* and *gr2a*) in *A. burtoni* females, quantitative polymerase chain reaction (qPCR) was performed on the liver samples from mouthbrooding, gravid, starved, and fed females. Levels of the reference gene *β -actin* were also measured in each sample to use for normalization. Forward and reverse primers for *hsp70*, *hsp90*, and the glucocorticoid receptors were created based on available *A. burtoni* sequences in NCBI GenBank and were commercially synthesized (Invitrogen) (Table 1). The glucocorticoid receptors *gr1* and *gr2a* were chosen because these receptors are identified in most teleost fishes (Greenwood *et al.*, 2003). qPCR was run on 20 μ L duplicate reactions of cDNA template, gene-specific primers, and Quantabio Sybr green fast mix using a BioRad CFX Connect Real-Time System using the following conditions: 3 minutes at 95°C followed by 45 cycles of 95°C, 60°C, and 72°C for 30 seconds each, ending with a melting curve analysis starting at 95°C and decreasing by 0.5°C each cycle until it reaches 50°C.

qPCR results were analyzed using the PCR Miner software (Zhao and Fernald, 2005). The levels of the reference gene *β -actin* did not differ among condition groups (ANOVA, $p > 0.05$), indicating that it is appropriate for this study. The mRNA levels of *hsp70*, *hsp90*, *gr1*, and *gr2a* were determined by normalizing them to the reference gene *β -actin*, using the following formula: relative target gene mRNA

levels = $[1/(1+E_{\text{target}})^{\text{CT}_{\text{target}}}] / [1/(1+E_{\text{geomean}})^{\text{CT}_{\text{geomean}}}]$, where E is the average efficiency of the gene (efficiencies > 0.85) and CT is the average cycle threshold of the replicate sample. Another reference gene, eukaryotic translation elongation factor 1 alpha 1 (*ef1a*), was initially used to determine mRNA levels of the target genes but was later replaced with *β-actin* due to difficulties in obtaining expression levels in all liver samples.

Table 1: Primer sequences used for qPCR of *hsp70*, *hsp90*, glucocorticoid receptors, and *β-actin*

Gene	Forward (5'→3')	Reverse (5'→3')	Product Size (bp)
<i>hsp70</i>	GTGCAGAACGCAGAGAAA	TCTCGACCACCTTCTTCTT	163
<i>hsp90</i>	CTTGGGAAAGCGGTCAAT	GTGACCCTTGGCTCTTTATC	185
<i>gr1</i>	TGCCTCTGTCCTGCCACCGTAG	AGTCGTCTGCGTCTGAAGTAACTG	109
<i>gr2a</i>	CATCAGAGCCCACCATTAGCAACA	GGTTCTATGGCCTTCAGTATGGA	103
<i>β-actin</i>	AAGATGAAATCGCCGCACT	GGGTACTTCAGGGTCAGGATA	205

Statistical Analysis

To compare the expression levels of *hsp70*, *hsp90*, *gr1*, and *gr2a* among mouthbrooding, gravid, starved, and fed female groups, a one-way ANOVA and post-hoc Tukey test was used. All data sets were checked for outliers using Iglewicz and Hoaglin's Two-Sided Test (*conchart.com*), and outliers were removed prior to statistical testing. Due to low sample sizes in some groups that reduces the power of detecting differences with ANOVA, I also performed t-tests between 2 variables in some cases to further explore the data. Correlations between variables (e.g., gene expression levels, GSI, HSI, condition factor) were also tested with Pearson correlations. Statistical significance was determined at $p < 0.05$.

RESULTS

Expression of heat shock proteins

To examine how heat shock proteins are expressed in female cichlids among different reproductive and metabolic states, qPCR for *hsp70* was used. I initially planned to also measure *hsp90*, but this gene did not amplify successfully in many liver samples, so I was unable to use it for comparison. Overall, there were no significant differences in *hsp70* expression among mouthbrooding, fed, gravid, and starved females (one-way ANOVA, $F_{(3,23)} = 1.621$, $p = 0.212$). Because some groups had low sample sizes, I also performed two-way comparisons between groups with t-tests to further explore the data. From these tests, mouthbrooding females had significantly higher relative mRNA levels of *hsp70* compared to gravid females (Figure 2; t-test, $t = 2.3531$, $p = 0.03811$). Gravid females also had significantly higher *hsp70* expression levels than starved females (Figure 2; t-test, $t = 2.4173$, $p = 0.04657$).

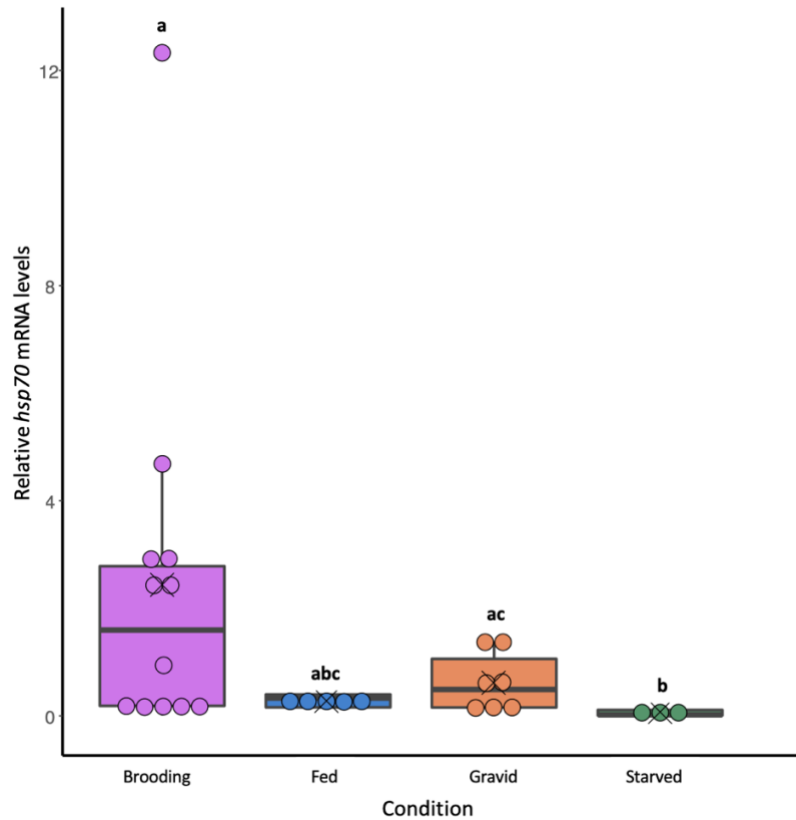


Figure 2. Relative *hsp70* mRNA levels of mouthbrooding, fed, gravid, and starved *A. burtoni* females. Mouthbrooding and gravid females had significantly higher levels of *hsp70* than starved females. Data is represented in box plots, with solid lines indicating the mean and box edges indicating the 25th/75th percentiles. Whiskers indicate the 10th/90th percentiles, and dots indicate 5th/95th percentiles. Different letters represent statistical significance at $p > 0.05$. Sample sizes: brooding = 12, fed = 5, gravid = 7, starved = 3.

Expression of glucocorticoid receptors

Relative expression levels of glucocorticoid receptors, *gr1* and *gr2a*, were also measured using qPCR. Levels of *gr1* were higher in mouthbrooding females compared to gravid females (ANOVA, $F_{(3,23)} = 4.013$, $p = 0.0196$), but there were no significant differences among other conditions. After pairwise comparisons, mouthbrooding females also had higher *gr1* expression levels than starved females (Figure 3A; t-test, $t = 2.7546$, $p = 0.01843$). Fed females had higher levels of *gr1* than gravid (t-test, $t = 7.2839$, $p = 0.003769$) and starved females (t-test, $t = 5.6075$, $p = 0.002646$).

Overall, there were no significant differences in the expression of *gr2a* among all conditions (ANOVA, $F_{(3,30)} = 2.052$, $p = 0.128$). However, mouthbrooding females had higher levels of *gr2a* than gravid females when both groups were compared (Figure 3B; t-test, $t = 2.4091$, $p = 0.0302$). Pairwise comparisons between all other conditions revealed no significant differences in *gr2a* expression.

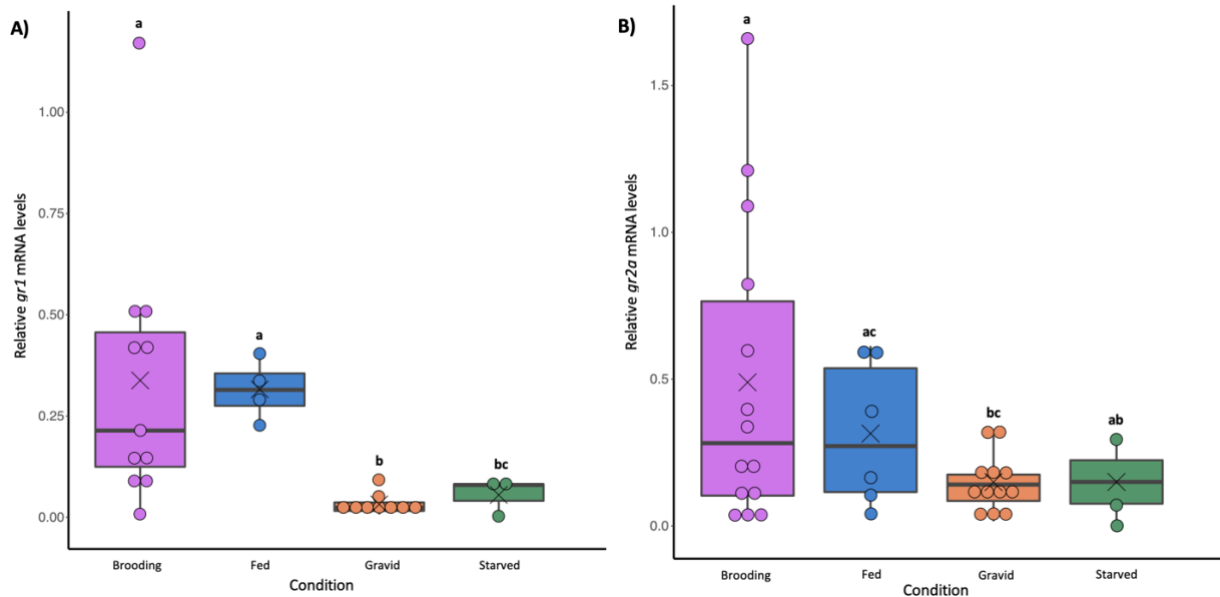


Figure 3. Relative *gr1* and *gr2a* mRNA levels of mouthbrooding, fed, gravid, and starved *A. burtoni* females. A) Mouthbrooding and fed females had higher expression of *gr1* than gravid and starved females. Sample sizes: brooding = 11, fed = 4, gravid = 9, starved = 3. B) Mouthbrooding females had higher expression of *gr2a* than gravid females. Data is represented in box plots. Solid lines indicate the mean, and box edges indicate the 25th/75th percentiles. Whiskers indicate the 10th/90th percentiles, and dots indicate the 5th/95th percentiles. Different letters represent statistical significance at $p > 0.05$. Sample sizes: brooding = 14, fed = 6, gravid = 12, starved = 3.

Relationship Between HSPs and GRs

There was a positive correlation between *hsp70* and *gr1* in mouthbrooding females (Figure 4A; $r = 0.970649$, $p = 6.183e-05$) but not in fed, gravid, or starved females ($r_{\text{Fed}} = -0.329535$, $p_{\text{Fed}} = 0.6705$, $r_{\text{Gr}} = -0.705132$, $p_{\text{Gr}} = 0.5018$, $r_{\text{St}} = -0.250117$, $p_{\text{St}} = 0.8391$). In addition, there was a negative correlation in the expression of *hsp70* and *gr2a* in fed females but no other correlations among other conditions (Figure 4B; $r_{\text{Fed}} = -0.936439$, $p_{\text{Fed}} = 0.01905$, $r_{\text{Br}} = 0.4425875$, $p_{\text{Br}} = 0.2002$, $r_{\text{Gr}} = 0.2510241$, $p_{\text{Gr}} = 0.6838$). There

was no correlation between *gr1* and *gr2a* within any female group (Figure 4C; $r_{Br} = 0.5485289$, $p_{Br} = 0.08059$, $r_{Fed} = 0.4178357$, $p_{Fed} = 0.5822$, $r_{Gr} = 0.2698951$, $p_{Gr} = 0.518$).

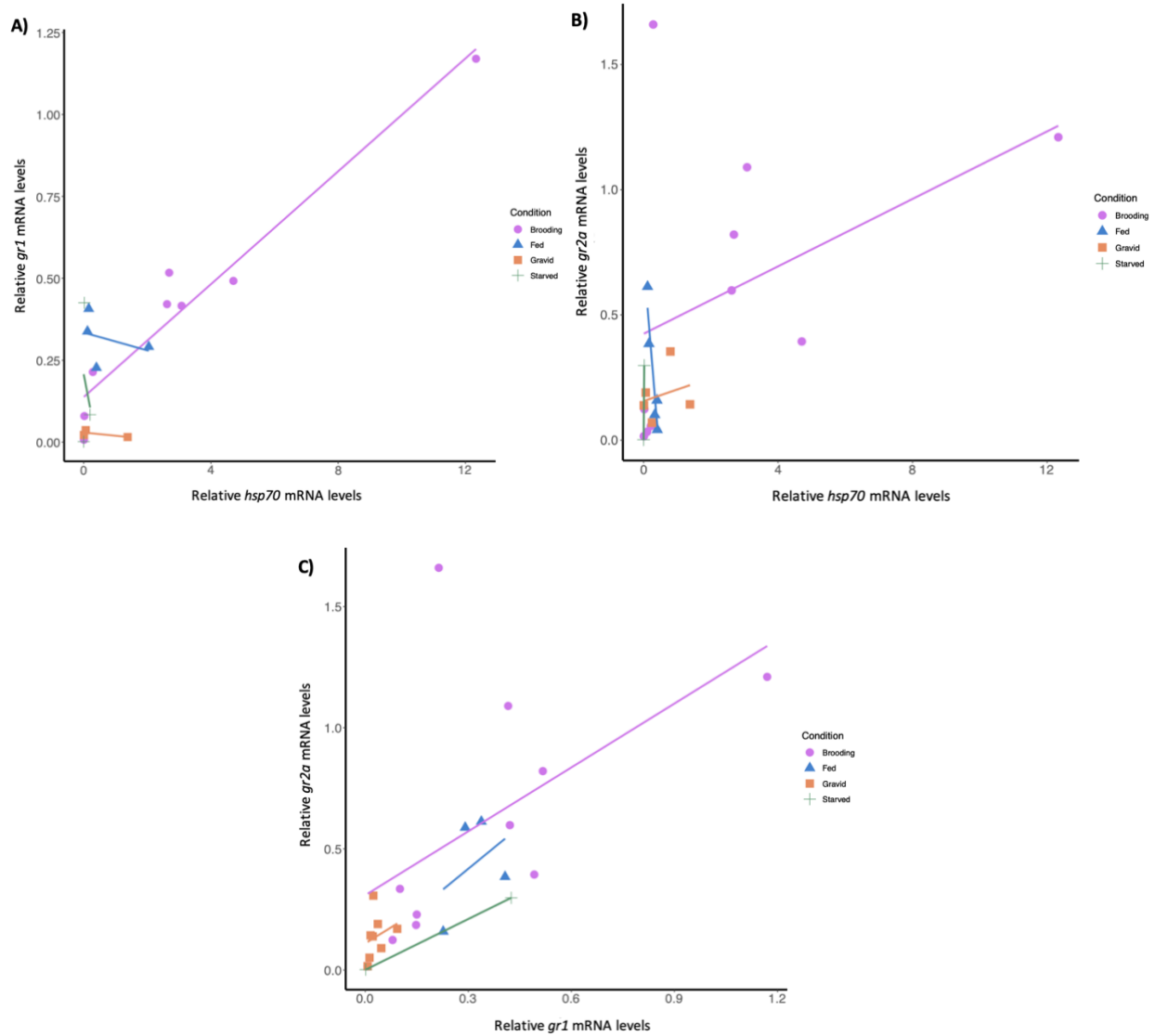


Figure 4. Correlations between *hsp70*, *gr1*, and *gr2a* in mouthbrooding, fed, gravid, and starved females. A) Levels of *gr1* and *hsp70* are positively correlated with each other in mouthbrooding females. B) Levels of *gr2a* and *hsp70* are positively correlated with each other in fed females. C) Levels of *gr1* are not correlated with levels of *gr2a* in any group. Points indicate individual fish shown with regression lines.

GSI, HSI, and Condition Factor

Gravid females had a higher gonadosomatic index (Figure 5A; ANOVA, $F_{(3,42)} = 70.81$, $p < 2e-16$) and hepatosomatic index (Figure 5B; ANOVA, $F_{(3,42)} = 13.77$, $p = 2.15e-06$) than all other conditions. In addition, gravid females had a higher condition factor than mouthbrooding and starved females (Figure 5C; ANOVA, $F_{(3,37)} = 6.938$, $p = 0.000805$). There were no correlations between *hsp70* and GSI (t-test, $t = -0.80237$, $p = 0.4299$), HSI (t-test, $t = -1.6309$, $p = 0.1154$), or condition factor (t-test, $t = -0.67197$, $p = 0.5078$) across all conditions. There was a negative correlation between *gr1* and GSI ($r = -0.4201483$, $p = 0.02911$) and between *gr1* and HSI ($r = -0.4056091$, $p = 0.03581$). On the other hand, there was no correlation between *gr1* and condition factor ($r = -0.2015352$, $p = 0.3134$). Finally, there were no correlations between *gr2a* levels and GSI, HSI, or condition factor (all $p > 0.05$).

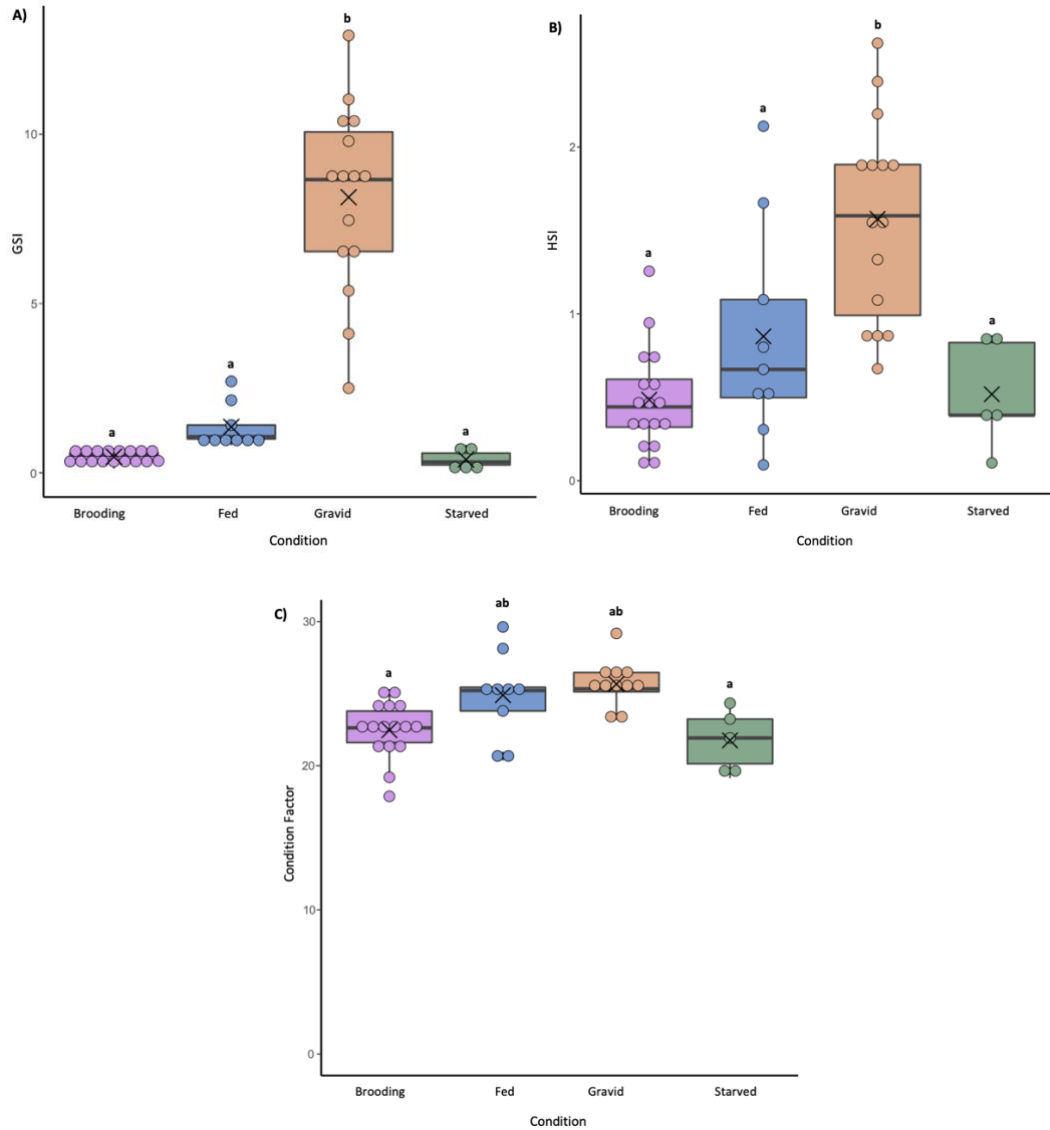


Figure 5. Measures of GSI, HSI, and condition factor in mouthbrooding, fed, gravid, and starved females. A) Gravid females have a higher GSI than all other conditions. Sample sizes: brooding = 17, fed = 9, gravid = 15, starved = 5. B) Gravid females have a higher HSI than all other conditions. Sample sizes: brooding = 17, fed = 9, gravid = 15, starved = 5. C) Gravid females have a higher condition factor than mouthbrooding and starved females. Sample sizes: brooding = 16, fed = 9, gravid = 11, starved = 5. Data is represented in box plots. Solid lines indicate the mean, and box edges indicate the 25th/75th percentiles. Whiskers indicate the 10th/90th percentiles, and dots indicate the 5th/95th percentiles. Different letters represent statistical significance at $p > 0.05$.

DISCUSSION

Through quantification of heat shock proteins and glucocorticoid receptors, this study examined how the female cichlid fish, *Astatotilapia burtoni*, responds to the stress induced by reproduction and metabolic demands. While mouthbrooding females had higher expression levels of *hsp70* than gravid females, gravid females also had elevated levels of *hsp70* compared to starved females (Figure 2). Although gravid females do not experience the stress induced by mouthbrooding, there may be other factors that contribute to stress in this reproductive state, such as making the yolk for developing eggs, which involves the liver, competition amongst females for mating opportunities, and dealing with chases and courtship attempts from courting males. Furthermore, the differences in *hsp70* expression levels among mouthbrooding, gravid, and starved females suggest that both reproduction and the parental care phase may be triggers in elevating levels of heat shock proteins in cichlids. Heat shock proteins, particularly *hsp70*, provide protection against stressors by preventing apoptosis of cells through the inhibition of caspases (Jäättelä, 1999; Li *et al.*, 2000). Considering this and the upregulated levels of *hsp70* in mouthbrooding females, the induction of heat shock proteins may help animals survive periods of stress by directly protecting cells against various stressors.

The expression of two glucocorticoid receptors, *gr1* and *gr2a*, were also measured to understand their relationship with heat shock proteins in the stress response. Mouthbrooding females had higher levels of *gr1* than gravid and starved females and higher levels of *gr2a* than gravid females (Figure 3A, B). Additionally, fed females had increased expression of *gr1* compared to gravid females. A study in zebrafish showed that the knockout of glucocorticoid receptors causes problems in reproduction, such as reduced fertility (Maradonna *et al.*, 2020). Considering the increased expression of *gr1* and *gr2a* in mouthbrooding females, as well as the findings from previous studies, glucocorticoid receptors in the liver may play a role in reproductive processes. Further, because these mouthbrooding females are in a starvation state, upregulation of glucocorticoid receptors in the liver may help to

mobilize glucose reserves during this metabolically stressful time to maintain homeostatic processes or processes related to brood care such as churning the developing eggs in the mouth. It would be interesting to measure levels of circulating cortisol in these same animals as a measure of the stress response and to test whether cortisol levels are correlated with expression of glucocorticoid receptors in the liver.

Although both mouthbrooding and starved females were deprived of food prior to collecting the liver samples, elevated expression levels of either *hsp70* or *gr1* and *gr2a* were only seen in mouthbrooding females. This suggests that it may not be the starvation itself that causes upregulation of these genes, but there are additional stressors faced by the parental mouthbrooding females. Similarly, a previous study in *A. burtoni* showed that some (e.g., circulating sex-steroids), but not all (e.g., brain gene expression), of the physiological changes that occur during mouthbrooding are consequences of the food deprivation (Grone *et al.*, 2012). Using ps6 immunohistochemical analysis also revealed a difference in neural activation patterns in brain regions closely associated with maternal care between mouthbrooding and food-deprived females (Maruska *et al.*, 2020), further indicating that maternal care-related factors play a larger role than mouthbrooding-induced starvation. Thus, there are likely different functions of both heat shock proteins and glucocorticoid receptors in the liver that are associated with starvation versus maternal demands.

There were no correlations between the two glucocorticoid receptors, but correlations between *hsp70* and *gr1* and *gr2a* were identified in some of the female groups. While the role of both heat shock proteins and glucocorticoid receptors in the stress response was previously established, these findings suggest that they also work together in producing the response. A similar relationship between *hsp70* and glucocorticoid receptors is found in the rainbow trout, particularly that the receptor heterocomplex in liver tissue contains bound *hsp70* (Basu *et al.*, 2003). There was a direct relationship between cortisol levels and the ratio of *hsp70* and glucocorticoid receptors, suggesting that exposure to stress triggers

the expression of both heat shock proteins and glucocorticoid receptors. Furthermore, glucocorticoid receptors may play a role in increasing the cellular stress threshold by causing an increase in *hsp70* and *hsp90* (Vijayan *et al.*, 2003), which could ultimately help animals better tolerate stress.

While this study focused on the localization of heat shock proteins in the liver, these proteins can also be released into the circulation following stress. For example, extracellular heat shock proteins are involved in the immune response and inflammation, inducing the activation of macrophages, dendritic cells, and monocytes (Calderwood *et al.*, 2007; De Maio and Vazquez, 2013). Therefore, measuring the levels of heat shock proteins in plasma or other tissues/organs may allow for a more definitive representation of expression levels among different reproductive states and their tissue-specific roles contributing to homeostatic regulation under different stressor conditions.

In conclusion, stress caused by mouthbrooding-induced starvation may be countered by the expression of heat shock proteins and glucocorticoid receptors in cichlid fish. Further, my results indicate that these molecular pathways in the liver may have different functions related to starvation alone compared to mouthbrooding-induced starvation accompanied by maternal care. Measuring plasma cortisol levels in mouthbrooding females and observing the relationship between cortisol and heat shock proteins may also provide more insight into the stress response, as they are both important indicators of stress. While this study focused only on females, it would also be interesting to examine the role of heat shock proteins and glucocorticoid receptors in males, which also show status-dependent changes in metabolism and reproductive potential. For example, how might levels of heat shock proteins and glucocorticoid receptors differ with dominant and subordinate status in male *A. burtoni*? Dominant males often experience higher reproductive success and fitness than subordinate males, but also experience the stressors of defending a territory and courting females (Maruska, 2014), suggesting that social status may play a role in triggering a stress response in male cichlids. Studies such as this will provide a better understanding of how animals react to stress, what cellular and molecular mechanisms

are involved, and how they undergo changes to overcome these challenges and maintain homeostasis under constantly changing conditions.

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