STRESS AND SOCIALITY

THE STRESS
OF
A COOPERATIVE BREEDER

By

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ABSTRACT

In this thesis I examined behavioural, physiological, and molecular aspects of the stress response of the highly social cichlid *Neolamprologus pulcher*. Through this work, I established that dominant individuals within a group have higher baseline stress levels (as measured by plasma circulating cortisol concentrations) than subordinate group members, and hypothesize that this is due to the high demands placed on dominant individuals in both acquiring and maintaining their dominance status. Additionally, social behaviours, and activity levels were positively correlated with stress levels in subordinate males but these correlations were not observed in any other social class. Life history traits of males may explain this pattern, as subordinate males are arguably the social class with least stability in a group, and may need to appease dominant individuals in order to be allowed to stay; this may in turn cause stress. I was also able to establish that while dominant individuals had higher resting cortisol levels than subordinates, they were in no way maximal, as the application of a 10 minute stressor caused large increases above resting levels (>10 fold in magnitude) in circulating cortisol levels of both social classes and in both sexes. As an extension to the characterization of the stress response in *N. pulcher*, we examined differences in corticosteroid receptor levels between dominants and subordinates. This will paint a much fuller picture of the stress response in *N. pulcher* and highlight differences and similarities between stress responses in each social class, both physiologically and at the molecular level.

In a second experiment, dominant female breeders were repeatedly stressed to assess possible maternal and offspring fitness costs. Through this manipulation we found that stressing females resulted in a longer interval between spawning events, and decreased maternal growth rates. Additionally there was a significant decrease in the number of eggs laid, as well as egg size in stressed mothers compared to those left unstressed. Helpers within a group seemed to have no effect on the above-mentioned characteristics, however mothers without helpers released highly variable cortisol concentrations during the first and second lay, while those with helpers saw less variability in the concentration of cortisol they released into eggs.

The results presented in this thesis shed light on the stress responses of *N. pulcher*, highlighting the impacts that within-group social dynamics have on stress levels, and their potential impacts on maternal and (possibly) offspring fitness.

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THESIS ORGANIZATION AND FORMAT

The organization of this thesis is in a sandwich format, consisting of 4 chapters and an appendix. **Chapter 1** outlines the general relevance of my research, my aims, study species and the structure of the thesis. **Chapter 2** is a manuscript that is in press, and **Chapter 3** is a manuscript that will soon be submitted for publication. The **appendix** consists of an introduction, methods and very short results section of a third manuscript that will be completed and submitted for publication. **Chapter 4** summarizes and provides a context for the research outlined in previous chapters, and suggests future areas of study.

CHAPTER 1: Sociality, the stress response, and their effects on maternal and offspring fitness.

Author: Viktoria R. Mileva

CHAPTER 2: The stress response of the highly social African cichlid *Neolamprologus pulcher*.

Author: Viktoria R. Mileva, John L. Fitzpatrick, Susan Marsh-Rollo, Kathleen M. Gilmour, Chris M. Wood, Sigal Balshine

Publication: Physiological and Biochemical Zoology (in press)

Comments: This manuscript is based on work done by VRM in 2007, and JLF in 2006. VRM performed behavioural analyses, cortisol analyses, and was responsible for writing the manuscript, under the supervision of SB, CMW, and KMG. SMR helped with data collection.

CHAPTER 3: Effects of maternal stress on egg characteristics and cortisol in a cooperatively breeding fish.

Author: Viktoria R. Mileva, Kathleen M. Gilmour, Sigal Balshine

Publication: Manuscript will be submitted to Journal of Comparative Physiology B

Comments: VRM was responsible for conducting this study, analyzing the data, and writing the manuscript, under the supervision of KMG and SB.

APPENDIX 1: Differential expression of corticosteroid receptor mRNA in a cooperatively breeding cichlid.

Author: Viktoria R. Mileva, Tammy M. Rodela, Sigal Balshine, Kathleen Gilmour

Publication: Manuscript will likely be submitted to General and Comparative Endocrinology

Comments: VRM conducted the study, analyzed data, and wrote the manuscript under the supervision of KMG and SB. TMR also collected data, and helped write the manuscript under the supervision of KMG.

CHAPTER 4: Insights into the stress biology of *Neolamprologus pulcher*, and potential future research.

Author: Viktoria R. Mileva

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

SOCIALITY, THE STRESS RESPONSE, AND THEIR EFFECTS ON MATERNAL AND OFFSPRING FITNESS.

In this thesis I studied responses to stress in a cooperatively breeding cichlid fish from Lake Tanganyika, Zambia, Africa. In this chapter I place my M.Sc. research into a general context, by providing a brief introduction to stress biology in general and to cooperative breeding (a type of breeding system) as observed in a wide variety of taxa. The response to stress in cooperatively breeding species is rarely examined and this thesis provides one of the first attempts to do so in a cooperatively breeding fish species. I also provide a natural history description of the study species, *Neolamprologus pulcher*, used in the three studies contained in this thesis.

1.1 A BRIEF HISTORICAL PERSPECTIVE AND OVERVIEW OF STRESS BIOLOGY

The endocrinologist Hans Selye first postulated the "The General Adaptation Syndrome" in the early 20th century (see Selye 1936). In his book "The stress of life" (1956), he provided further insight, through his pioneering experimental work, on how animals respond to stressors. These ideas have since been studied and refined by prominent endocrinologists and neuroendocrinologists, including Bruce McEwen and his student

Robert Sapolsky, and have led to our current understanding of what is now described as a "stress response".

The stress response is characterised as a suite of physiological changes evoked by a stressor to allow the animal to respond to situations that would otherwise result in harmful perturbations of homeostasis. Generally the stress response can be thought of as having three stages, with the first (primary stress response) being the rapid release of the stress hormones, catecholamines (including epinephrine and norepinephrine) and glucocorticoids (such as cortisol and corticosterone). The second stage comprises the physiological responses elicited by the stress hormones, including the suite of changes commonly termed the "fight-or-flight" response, where energy reserves are mobilized, lipid metabolism is increased, blood flow to locomotory muscles and the heart is increased, and conversely blood flow to non-essential organs including the gut and gonads is decreased; in effect, a shunting of oxygen and energy away from processes which are not directly required for survival at that time (Charmandari et al. 2005). In general, catecholamines are responsible for producing rapid, transient responses (the "alarm" phase of Selye's stress syndrome) while glucocorticoids are mobilized more slowly and cleared less rapidly, sustaining longer-term physiological responses to stress (the "resistance" phase postulated by Selye). Finally, the tertiary stage is reached only in cases of prolonged glucocorticoid production, and its effects are visible at the whole-organism level. A prolonged increase in circulating glucocorticoids has been linked to decreased growth rates, suppressed reproduction, and ultimately, reduced fitness of the

organism (for examples see Carragher et al. 1989; McCormick 1998; Salvante and Williams 2003; Cyr and Romero 2007).

Physiologically, the stress response begins with the perception of a stressor. This is followed by activation of the sympathetic arm of the autonomic nervous system (and concomitant suppression of the parasympathetic division) as well as activation of the hypothalamic-pituitary-adrenal (HPA; in tetrapods) or hypothalamic-pituitary-interrenal (HPI; in fish) axis. The sympathetic branch of the autonomic nervous system is responsible for the fight-or-flight response, eliciting the responses noted above both via sympathetic nerve pathways and by triggering the secretion into circulation of catecholamine hormones from chromaffin cells (Douglass and Poisner 1966; Wendelaar Bonga 1997; Moyes and Schulte 2006). Glucocorticoid stress hormones are synthesized and released as the end result of activation of the HPA or HPI axis. The first step in this pathway is the release of corticotropin releasing factor (CRF, the principal trigger of the HPA/HPI axis) and arginine vasopressin (AVP; mammals) or arginine vasotocin (AVT; other vertebrates, including fish), from neurons in the paraventricular nucleus of the hypothalamus (see Chrousos 1997; Charmandari 2005). Upon binding to receptors in the anterior pituitary, these tropic neuropeptides cause the release of adrenocorticotropic hormone (ACTH). ACTH is transported by the circulation to the glucocorticoid-synthesizing interrenal cells, which are found in proximity to the kidney, although the specific location varies among vertebrates (e.g. adrenal cortex in mammals, scattered cells in the head kidney region of fish) (see Moyes and Schulte 2006). Binding of ACTH to interrenal cell MC2R receptors stimulates the synthesis of GCs (generally cortisol in fish,

amphibians and most mammals or corticosterone in birds, reptiles and many rodents) (Moyes and Schulte 2006). While this cascade causes the secretion of GCs into the bloodstream to be carried to target tissues, the GCs also act on the hypothalamus to reduce secretion of AVP/AVT and CRF, thereby acting as a negative feedback loop to prevent the overproduction of GCs. Glucocorticoid-mediated responses were the main focus of this thesis.

In target tissues, glucocorticoids exert their effects by interacting with corticosteroid receptors. In mammals, two corticosteroid receptor types occur, the glucocorticoid receptor (GR; binding glucocorticoids) and the mineralocorticoid receptor (MR; binding the mineralocorticoid aldosterone) (Moyes and Schulte 2006). Responses elicited by GR activation typically are those that function in the regulation of metabolism, whereas MR functions include the regulation of salt and water balance (see Moyes and Schulte 2006; Lu et al. 2006). In most fish, by contrast, there are two GRs (1 and 2) as well as one MR (reviewed by Prunet et al. 2006, Bury and Sturm 2007; for primary publications see Ducouret et al. 1995, Colombe et al. 2000, Bury et al. 2003, Greenwood et al. 2003, Sturm et al. 2005). Interestingly, however, fish lack aldosterone and therefore cortisol is thought to be the primary ligand of all three corticosteroid receptors (reviewed by Bury and Sturm 2007; Prunet et al. 2006). The roles of the different corticosteroid receptors in fish remain poorly defined. The two GRs differ in their affinity for glucocorticoids, as well as in molecular sequence and general tissue distribution, suggesting that they may each have distinct physiological functions (Stolte et al. 2006). GRs in general (i.e. without distinguishing between GR1 and GR2) are known to be

involved in the regulation of metabolism and appear also to have some function in water and salt balance (reviewed by Wendelaar Bonga 1997, Mommsen et al. 1999). While specific functions have not yet been ascribed to the fish MR, several studies suggest that it may play a role in regulating branchial ionocyte prevalence during changes in salinity (Sloman et al. 2001; Scott et al. 2005). Much work remains, however, to fully characterize the ligands and functions of the fish MR.

1.2 COOPERATIVE BREEDERS

Cooperatively breeding species are those in which parents and other individuals (generally called helpers) raise offspring together. In many cases these helpers are offspring of the individuals they are helping, but they do not always have to be related (for examples see white-browed scrubwrens, *Sericornis frontalis*, Magrath and Whittingham 1997; and pied kingfishers, *Ceryle rudis,* Reyer 1980). Cooperative breeding is a breeding system found in around 220 species of birds (see Stacey and Koenig, 1990), 120 mammalian species (Riedman 1982) and 19-21 fish species (Heg and Bachar 2006).

A number of different explanations exist for the evolution of cooperative breeding (see Stacey and Koneig 1990; Krebs and Davies 1997 for reviews). One of the best accepted theories is that of "kin selection". Kin selection was first described by Hamilton (1963) and postulates that genes selecting for altruism will only spread through a population if the cost (c) to the actor is less than the benefit (b) gained by the recipient, weighted by relatedness (r). The higher the relatedness (r) between the actor and

recipient, the higher the likelihood that these genes will spread; this can be summarized by the equation rb>c (Hamilton 1963). Another well accepted theory for the evolution of cooperation is "reciprocity" in which an actor performs an altruistic act, under the expectation that the recipient will, in the future 'return the favour' (Trivers 1971). In order to provide assistance, helpers must remain in a territory and not disperse to breed independently. A well accepted theory for why helpers stay, help incorporates kin selection, and is known as the "ecological constraints theory" (Emlen 1982). This theory postulates that helpers will, through staying to help, increase their inclusive fitness (kin selection) when dispersal would either be unsafe, or surrounding territory quality is too poor to confer direct fitness benefits (high cost or low benefits of dispersal). By staying a helper can maximize fitness at the time, and disperse when opportunities are more favourable or even inherit the natal territory (see Krebs and Davies 1997). Another theory is known as the "life history hypothesis", and suggests that the evolution of cooperation is more likely to evolve in species that are k strategists (i.e. long lifespan, longer time to mature, etc.) than those that are r strategists (See Arnold and Owens 1998; Hatchwell and Komdeur 2000 for reviews). The life history hypothesis suggests that low adult mortality and a long lifespan result in little opportunity for young to find a territory of their own due to habitat saturation (again the low benefits of dispersal lead to helpers staying around their parents where they can gain inclusive fitness by helping them).

Generally cooperatively breeding species live in groups forming a social hierarchy, containing both dominants (breeders) and subordinates (helpers) (see Creel 2001 for some examples). The dominant individuals within a group generally have all of

the reproductive output (e.g. naked mole rats, *Heterocephalus glaber*, see Clarke and Faulkes 1997; dwarf mongoose, *Helogale parvula*, Creel 2005) however in some cooperative breeders subordinate reproduction is observed and can even be the norm (e.g. meerkats, *Suricata suricatta,* see Young et al. 2006; and banded mongoose, *Mungos mungo,* Gilchrist 2006).

Most research conducted to date on cooperative breeders has employed mammals and birds. Much less work has been conducted on cooperatively breeding fish species but the most well studied cooperative breeding fish, *Neolamprologus pulcher,* is the study species used throughout this thesis.

1.2.1 *NEOLAMPROLOGUS PULCHER NATURAL HISTORY*

Neolamprologus pulcher is a cooperatively breeding cichlid fish species endemic to the southern shores of Lake Tanganyika, Africa. These fish live in rocky territories in social groups consisting of a male and female dominant breeder and 1- 20 subordinate helpers (Taborsky 1984; Balshine-Earn et al. 1998; Balshine et al. 2001; Heg et al. 2005). Each group inhabits a territory of approximately 3150cm^2 size (Balshine et al. 2001) and has a size-based dominance hierarchy, with the two breeders being the largest, and the helpers decreasing in size and consequently social status. Breeders within a group are the only fish to reproduce (Stiver et al. 2009, but see Dierkes et al. 1999; Heg 2006 for alternative interpretation based on laboratory findings). Helpers participate in territory defence, maintenance, and brood care (Taborsky and Limberger 1981). Additionally, some dominant males in the wild are known to hold multiple territories, with one study

(Desjardins et al. 2008) suggesting that out of the study population, approximately 73% of the groups were polygynous (females in these groups shared a male with females in other groups; each group had its own set of helpers).

N. pulcher exhibit a wide array of well characterised and stereotypical behaviours including aggressive, submissive, non-aggressive social, and reproductive behaviours (see Sopinka et al. 2009 for a recent ethogram for this species). Their unusual breeding system coupled with the complexity of their social interactions, and their ease of maintenance in a laboratory setting has made *N. pulcher* an ideal study organism for a series of behavioural, physiological, and molecular studies. For example studies have focused on the benefits of helpers (Taborsky 1984, 1985, Balshine et al. 2001), predictions for the evolution of cooperation (Balshine-Earn et al 1998, Bergmüller et al. 2005, Heg 2008), relatedness, paternity, and sperm competition (Dierkes et al. 1999, Stiver et al 2004, 2008, 2009, Fitzpatrick et al. 2006), and for hormones and neuropeptides in relation to social status (Buchner et al. 2004, Aubin-Horth et al. 2007, Desjardins et al. 2005, 2008, Taves et al 2009). Work reported in **Chapter's 2, 3,** and the **Appendix 1** fall into the last category.

Despite their attractiveness as a study organism, several drawbacks are associated with using *N. pulcher*, particularly for physiological approaches. Owing to their small size, *N. pulcher* must be humanely sacrificed to acquire blood for analysis of cortisol or other parameters, and very little blood can be obtained (~10-25μl), especially from small subordinate helpers. It is therefore impossible to take repeated blood samples from a single fish, as would be required to measure changes in circulating hormone

concentrations over time. Additionally, measurements of blood catecholamine concentrations are problematic because blood samples cannot be obtained very rapidly from undisturbed fish – normally this is accomplished through the use of a catheter implanted into a blood vessel (see Perry et al. 1996 for example), a procedure that is virtually impossible with *N. pulcher*. Similarly, administration of chemical agents (e.g. cortisol) to fish of such small size can be challenging. Nevertheless, many of these difficulties can be overcome through ingenuity, permitting the interactions between behaviour and physiology to be studied in this interesting species. This thesis represents one such study.

1.3 AIMS OF THIS THESIS

By using *N. pulcher* as a study organism we investigated the stress biology of a cooperative breeder. The aims of this thesis were two-fold:

1. To investigate whether dominant versus subordinate individuals exhibited different stress hormone levels and how these individuals responded to an experimental stressor (**Chapter 2**). This was accomplished using behavioural and physiological data. Additionally we investigated whether there were any underlying corticosteroid receptor differences between dominant and subordinate, male and female *N. pulcher* (**Appendix 1**). We intend to eventually relate corticosteroid receptor differences to plasma cortisol concentrations and social behaviours.

2. To investigate whether chronic stress on dominant breeding females influences maternal and offspring fitness, and whether the costs of stress might be dampened by the presence of helpers (**Chapter 3**).

1.4 STRUCTURE OF THIS THESIS

Chapter 2 was important in establishing baseline levels of the circulating glucocorticoid, namely cortisol, and how these differed between dominant and subordinate fish, as well as between the sexes. In most animals, typically subordinate individuals have higher circulating basal glucocortoids (for examples see Virgin and Sapolsky 1997, Sloman et al. 2001) probably because they have less access to high quality resources, including territories and food (see Krebs and Davies 1997, Pg. 262). However, in many cooperatively breeding species, the opposite pattern has been observed; dominants have higher levels of circulating cortisol. Goyman and Wingfield (2004) have argued that the difficulty of ascending and maintaining dominance status results in higher levels of cortisol in dominants, in cooperative breeding species. Thus with **Chapter 2** we set out to examine whether dominant or subordinate *N. pulcher* had higher cortisol levels. We predicted that because acquisition and maintenance of dominance status is extremely difficult, and subordinates rarely reproduce, dominant individuals of both sexes would have higher baseline cortisol levels than male and female subordinates. Additionally, in **Chapter 2** we examined correlations between specific well-characterized behaviours and cortisol levels. For example, we explored whether more aggressive fish have higher

cortisol levels than those fish that were less aggressive, and how submission related to cortisol levels. Finally, by acutely stressing fish, we were able to examine whether: 1) dominant and subordinate individuals of either sex responded differently to an experimental stressor, and 2) certain behaviours correlated with a heightened or lowered response to stress, in both social status categories and sexes.

The data reported in **Appendix** 1 are a direct extension of **Chapter 2** in which the work was taken one step further to look at corticosteroid receptor levels (GR1, GR2 and MR) in the brain and liver of dominant and subordinate fish. In the absence of antibodies or assays that are specific for the different receptor types, we opted to assess receptor mRNA expression. Although the degree to which receptor mRNA expression represents tissue receptor levels is uncertain, examination of mRNA expression is at present the only method available to separate the different corticosteroid receptors. These two studies together enable us to predict whether the differential cortisol concentrations found in blood plasma between the social classes (**Chapter 2**) were indeed indicative of variation in stress responses with social status.

Chapter 3 focuses on the effects of maternal stress on both maternal fitness (as measured by growth rate, the number of eggs laid and the inter-spawn interval) and predicted offspring fitness (as measured by cortisol levels found in eggs, as well as egg size). It is very likely that mothers in the wild experience different stress levels, due to the differential predator pressure depending on where their territory is located and differential social pressure depending on the number, sexes and behaviour of helpers present. Characterizing maternal and predicted offspring fitness in experimentally stressed vs.

control mothers allowed us to investigate the deleterious effects of repeated stress on mothers, and whether being part of a social group would help dampen or worsen these effects.

Chapter 4 summarizes my research findings and provides possible implications of my work, as well as providing ideas for future studies based on these findings.

CHAPTER 2

THE STRESS RESPONSE OF THE HIGHLY SOCIAL AFRICAN CICHLID *NEOLAMPROLOGUS PULCHER*

2.1 ABSTRACT

In group-living species, dominant individuals are frequently aggressive towards subordinates, and such dominant aggression can lead to chronic stress, higher glucocorticoid levels, and decreased fitness for subordinates. However, in many cooperatively breeding species it is surprisingly the dominants rather than the subordinates that exhibit higher levels of glucocorticoids, a possible consequence of the demands of maintaining high social rank and socially suppressing the reproduction of other group members. This study investigates the relationship between social status and circulating plasma cortisol in groups of the cooperatively breeding African cichlid, *Neolamprologus pulcher.* Baseline (resting) levels of cortisol were quantified, as was the cortisol response following an acute stressor. Dominants had the higher cortisol concentrations, and these were not related to their social behavior. Cortisol concentrations correlated (positively) with social behaviors and general activity levels only in subordinate males, arguably the individuals with the least stability in the social group. No status-dependent differential responses to acute stress were detected, suggesting that the status-induced chronic stress has little impact on the capacity to mount a full stress response to large-scale, life-threatening risk.

2.2 INTRODUCTION

The sight of a predator, the need for shelter, the lack of food, or interacting with a more dominant or aggressive conspecific, may all cause significant stress in individuals. Across vertebrates, an integral component of the stress response is mediated by the hypothalamic-pituitary-adrenal axis (HPA), or the hypothalamic-pituitary-interrenal axis (HPI) in fish. This axis helps the animal to regain homeostasis by releasing glucocorticoid (GC) hormones (i.e. cortisol or corticosterone) into the bloodstream. In turn, GCs mobilize fatty acids and liver glycogen to provide the energy needed to deal with the stressor. While such stress responses are adaptive in the short-term, chronic stress can lead to decreases in immune function, reproduction, growth, and ultimately, fitness (for reviews see Wendelaar Bonga 1997; Chrousos 1998).

Subordinate individuals within many species exhibit chronically elevated GC levels while dominants commonly have lower levels (e.g. olive baboons, *Papio Anubis,* Virgin and Sapolsky 1997; rainbow trout, *Oncorhynchus mykiss,* and brown trout, *Salmo trutta*, Sloman et al. 2001; for review see Creel 2001). However, in many cooperatively breeding species the opposite pattern has been observed, with dominants exhibiting higher GC levels than subordinates (e.g. Florida scrub-jay, *Aphelocoma coerulescens* Schoech et al. 1991, but see Schoech et al. 1997; African wild dogs, *Lycaon pictus,* and dwarf mongoose, *Helogale parvula,* Creel et al. 1996; common marmoset, *Callithrix jacchus,* and cotton top tamarin, *Saguinus oedipus;* reviewed in Abbott et al. 2003; for review see Creel 2001). In cooperatively breeding species, subordinates help raise

offspring of dominants, often forgoing reproduction themselves, either by design or by force.

In most social species, both dominants and subordinates can realize some reproductive success, while in many cooperatively breeding species, it is only the dominants that breed (despotic or skewed reproduction) and subordinate reproduction is suppressed. In the few cooperatively breeding species in which both dominants and subordinates reproduce within a group (egalitarian reproduction), subordinates tend to have higher cortisol levels, just as seen in the vast majority of non-cooperatively breeding social and non-social species. These patterns are apparent in Creel's (2001) comparative review of GC's across cooperatively breeding species. More recently, Goymann and Wingfield (2004) have proposed that allostatic load (or the relative physiological costs of achieving and maintaining dominance status) best explains the observed species differences in status-related stress responses. Note that the concept of allostatic load and its quantification can easily and naturally encompass the degree to which subordinates are prevented from reproducing. In general, when the allostatic load is high, dominants will have higher cortisol. When allostatic load is low, subordinates are expected to have higher cortisol levels, or no status-related differences in cortisol levels are expected.

The main goals of this study were to elucidate the interactions between circulating cortisol levels, sex, and social status in relation to allostatic load under baseline, or resting, conditions as well as following an acute stressor in the cooperatively breeding cichlid fish, *Neolamprologus pulcher*, from Lake Tanganyika. This fish lives in permanent social groups consisting of a dominant breeding male and female together with

anywhere from one to 20 subordinate helpers (on average groups have five subordinates, Taborsky and Limberger 1981; Balshine et al. 2001; Heg et al. 2005). *N. pulcher* subordinate helpers assist dominant breeders in territory maintenance, defence and brood care (Taborsky 1984; Brouwer et al. 2005). *N. pulcher* dominants are frequently aggressive towards subordinate group members (laboratory data: 0.7± 0.01 aggressive acts/min) while subordinates rarely show aggression towards dominants (laboratory data: 0.04 ± 0.01 aggressive acts/min), and dominant individuals claim the majority if not all of the reproductive opportunities in natural social groups in the wild (Dierkes et al. 2005; Fitzpatrick et al. 2006; Stiver et al. 2009; but see Dierkes et al. 1999; Heg et al. 2006 for some contrary evidence based on captive laboratory groups). As well, achieving and maintaining a dominant breeding position is extremely challenging in *N. pulcher* where there are many more subordinate helpers than vacant breeding positions (Balshine-Earn et al. 1998; Stiver et al. 2006; Fitzpatrick et al. 2008), and thus the allostatic load for dominant breeders is expected to be higher than for subordinate helpers (see results, Table 2.1, for our allostatic load calculation for dominant and subordinate *N. pulcher).* Moreover, a new model by Rubenstein and Shen (2009) suggests that the allostatic load of dominants will increase with increased numbers of subordinates. Based on the allostatic load hypothesis, and consistent with the reproductive skew cortisol framework, we predicted that dominant *N. pulcher* would have higher cortisol levels than subordinates. We also predicted that dominant breeders in large groups with more subordinates would have higher cortisol levels than dominant breeders in smaller groups.

We further predicted that high levels of aggression and social policing (often observed in more dominant individuals) would be associated with higher baseline cortisol levels. This prediction was shaped by previous work on the physiological costs of social living in *N. pulcher*. For example, Buchner et al. (2004) reported that among size-matched and sex-matched *N. pulcher* subordinates, the more dominant subordinates tended to have higher plasma cortisol concentrations than the subordinates lower down in the hierarchy. Bender et al. (2006) found that more submissive male subordinates exhibited lower cortisol levels. We also examined the status dependent effect of an acute stressor (confining fish in a net for 10 minutes) on the cortisol response, an approach never before utilized in *N. pulcher* and only rarely examined in cooperative breeders (but see Creel 2005; Schoech et al. 1997, 2007; and Rubenstein 2007 for some illuminating exceptions). The pattern found in non-cooperative species suggests that a high pre-existing cortisol level would attenuate an individual's ability to mount an additional, acute cortisol response (for examples see Barton et al. 1986; Rotllant et al. 2000; Reeder et al. 2004). Similarly, using the cooperative superb starling, *Lamprotornis superbus*, Rubenstein (2007) found that subordinates had higher handling stress GC responses but only in times of low rainfall and presumably food shortage. In more favourable years, dominant and subordinate superb starlings had similar responses to handling. Schoech et al. (1997) too found no difference in mounting of a stress response in dominant breeder vs. subordinate helper Florida scrub-jays, another cooperative breeder. Thus, based on these results, we predicted either no difference in dominant and subordinate responses to an acute stressor, or that an acute stressor would cause a larger cortisol response in the

social class with the lower pre-existing cortisol levels (i.e. in our predictions, subordinate helpers).

2.3 MATERIALS AND METHODS

2.3.1 Study population and holding conditions

The study was conducted using a colony of *N. pulcher* held at McMaster University, Hamilton, Ontario, Canada. Fish in this experiment were descendants of fish caught in Lake Tanganyika, Zambia in 2001 and 2002. Genetic relatedness in these laboratory social groups is thought to be roughly similar to field populations for two reasons: 1. In the wild, these fish are highly philopatric; and 2. New breeding pairs are regularly formed in the laboratory. In total, 30 social groups were observed and sampled, with each group containing a dominant breeding pair and subordinate helpers (mean group size ± se was 10.8 ± 0.8 individuals). In each tank, the two dominant fish and two large subordinate helpers (>4 cm SL) were selected for focal observations and eventual cortisol sampling. The dorsal fins of these individuals were clipped in a unique pattern, three to seven days before observations began, so that all four fish could be clearly distinguished from one another. Fin clipping does not adversely affect behaviors, and the fish return to normal social behavior within 1-2 minutes of being clipped (Stiver et al. 2004). Also in other fish species, following an acute stressor such as handling or marking stress, cortisol levels are known to return to baseline after 24 hrs (Pickering and Pottinger 1989).

A total of 115 fish were used for the two experiments (see below); 30 dominant males; 30 dominant females and 55 subordinate helpers. In experiment 1, one group did

not have a second large subordinate while in experiment 2, four groups did not have a second large subordinate. Across all groups, the dominant breeder males were on average 70.3 ± 1.2 mm (mean ± se) in standard length, dominant breeder females were 64.5 ± 1.1 mm long, and subordinates were on average 50.8 ± 0.8 mm long. Each social group was housed individually in a 189 L tank containing ~2 cm of coral sand, two foam filters, air-stones, a heater, a thermometer, two upside down flower-pot halves (to provide shelter, and a place on which to lay and fertilize eggs), and two mirrors (used to alleviate excessive aggression towards group members). Water temperature was kept constant at 26 ± 2°C and a 13:11 h light:dark cycle was maintained. Groups were fed *ad libitum* daily with Nutrafin® basix Large Flake commercial cichlid food.

2.3.2 Experiment 1. Baseline cortisol levels

This experiment was conducted in August 2006 and in August and September of 2007. Ten male and ten female dominant breeders were used in each year. In 2006, all but one of the subordinates removed from a tank to measure baseline cortisol levels were female (n=20) and in 2007 all subordinates observed and measured were male (n = 19). Three to seven days after (fin) clipping the fish, three focal watches were performed on the four marked fish (dominant breeder male, dominant breeder female and two large subordinate helpers). Focal observations on each fish in each social group were made for a total of 30 minutes (3 x 10 min watches) and behaviors were then averaged among the three watches to yield the number of observed behaviors per 10 min for each fish. Observers sat still for 3-5 minutes prior to recording behavior to habituate the fish to their presence, and always

remained at a distance of 1.5 m from the tank. Observations were conducted between 8:00 and 13:00 to control for observed diurnal fluctuations in behavior in this species (Desjardins et al. in prep). To minimize possible time of day variation in plasma cortisol levels (Davis et al. 1984; Barton et al. 1986), all blood sampling was performed between 13:30 and 15:30.

Based on detailed ethograms that have been published elsewhere for this species (Taborsky 1984; Buchner et al. 2004; Sopinka et al. 2009), we documented all behaviors including aggression (ramming, chasing, biting, etc), submission (submissive postures and displays), affiliative behaviors (soft touches and parallel swims, follows), and workload or care behaviors (visiting the brood chamber, micronipping eggs, defence and guarding of young, fanning, territory maintenance activities such as carrying and digging, etc). Additionally, time spent by the focal fish behind filters and in the brood chambers, as well as the frequency that each individual performed locomotive (swimming, darting) and maintenance activities (feeding, yawning, and scraping) were recorded.

Following the final observational period, all four fish were quickly netted and sacrificed (as described in the Ethical Note below), and blood was collected in 10 µl heparinised micro-capillary tubes following caudal severance. Multiple micro-capillary tubes were used depending on the size of the fish, in order to collect as much blood as possible (typically, 10-25 µL of blood were collected). All blood was collected within 2 min of the initial approach towards the tank (mean ± se = 112 ± 2 s). During dissection, sex was confirmed. Blood samples were centrifuged at 8,000 g for 10 min and plasma was separated and stored at -80°C for later analysis of cortisol concentration.

2.3.3 Experiment 2. Acute stress induction

This experiment was conducted in 2007. We used 10 social groups for this study (total number of fish observed and processed = 36: breeder males = 10, breeder females = 10, female helpers = 10, male helpers = 6). All conditions and procedures in this experiment mimicked those used in experiment 1, however one important addition was made. At the end of the last observational watch, all four fish were individually netted as quickly as possible, as described above, except that the fish remained confined in the net in water for 10 min. We chose 10 minutes of confinement for *N. pulcher* based on similar approaches used in other species (see Gamperl et al. 1994). Confinement for thirty minutes was enough to elicit a maximal stress response in salmonids (Gamperl et al. 1994), which are cold-water fish that are burst swimmers, whereas *N. pulcher* is a tropical fish that does not normally swim for long periods or distances. Additionally, a recent study (Alderman and Bernier 2009) used a 15 min stressor for another small freshwater tropical fish species, the zebrafish, *Danio rerio*. Following this acute stress, fish were sacrificed (see Ethical Note below) and blood was sampled (within 111 ± 2 s following the removal of fish from the net).

2.3.4 Analysis of cortisol concentrations

Assessment of cortisol concentration was performed on 10 µl plasma samples using a commercial ^{125}I RIA kit (DiaSorin, USA; CA-1529) as per the manufacturer's instructions. Each fish had very little plasma (typically 5-15 µl of blood plasma could be

obtained from a single fish, and the cortisol assay requires 10 µl) and thus we were unable to analyze samples in duplicate. Where plasma samples were lower than 10 µl, a dilution factor was taken into account in calculating cortisol concentration. The sensitivity of the RIA is listed as 2.1 ng ml^{-1}, while the inter-assay variation (% CV) is 8.8-9.8 %. In our hands, the intra-assay variation (% CV) was 1.5-8.8 %. Cortisol analysis by this method has been validated for *N. pulcher* in previous studies (Buchner et al. 2004) and in other cichlid species, e.g. *Astatotilapia burtoni* (Fox et al. 1997).

2.3.5 Statistical Analyses

An aggression composite, a submission composite, and a dominance index were calculated as follows. The aggression composite was based on the formula: Σ(Aggressive acts given) + Σ(Submissive acts received). The submission composite was calculated for each fish using the formula: Σ(Aggressive acts received) + Σ(Submissive acts given). A dominance index for each focal fish was created using the sum of aggressive/dominant behaviors minus the sum of submissive behaviors (see Aubin-Horth et al. 2007 for an example of this well-used index). Overall activity levels were scored as all aggressive, submissive, social, and care/workload behaviors performed.

All statistical analyses were performed using JMP IN 5.0, and Microsoft Excel 2007. Data were tested for normality and transformed when necessary; where transformation did not normalize the data, an alternative non-parametric test was used. ANOVAs were employed to examine the effect of sex and status on cortisol concentrations; interactions that were not significant were removed from the models. As

we predicted a priori that dominant individuals would have higher cortisol levels than subordinates, we used one-tailed tests to compare baseline cortisol levels of dominant vs. subordinate individuals. All other tests were two-tailed.

To fully explore the influence of status, year and group size on cortisol levels we performed a GLM analysis and removed all non-significant interactions. We observed a strong year effect despite the fact that the fish were held under identical conditions in the same lab, were fed the same food, and were held under the same temperatures and light regimes. Fish were handled in an identical manner by the same group of researchers. We do not know what caused the year differences so we statistically controlled for year effects by using year as a covariate in a GLM and by using paired Wilcoxon and t-tests to examine cortisol concentrations; these controlled for year effects and for potential group/tank effects in cortisol concentrations by only comparing individuals differing in status within the same social group (see results). Note that cortisol values for acutely stressed fish were compared with baseline cortisol levels for fish from the same year.

One fish (a dominant female) was excluded from analysis of baseline cortisol as an extreme outlier (see outlier analysis Sokal and Rohlf, 1995 pg. 406-407). Inclusion of data for this fish would have increased the significance of results (to $p=0.027$ for the status and cortisol analysis; see results section below). Note that body mass and length were strongly linked with social status; dominants were always bigger than subordinates in their group (Wilcoxon rank-sums test: body mass $\chi^2_1= 69.79$, $p<0.0001$; body length, $\chi^2_1=70.77$, $p<0.0001$). However, body size was not used as a covariate in any analyses

because circulating cortisol concentrations are expected to be independent of mass (Hartman et al. 1943).

2.3.6 Ethical Note

To sacrifice fish quickly, individuals were killed with a firm cephalic blow. The procedures described conform to the protocols approved by the Animal Research Ethics Board of McMaster University (AUP # 06-10-59) and the Canadian Council for Animal Care guidelines.

2.4. RESULTS

2.4.1 Experiment 1. Cortisol concentrations at baseline: the influence of status and sex

Overall, cortisol levels were higher in dominants than subordinates (Figure 2.1a). Along with the status difference, there was also a significant year effect, but group size had no effect on cortisol levels (GLM, overall model: $F_{3,74}$= 8.08, $p < 0.0001$; year $F_{1,74} = 17.13$, $p < 0.0001$; status, $F_{1,74} = 4.22$, $p = 0.04$; group size $F_{1,74} = 2.64$, $p = 0.11$). When we controlled for the year effect by pairing dominants and subordinates within a social group, again dominants had higher cortisol levels (Wilcoxon signed ranks test, $W_{19} = 46.00$, $p = 0.045$). Within a social status category, cortisol concentrations did not differ with rank. For example, the two dominants from the same social group (male and female of a breeding pair) had similar cortisol levels (Wilcoxon signed ranks, $W_{19} = -18.5$, $p = 0.75$) despite the fact that male breeders are dominant to female breeders. Also, in each group the subordinates of higher rank (H1) had similar cortisol concentrations to the

subordinates of lower rank (H2, paired t-test, $t_{18} = 0.05$, $p = 0.96$). In general, males and females did not differ in cortisol concentrations (2-way ANOVA, effect of sex: $F_{1,75} = 0.82$, $p = 0.37$; Figure 2.1b).

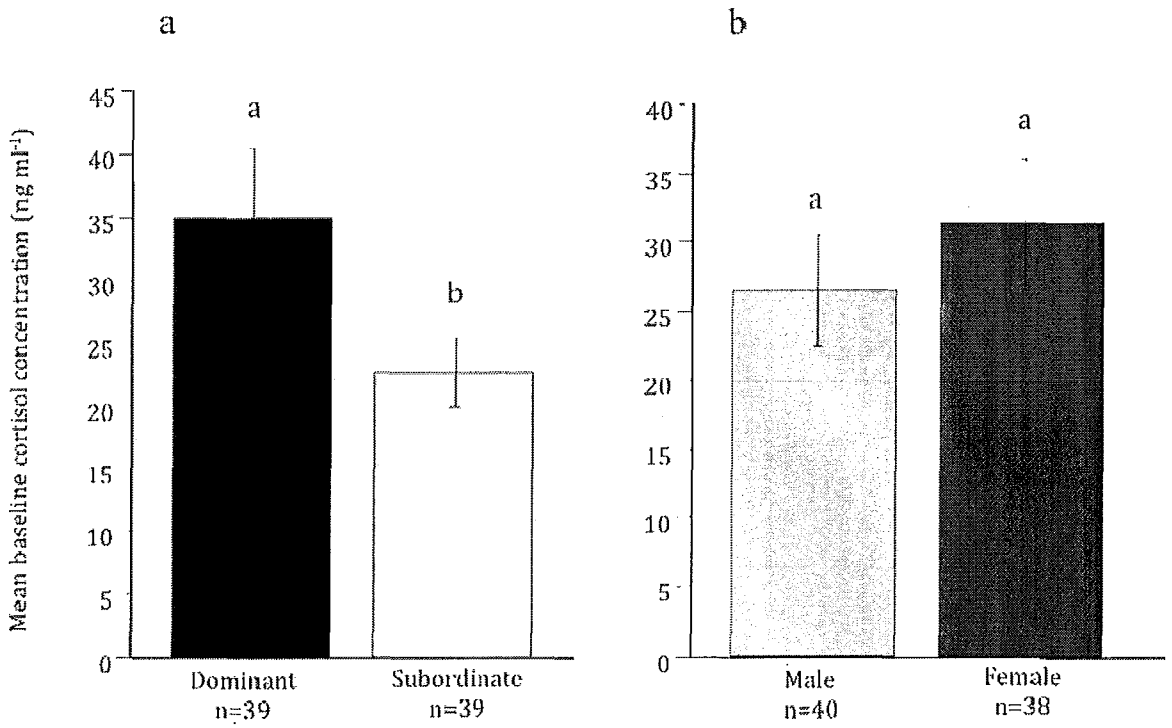

Figure 2.1. **(a)** Mean baseline cortisol concentration (± se) in dominant and subordinate individuals pooled across both experimental years. **(b)** Mean baseline cortisol concentrations (± se) in male and female fish in both experimental years. Letters above each graph denote significant differences at $\alpha=0.05$ after controlling for year effects.

Table 2.1. The allostatic loads and cortisol levels of male and female *N. pulcher.* This table was adapted from a table in Goymann and Wingfield (2004) for other cooperative breeders and the assignment of allostatic loads followed their criteria. Dominance acquisition costs for males are higher than females, because males are unlikely to inherit a territory (Stiver et al. 2006; Balshine and Buston 2008), must disperse farther to breed (Stiver et al. 2004; 2007), and have shorter tenure as breeders compared to females (Stiver et al. 2004). The costs of maintaining dominance are high and roughly equivalent for both males and females as they both defend the territory and 'police' within a territory (i.e. keep subordinates in check, Fitzpatrick et al. 2008). Dominant female breeders are usually more active in territory defence against heterospecific predators and space competitors (Balshine et al. 2001; Desjardins et al. 2008), however dominant males constantly defend territories from takeovers by other conspecific males (Desjardins et al. 2008; John Fitzpatrick, unpublished data). Both female and male subordinates within a territory receive moderate threats from dominants, but as there are several helpers in each group (mean =5, range 1-20, Balshine et al. 2001; Heg et al. 2005), the aggression received from dominants is diluted. Also subordinates can hide in shelters and/or leave the group to avoid aggression (Bergmüller et al. 2005). Thus, we assume the coping costs for subordinates are low. No effective bias exists in the control of resources in *N. pulcher*, as dominants and subordinates both feed in the water column and hence do not compete for food (Balshine et al. 2001) and shelter, another key resource on the territory, is plentiful and shared (Balshine-Earn et al 1998). Due to year differences in cortisol levels, subordinate males from 2007 were compared with dominant males from 2007, while subordinate females from 2006 were compared with dominant females from 2006.

Soc. Status	Dominance acquisition costs	Dominance maintenance costs	Subordinance costs: threats from dominants	Subodordinance coping costs	Strong bias in resource control	Sum of allostatic load	Ratio of allosatic load (dom/sub)	GC levels in dominants and subordinate (mean)	Ratio of GC concentration (dom/sub)
Dominant male	2	3				5	1.66	25.92	1.83
Subordinate male			2	1	0	3		16.12	
Dominant female	1	3				4	1.33	42.0	1.38
Subordinate female			2	1	0	3		30.41	

2.4.2 Experiment 2. Cortisol following an acute stressor

Acutely stressed fish exhibited markedly higher cortisol concentrations than fish for which baseline cortisol levels were determined (Mann-Whitney U test, $\chi^2_1 = 54.72$, $p < 0.0001$; Figure 2.2a). However, stressed dominants and stressed subordinates elevated cortisol concentrations to a similar extent (ANOVA, $F_{1,34} = 0.59$, $p = 0.45$; within group paired t-tests, $t_9 = -1.15$, $p = 0.28$; Figure 2.2b). Following a 10 minute acute stressor, males tended to have higher cortisol concentrations than females but this difference did not reach significance (ANOVA, $F_{1,34} = 3.20$, $p = 0.08$, Figure 2.2c).

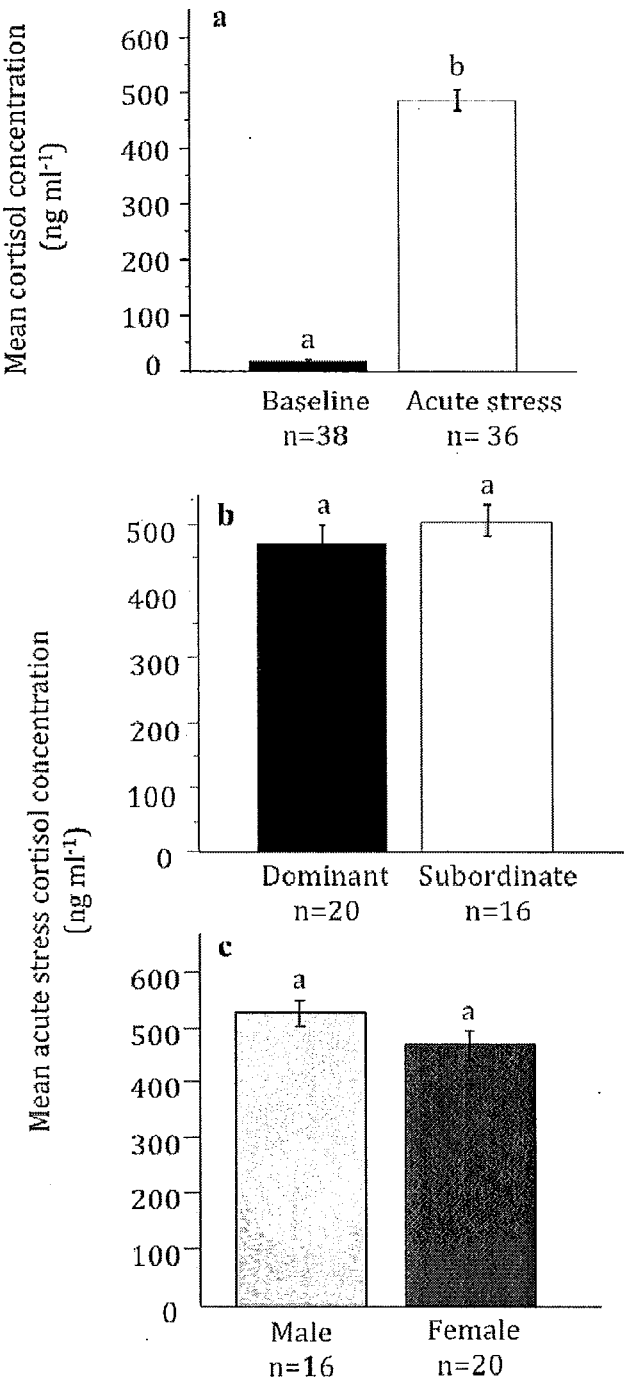

Figure 2.2 (a) Mean cortisol concentration (±se) of all baseline and acutely stressed fish in 2007. **(b)** Mean cortisol concentration (±se) of acutely stressed dominant and subordinate *N. pulcher* of either sex. **(c)** Mean cortisol concentration (±se) of acutely stressed male and female *N. pulcher* of both status classes. Letters over error bars denote significant differences at α=0.05.

2.4.3 Social behaviours

Dominant breeders (both sexes) had higher dominance indices than subordinate helpers (Wilcoxon rank-sum test, $\chi^2 = 43.43$, $p < 0.0001$; Figure 2.3). Dominants were more aggressive and received more submission (had higher aggression composite scores) than male and female subordinates, while subordinates were more submissive and received more aggression (see Table 2.2). Dominant breeders of both sexes exhibited more workload/care behaviors than subordinate helpers ($\chi^2 = 13.35$, $p = 0.0003$), a pattern that has been reported previously in the field (Desjardins et al. 2008). Female and male subordinates had similar workload/care scores ($\chi^2 = 1.15$, $p = 0.28$), as did dominant female and male breeders ($\chi^2 = 0.002$, $p = 0.96$). There were no differences in the total number of affiliative interactions performed between dominants and subordinates ($\chi^2 = 2.084$, $p = 0.15$) or between males and females ($\chi^2 = 0.02$, $p = 0.90$).

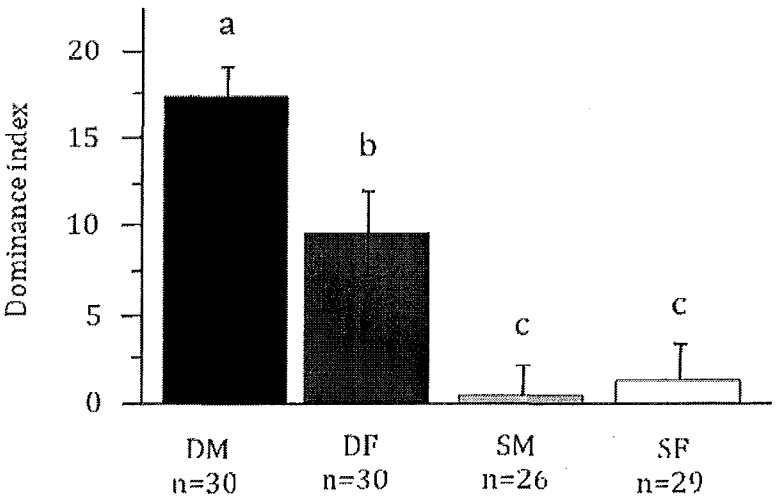

Figure 2.3. Mean dominance indices (± se), calculated as the sum of dominant behaviors minus the sum of submissive behaviors for (DM) dominant males, (DF) dominant females, (SM) subordinate males, and (SF) subordinate females. Letters over error bars denote significant differences at $\alpha=0.05$.

Table 2.2. Behavioral composite scores (mean ± se; number of acts per 10 min) for all fish used. The dominant male and female breeders had significantly higher levels of aggression and lower levels of submission than subordinate individuals. Note, there were no differences in the frequency of the behavioral categories across experiments and years, and therefore the behavioral observations were pooled for these analyses. The behaviors that are included in each category are described in detail in the methods section above. Wilcoxon statistic (χ^2) is reported as well as its corresponding p-value.

Behavior		Dominant males (n=30)	Dominant females (n=30)	Subordinate males (n=26)	Subordinate females (n=29)	χ^2	p
Aggression composite		18.4±2.00	15.7±1.8	8.0±1.3	10.4±1.8	24.7	<0.0001
Submission composite		0.9±0.3	5.8±1.0	7.5±1.00	8.9±0.8	58.7	<0.0001
Affiliative	*Given*	1.8±0.5	2.7±0.4	1.6±0.3	1.3±0.3	7.4	0.06
	Received	1.7±0.3	1.6±0.3	0.7±0.3	1.1±0.4	15.1	0.002
Workload composite		5.1±1.1	12.1±7.4	2.1±0.7	3.7±1.0	14.5	0.002

2.4.4 Social behaviours and baseline cortisol

In subordinate males, overall activity levels were significantly positively correlated with cortisol levels (Spearman's rank correlation test, Rho = 0.76, p = 0.0002). Following sequential Bonferonni corrections for multiple comparisons (Rice 1989), of all the social behaviors examined, only affiliative behaviors (non-aggressive social interactions with other group members) remained significantly (positively) correlated with cortisol levels (Rho = 0.75, p = 0.0002; Figure 2.4) although prior to the correction for multiple comparisons, aggression and submission performed were also positively correlated with cortisol levels (aggression, Rho = 0.57, p = 0.01; submission, Rho = 0.53, p = 0.02). None of the behavioral categories were significantly related to baseline cortisol concentrations when all fish were examined together. This was also the case when we examined

dominant breeding males, dominant breeding females and female subordinates separately (all $p > 0.05$).

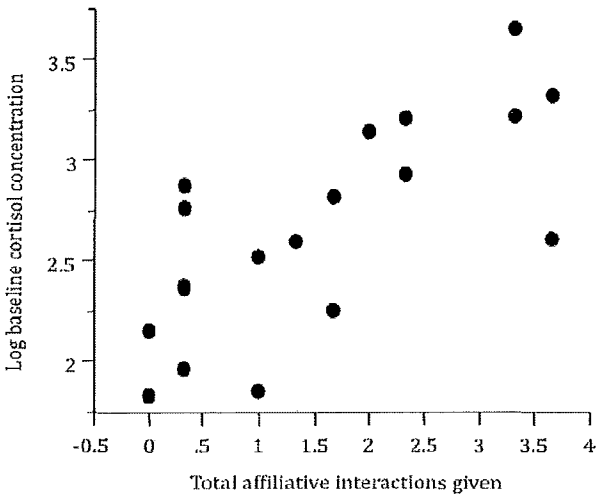

Figure 2.4. Correlation between baseline cortisol concentration ng ml^{-1} (log transformed) and total affiliative interactions given as an average of three 10 min watches, by subordinate males.

2.4.5 Social behaviours and cortisol levels following acute stress

As observed in the baseline condition, social behaviors were not correlated with cortisol levels following an acute stressor when we examined all fish together. Within dominant breeding males and dominant breeding females, fish that performed more care had lower cortisol levels following a stressor (Males, Rho = -0.76, p = 0.01; Females, Rho = -0.64, p = 0.05). Interestingly, although subordinate males that were more active and affiliative had higher cortisol levels in the unstressed condition (see above), following an acute stressor the opposite trend was observed, with subordinate males that performed a high frequency of affiliative displays having lower cortisol levels (Rho = -0.81, p = 0.05). However, stringent Bonferroni corrections for multiple comparisons removed

significance from all of these analyses.

2.5 DISCUSSION

In this study, we have documented one aspect of the stress response in an African cichlid fish with a cooperative breeding system, and have shown that cortisol differences are based on the position a fish occupies in a social hierarchy but not on its sex. Dominant *N. pulcher* exhibited higher baseline cortisol concentrations than subordinate helpers. Additionally we discovered a relationship between behavior and cortisol (in subordinate males), and these patterns may be important when examining the consequences of status and sex differences in stress responses, general physiology, and reproductive success in complex social groups. After a 10 minute acute stressor, cortisol levels did not reflect the social status-dependent cortisol differences observed in unstressed animals, broadly suggesting that mounting of a stress response is similar in dominants and subordinates.

This study provides a teleost example supporting the allostatic load hypothesis. In *N. pulcher*, as seen in many other cooperative breeders, a high allostatic load in dominants compared to subordinates was associated with higher circulating GCs in dominants (Goymann and Wingfield 2004; Table 1). Dominance status in *N. pulcher* is difficult to achieve and few fish manage to inherit a position or successfully take over a breeding position via competition (Balshine-Earn et al. 1998; Balshine et al. 2001; Stiver et al. 2006; Desjardins et al. 2008; Fitzpatrick et al. 2008). Perhaps somewhat surprisingly given the allostatic load concept, group size did not influence cortisol levels of dominants (see Rubenstein and Shen 2009 for predictions).

An alternative explanation for the observed higher cortisol detected in dominant fish, is that cortisol levels may simply increase when a fish reproduces. Cortisol levels are known to be associated with spawning and the production of gametes in other species (brown trout, *Salmo trutta,* Pickering and Christie 1981; catfish, *Heteropneustes fossilis,* Lamba et al. 1983). In *N. pulcher*, subordinates are either reproductively immature or reproductively suppressed (see Fitzpatrick et al. 2006). Therefore, reproduction itself may drive the social status-related difference observed in circulating cortisol concentration. The most dominant pair in the group breeds in *N. pulcher* whereas other more subordinate individuals do not, and therefore it remains possible that either or both factors may be driving the relationship between circulating cortisol levels and status.

No sex differences in cortisol concentrations were detected in this study. To date, the vast majority of studies on status-related stress have not examined sex differences. This may be because in many cooperatively breeding species, subordinate helpers are only one sex (but see Jarvis 1981; Komdeur 1994; Carlson et al. 2004). No corticosterone level differences were found between subordinate males and subordinate females in the cooperatively breeding African striped mouse, *Rhabdomys pumilio*, during either the breeding or the non-breeding season (Schradin 2008). However, dominant males had significantly lower corticosterone levels during the breeding season than dominant females (Schradin 2008). In the dwarf mongoose, dominant females had higher baseline cortisol levels than subordinates (Creel et al. 1996; Creel 2005). Additionally, acutely stressed dominant females showed the highest cortisol rise while dominant males showed little increase (Creel et al. 1996; Creel 2005). In both wild dogs and wolves, dominant

individuals had higher baseline cortisol levels than subordinates, but males and females within a social rank had similar cortisol levels (Creel 2005). Among non-cooperatively breeding species too, sex differences in cortisol levels are variable with some studies finding females having higher baseline cortisol levels, even after acute stressors (Siberian hamsters *Phodopus sungorus,* Bilbo and Nelson 2003), while other studies report higher baseline cortisol levels in males (the variable flying fox, *Pteropus hypomelanus,* Reeder et al. 2004). There are also studies that report no sex differences in either baseline or acutely stressed conditions (for review see Touma and Palme 2005). Diurnal fluctuations, the time of the reproductive cycle (rats, *Rattus norvegicus*, Critchlow et al. 1963), as well as female versus male HPA axis differences (humans, *Homo sapiens*, Kudielka and Kirschbaum 2005) have been put forth to explain these discrepancies. Previous findings in salmonids suggest that androgens suppress the stress response while estrogen can enhance it (Pottinger et al. 1996). In *N. pulcher,* testosterone was higher in females, while the 11-ketotestosterone/testosterone ratio was higher in males (Taves et al. 2009). The high levels of androgens in both males and females may partially explain the lack of sex differences observed.

Why did male but not female subordinates show correlations between social behavior and cortisol levels? In *N. pulcher*, female subordinates are likely to inherit a territory while male subordinates tend to disperse into new territories (Stiver et al. 2004; Bergmüller et al. 2005; Stiver et al. 2006; 2008). Hence, because males eventually leave their natal groups and are less related to the same sex breeder, male subordinates may be experiencing more stress compared to female subordinates, as males may be under a

greater threat of eviction, and this might explain the links between social behaviors and plasma cortisol in subordinate males.

Bender et al. (2006) found that more submissive subordinate *N. pulcher* males had the lowest cortisol concentrations, which is effectively the opposite of our observations. However, it is possible that this discrepancy is due to the different procedure used for measuring cortisol between studies. We used plasma cortisol (a concentration measurement). Bender et al. (2006) removed each focal fish from its tank (a potential stressor), placed it in a set volume of water, and assayed the cortisol excreted by the fish into the water (a production measurement) during a 1 hr period. If the fish examined by Bender et al. (2006) were indeed stressed, then our results align. Following a stressor the more affiliative subordinate males in our experiment did have the lower cortisol levels.

Exposure to a standardized stressor (confinement in a net for 10 minutes) did not differentially affect circulating cortisol levels in dominants vs. subordinates, or in males vs. females. It is possible that the mechanism of stress used in this experiment elicited a stronger cortisol response than would occur with threats encountered in this species' natural environment. As reviewed by Gamperl et al. (1994), following a prolonged confinement stressor (~30 min), all salmonid species studied had circulating levels of cortisol below those of *N. pulcher.* After 30 min of severe confinement, plasma cortisol levels in salmonids ranged from ~100 to ~260 ng ml^{-1} (Gamperl et al. 1994), while in *N. pulcher* a 10 minute confinement caused cortisol to rise to 491 ng ml^{-1} (Figure 2a). This suggests that net confinement is a very strong stressor for *N. pulcher,* and

correspondingly, any status or sex differences in cortisol may have been 'washed out' due to the severity of this stressor.

To further explore the relationship between social status and cortisol responses to a perceived threat, a follow-up experiment could be to introduce naturalistic intruders that represent differing levels of risk and danger (see Desjardins et al. 2008 for a similar protocol), and then measure changes in circulating cortisol levels. Arguably, a same sex intruding conspecific would pose greater threat to a dominant individual of the group than to a subordinate (in terms of group stability, offspring survival, etc.) but a heterospecific predator (of adult fish) would be equally stressful and dangerous to each social class. Also, we measured the impact of the stressor only at one time point (10 minutes). It is possible that status (or sex) differences in cortisol levels could have emerged were the stress response followed over a longer time course. It is also possible that while cortisol concentrations rise to the same levels, they may rise (or decrease following a stressor) more rapidly in one social class or sex than the other, a possibility that could be addressed by assessing cortisol turnover rates. Unfortunately, because of *N. pulcher*'s small size and the need to perform a terminal bleed to examine plasma cortisol levels, we currently do not have a stress response curve for this species, another obvious area for future study.

It is also important to note that while dominant *N. pulcher* exhibit higher baseline cortisol levels, their similar responses to an acute stressor provide evidence that they do not have an attenuated cortisol response. Such an attenuated stress response has been shown in juvenile rainbow trout (Barton et al. 1986) and gilthead sea bream, *Sparus aurata* (Rotllant et al. 2000); in both cases, acute stress in chronically stressed individuals

did not lead to higher cortisol levels. In yellow perch, *Perca flavescens,* individuals that have been chronically exposed to contaminants (Hontela et al. 1995; Hontela 1998) do not respond to acute stress with higher cortisol levels perhaps because of the negative feedback action of cortisol on the HPI axis (Barton 2002) or because of the action of the contaminant itself. The addition of an acute stressor in this study provides a 'snapshot' of the stress response exhibited by dominant and subordinate *N. pulcher*, but a fuller picture of HPI axis function would be beneficial. Future study could involve characterising corticosteroid receptors, and the time course and magnitude of cortisol responses to various acute stressors. Additionally, measuring levels of gonadal hormones may be important to elucidating the interplay between the HPI and HPG axes.

In conclusion, the concept of allostatic load appears to be a useful indicator of stress levels, and visa versa, especially in cooperative breeders. With more empirical evidence the concept will be further tested and refined, and promises to be an important tool in determining the interplay between sociality and stress.

2.6 ACKNOWLEDGEMENTS

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CHAPTER 3

EFFECTS OF MATERNAL STRESS ON EGG CHARACTERISTICS IN A COOPERATIVELY BREEDING FISH

3.1 ABSTRACT

Elevated stress levels experienced by a mother can compromise her reproductive success and that of her offspring. In this study, we investigated whether chronically stressed mothers experienced such effects using a cooperatively breeding fish species, in which allocare by helpers at the nest may diminish the negative effects of maternal stress. Using *Neolamprologus pulcher*, a social group-living cichlid fish from Lake Tanganyika, we observed the effects of experimentally increased stress levels on female reproductive success (measured as inter-spawn interval, and number of eggs) as well as egg characteristics including size and cortisol concentrations. Stress levels were manipulated by repeated exposure to the acute stresses of chasing and/or netting. Stressed mothers had longer inter-spawn intervals and laid fewer, smaller eggs. Although no differences in egg cortisol concentrations were detected between control and stressed mothers, egg cortisol concentration fell between spawns for control but not stressed mothers. No effect of helper number was detected for any parameter examined, except there appeared to be a smaller change in cortisol content in the eggs, between the first and second lay, in groups with helpers present. Our results suggest that stress imposes fitness costs on mothers, and

allocare does not appear to alleviate or dampen the negative effects of such maternal stress.

3.2 INTRODUCTION

Stress, resulting from sudden perturbations (acute) or from constant challenges (chronic) in the environment, induces a suite of important physiological and behavioural responses. Stressful conditions experienced early in life (or in utero) may have profound impacts later in life (Contreraz-Sánchez et al. 1998; Hayward and Wingfield 2004; Ostrand et al. 2004; Rondó et al. 2003). In this study, we used a cooperatively breeding fish species to explore whether stress experienced by mothers projects onto the next generation. Responses to stress have been shaped by natural selection to enhance an organism's ability to cope with the stressor and to eventually regain homeostasis (Chrousos 1997; Charmandari et al. 2005). A key component of the physiological stress response is the mobilization of glucocorticoid (GC) stress hormones (cortisol or corticosterone) via activation of the hypothalamic-pituitary-adrenal (HPA, in tetrapods; in fish the HPI or hypothalamic-pituitary-interrenal) axis. The ability to mount an appropriate stress response is generally considered beneficial. However, a prolonged elevation of GCs in response to long-term exposure to stressors can have detrimental effects on an individual, including reductions in growth and immune function, and the suppression of reproduction (Chrousos, 1997; Charmandari et al. 2005; Wendelaar Bonga 1997). Here, we examined the effects of chronic maternal stress (induced by repeated exposure to an acute stressor) on reproductive rates, fecundity, and egg characteristics.

To our knowledge, the potential fitness costs of maternal stress have not yet been experimentally manipulated in cooperatively breeding species (but Schmaltz et al. personal communication, has collected data on yolk corticosterone concentration in the cooperatively breeding smooth billed ani, *Crotophaga ani*). Cooperative breeders are group-living species, in which subordinate group-members forgo reproduction to help raise the offspring of dominant individuals. We hypothesize that the effects of maternal stress on offspring number and quality may be dampened in a cooperative breeder because of the helpful effects of having subordinates participate in care (allocare). To date, the impact on reproductive success of maternal stress during gestation has been studied in several species that are not cooperative breeders. It results in lowered birth-weight in human, *Homo sapiens*, infants (Rondó et al. 2003), slower juvenile growth rates in Japanese quail (*Coturnix coturnix japonica*, Hayward and Wingfield 2004), smaller egg clutches in moor frogs (*Rana arvalis,* Räsänen et al. 2008), and smaller eggs and young in rainbow trout (*Oncorhynchus mykiss*, Campbell et al. 1992; Contreraz-Sánchez et al. 1998).

To address the impact of maternal stress on reproductive fitness in a cooperative breeder, we used the cichlid fish, *Neolamprologus pulcher*, endemic to Lake Tanganyika, Zambia. *N. pulcher* live in social groups organized into linear dominance hierarchies based on size, with a breeding male and female pair at the top of the hierarchy and 1-20 subordinate helpers (Taborsky and Limberger, 1981; Balshine et al. 2001; Heg et al. 2005). The dominant breeders remain in their position for 3-12 months (Stiver et al. 2004) and subordinates rarely if ever breed in the wild (Fitzpatrick et al. 2008, Stiver et al.

2009). *N. pulcher* groups live in communally defended rocky territories clustered together into subpopulations at 10-45 metres depth (Stiver et al. 2008). In these territories, female breeders may be exposed to a number of potential environmental and social stressors. First, some individuals experience elevated predation pressure (Balshine et al. 2001). Second, individuals may need to defend their territories and positions against encroaching, neighbouring conspecifics as well as heterospecific space competitors. Finally, social stress can occur within the group from more dominant individuals or as a result of the need to police subordinates. Exposure to such stressors has the potential to repeatedly raise GC levels and therefore could have fitness implications for both mother and offspring.

We made a series of predictions for the effects of maternal repeated stress on reproductive fitness in *N. pulcher* based on findings in the literature. One, we predicted that stressed mothers would take longer to spawn. In rainbow trout, both chronic and acute stress caused delays in ovulation and spawning (Campbell et al. 1992; Contreraz-Sánchez et al. 1998). Two, we predicted that stressed mothers would lay fewer, smaller eggs. Chronic stress was found to reduce egg mass in rainbow trout (Campbell et al. 1992) although no relationship was detected in zebra finches, *Taeniopygia guttata*, among corticosterone (used as an index of stress), egg mass, and clutch size (Salvante and Williams 2003). Three, we predicted that cortisol levels would be higher in the eggs of stressed mothers compared to those of unstressed (control) mothers. Similar findings of maternal transfer of GCs have been observed in egg yolk of Japanese quail (Hayward and Wingfield 2004) and in the eggs of coho salmon, *Oncorhynchus kisutch* (Stratholt et al.

1997). Finally, we predicted that the presence of helpers would decrease the stress load placed on breeder females and therefore that females with more helpers would exhibit a dampened response to our experimental stressor. Russell et al. (2003) found that helper number was positively correlated with female mass, which in turn positively influenced offspring fitness, in another cooperative breeder, the meerkat, *Suricata suricatta.*

3.3. METHODS

3.3.1 Fish husbandry and housing

All fish used in this experiment were held at McMaster University, Hamilton, Ontario, Canada. They were either descendants of wild-caught breeding pairs captured in 2002, or were wild-caught breeding pairs from early 2008, captured at the southern tip of Lake Tanganyika. Fish were kept in social groups consisting of a dominant male and female with 0-4 subordinates of either sex. Each 189L tank contained a ~2cm layer of sand, a water heater, a thermometer, two mirrors, and two flowerpot halves (to deposit eggs on), and was aerated using two foam filters. Room temperature was kept at 24 ± 2 °C, with each tank held at 26 ± 2°C. The light: dark cycle was maintained at 13:11h, and each group was fed Nutrafin® basix Large Flake commercial cichlid food once daily, in the morning.

3.3.2 Experimental setup and egg collection

In each tank, the two flowerpot halves on which the fish could spawn were lined with a sheet of roughened flexible transparent plastic. Tanks were monitored daily for spawning and once spawning was noted, tanks were assigned to either the "stressed" or

"control" treatment in an alternating fashion. This first spawning day was considered Day 1 of the experiment. To encourage subsequent spawning, any young <2 cm in length, from previous spawning events were removed on this day, a process that required a maximum of 7 min. To ensure that all tanks were equally disturbed by this process, even tanks without young were subjected to a net continuously being dipped and run through the water for 7 min.

On Day 1, the plastic sheet was removed, eggs (spawn 1) were dried carefully with kimwipes, and the eggs were photographed together with a ruler, using a digital SLR camera (Canon EOS Rebel 300D). The eggs were then gently scraped off the plastic surface, weighed to the nearest 0.001g, and stored at -80° C for later analysis. The dominant female was also caught on Day 1, and measured for standard length and body mass. Fulton's body condition factor [100 x (body mass/ standard length^3)] and specific growth rate [100 x (lnM2 - lnM1)(t2 - t1)$^{-1}$] were calculated for each female. In the calculation of specific growth, M2 represented the female's mass at the second spawn (end of the experiment), M1 was the female's mass at first spawn (beginning of the experiment), and t2-t1 was the inter-spawn interval.

The ten females assigned to the control treatment were not subjected to any stressor other than the regular weekly tank cleanings, and were simply fed daily. Five dominant breeder females assigned to the "stressed" treatment were subjected to a daily stressor for up to 80 days. Females were chased with a net for 2-5 min twice per day for up to 50 days (once in the morning before 12pm, and once in the afternoon, allowing more than 3 h between chasing events). If by the end of 50 days no spawning had

occurred, the stressor was changed to netting for 10 min, once daily for 30 days. Two additional dominant breeder females were added to the experiment at this point, i.e. the only stressor imposed was netting for 10 min once daily. After 30 days of daily netting, the stressor was further reduced to netting 3 times per week for 10 min for all seven females. Both chasing (J. Fitzpatrick unpublished data) and netting for 10 min (Mileva et al. in press; **Chapter 2**) have been shown to elevate cortisol concentrations significantly in *N. pulcher,* and presumably elevate catecholamine levels also, as seen in other fish (see Gamperl et al. 1994; Reid et al. 1998; Perry and Bernier 1999). Repeated exposure to acute stressors was used in the present study to elevate cortisol levels. Cortisol levels probably cycled, rising with each application of the acute stressor and then gradually falling again. The day that females spawned for the second time was the last day of the experiment. At this time all females were caught and re-measured, and their eggs removed again (spawn 2) as described above.

3.3.3 Fecundity and egg size analyses

Egg images were analysed using ImageJ v1.42 software (Wayne Rasband, NIH, USA, available at http://rsb.info.nih.gov/ij). Eggs were counted from the digital images, and length and width were measured for 20 randomly chosen eggs per clutch. Five large egg batches were not deposited in a complete monolayer, making it more difficult to accurately count eggs. Hence a second observer re-counted these five clutches and the two egg counts were then averaged. The difference between the two observers' egg counts was on average 21.2 ± 6.8 eggs, or 8.3% of the total egg number. One female's

second clutch included some visible egg-shells/scars (and some of these eggs were too soft to collect); these eggs and scars were counted in the fecundity analyses but were not included in egg size analyses. To compare laboratory fecundity with fecundity in wild fish, we analyzed the preserved ovaries of twenty dominant breeders from Lake Tanganyika. The 20 females were on average 5.16 ± 0.04 cm in standard length (ranging from 4.85 to 5.57 cm) and on average weighed 3.5 ± 0.06 g (with a range of 3.00 to 3.99 g); they were collected in Kaskalawa Bay between February and April 2005, and preserved in ethanol (see Stiver et al. 2006 or Fitzpatrick et al. 2006 for further details of the field site and collection methodology). Each ovary was gently teased apart using forceps and eggs were counted under an external dissection microscope light source (Volpi, NCL 150).

3.3.4 Egg cortisol analysis

Cortisol was extracted from egg samples using the methods described by Alsop and Vijayan (2008). In brief, cortisol was extracted from homogenized egg samples three times using 5ml of diethyl ether each time. Following evaporation of the ether, samples were reconstituted in either 200µl (for all egg masses less than 150mg) or 600µl (for all egg masses above 150mg) of enzyme immunoassay buffer (see below) and kept at 23°C for 2-4 h, with occasional vortexing. Reconstituted samples were diluted further and used for cortisol analysis, which was carried out in duplicate with a colorimetric 96-well enzyme immunoassay kit (Cayman Chemicals, cat# 582121, Ann Arbor, MI, USA). Measured cortisol concentrations were adjusted for dilution factors, and corrected for egg

mass so as to be expressed in units of pg cortisol per mg egg mass. Cortisol extraction efficiency was 125% and the detection limit of the cortisol kit was 12pg/ml.

3.3.5 Statistical analyses

All statistical analyses were performed using JMP IN 5.0 (SAS Institute) and Microsoft Excel 12.0 2008 for Macintosh (Microsoft Corporation). Analysis of variance (ANOVA), Student's *t*-tests and Pearson's r correlations were performed as appropriate where data were normally distributed and of equal variance (the latter applied only for ANOVA and Pearson's r correlations analyses); where data did not meet these assumptions, equivalent non-parametric tests were employed. All *t*-tests assumed unequal variance. Non-significant interaction terms were removed from multi-factor models. To account for differences in mass among breeding females, female mass was used as a variable in each model, when testing for differences between treatments in fecundity, egg size, and egg cortisol concentration. Finally, *N. pulcher* eggs are elongated spheres, and hence the effective diameter of each egg was calculated by means of the widely-used formula, cube root of length x width^2 (Coleman and Galvani, 1998).

3.3.6 Ethical note

No fish were harmed in this experiment, and no injuries or fatalities occurred. The experimental procedures used for chasing and netting fish as well as egg sampling were reviewed and approved by the Animal Research Ethics Board of McMaster University (Animal Utilization Protocol # 06-10-59) and adhere to the animal handling guidelines specified by the Canadian Council for Animal Care.

3.4 RESULTS

3.4.1 Female morphology

Prior to the stress manipulation, control breeder females and females in the stress treatment did not differ in body mass, standard length or body condition (body mass: Mann Whitney U test, $U_{15} = 0.61$, $p = 0.44$; standard length: t-test, $t_{15} = 1.41$, $p = 0.18$, body condition, t-test, $t_{15} = -1.18$, $p = 0.26$; Table 3.1). Over the course of the experiment (controlling for differences in spawning interval, see below), females in the stressed treatment gained less body mass, in fact some lost mass, compared to non-stressed breeder females (ANOVA, Overall model $F_{2, 14} = 3.342$, $p = 0.07$; Effect of Treatment $F_{1, 14} = 6.44$, $p = 0.02$). In terms of specific growth rates, stressed females had a lower daily growth rate than control females (Mann Whitney U test, $U_{15} = 4.20$, $p=0.04$), however their body condition was not different from that of control females at the second lay ($t_{15} = 0.41$, $p = 0.69$). In control females, body mass and inter-spawn interval were strongly positively correlated (Spearman's rho = 0.89, $p = 0.0005$). However, females in the stressed treatment did not show this relationship (Pearson's r, $r^2 = 0.08$, $p = 0.53$).

Table 3.1. Characteristics of control (n = 10) and experimentally stressed (n = 7) mothers at 1st and 2nd lay, and the specific growth rate. SL refers to the standard length and is a common length metric in fisheries research. K refers to body condition, see methods for how this and specific growth rates are calculated.

Variable	Control (mean ± se)	Experimental (mean ± se)
Body mass at 1st lay (g)	8.95 ± 0.55	8.35 ± 0.54
Body mass at 2nd lay (g)	9.48 ± 0.66	8.07 ± 0.59*
SL at 1st lay (cm)	6.64 ± 0.14	6.39 ± 0.10
SL at 2nd lay (cm)	6.83 ± 0.15	6.52 ± 0.14
Fulton's K at 1st lay	3.04 ± 0.08	3.17 ± 0.08
Fulton's K at 2nd lay	2.95 ± 0.09	2.89 ± 0.11
specific growth rate	0.10 ± 0.07	-0.06 ± 0.03*

* denotes significance between control and experimental females, $p < 0.05$.

3.4.2 Spawning Rates

In the wild, female *N. pulcher* can spawn every month and they will spawn even more frequently (every two weeks) in the laboratory, where ample food is available (Balshine, unpublished data). In the present experiment, the mean inter-spawn interval was 42.5 days, ranging from 13 to 120 days. Females in the stressed treatment took significantly longer to spawn a second time than did non-stressed females (Mann-Whitney U test, U = 6.70, p= 0.01; Figure. 3.1).

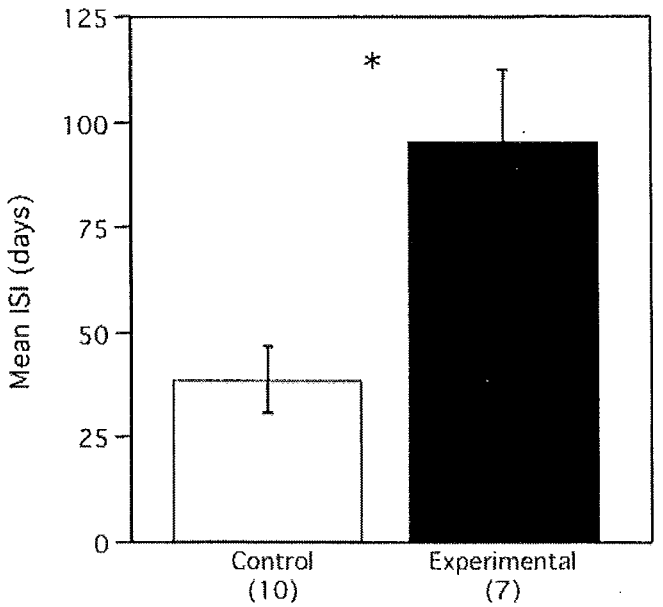


Figure 3.1. The interval between the first and second egg laying events (inter-spawn interval, ISI) for *N. pulcher* breeder females in control and stressed experimental treatment groups. Values are means ± se; *N* for each experimental group is indicated in parentheses. The asterisk indicates a significant difference between control and experimental groups.

3.4.3 Fecundity

Fecundity in laboratory females varied from 21 to 408 eggs per clutch with an overall mean of 133.9 ± 14.3 eggs per clutch. This fecundity is in striking contrast to that in the field, where 20 ovarian samples from ripe female breeders revealed a mean (ovarian) clutch size of 42.7 ± 2.6 eggs, with a range of 23 to 61 eggs per female.

On Day 1 (in the first spawns before any stressors were applied), egg number did not differ between treatments (t-test, $t_{15} = -0.57$, $p = 0.58$; Figure 3.2a). However, when we compared fecundity at the second spawn (end of the experiment), stressed females laid significantly fewer eggs than did control females (t-test, $t_{15} = 2.21$, $p=0.04$; Figure 3.2a).

Interestingly, although both stressed and control females laid fewer eggs in their second spawn compared to their first spawn, the reduction in egg number between spawns was significantly greater for stressed mothers than for control mothers when we controlled for specific growth rate (ANOVA, Overall model $F_{2,14}$=3.33, p=0.07, Effect of treatment, $F_{1,14}$=5.92, p=0.03, Effect of strategic growth rate $F_{1,14}$=3.17, p=0.10 ; Figure 3.2b).

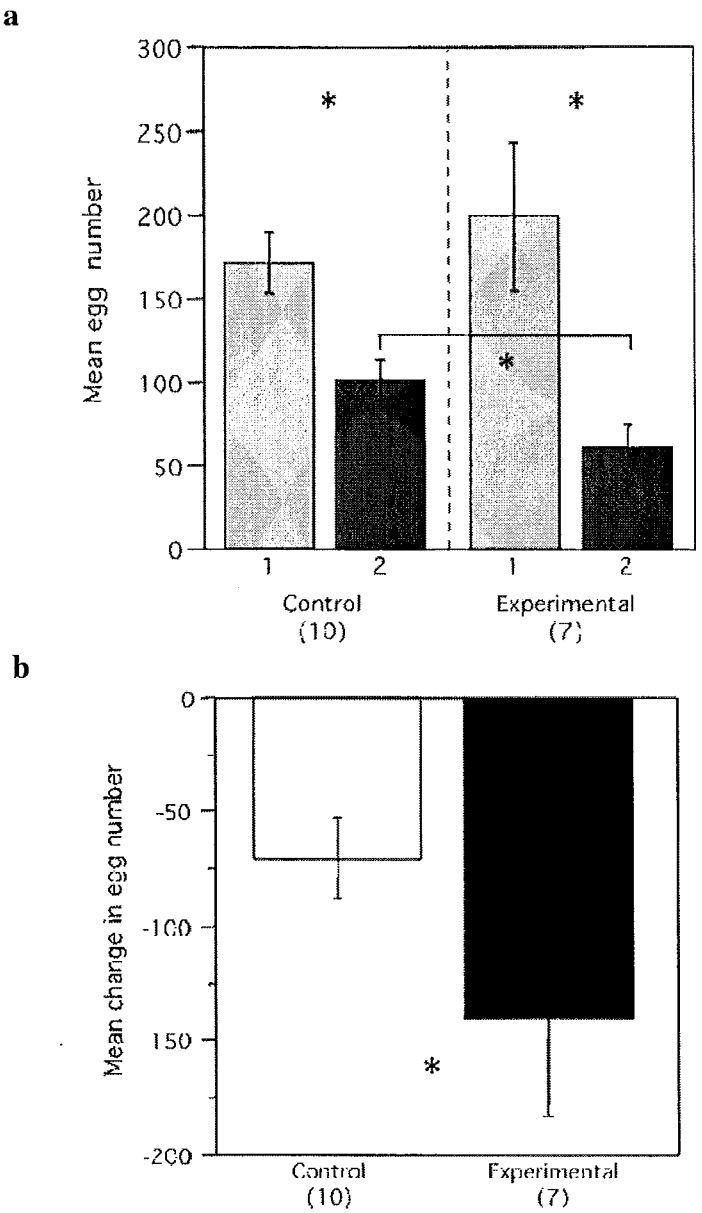


Figure 3.2. **(a)** Clutch sizes (mean number of eggs per clutch ± se) for the first and second lay of *N. pulcher* breeder females assigned to control and stressed experimental conditions. *N* for each experimental group is indicated in parentheses. Asterisks indicate a significant decrease in clutch size between spawns for each experimental group (control: t-test, $t_{15} = 3.21$, p=0.01; experimental: t-test, $t_{15}= 2.95$, p=0.02). (**b**) the mean change in egg number between first and second spawns of both control and experimental conditions..

3.4.4 Egg Size

Stressed females laid eggs of lower mass in their second spawn as compared to their first spawn (ANOVA, overall model: $F_{2, 11}$= 4.03, p=0.05; Effect of 1st vs 2nd spawn on egg mass $F_{1, 11}$= 7.94, p=0.02; Effect of female body mass $F_{1, 11}$= 0.40, p=0.54; Figure 3.3a), whereas unstressed females laid eggs of similar mass across the two spawning events (ANOVA, overall model: $F_{2, 17}$= 0.20, p=0.82; Effect of 1st vs 2nd spawn on egg mass $F_{1, 17}$= 0.40, p=0.53; Effect of female body mass $F_{1, 17}$= 0.02, p=0.87; Figure 3.3a). Calculation of the change in egg mass between the first and second spawns illustrated this significant difference further (Figure 3.3b).

Egg diameter averaged 1.24 mm ± 0.02 across all females, and ranged between 1.02 and 1.40 mm. Controlling for body mass, egg diameter was smaller in the second spawn compared to the first spawn of stressed females (ANOVA, Overall model: $F_{2,11}$ = 4.03, p=0.05; Effect of 1st vs. 2nd lay on egg diameter, $F_{1,11}$ = 7.94, p = 0.02; Effect of female body mass, $F_{1,11}$ = 0.40, p=0.54; Figure 3.3c) but no such decrease was observed in control females (ANOVA, Overall model $F_{2,17}$ = 0.20, p = 0.82; Effect of 1st vs. 2nd lay on egg diameter $F_{1,17}$ = 0.40, p=0.54; Effect of female body mass $F_{1,17}$ = 0.02, p = 0.89; Figure 3.3c). Overall, however, the change in egg diameter from one spawn to the next did not vary between treatments (Mann-Whitney U test, U=2.14, p=0.14; Figure 3.3d).

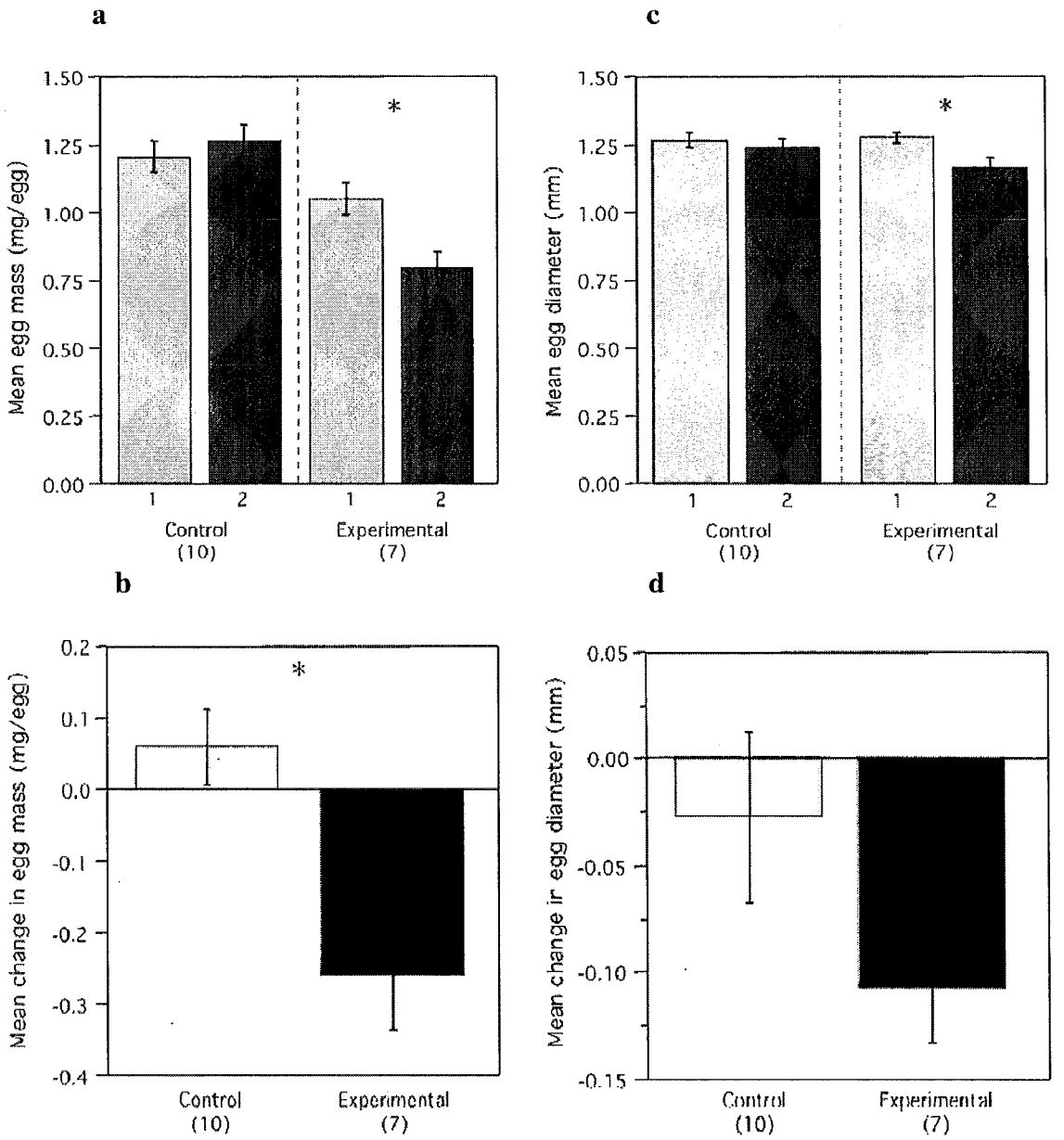
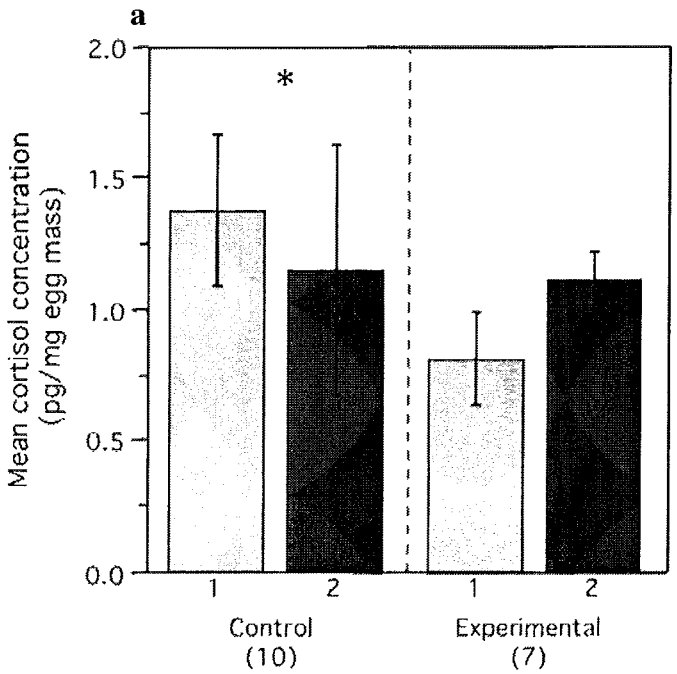


Figure 3.3. Data on egg size (mass and diameter) are presented for the first and second lay of *N. pulcher* breeder females assigned to control and stressed experimental conditions. **(a)** Egg mass (mg/egg) and **(b)** the mean change in egg mass (mg/ egg) between control and experimental conditions. **(c)** Effective egg diameter (mm) and **(d)** the mean change in egg diameter (mm) for both control and experimental conditions. Values are means ± se; *N* for each experimental group is indicated in parentheses. Asterisks indicate significance at $p < 0.05$

3.4.5 Cortisol levels in eggs

At the first lay, eggs from both control and stressed mothers had similar concentrations of cortisol (Mann-Whitney U test, U=2.44, p=0.12). Cortisol levels in eggs from control females fell significantly from the first to the second spawn (U=4.48, p=0.03; Figure 3.4a) while eggs from females in the stressed treatment maintained similar cortisol levels in both the first and second spawns (T-test, t= -1.43, p=0.18; Figure 3.4a). Examination of the change in cortisol between spawns did not reveal any significant difference between the control and stress treatment groups (T-test, t=1.01, p=0.33; Figure 3.4b).



b

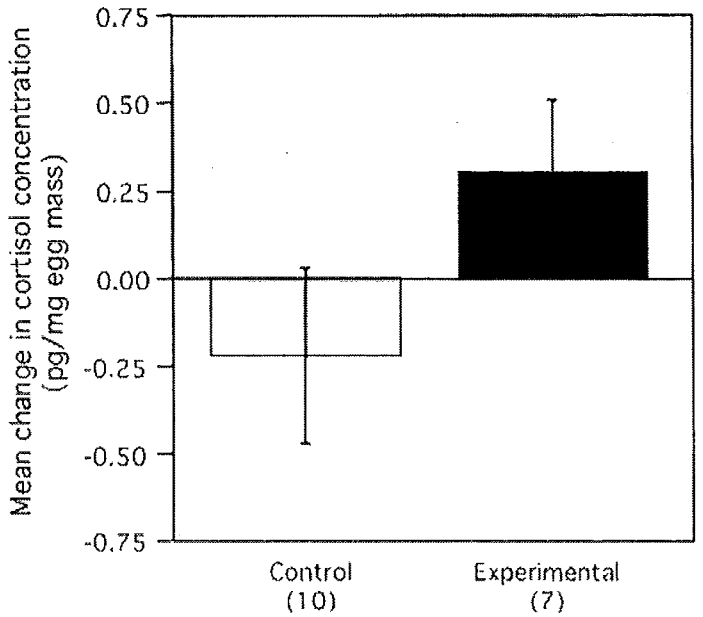


Figure 3.4. (a) Data on egg cortisol concentrations (pg/mg egg mass) are presented for the first and second spawn of *N. pulcher* breeder females assigned to control and stressed experimental conditions. Note that in the control females egg cortisol significantly decreased between the first and second spawn but egg cortisol levels remained high in stressed females (see discussion for likely explanation). **(b)** The mean change in cortisol concentration (pg/ mg egg mass). Values are means ± se; *N* for each experimental group is indicated in parentheses. The asterisk indicates significance at $p<0.05$.

3.4.6 Helper Effects

Helper number had no effect on any egg characteristic (all $p> 0.05$, Table 3.2). To increase the likelihood of detecting a helper effect, data for stressed and non-stressed females were pooled for this analysis. No effect of helper number was observed but there was a trend for helper number to be negatively related to the change in cortisol levels (Table 3.2). Also, because no other effect of helper number was detected, an additional analysis was carried out where groups with helpers (any number from 1 to 4) were compared to those without helpers. Again, the presence of helpers had no effect on egg number, mass or diameter. Helper presence did affect egg cortisol levels, where the

presence of a helper resulted in a smaller change in egg cortisol levels between spawns than was measured in the absence of a helper (Table 3.2).

Table 3.2. The effects helpers had on the change in egg characteristics when pooling conditions and treating helpers either as a continuous or nominal variable.

Helpers effects*	**Change in egg number**	**Change in mass (mg/egg)**	**Change in diameter (mm)**	**Change in cortisol (pg/ml egg mass)**
Pooled conditions with helpers as a continuous variable	Rho=-0.07, p=0.78	Rho=0.12, p=0.67	Rho=0.16, p=0.53	Rho=-0.47, p=0.06
Pooling conditions with helper presence (Yes/No)	t-test, t=-0.63, p=0.55	t-test, t=-0.68, p=0.51	t-test, t=-0.95, p=0.36	Mann-Whitney U =5.95, p=0.01

* All experimental groups had n=7, and control had n=10.

3.5 DISCUSSION

This study provides clear evidence that *N. pulcher* breeder females repeatedly exposed to acute stress experienced a decline in fitness. Stressed *N. pulcher* mothers experienced longer intervals between spawning events, and laid fewer eggs of smaller mass and diameter than control mothers. Egg cortisol levels fell between spawns in control mothers, but not in stressed mothers. The results suggest that not only is maternal fitness influenced, but that maternal stress may have repercussions for offspring fitness owing to the production of smaller eggs. Finally, the data suggest that neither the presence nor the number of subordinate helpers played a strong role on how mothers invested in gametes. However, when we pooling both stressed and control mothers, we observed that mothers with helpers versus those without, had smaller fluctuations in cortisol concentrations in eggs, suggesting that helpers may serve to reduce maternal cortisol levels. To our

knowledge, this is among the first studies to experimentally manipulate stress levels in a cooperative breeder, and explore the affects of allocare on stress responses.

Repeated acute stress is likely to raise both catecholamine and glucocorticoid levels in blood plasma (Reid et al. 1994; Perry et al. 1996; Perry and Bernier 1999), although data for the effect of acute stress on catecholamine concentrations in *N. pulcher* are not available owing to the small size of this species. With each presentation of a stressor, cortisol and catecholamine levels are expected to rise, and then gradually return to baseline (for an example in rainbow trout, see Pickering and Pottinger 1989). With time, the magnitude of the hormone elevation may have declined, as repeated stress often results in attenuation of the glucocorticoid response (see for example the work of De Boer et al. 1990 on rats, and that of Barton et al. 1987, Perry et al. 1996, Jentoft et al. 2005 on rainbow trout). The repeated stressors of chasing and netting were chosen in the present study for practical reasons. Commonly used chronic stressors such as crowding (Pickering and Stewart 1984; Montero et al. 1999; Ramsay et al. 2006) cannot be applied to a single individual within a group and therefore would have affected the entire social group and its dynamics. A similar argument applies to the use of cortisol-treated feed, an approach often used to chronically elevate plasma cortisol in the absence of a chronic stressor (see Barton et al. 1987). Additionally, elevation of cortisol using an intraperitoneal implant, another commonly used approach (for example see McCormick 1998), is problematic in fish of such small size. Barton et al. (1987) found that repeated stress evoked physiological changes comparable, although smaller in magnitude, to those elicited by chronic elevation of cortisol through the use of cortisol-treated food, and we

therefore adopted this approach, enabling the breeder female to be selectively stressed while keeping each *N. pulcher* social group intact.

In this study, experimentally stressed females exhibited reduced growth rates in comparison to control fish. Decreased growth rates in response to repeated stress have been reported in other fish species (eg. rainbow trout, Barton et al. 1987; Eruasian perch, *Perca fluviatilis*, Jentoft et al. 2005; Atlantic salmon, *Salmo salar*, McCormick et al. 1998). When individuals are chronically or repeatedly stressed, tradeoffs occur between growth and survival (Schreck et al. 2000; Wendelaar Bonga 1997). Mobilization of energy reserves by stress hormones is thought to contribute to this phenomenon (reviewed by Gilmour et al 2005). In addition, acute and chronic stress cause decreases in food intake (e.g. brown trout, *Salmo trutta,* Pickering et al. 1982; rainbow trout, Øverli et al. 2006; see reviews by Bernier and Peter 2001, Bernier 2006), and it is possible that such appetite suppression may impact growth rates. All aquaria in the present study were fed *ad libitum*, but we were unable to measure specific food intake by the breeder females.

Our results also indicated that a delay in reproduction occurred as a result of repeated stress, and this confirms previous findings in fish (Carragher et al. 1989; Campbell et al. 1992), ungulates (Dobson and Smith, 2000), and birds (Reynard and Savory 1999; Salvante and Williams 2003; Schoech et al. 2009). The mechanisms underlying the tendency for stress to delay reproduction have been studied in fish, where acute stress has been found to inhibit aspects of the fish endocrine system pertaining to reproduction (Pankhurst and Van der Kraak 1997; Pankhurst and Van Der Kraak 2000). For example, Carragher et al. (1989) found that female brown and rainbow trout given

cortisol implants had lower levels of circulating vitellogenin and estradiol, as well as smaller gonads. More recently, Pankhurst and Van der Kraak (2000) reported that sexually mature female rainbow trout showed a decrease in plasma testosterone and slight decreases in estrogen following exposure to an acute stressor.

In addition to the increased inter-spawn interval, exposure to repeated stress resulted in the production of fewer, smaller eggs by *N. pulcher* breeder females. In both stressed and control treatment groups, clutch size decreased from the first to the second lay. It is likely, however, that this effect was due to the inadvertent provision initially of an inadequate spawning substrate. Originally, smooth, clear transparencies were installed in the brood chambers for the collection of eggs. It quickly became apparent that fish were not spawning as usual under these conditions, and the smooth plastic was therefore replaced with roughened transparencies. Following this substitution, the fish began to spawn almost immediately, laying very high numbers of eggs (9/17 fish laid greater than 150 eggs, with two individuals laying 300+; laboratory estimates suggest 100-300 eggs per clutch, Taborsky et al. 1984, Sigal Balshine unpublished data) presumably because eggs had been sequestered while no appropriate substrate was available for laying. Even with this methodological confound, we still observed a greater decrease in egg number in stressed females than control females and a significantly greater drop in egg mass in the experimentally stressed females vs. controls. Other studies have also linked maternal stress to smaller gametes, smaller young, and higher juvenile mortality (Coleman and Galvani, 1998; McCormick 1998; Contreras-Sánchez et al. 1998; Ostrand et al. 2004, Campbell et al. 1992). Fewer gametes may be attributed to the allocation of resources to

maintaining homeostasis, and away from reproduction. If reproduction occurs at all, the output is fewer, smaller eggs (as seen in this study), likely at least in part because of the negative effects cortisol has on vitellogenin and estradiol production (see Carragher et al. 1989). In this experiment, eggs were used to quantify cortisol levels, and hence were not allowed to hatch for young to mature. It would be valuable for future studies to explore the impacts of decreased egg size on offspring, in terms of survival rates and later life social and reproductive repercussions.

Several studies (Stratholt et al. 1997; McCormick 1998; Eriksen et al. 2006) have found that high maternal stress and/or cortisol levels results in elevated egg cortisol concentrations. In the present study, no difference was detected in the cortisol concentrations of eggs produced by stressed vs. control *N. pulcher* breeder females. However, whereas egg cortisol levels decreased significantly from the first to the second spawn in control mothers, the cortisol content of eggs produced by stressed mothers remained constant or tended to increase. It is possible that the sequestering of eggs, because of the inappropriate spawning substrate noted above, resulted in elevated maternal and hence egg cortisol concentrations. With this scenario, the subsequent fall in egg cortisol concentrations in the control group would reflect re-establishment of normal spawning conditions, while continued high cortisol concentrations in eggs from stressed females reflected the impact of the stress exposure. Additionally, cortisol concentrations in fish eggs and embryos follow a U-shaped curve between laying and hatching, starting with cortisol that is deposited by the mother. Cortisol concentrations then decrease until hatching, and increase post-hatch (de Jesus et al. 1991; Barry et al. 1995; Alsop and

Vijayan 2008). Hatching in *N. pulcher* occurs 3-4 days after spawning (Balshine-Earn 1998; Taborsky et al. 2007). In the present study, eggs were collected between 1 and 24 h post-spawn (this variable was not monitored) and therefore differences in egg cortisol concentration reflecting differences in incubation time may have caused increased variation in the data, which would make treatment effects difficult to detect when coupled with our small sample sizes. Clearly, the impact of maternal stress on egg cortisol levels in *N. pulcher* warrants further investigation, employing larger sample sizes.

Typically, in highly social species (eg. Wolves, *Canis lupus*, Creel 2005; meerkats, *Suricata suricatta*, Young et al. 2006; or Florida scrub jays, *Aphelocoma coerulescens*, Schoech et al. 2009), it is extremely challenging to manipulate maternal stress in an experimentally consistent manner and investigate its effect on maternal reproductive success and offspring characteristics. In contrast, in this study we used *N. pulcher*, a highly social species that performs naturalistic behaviour in the laboratory and facilitates such investigations on stress responses in social species. Our results suggest that the presence of additional individuals in a social group reduces the variation in amount of cortisol that mothers transfer to eggs between spawns. Future studies that rigorously manipulate the helper numbers are needed to further explore the impacts of cooperative living on maternal stress levels and on offspring survival, growth rates, and social status.

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APPENDIX 1

DIFFERENTIAL EXPRESSION OF CORTICOSTEROID RECEPTOR mRNA IN A COOPERATIVELY BREEDING CICHLID

A.1 INTRODUCTION

Neolamprologus pulcher is a cooperatively breeding cichlid fish that is endemic to Lake Tanganyika, Zambia. *N. pulcher* lives in stable social groups consisting of a dominant breeder male and female together with 1-20 subordinate helpers of either sex (Taborsky and Limberger, 1981; Balshine et al. 2001; Heg et al. 2005). The effects of status and/or environment on stress responses in socially-living species have been the subject of concentrated research interest (Creel 2001; Goymann and Wingfield 2004; Schoech et al. 2007; Young et al. 2006). Within many species, subordinate but not dominant individuals exhibit chronic elevation of glucocorticoid (GC) stress hormones, but the opposite pattern has been observed in many cooperatively breeding species, with dominant individuals exhibiting higher GC levels than subordinates (see Creel 2001; Goymann and Wingfield 2004, for review). Recently, Mileva et al. (in press; **Chapter 2**) found that, like other cooperative breeders (see Creel et al. 2001), dominant *N. pulcher* exhibit higher cortisol levels than subordinates.

Circulating GC hormone levels are not, however, the sole determinant of stress responses. The effects of corticosteroid hormones in fish, as in other vertebrates, are mediated through intracellular receptors that act as ligand-dependent transcription factors

(reviewed by Mommsen et al. 1999; Prunet et al. 2006; Stolte et al. 2006; Bury and Sturm 2007). Although the relationship between stressors and circulating plasma levels of GCs has been studied extensively, the regulation of corticosteroid receptors is much less well understood. Individual differences in stress responses are likely to depend on the number and affinity of corticosteroid receptors. For example, Shrimpton and McCormick (1999) reported that the responsiveness of rainbow trout, *Oncorhynchus mykiss*, gill tissue to cortisol *in vitro* was directly related to the concentration of gill corticosteroid receptors. Moreover, binding studies using radiolabelled ligands have suggested that the number and/or affinity of corticosteroid receptors can be altered by a variety of treatments including hormone manipulation (Shrimpton et al. 1995; Shrimpton and McCormick 1999), exposure to stressors (Shrimpton and McCormick 1999) and changes in salinity (Dean et al. 2003).

Molecular characterization of corticosteroid receptors revealed that fish express both glucocorticoid (GR) and mineralocorticoid (MR) receptors (Ducouret et al. 1995; Colombe et al. 2000; reviewed by Prunet et al. 2006; Bury and Sturm 2007). Teleost fish are unique in that most species (but see Alsop and Vijayan 2008 for an exception) possess two GRs, likely as a result of a gene duplication event that occurred around 335MYA (Greenwood et al. 2003; Bury et al. 2003). The two GRs differ in molecular sequence, ligand affinity, transactivation properties and tissue distribution, but their functional significance remains uncertain (Stolte et al. 2006). Additionally, MRs in teleost fish are probably activated by cortisol (McCormick et al. 2008) since unlike in tetrapods, fish cannot synthesize aldosterone (Bury and Sturm. 2007). The roles of teleost MRs remain

elusive. Much of our knowledge of teleost corticosteroid receptor tissue distribution rests on mRNA expression data, since isoform-specific antibodies are not yet available for probing corticosteroid receptor protein distribution. Based on mRNA expression, teleost GRs and MRs have been found in all tissues examined to date (Bury et al. 2003; Greenwood et al. 2003; Sturm et al. 2005). Greenwood et al. (2003) noted that in *Astotilapia burtoni*, a cichlid species closely related to *N. pulcher*, MR mRNA expression was higher than GR expression in the brain, but lower in the liver, kidney, spleen and other organs. These researchers also reported differential distributions of the two GR isoforms (1 and 2), with GR2 being expressed more highly in the liver and gill (Greenwood et al. 2003).

Recent work suggests that corticosteroid receptor mRNA expression is responsive to a variety of factors, including chronic stress due to high stocking density and/or elevated cortisol levels (Vijayan et al. 2003; Terova et al. 2005; McCormick et al. 2008). In particular, experimental elevation of cortisol concentrations has been associated with increased GR1 mRNA expression in the gill and liver of rainbow trout (Vijayan et al 2003; McCormick et al 2008). In contrast, high stocking density and an associated elevation of circulating cortisol resulted in lowered liver GR mRNA expression (Terova et al 2005). In the present study, corticosteroid receptor mRNA expression was explored as a function of social status within a cooperatively breeding fish species, *N. pulcher.* Based on our previous finding on this species of dominant individuals (breeders) exhibiting higher cortisol levels than subordinates (helpers) (Mileva et al. in press; **Chapter 2**), we predicted that GR mRNA expression would also differ between dominant

and subordinate fish. Corticosteroid receptor mRNA expression was examined in the brain because differences in social status likely originate in the brain. For example, the hormone AVP, an important part of the HPA/HPI pathway, is important in modulating aggression in male field sparrows, *Spizella pusilla* (Goodson 1998) and agonistic behaviour in golden hamsters, *Mesocricetus auratus* (Ferris and Delville 1994). We also looked at mRNA exression in the liver, as it plays a key role in both growth and metabolic responses to stress, both of which differ or are expected to differ between breeder and helper *N. pulcher* (Taborsky 1984; Sopinka et al 2009; Mileva et al. in press, **Chapter 2**).

A.2 MATERIALS AND METHODS

A.2.1 Experimental animals

A breeding colony of adult *N. pulcher* held at McMaster University, Hamilton, Ontario, Canada was used. All fish used in this experiment were descendents of male and female breeding pairs caught in 2001 from southern Lake Tanganyika. Fish were housed in 189 L freshwater tanks enriched with two mirrors to prevent in-group aggression, two upside down flowerpot halves for egg laying and shelter, a heater, thermometer, 2 foam filters with airstones, and ~2 cm of coral sand substrate. The light: dark cycle was kept constant at 13:11 hrs and water temperatures were maintained at 26± 2°C. Fish were fed *ad libitum* daily with Nutrafin® Basix Large Flake commercial cichlid food.

A.2.2 Experimental protocol

Each tank housed a social group consisting of a male and female dominant breeding pair and 1-20 subordinate helpers (mean group size ± standard error, 10.1 ± 1.5; total of 7 tanks). Four focal fish, namely the dominant breeding pair and the two largest subordinate helpers, were identified through the use of detailed ethograms for this species (Taborsky 1984, Buchner et al. 2004, Sopinka et al. 2009). Three to seven days prior to behavioural watches, the focal fish were weighed, standard length was measured, and the dorsal fins were clipped in unique patterns so as to be able to distinguish the fish from one another. Fin clipping does not adversely affect behaviour (Stiver et al. 2004). Behavioural watches were carried out between 8:00 and 13:00 in three 10-minute intervals for each fish. Following the behavioural watches (from 13:30-15:30), focal fish were quickly captured and placed in an ice-bath for 5-10 seconds, then killed by a firm cephalic blow. Whole brain and liver tissues were quickly extracted and immediately frozen in liquid nitrogen; then stored at -80°C until analysis of GR1, GR2, MR and 18S mRNA expression. The tissue mRNA expression investigated in this paper was collected from a subset of the fish investigated in Mileva et al. (in press; **Chapter 2**), and all fish sizes, behaviours and cortisol concentrations used in this study are reported here (Table A.1).

A.2.3 Isolation of partial corticosteroid receptor sequences

A.2.4 Molecular analysis

Real-time RT-PCR was used to assess liver and brain mRNA expression of the three corticosteroid receptor isoforms (GR1, GR2 and MR) as a function of social status.

Tissue samples were homogenized using a 21-gauge needle attached to a 3 mL syringe until the mixture could pass easily through the needle. Total RNA was extracted from liver and brain tissues of focal fish using TRIzol reagent (Invitrogen) according to the manufacturer's specifications. The concentration and quality of RNA was analysed using a NanoDrop 1000 spectrophotometer (ThermoFisher Scientific) and no sample A_{260}/A_{280} ratios were below 1.6. Prior to cDNA synthesis, RNA was treated twice with amplification grade DNase (Invitrogen) according to the manufacturer's instructions to eliminate genomic DNA contamination. Unusually high levels of genomic DNA contamination in all RNA samples necessitated the double DNase treatment. First strand cDNA synthesis was then performed on 0.5 μg of DNase-treated total RNA using RevertAid H-minus M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer's instructions, and random primers (0.2 μg per reaction).

Primers for real-time RT-PCR analysis (Table A.2), with the exception of 18S, were designed from GenBank sequences for *N. pulcher* using Genetool software (Biotools, Inc.). Primers were designed to generate amplicons of approximately 110-220 base pairs (bp) and were selected for annealing temperatures of 58°C. Primers for the control gene, 18S, were designed (using Genetool software) based on the GenBank sequence for 18S from *Oreochromis esculentus* (Table A.2). The specificity of all primer pairs was confirmed by sequencing of amplicons. Gel purified products were cloned (using QIAquick gel extraction kit, Qiagen), ligated into a plasmid (pDrive vector, Qiagen), and amplified in DH5α competent cells (Invitrogen). Plasmid cDNA was isolated (QIAprep Spin Miniprep kit, Qiagen), and clones were sequenced and identified.

All real-time RT-PCR reactions were performed using a SYBR green master mix kit (Stratagene) and Mx3000P Real-Time PCR System with associated MxPro 4.01 software (Stratagene). The composition of the reaction, as well as the settings used for the thermocycler, were those suggested by the manufacturer with the exception that the reaction volume was scaled to 12.5 μL from 25 μL. Standard curves were generated for all genes using pooled liver cDNA to assess the efficiencies of the primer reactions. Pooled samples were serially diluted (1 in 5) in RNase/DNase-free water (Sigma) for a total of 6 standards, including a no-template control. The resulting cycle threshold (*Ct*) values were plotted against relative template concentration and the relationship was described by linear regression. Efficiencies were deemed acceptable if they fell between 85 and 115% and had an R^2 value ≥ 0.97. A set of 'no reverse transcriptase' control templates was analyzed in addition to the samples to ensure that the generated amplicons did not originate from genomic DNA contamination. These control templates were produced by omitting the reverse transcriptase enzyme during cDNA synthesis and were included in each plate together with 'no template' controls (where water was included in the reaction as template instead of cDNA). Dissociation curves were generated and evaluated for each reaction to ensure that only one PCR product was amplified and contributed to the measured *Ct* value. All sample reactions were carried out using 2 μL of template in 12.5 μL reactions. Relative mRNA expression of the gene of interest against the reference gene 18S (template diluted 1000-fold) was determined according to the modified delta delta *Ct* method (Pfaffl 2001). For purposes of comparison across social status, mRNA expression of each focal individual (breeder female, breeder male, helper 1

and helper 2) was calculated relative to the mRNA expression of the gene of interest in the breeder female group.

A.2.5 Statistical analysis

Values are means ± 1 SEM. One-way analysis of variance (ANOVA) was used to test for statistically significant differences in mRNA expression among social groups, for each gene, in both the brain and liver.

A.3 RESULTS

Table A.1. Aggression, submission and workload composite scores for breeder males (BM), breeder females (BF), helper females (HF) and helper males (HM) used in this study. Composite scores were calculated as in Mileva et al. (in press; **Chapter 2**). Brackets refer to sample sizes.

Variable	BM (7)	BF (7)	H1 (6)	H2 (8)
Aggression composite	17.1 ± 4.7	11.8 ± 1.7	7.4 ± 2.2	3.5 ± 1.1
Submission composite	0.5 ± 0.5	4.7 ± 1.1	6.6 ± 1.6	6.5 ± 2.0
Workload composite	6.9 ± 2.2	4.6 ± 1.6	0.1 ± 0.1	0
Cortisol concentration*	45.5 ± 15.8	45.9 ± 22.1	31.7 ± 9.0	24.7 ± 9.4

*Cortisol concentrations different between years. See Mileva et al. in press; **Chapter 2.**

Table A.2. Forward (F) and reverse (R) real-time RT-PCR primers used to analyze corticosteroid receptor mRNA expression in *N. pulcher* brain and liver tissue.

Gene	Accession #	Primer	Sequence 5' – 3'
GR1	EF661651	F	TGC CTC TGT CAC TGC CAC CGT AG
		R	AGT CGT CTG CGT AAG TAA CTG
GR2	EF661652	F	GCA CCA GAG CCC ACC ATT AGC AAC AT
		R	CTT GGC CCA CTT GAC TGC AGA GAC A
MR	EF661650	F	GGG CTC TAA GGA TGG CCA AAC TG
		R	CAG ATG GAG GGC AGA AAA GGT
18S	AF337051	F	ATG GCC GTT CTT AGT TGG TG
		R	CTC AAT CTC GTG TGG CTG AA

All sequences are listed in the 5' – 3' direction. Reverse primers (R) are listed as the reverse complement sequence of the original DNA template.

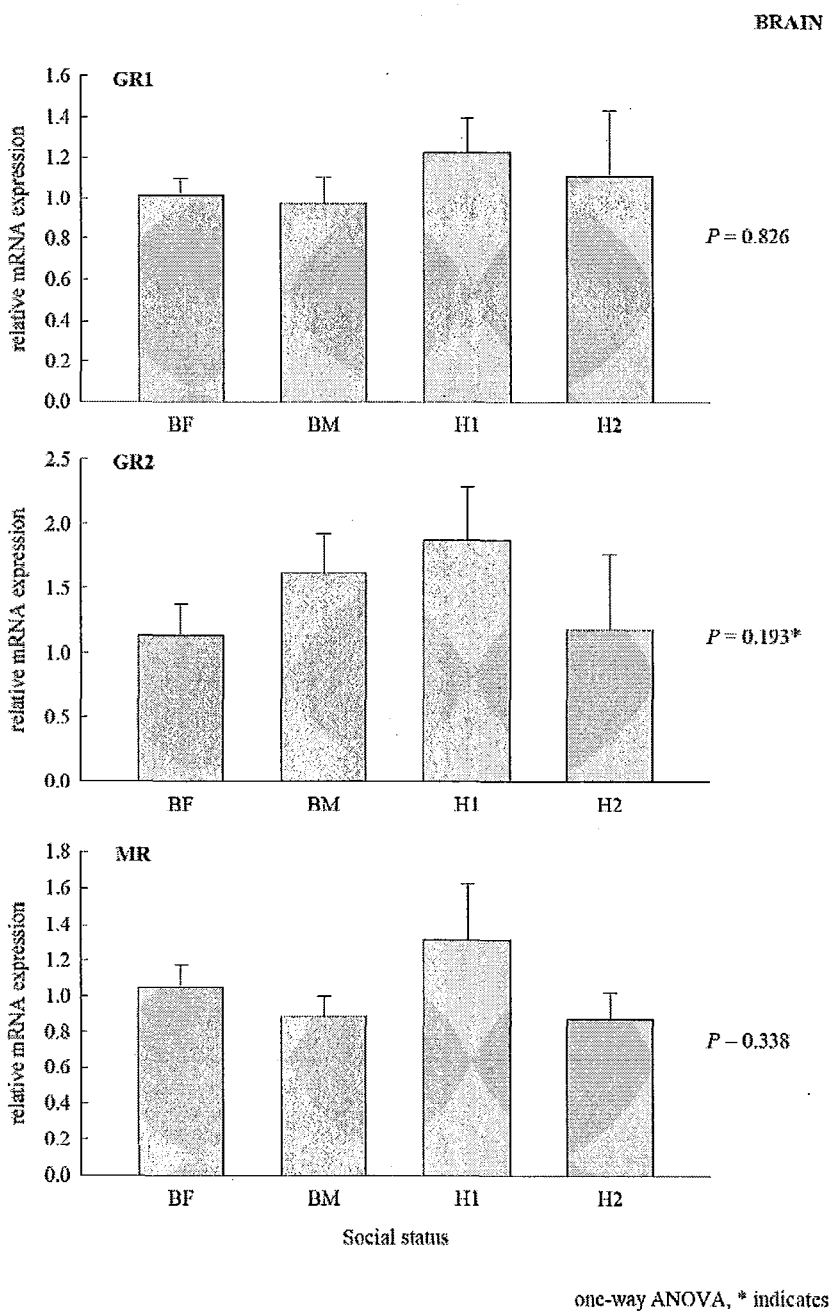

Figure A.1. Relative mRNA expression GR1, GR2, and MR in the brain of breeder females (BF, n=7), breeder males (BM, n=7), Helper 1 (H1, n=6), and Helper 2 (H2, n=8).

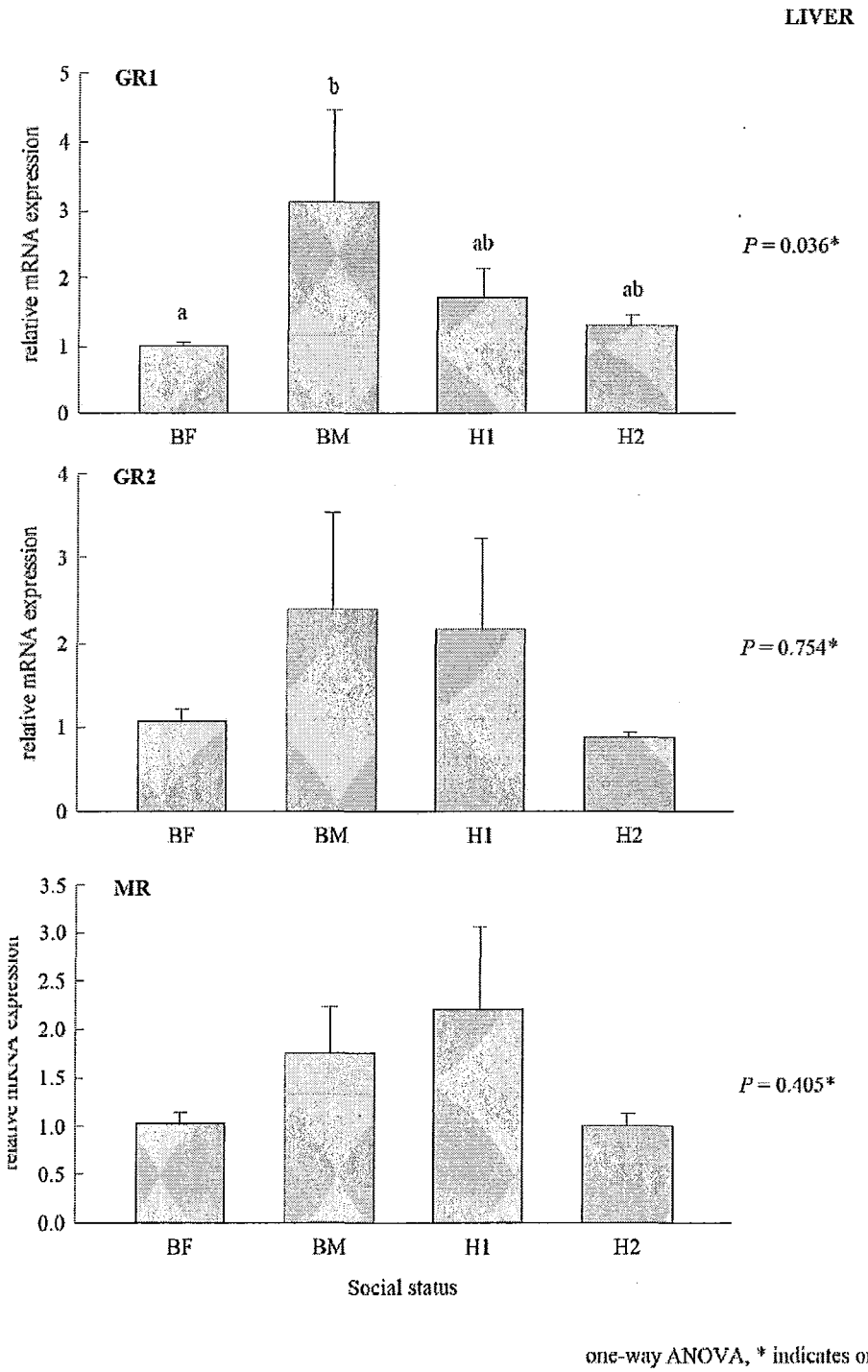

Figure A.2. Relative mRNA expression GR1, GR2, and MR in the liver of breeder females (BF, n=7), breeder males (BM, n=7), Helper 1s (H1, n=6), and Helper 2s (H2, n=8).

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CHAPTER 4

INSIGHTS INTO THE STRESS BIOLOGY OF *N. PULCHER*, AND POTENTIAL FUTURE RESEARCH

With this thesis, I have provided evidence that dominant *N. pulcher* have higher baseline circulating glucocorticoid levels than subordinates **(Chapter 2).** We believe that this situation reflects higher allostatic loads placed on dominant individuals (i.e. greater difficulty acquiring and maintaining dominance status). Additionally, we found that following a 10 min acute stressor both subordinate and dominant individuals had elevated cortisol levels to a similar degree. This result, coupled with our finding that stressed fish of both classes have dramatic cortisol increases when stressed, suggests that dominant breeders do not attenuate their stress response and are not at 'ceiling' prior to stress manipulation. Total activity levels and social interactions were revealed to correlate positively with cortisol levels at baseline, but only in subordinate males. This result leads us to conclude that because life history traits in male and females subordinates differ, with males rarely inheriting territories and therefore needing to disperse, such social interactions are in effect serving to appease dominant breeders in an attempt to be allowed to stay within the territory (for example see Bergmüller et al. 2005). With more information on the corticosteroid receptors and their distribution in male and female, dominant and subordinate *N. pulcher,* we will be able to investigate how circulating glucocorticoids are affected by and/or affect receptor levels **(Appendix 1).** We can also

link these findings directly to behaviour to paint a fuller picture of the stress response in this cooperative breeder.

Studies of maternal stress have, to our knowledge, never been conducted in cooperatively breeding species, but can help us uncover potential benefits and costs of helpers that have not yet been investigated. By repeatedly stressing some dominant breeder females and not others we witnessed a decrease in the growth rate and a greater inter-spawn interval in stressed females compared with controls. When egg laying did occur, stressed females laid fewer eggs, and these were of smaller mass and diameter (**Chapter 3**). There appeared to be no effect of helper number on any of the parameters examined above, except that eggs laid by females in groups with helpers showed lower cortisol concentrations. We have effectively shown that repeated stress causes reproductive changes in *N. pulcher*, which could have negative impacts on both the mother's fitness, and that of her offspring. Most interestingly, and helper presence does not seem to dampen these negative impacts.

Future studies, using larger samples sizes, would benefit from investigating the potential costs/benefits of varying helper numbers within a group (i.e. greater than 4), with respect to maternal stress. Additionally, by allowing eggs of stressed and control mothers to hatch, we would acquire direct information on mortality rates, which could be linked back to maternal fitness. Allowing these fry to grow, could illuminate differences in growth rates, behaviours, response to stressors, and to speculate wildly, even point to the development of different behavioural syndromes.

Thus in **Chapter 2** I characterised the stress response of individuals making up a social group, and in **Chapter 3** I was able to focus on the potential impacts of stress transmitted between the generations. The social system results in their being higher stress in dominant breeders (**Chapter 2**) and this may influence reproductive output, especially in the dominant breeding female (**Chapter 3**).

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