PHYSIOLOGY OF SOCIAL ASCENSION IN A GROUP-LIVING CICHLID FISH

PHYSIOLOGY OF SOCIAL ASCENSION IN A GROUP-LIVING CICHLID FISH

By

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ABSTRACT

Animals living in groups often form social hierarchies as a result of competition over limited resources. While the behavioural and physiological consequences of social rank in stable groups have been well studied, few investigations have considered how these traits vary during times of social transition. In this thesis, I used a group-living cichlid fish, *Neolamprologus pulcher*, to investigate how subordinate males adjust their physiology as they ascend to social dominance. Specifically, I focused on the mechanisms responsible for the regulation of stress (Chapter 2) and growth (Chapter 3). In Chapter 2, I found that subordinate males rapidly assumed behavioural dominance in their social groups following an experimentally created vacancy. However, 72 h post ascension ascenders had higher transcript abundance of key steroidogenic genes in the head kidney and maintained elevated circulating cortisol levels, consistent with cortisol levels of subordinates. Cortisol production appeared to decline as groups regained social stability, because cortisol levels were lowest in ascenders that performed few aggressive acts towards their group mates. Ascenders also had higher transcript abundance of glucocorticoid receptors 1 and 2 in the preoptic area of the brain, supporting an enhanced capacity for negative feedback regulation of cortisol production. In Chapter 3, I found that ascending males became more active, performed more social behaviours, and increased their growth. These energetically-costly adjustments seemed to be fuelled by utilizing onboard energy stores, because ascenders exhibited a 50% reduction in liver glycogen reserves but did not increase food intake. In addition, faster growth during social ascension appeared to be mediated by reduced production of insulin-like growth factor binding proteins, likely increasing

bioavailability of the growth stimulating hormone, insulin-like growth factor 1. Overall, the results of my thesis enhance our understanding of the regulatory mechanisms responsible for physiological differences observed in social hierarchies, and provide insight into how these regulatory mechanisms change during periods of social transition.

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THESIS ORGANIZATION AND FORMAT

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CHAPTER 1: General introduction

*Author***:** Brett M. Culbert

CHAPTER 2: Stress axis regulation during social ascension in a group-living cichlid fish

*Authors***:** Brett M. Culbert, Kathleen M. Gilmour and Sigal Balshine

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*Comments***:** BMC, KMG, and SB conceived and designed the experiments. BMC collected the data, conducted the analysis, and wrote the first draft of the manuscript under the supervision of KMG and SB. Reprinted with permission.

CHAPTER 3: Physiological regulation of growth during social ascension in a group-living fish

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CHAPTER 4: General discussion

*Authors***:** Brett M. Culbert

Chapter 1: General introduction

Brett M. Culbert

1.1 Introduction

Humans have long been fascinated by the social behaviours of other animals (Alexander, 1974); an interest that is likely sparked by our own social nature (Gintis, 2000). One of the most intriguing and complex social behaviours is cooperation (Axelrod and Hamilton, 1981). Cooperation occurs when individuals interact in a coordinated fashion to accomplish a task, such as communal rearing of offspring (Clutton-Brock, 2002; Taborsky and Taborsky, 2015). In cooperatively breeding species, individuals live in behaviourally stratified social groups in which the members of the group collectively provide care for offspring, even when the young are not their own (Cant, 2012). Most animals are much less social, with some even living the majority of their lives in solitude (Brown and Orians, 1970; Sandell, 1989). These differences in social tendencies across species have long puzzled scientists; in fact, the exact factors driving the evolution of complex social lifestyles remains one of the most intriguing questions in science (Pennisi, 2005). Recent incorporation of physiological approaches and techniques into behavioural studies has proven key in advancing our understanding of the proximate mechanisms regulating sociality (Goodson, 2013; Soares et al., 2010). Despite these advances, there remains a great deal that is unknown regarding the mechanistic underpinnings regulating social behaviours. My thesis addresses this knowledge gap, and in this general introduction chapter I provide context and background for my subsequent data chapters.

1.2 Social Groups and Hierarchies

A common consequence of a social lifestyle is the formation of social groups (Krause and Ruxton, 2002). In fact, living in social groups is thought to be an evolutionary precursor for more complex social societies, such as those that show cooperative breeding (Dey et al., 2017; Drobniak et al., 2015; Griesser et al., 2017). As a result of the high frequency of repeated interactions among members of social groups, dominance hierarchies often form (Boone, 1992; Cummins, 2015). Dominance hierarchies provide social order to groups, allowing individuals to establish relationships with one another that reflect their relative social position. Typically, individuals that are larger, stronger, more aggressive, older, and/or more experienced become dominant within hierarchies (Adams and Huntingford, 1996; Jonart et al., 2007; Reddon et al., 2011; Sevenster and Bakker, 1983; Thavarajah et al., 2014). Hierarchies ultimately reduce conflict, as rates of aggression tend to decrease following hierarchy establishment (Meese and Ewbank, 1973; Verbeek et al., 1999). By reducing the time and energy spent squabbling, individuals can spend more time performing acts that benefit themselves, and the group as whole, such as predator vigilance (Evans et al., 2016; Roberts, 1996) and workload sharing (Ausband et al., 2016; Balshine et al., 2001; Dornhaus et al., 2008).

Although the formation of hierarchies dampens aggression, subordinates often still continue to receive low levels of aggression from dominants, and will commonly perform submissive acts to appease dominants (Bernstein, 1981; Taborsky and Oliveira, 2012). In addition, subordinates can suppress their reproductive capabilities to further

reduce the threat that they represent to dominants. However, social structure is not permanent, and if resource density or social circumstances change, subordinates can eventually become dominant.

Occasionally, a subordinate will be presented with the opportunity to rapidly ascend and become socially dominant. Typically, this occurs as a result of a predatory event of a dominant (Balshine et al., 2001), the formation of a new group (Brockelman et al., 1998; Milich et al., 2018; Woolfenden and Fitzpatrick, 1978), or via the overthrow of a previously dominant individual (Brockelman et al., 1998; Doolan and Macdonald, 1996; Milich et al., 2018; Sapolsky, 1983). During this acute window of opportunity, subordinates must make quick adjustments to be able to assume dominance, as competition for social dominance is fierce. Subordinates wishing to ascend to a dominant position must outcompete subordinates from other social groups, as well as subordinates from within their own group to assume dominance status. They may also have to outcompete individuals that are already dominant in another social group, that wish to expand their territory (Ausband et al., 2017; Schoepf and Schradin, 2012; Stiver et al., 2006). As such, subordinates have to be able to rapidly and accurately assess changes in their specific social environment (Burmeister et al., 2005; Chen and Fernald, 2011; Grosenick et al., 2007), and promptly adjust their behaviour and physiology accordingly (Alonso et al., 2012; Buston, 2003; Dengler-Crish and Catania, 2007; Fitzpatrick et al., 2008; Maruska, 2015). Such periods of social flux are often stressful.

1.3 Stress axis

When an animal is challenged, a suite of physiological changes occur to help the animal overcome the threat. These changes are broadly referred to as the stress response. During the initial moments of a stress response, the "fight-or-flight" response is evoked, increasing production of the catecholamine stress hormones, epinephrine and norepinephrine (Charmandari et al., 2005). These hormones aid in overcoming acute challenges by increasing energy mobilization, and altering cardiovascular and respiratory function (Fabbri et al., 1998; Reid et al., 1998). Additionally, the hypothalamic-pituitary-adrenal (HPA; mammals and birds) or hypothalamic-pituitary-interrenal (HPI; reptiles, amphibians, and fish) stress axis becomes activated, ultimately resulting in increased production of glucocorticoid stress hormones (Figure 1.1). In fish, activation of the stress axis causes corticotropin-releasing factor (CRF) to be released by neurons that project from the preoptic area of the hypothalamus onto corticotropes within the anterior pituitary (Aguilera, 1998). Stimulation of the corticotropes by CRF causes production of proopiomelancortin, a polypeptide precursor to several hormones, including adrenocorticotropic hormone (ACTH) (Wendelaar Bonga, 1997). When ACTH is synthesized and released into the blood, it circulates until it binds melanocortin 2 receptors (MC2R) located on steroidogenic interrenal cells in the head kidney (Fridmanis et al., 2017). Following the binding of ACTH to MC2R, synthesis of glucocorticoid hormones from the precursor cholesterol is stimulated. Glucocorticoid synthesis is rate-limited both by the movement of cholesterol across the mitochondrial membrane—which is regulated by steroidogenic acute regulatory protein (StAR; Tokarz et al., 2015)—as

well as the cleavage of cholesterol to pregnenolone—which is catalyzed by cytochrome P450 side-chain cleavage enzyme (P450scc; Payne and Hales, 2004). In general, the most abundant glucocorticoid in mammals and fishes is cortisol, while corticosterone is more abundant in amphibians, birds, reptiles, and rodents (Sopinka et al., 2015).

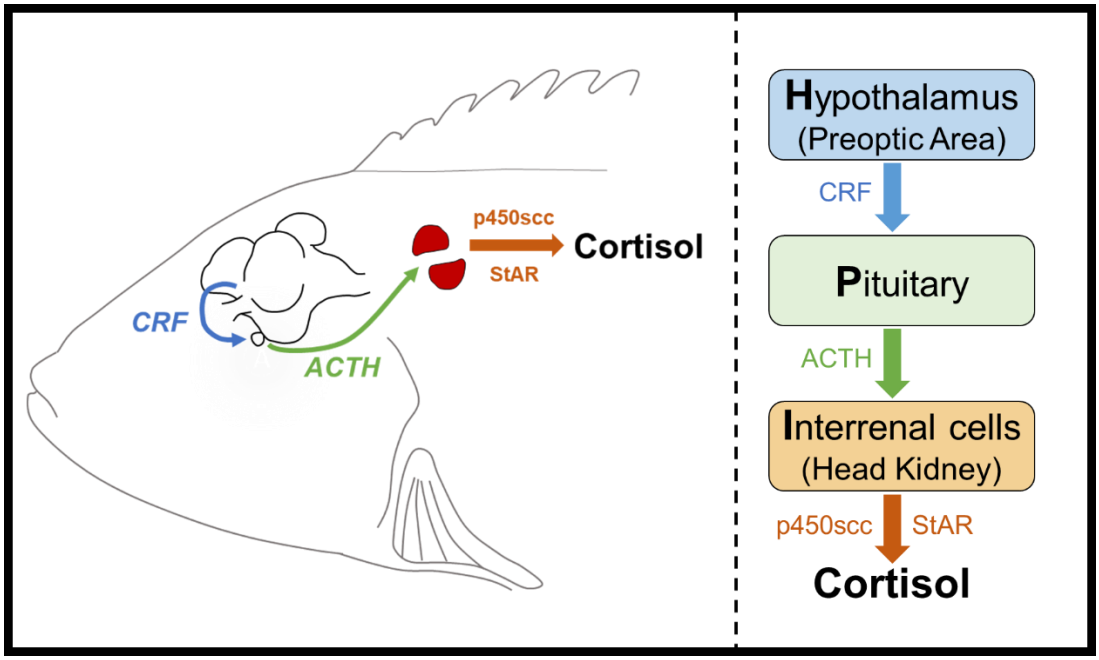

Figure 1.1 Schematic of the hypothalamic-pituitary-interrenal stress axis in fish. In response to a stressor, corticotropin-releasing factor (CRF) from the preoptic area stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary. Following this, ACTH circulates to the head kidney and stimulates cortisol production, which is rate-limited by the combined actions of cytochrome p450 side-chain cleavage enzyme and steroidogenic acute regulatory protein.

Once a stressor subsides, glucocorticoid levels can take several hours, or even days, to return to pre-stressor values (Cnaani and McLean, 2009; Jeffrey et al., 2014; Skrzynska et al., 2018). During this period, glucocorticoids aid in metabolic recovery by favouring the replenishment of glycogen stores that were mobilized by catecholamines

during the initial "fight-or-flight" response (Mommsen et al., 1999). However, if stressors persist, glucocorticoid production will remain elevated chronically, and this can have widespread negative effects, including reduced immune function (Cain and Cidlowski, 2017), impaired growth (Allen, 1996; Gregory and Wood, 1999), suppression of feeding (Gregory and Wood, 1999; Simpson et al., 1974), and hyperglycaemia (Clore and Thurby-Hay, 2009; Mommsen et al., 1999).

In groups with social hierarchies, subordinates often maintain elevated glucocorticoid levels compared to dominants, indicating that subordinates are chronically stressed (Abbott et al., 2003; Gilmour et al., 2005). However, among cooperative breeding species dominants sometimes have higher glucocorticoid levels than subordinates (Creel, 2001; Creel et al., 2013; Goymann and Wingfield, 2004). Elevated cortisol production in dominants is thought to reflect the allostatic demands associated with maintaining a dominant position in large groups, such as the policing of large numbers of subordinate group members (Rubenstein and Shen, 2009). Although many studies have investigated the effects of social status on glucocorticoid production, far less is known about glucocorticoid regulation during ascension from subordinate to dominant status. Studies conducted on numerous species, including olive baboons (*Papio anubis*; Sapolsky, 1992; Virgin and Sapolsky, 1997), chacma baboons (*Papio hamadryas ursinus*; Engh et al., 2006), spotted hyenas (*Crocuta crocuta*; Van Meter et al., 2009), Japanese quail (*Coturnix coturnix japonica*; Guibert et al., 2010), and cichlid fishes (*Astatotilapia burtoni;* Huffman et al., 2015; Maruska et al., 2013), suggest that social ascension, and social transitions more generally, can cause increased glucocorticoid

production. However, the precise mechanisms regulating the stress axis during these transition periods remain unclear. Additionally, few studies have assessed how physiological processes that are influenced by both glucocorticoids and social rank, such as somatic growth (Culbert and Gilmour, 2016; Dengler-Crish and Catania, 2007; DiBattista et al., 2006; Heg, 2010), are modulated during ascension.

1.4 Growth

Body size is one of the most important physical traits for all animals. When competing for limited resources, such as food and shelter, larger animals are typically more successful than smaller ones (Abbott et al., 1985; Schuett, 1997; Tokarz, 1985). Consequently, many hierarchies are sized-based (Ang and Manica, 2010a; Buston, 2003; Lewis and Saliva, 1987), with larger individuals becoming dominant over smaller individuals. To reduce conflict, subordinates will sometimes restrict their growth and grow more slowly than dominants (Dengler-Crish and Catania, 2007; DiBattista et al., 2006; Heg et al., 2004; Hofmann et al., 1999), which can be accomplished by reducing food intake (Ang and Manica, 2010b; Koebele, 1985; Wong et al., 2008). In addition, elevated glucocorticoid production as a consequence of high rates of conflict may further supress growth (Allen, 1996; Gilmour et al., 2005; Gregory and Wood, 1999). However, when presented with an opportunity to ascend and become dominant, subordinates can rapidly enhance their growth which increases their chances of attaining dominance. This strategy has been observed in both fishes (Buston, 2003; Hofmann and Fernald, 2000; Wong et al., 2008) and mammals (Dengler-Crish and Catania, 2007; Huchard et al.,

2016; Russell et al., 2004; Thorley et al., 2018), yet few studies have investigated the physiological mechanisms facilitating this rapid growth during ascension (Hofmann and Fernald, 2000; Trainor and Hofmann, 2007).

In general, the physiological regulation of growth is a complex process, integrating signals from many endocrine systems, including growth hormone (Norbeck et al., 2007; Reindl and Sheridan, 2012), somatostatin (Klein and Sheridan, 2008; Tannenbaum and Epelbaum, 2011), and insulin (Barbieri et al., 2003; Caruso and Sheridan, 2011). However, the insulin-like growth factor (IGF) system has emerged as one of the most influential regulators of growth (Duan et al., 2010; Froesch et al., 1985; Wood et al., 2005).

The IGF system consists of two ligands (IGF-1 & -2), two receptors (IGF1-R & IGF2-R), and seven binding proteins (IGFBP 1-7; Duan et al., 2010; Duan and Xu, 2005; Evdokimova et al., 2012). To date the majority of studies investigating the role of IGF in somatic growth have focused on the endocrine actions of IGF-1. Production of IGF-1 is stimulated by growth hormone (Froesch et al., 1985), with the majority of circulating IGF-1 being produced by the liver (Ohlsson et al., 2009; Sjogren et al., 1999). In circulation, >99% of IGF-1 is bound by IGFBPs (Shimizu et al., 1999). Together, the seven IGFBPs modulate the biological actions of IGF-1 either by inhibiting (IGFBP-1, -2,-4, -6, -7) or potentiating (IGFBP-3, -5) the ability of IGF-1 to bind IGF-R (Duan and Xu, 2005; Evdokimova et al., 2012; Shimizu and Dickhoff, 2017). When IGF-1 binds IGF1-R it activates a suite of physiological responses, including increasing cellular proliferation in myoblasts and osteoblasts (muscle and bone building cells) (Duan et al.,

2010; Tiago et al., 2011), and stimulating somatic growth (Beckman, 2011; Wood et al., 2005).

Despite the clear involvement of the IGF system in the regulation of growth, its role during socially-mediated growth has not been well investigated. I am aware of only two studies that have assessed the role of IGF-1 in socially-mediated growth—in southern pudu deer (*Pudu puda*; Bartoš et al., 1998) and Nile tilapia (*Oreochromis niloticus*; Vera Cruz and Brown, 2007)—and neither measured levels of IGFBPs. Thus, the IGF system represents an understudied but prime candidate for advancing our knowledge of the physiological factors responsible for socially-mediated growth. In this thesis I investigate how growth and the glucocorticoid stress axis are regulated during ascension in a small cichlid fish, *Neolamprologus pulcher*.

1.5 Study species: *Neolamprologus pulcher*

Neolamprologus pulcher is a cichlid fish endemic to Lake Tanganyika in Africa (Figure 1.2). These fish are distributed throughout the rocky littoral zone of the lake (Konings, 2015) and live in colonies that consist of 2-200 distinct groups (Hellmann et al., 2015). Groups contain up to 20 adults (Balshine et al., 2001) and fish spend their entire lives as part of a group. Groups are structured in a linear, size-based hierarchy where the largest male and female are dominant over the smaller subordinate fish (Dey et al., 2013; Wong and Balshine, 2011). The majority of young that are produced within groups belong to the dominant breeding pair, however, subordinate reproduction is sometimes tolerated (Dierkes et al., 1999; Heg et al., 2009; Hellmann et al., 2015; Stiver

et al., 2009). Subordinate group members provide allocare towards the offspring of the dominant breeding pair (Taborsky, 2016, 1984; Taborsky and Limberger, 1981), despite typically sharing an average of only ~15% genetic relation to dominant females and no relation to dominant males (Hellmann et al., 2016b; Stiver et al., 2005). Additionally, subordinates assist with territory maintenance, and will defend the territory from predators and intruding conspecifics (Desjardins et al., 2008; Ligocki et al., 2015; Taborsky, 1984). As such, *N. pulcher* is considered a cooperative breeder (Wong and Balshine, 2011). Only 21 species of fish are known to be cooperative breeders, all of which are Tanganyikan cichlids belonging to the Lamprologine tribe (Dey et al., 2017). Thus, cooperative breeding is performed by less than 0.5% of all fishes studied to date (Taborsky, 1994), making cooperative breeding exceedingly rare in fishes compared to mammals (~2%) and birds (~9%) (Cant, 2012).

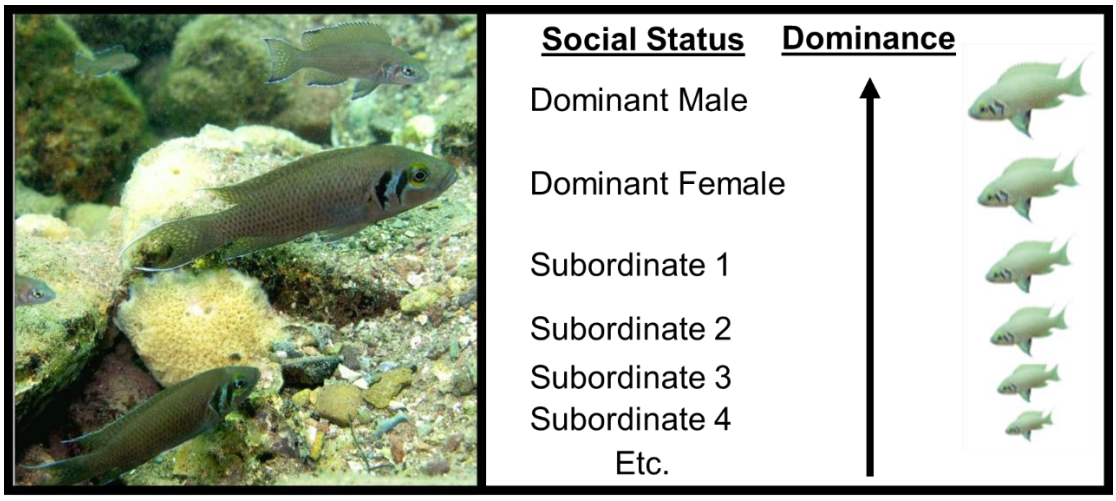

Figure 1.2 A social group of *Neolamprologus pulcher*. Groups are structured in a size-based dominance hierarchy, where larger individuals are dominant over the smaller subordinates.

In addition to their unusual social lifestyle, individuals of this species are small bodied, short-lived, and can easily be kept in a laboratory setting, causing *N. pulcher* to emerge as an ideal model species for behavioural ecologists interested in the evolution of cooperation and sociality. However, while the life-history and behaviour of *N. pulcher* have been well studied (Taborsky, 2016; Wong and Balshine, 2011), less is known about their physiology. More specifically, studies that have assessed physiological endpoints in *N. pulcher* have largely focused on identifying the physiological differences that exist between static social states: dominants and subordinates (Aubin-Horth et al., 2007; Bender et al., 2008, 2006; Hellmann et al., 2016a; Mileva et al., 2009; O'Connor et al., 2013; Reddon et al., 2015; Sopinka et al., 2009). In contrast, few investigations have focused on the physiological changes that occur during social ascension and the transition from one rank to another (Fitzpatrick et al., 2008; Sopinka et al., 2009).

1.6 Aims and structure of the thesis

In this thesis, I investigated the physiological mechanisms governing ascension to dominance in the group-living cichlid fish, *Neolamprologus pulcher*. I took an integrative approach, combining behavioural and physiological (biochemical, hormonal, and molecular) analyses to gain a holistic understanding of the mechanisms regulating social ascension. In Chapter 2, I examined how ascension to dominance influences glucocorticoid production, assessing whether regulation of the stress axis changes during ascension. In Chapter 3, I investigated how the physiological processes regulating growth are altered during ascension, focusing on the regulatory role of the insulin-like growth factor system,

as well as estimating the energetic costs associated with social ascension. Chapter 4 provides a general discussion that ties together the results of my thesis, detailing the implications of my work, as well as outlining some possible future directions. Collectively, these studies contribute to our understanding of the physiological mechanisms at play during social transitions, and provide insight into the proximate mechanisms regulating social hierarchies.

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Chapter 2: Stress axis regulation during social ascension in a group-living cichlid fish

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2.1 Abstract

Animals living in groups often form social hierarchies, with characteristic behaviours and physiologies associated with rank. However, when social opportunities arise and a subordinate ascends into a dominant position, quick adjustments are necessary to secure this position. Such periods of social transition are typically associated with elevated glucocorticoid production, but the precise regulation of the stress axis during these occasions is not well understood. Using the group-living cichlid, *Neolamprologus pulcher*, the effects of social ascension on the stress axis were assessed. Ascenders rapidly filled experimentally created vacancies, adopting a dominant behavioural phenotype within 72 h—elevating aggression, activity, and workload, while receiving high rates of affiliative behaviours from their group members. Despite assuming behavioural dominance within their groups, ascenders displayed higher cortisol levels than dominants three days post ascension. Additionally, compared to subordinates, ascenders had increased transcript abundance of steroidogenic acute regulatory protein (*star*) and cytochrome p450 side-chain cleavage enzyme (*p450scc*) in the head kidney, indicating activation of the stress axis. Cortisol levels were lowest in ascenders that displayed low rates of aggression, potentially reflecting the reestablishment of social stability in these groups. Increased transcript abundance of both glucocorticoid receptors *(gr1* and *gr2*) in the brain's preoptic area (POA) of ascenders compared to dominants suggested an enhanced capacity for cortisol regulation via negative feedback. Our results reveal a regulatory cascade of behavioural and physiological interactions and highlight the importance of investigating the underlying mechanisms regulating the stress axis.

2.2 Introduction

Living in a social group can provide a number of advantages, such as increased vigilance (Evans et al., 2016; Roberts, 1996), improved food acquisition (Evans et al., 2016; Ward and Zahavi, 1973) and workload sharing and load lightening (Ausband et al., 2016; Balshine et al., 2001; Dornhaus et al., 2008). Thus, by living in a group, individuals can save time and energy. However, social life also has costs. Conflicts within groups often arise over access to food, shelter, and reproductive opportunities (Milinski and Parker, 1991; Stockley and Bro-Jørgensen, 2011). Hierarchy formation, a common phenomenon in social groups, is thought to have evolved to reduce conflict over such limited resources. The most competitive individuals typically attain dominant positions, and secure primary access to resources, while less competitive individuals are subordinate and typically have less access to resources. Consequently, dominants and subordinates often differ considerably in terms of behaviour, physiology, health, and fitness (Sapolsky, 2005, 2004; Silk et al., 2003).

Levels of stress frequently vary with social rank and these rank-related physiological phenotypes have been well investigated (Creel, 2001; Creel et al., 2013; Goymann and Wingfield, 2004). However, the majority of studies only measure glucocorticoid levels, and not the mechanisms regulating glucocorticoid production. Moreover, the relationship between social rank and stress is often complex, influenced by a variety of factors. For example, during periods of social instability, challenges in rank order often are associated with elevated glucocorticoid levels (Sapolsky, 1992) but as rank is established glucocorticoids levels usually decrease (Engh et al., 2006; Van Meter

et al., 2009). To date, few studies have focused on the mechanisms regulating stress axis dynamics during periods of social instability, but such knowledge would strongly enhance our understanding of the factors influencing social stress.

To study the mechanisms regulating stress during social transitions, we used *Neolamprologus pulcher*, a cooperatively breeding cichlid fish that lives in permanent social groups. Groups consist of a dominant breeding pair and 1-20 subordinate helpers within a hierarchy (Wong and Balshine, 2011). Dominants are more aggressive (Fitzpatrick et al., 2008), more active (Sopinka et al., 2009), and perform more territory defense (Desjardins et al., 2008) compared to subordinates. When a dominant position becomes available, a subordinate can ascend to the dominant position (Balshine-Earn et al., 1998; Bergmüller et al., 2005; Fitzpatrick et al., 2008). The effects of social transition on cortisol production as yet have not been assessed in *N. pulcher*, although in *Astatotilapia burtoni*—a closely related cichlid, where transitions between territorial and non-territorial status occur repeatedly and reversibly—social ascension is associated with a rapid increase in circulating cortisol levels (<30 mins; Maruska, 2015) that can persist for 3 or more days (Huffman et al., 2015). However, the mechanisms underlying these changes in glucocorticoid production remain poorly understood.

We tested the hypothesis that periods of social ascension activate the entire stress axis, from the preoptic area (POA) of the brain where stress responses are initiated via release of corticotropin-releasing factor (CRF) to the head kidney, where cortisol is produced by interrenal cells. We removed dominant males from social groups, creating an opportunity for subordinate males to ascend in social rank and assume the dominant

position. We predicted that ascending males would rapidly adopt a dominant behavioural phenotype, and that this period would be associated with increased activation of the stress axis. Specifically, we measured and compared circulating cortisol levels as well as transcript abundance of stress axis genes in stable dominant, stable subordinate, and ascending males. In the POA, we targeted CRF (*crf*), which initiates activation of the stress axis following a stressor (Aguilera, 1998). We also measured transcript levels of the glucocorticoid receptors, which contribute to the regulation of cortisol production via negative feedback (Dallman et al., 1994). In most teleost fish, including *N. pulcher* (O'Connor et al., 2013), two isoforms of the glucocorticoid receptor exist (GR1 and GR2; Stolte et al., 2006), and therefore we measured both *gr1* and *gr2*. In the head kidney (analogous to the adrenal in mammals and birds), we measured transcript abundance of melanocortin 2 receptor (*mc2r*), steroidogenic acute regulatory protein (*star*), and cytochrome P450 side-chain cleavage enzyme (*p450scc*). We chose these genes because cortisol synthesis in steroidogenic cells is initiated when MC2R is activated by adrenocorticotropic hormone (Fridmanis et al., 2017), and is rate-limited by the conversion of cholesterol to pregnenolone, involving transfer of cholesterol across the mitochondrial membrane, which is regulated by StAR (Tokarz et al., 2015), and its cleavage to pregnenolone, which is catalyzed by P450scc (Payne and Hales, 2004).

2.3 Materials and methods

2.3.1 Experimental animals

The experiment was conducted from November 2016 – April 2017 at McMaster University in Hamilton, Ontario, Canada. Fish were laboratory-reared descendants of wild-caught *Neolamprologus pulcher* from Lake Tanganyika, Africa. Social groups consisting of a dominant breeding male-female pair, 1–3 large helpers (standard length (SL) > 4.5 cm), and 1–4 small helpers (SL < 4 cm) were held within 189 litre aquaria. All social groups (n=20) had been together for at least a month and had produced young prior to any experimental manipulation. Each fish was given a unique dorsal fin clip for identification, which does not adversely affect behaviour (Stiver et al., 2004). Each aquarium contained two large sponge filters, a heater, 3 cm of coral sand for substrate, two terracotta flowerpot halves, two mirrors, and two PVC tubes as shelter. Water was kept at 27°C and a 13L:11D photoperiod was maintained throughout the experiment. Fish were fed 1% combined group body weight daily with NorthFin floating cichlid pellets (1 mm; Canadian Aquatic Feed Inc., Toronto, ON, Canada). All experimental protocols were approved by the Animal Research Ethics Board of McMaster University (Animal Utilization Protocol No. 14-02-05), and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) regarding the use of animals in research and teaching.

2.3.2 Experimental protocols

Thirty-two focal fish were targeted in this experiment. At the beginning of the experiment (Day 0), all individuals within a group were weighed and measured, and each

group was randomly designated as control (*N*= 8; average of 6.75 ± 0.45 group members) or treatment (*N* = 12; average of 6.83 ± 0.42 group members). Behavioural observations (see Section 2.3) were recorded using a video camera (Canon VIXIA HF S200) on Days 10, 11, 13, and 14, and later scored. In treatment groups, dominant males were removed and sampled (mass = 7.45 ± 0.35 g, SL = 6.73 ± 0.18 cm, mean ± SEM; *N* = 12) on the morning of Day 11—providing an opportunity for subordinate males within these groups to ascend to the dominant position. On the morning of Day 14, ascending males were removed and sampled (mass = 5.21 ± 0.38 g, SL = 5.86 ± 0.17 cm; *N* = 8). In four of the twelve treatment groups, a clear dominant male had not emerged by Day 14 and therefore target ascending males were not collected from these groups. In control groups, subordinate males were removed and sampled on the morning of Day 11 (mass = 3.79 ± 0.31 g, SL = 5.23 ± 0.12 cm; *N* =12). Note that in four control groups, two stable subordinate males were sampled.

2.3.3 Behavioural analyses

Fish were given a 5 minute acclimation period following placement of the camera in front of their tank. Focal fish were then continuously recorded for 10 minutes and all behaviours performed or received were scored (see Sopinka et al., 2009 for a detailed species-specific ethogram). A dominance index (see Fig. 2.1A) was determined for each focal fish by subtracting the combined number of aggressive acts (chases, bites, rams, opercular flares, aggressive postures, and lateral displays) received and submissive acts (flees, and submissive postures and displays) given from the total number of aggressive acts given and submissive acts received ($DI = (Agg_{Given} + Sub_{Rec}) - (Agg_{Rec} + Sub_{Given})$,

see Fitzpatrick et al., 2008). The total number of affiliative acts (follows, parallel swims, and soft touches) received from all group members was also determined (see Fig. 2.1B). A workload index (see Balshine et al., 2001) for each fish was assigned by combining the number of visits to the brood chamber, the number of territory maintenance behaviours (digs and carries—the act of picking up and moving substrate with their mouths; see Fig. 2.1C), and defensive aggressive acts performed towards a mirror and neighbours. To assess locomotor activity, tanks were visually split into 12 quadrats using a grid and the number of times each fish crossed between quadrats was counted (see Fig. 2.1D). Behaviours are reported as averages of the two observation periods (i.e. Days 10/11 or Days 13/14). Following the initial behavioural recordings on Days 10 and 13, an unfamiliar male conspecific (SL = 5.82 ± 0.14 cm) was placed in the centre of the tank within a clear perforated plastic tube (see Fig. 2.1E) and the number of aggressive defense acts performed towards the intruding conspecific was recorded over a 10 minute period.

2.3.4 Tissue sampling

Fish were rapidly netted and killed via terminal anaesthesia (0.5 g L^{-1} ethyl-p-aminobenzoate; Sigma-Aldrich, Oakville, ON, Canada), and mass and standard length were recorded. All fish were sampled between 0800 h and 1100 h to avoid diurnal fluctuations in cortisol production. Blood was collected within 2-3 minutes via caudal severance into heparinized micro-hematocrit capillary tubes (Thermo-Fisher Scientific, Ottawa, ON, Canada) and centrifuged (4,750 *g* for 3 minutes). Plasma was flash frozen in liquid nitrogen and stored at -80°C for later analysis of cortisol concentrations. Sex was

confirmed via examination of the gonads, and the head kidney and POA were dissected out, flash frozen, and stored at -80°C for later analysis of transcript abundance.

2.3.5 Cortisol quantification

Circulating cortisol levels were measured using an enzyme-linked immunosorbent assay (EIA; Neogen, Lexington, KY, USA) following the manufacturer's protocol. The kit has a detection limit of 0.04 ng mL^{-1}. Based on preliminary trials, plasma samples were diluted 50x with Milli-Q water (EMD Millipore, Etobicoke, ON, Canada) prior to analysis. Samples were assayed in duplicate with an intra-assay variation of 2.8% (% CV). We were unable to collect blood from three subordinates, and therefore plasma cortisol was not determined for these fish.

2.3.6 Transcript abundance analysis by real-time RT-PCR

Tissues were homogenized on ice in TRIzol reagent (Invitrogen, Burlington, ON, Canada) using a sonicator (Sonic Dismembrator Model 100; Thermo-Fisher Scientific) and total RNA was extracted according to the manufacturer's protocol. Extracted RNA was quantified (NanoDrop 2000c UV-Vis Spectrophotometer; Thermo-Fisher Scientific) and complementary DNA (cDNA) was generated using a QuantiTech Reverse Transcription Kit (Qiagen, Toronto, ON, Canada) following the manufacturer's protocol.

Gene specific primers (Table 2.1) were used to assess changes in transcript abundance by semi-quantitative real-time RT-PCR. Previously published primers were used when available. Primers for target genes in the head kidney (*p450scc, star,* and *mc2r*) were designed using Primer-BLAST (NCBI; Ye et al., 2012) based on predicted

sequences for *Neolamprologus brichardi*. *N. brichardi* is considered to be a subspecies of *N. pulcher* (Taborsky and Grantner, 1998), and genetic evidence suggests that *N. brichardi* and *N. pulcher* may be the same species (Duftner et al., 2007). To confirm primer specificity, pooled PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced (StemCore Laboratories, Ottawa, ON, Canada).

All real-time RT-PCR reactions were carried out in duplicate using a Rotor-Gene SYBR Green PCR Kit (Qiagen) and a Rotor-Gene Q real-time PCR system (Qiagen), following the manufacturer's protocol with the exception that reaction volumes were scaled to 10 μL from 25 μL. Each reaction contained 5 μL SYBR 2x PCR mix, 1 μL of combined forward and reverse primers (10 μM of each), 3 μL of RNase/DNase free water, and 1 μL of cDNA template. Cycling parameters consisted of a 5 minute activation step at 95 ºC, followed by 40 cycles consisting of a 5 second denaturation step at 95 °C, and a combined 10 second annealing and extension step. Standard curves were developed for each primer set using serial dilutions (4x) of pooled cDNA from each individual, and conditions were adjusted to optimize the efficiency of each reaction. Negative controls, including no template controls (where cDNA was replaced with water) and no reverse transcriptase controls (where RNA was treated as per other cDNA reactions but reverse transcriptase was replaced with water) were included. Melt curves were performed at the end of each run to confirm the presence of a single product, as well as the absence of primer dimers. Transcript abundance was calculated relative to the subordinate group using the modified delta-delta Ct method (Pfaffl, 2001), normalizing to mRNA abundance of the reference gene 18S, which did not vary among groups.

2.3.7 Statistical analysis

Statistical analyses were performed using R (version 3.3.2, R Core Team, 2014). All data are presented as means ± 1 standard error of the mean (SEM) and a significance level (α) of 0.05 was used for all tests. When data did not meet the assumptions of normality and/or equal variance, data were transformed, or if the data could not be transformed to meet the assumptions, then equivalent non-parametric analyses were performed. To assess behavioural changes in ascending fish prior to, and after removal of the dominant male, paired Student's t-tests or Wilcoxon signed-ranks tests were performed. To investigate behavioural and physiological differences across dominant, subordinate and ascending fish, general linear mixed models (GLMM) were fit using the lmer function in the 'lme4' package (Bates et al., 2015). To account for non-independence of animals sampled from the same group, group id was included as a random factor in all models. When overall differences were detected using the Anova function in the 'car' package (Fox and Weisberg, 2011), Tukey's HSD post hoc analysis was performed using the glht function in the 'multcomp' package (Hothorn et al., 2008).

2.4 Results

2.4.1 Behaviour

Following dominant removal, ascending males became more aggressive and less submissive, resulting in higher dominance index scores (Fig. 2.1A; Paired t-test, $t_7 = 2.96$, $p = 0.02$). Ascenders also tended to receive more affiliative acts from other group members (Fig. 2.1B; Wilcoxon signed-rank test, $W_7 = 15.00$, $p = 0.06$), increased their workload

(Fig. 2.1C; Paired t-test, t_7 = 2.93, p = 0.02), displayed higher locomotor activity (Fig. 2.1D; t_7 = 2.78, p = 0.03), and were more aggressive towards an intruding conspecific after dominant removal (Fig. 2.1E; t_7 = 5.17, p = 0.001),

Dominance index scores of stable dominants and ascending males did not differ from one another, but both were higher than those of subordinate males (Fig. 2.1A; GLMM, X^2 = 56.61, df = 2, p < 0.001). Similarly, dominant and ascending males received more affiliative acts from group members (Fig. 2.1B; X^2 = 19.85, df = 2, p < 0.001), had higher workloads (Fig. 2.1C; X^2 = 22.43, df = 2, p < 0.001), higher locomotor activity (Fig. 2.1D; X^2 = 37.57, df = 2, p < 0.001), and performed more aggressive acts towards an intruding conspecific (Fig. 2.1E; X^2 = 36.50, df = 2, p < 0.001) than subordinate males.

2.4.2 Circulating cortisol levels

Circulating cortisol levels varied across social ranks with subordinates displaying the highest plasma cortisol levels (Fig. 2.2B; GLMM, X^2 = 11.22, df = 2, p = 0.004). Dominants had lower cortisol levels than subordinates (p = 0.008) and ascenders (p = 0.02), and ascending males did not differ from subordinates (p = 0.89). Circulating cortisol levels of ascenders strongly and positively correlated with both the dominance index (Pearson's correlation, r = 0.765, p = 0.03) and aggression towards an intruding conspecific (r = 0.885, p < 0.001), with the most aggressive ascenders displaying the highest levels of cortisol.

2.4.3 Stress axis transcript levels

Transcript levels of key components of the HPI axis were elevated in ascending fish. In the POA, ascending fish had higher mRNA abundance of *gr1* (Fig. 2.2C; GLMM, X^2 = 6.23, df = 2, p = 0.04) and *gr*2 (Fig. 2.2D; X^2 = 12.31, df = 2, p = 0.002) than dominants. No differences in *crf* transcript abundance were detected (Fig. 2.2E; X^2 = 3.67, df = 2, p = 0.16). Interestingly, *crf* transcript abundance did not correlate with circulating cortisol levels (Pearson's correlation, r = -0.04, p = 0.82), but instead correlated positively with both activity (r = 0.45, p = 0.01) and dominance (r = 0.37, p = 0.04).

In the head kidney, mRNA abundance of the cholesterol transport protein *star* was higher in ascenders (Fig. 2.2F; GLMM, X^2 = 7.46, df = 2, p = 0.02) than subordinates (p = 0.02), but not dominants (p = 0.14). Similarly, transcript levels of the steroidogenic enzyme *p450scc* were affected by social status (Fig. 2.2G; X^2 = 6.19, df = 2, p = 0.04) with ascenders tending to have higher levels than both dominants (p = 0.06) and subordinates (p = 0.09). No differences in *mc2r* transcript levels were detected across social ranks (Fig. 2.2H; X^2 = 1.79, df = 2, p = 0.40).

2.5 Discussion

Periods of social transition and instability are typically considered to be both dangerous and stressful. Here, we show for the first time that social ascension modulates the stress axis at multiple levels; specifically, at the site of cortisol synthesis—the head kidney—and in the POA of the brain. Transcriptional changes at the head kidney indicate an enhanced capacity for cortisol production as a result of stress incurred during

ascension to the dominant position. However, social hierarchies stabilized rapidly within each group, as indicated by ascenders displaying increased levels of aggression, activity, and work, while receiving more affiliative acts—all characteristics associated with dominance in *N. pulcher* (Wong and Balshine, 2011). As hierarchies stabilized, ascenders began to suppress their cortisol levels, aided by an enhanced capacity for negative feedback of the stress axis in the POA.

Periods of within-group social instability are associated with high levels of aggression among group members and elevated glucocorticoid levels, as has been documented in mammals (e.g. olive baboons, *Papio anubis*; Sapolsky, 1992) and birds (e.g. Japanese Quail, *Coturnix coturnix japonica*; Guibert et al., 2010). However, as social order stabilizes, rates of aggression decrease and glucocorticoid levels typically fall (e.g. Maruska, 2015). In the lekking cichlid *Astatotilapia burtoni*, social change can cause acute (<30 mins; Maruska, 2015), or even chronic elevation of cortisol levels (3 days; Huffman et al., 2015). Similarly, increased levels of aggression and cortisol production were observed during an attempted dominance takeover within the hierarchy of a wild group of female chacma baboons (*Papio hamadryas ursinus*; Engh et al., 2006). We found that, relative to stable dominants, ascending males displayed elevated circulating cortisol levels, but there was considerable variability in the levels measured. Ascenders displaying the highest cortisol levels were the most aggressive, with the highest dominance index scores and high levels of defense displayed towards intruders. Such high levels of aggression may reflect continuing social instability, with ascenders attempting to solidify their new rank through aggressive interactions. In turn, social

uncertainty coupled with a high frequency of aggressive encounters may have resulted in higher levels of stress and hence circulating cortisol levels. Overall, these findings suggest that the hierarchies within some social groups had not yet stabilized 72 h following disruption, resulting in elevated levels of stress and aggression in ascenders from these groups.

Dominant males had relatively low circulating cortisol levels. This finding contrasts with a previous study where cortisol levels were higher in dominant than subordinate *N. pulcher* (Mileva et al., 2009). Dominants with higher cortisol levels are common in many cooperatively breeding species that have high reproductive skew, reflecting an elevated allostatic load associated with the need to reproductively suppress subordinates and maintain dominance (Creel, 2001; Creel et al., 2013; Goymann and Wingfield, 2004). However, allostatic load can also be influenced by the number of individuals in a group (Rubenstein and Shen, 2009), with larger groups translating to higher allostatic loads for dominants. Compared to Mileva et al. (2009), the social groups used in our study contained fewer individuals per group (6.83 ± 0.42 vs 10.8 ± 0.8 individuals in Mileva et al., 2009), meaning that dominants in our study had fewer subordinates to police. This factor may have reduced their allostatic load and hence stress level associated with dominance, offering a possible explanation for the difference between studies.

Most previous studies on stress axis regulation during social transitions have focused exclusively on glucocorticoid levels. In contrast, we investigated the mechanisms underlying social regulation of the stress axis and show that ascending males exhibit

elevated transcript abundance of *star*, with a similar trend for *p450scc*, in the head kidney compared to that of subordinate males. Cortisol production is rate limited by P450scc and StAR (Payne and Hales, 2004; Tokarz et al., 2015), thus increased transcript abundance suggests activation of the stress axis and enhanced capacity for cortisol synthesis. Similarly, elevated transcript abundance of *p450scc* and *star* in the head kidney of dominant rainbow trout (*Oncorhynchus mykiss*; Jeffrey et al., 2012) was attributed to the acute stress of hierarchy formation (Øverli et al., 1999), serving to facilitate responses to additional stressors. Hence, steroidogenic capacity at the head kidney (analogous to the adrenal in mammals and birds) appears to be socially regulated.

Ascending males also exhibited elevated *gr* transcript abundance in the POA compared to dominant males. Negative feedback of cortisol production via glucocorticoid receptor signalling in the brain occurs in mammals (Dallman et al., 1994), birds (Cornelius et al., 2018; Dickens et al., 2009) and fish (Alderman et al., 2012; Kiilerich et al., 2018). Dominants typically downregulate their stress response more quickly than subordinates (Jeffrey et al., 2014; Sapolsky, 1983), and our results suggest that this capacity may be even greater in ascenders. In an unstable social environment, it may be beneficial not only to be prepared to mount an immediate cortisol response to mobilize energy to overcome social challenges, but also to be able to rapidly downregulate the stress response, thereby avoiding the costs of chronically elevated cortisol (McEwen, 2008). Similarly, Taborsky et al. (2013) found that *N. pulcher* raised in the absence of a social group displayed elevated transcript abundance of *gr1* in the brain. *N. pulcher* typically live their entire lives as part of a social group, therefore, development in the

absence of a group constitutes chronic social instability. Taken together, these results suggest that enhanced negative feedback of the stress axis may serve an important regulatory role during periods of socially instability.

Although CRF is a critical component of the stress axis—stimulating adrenocorticotropic hormone production in the pituitary (Aguilera, 1998)—its involvement in the regulation of social stress remains unclear. While CRF expression increases in response to acute stressors, there is mounting evidence that the CRF system habituates during chronic social stress (Backström and Winberg, 2013). In mammals, chronic stress causes a shift from CRF to arginine vasopressin as the primary secretagogue of ACTH (Ma et al., 1997; Ma and Lightman, 1998; Pinnock and Herbert, 2001). Social ascension in the closely related cichlid, *A. burtoni*, is associated with increased whole brain transcript abundance of arginine vasotocin (AVT; the teleost homologue of arginine vasopressin) as well as elevated circulating cortisol levels (Huffman et al., 2015), suggesting that a similar switch from CRF- to AVT-driven ACTH production may occur during periods of social transition. However, production of CRF is not limited to the POA (Alderman and Bernier, 2007; Pepels et al., 2002), and not all CRF neurons originating in the POA project onto corticotropes (Zupanc et al., 1999). Thus, further research into the regulatory role of CRF during ascension is warranted. In addition to its role in stimulating ACTH production, CRF is also implicated in the regulation of a wide range of behaviours (Hostetler and Ryabinin, 2013), including aggression (Carpenter et al., 2009; Mele et al., 1987) and decision-making (Bryce and Floresco, 2016; Summers et al., 2017). Modulation of such behaviours is likely important

as social order is re-established following hierarchy disturbances. However, central injections of CRF also increase locomotion in many vertebrates (Lowry and Moore, 2006), including fish (Clements et al., 2002). Consistent with these observations, we observed a positive relationship between activity and *crf* transcript abundance in the POA. Therefore, CRF appears to be playing an immediate and central role in the regulation of behaviour during social ascension.

In conclusion, our findings provide novel insight into regulation of the stress axis during social ascension. Specifically, cortisol levels during social transitions reflect the impact of perceived stability within a group on stress axis activity. In turn, activity of the stress axis is tuned to allow such dynamic adjustments of cortisol, with enhanced capacity for cortisol production at the head kidney as well as enhanced capacity for negative feedback regulation at the POA. These results illustrate the importance and benefit of integrative studies investigating the mechanisms underlying social regulation of stress.

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Table 2.1 Gene specific primers used for real-time RT-PCR.

Gene	Primer Sequence (5' to 3')	Amplicon Size (bp)	Efficiency (%)	Annealing Temperature (˚C)	Accession Number	Reference
18s	F: ACAAGAAGAGACCTTCACCTGG	146	POA: 93	56	AF337051	O'Connor et al., 2013
	R: CTCAATCTCGTGTGGCTGAA		HK: 90	60		
crf	F: ATCACCTTCCATCTTCAACAG	204	POA: 93	60	JX134406	Taborsky et al., 2013
	R: CTGGACATCTCCATCATCTC					
gr1	F: GCTGATCAAGATGAAAGTGC	198	POA: 95	60	EF661652	Taborsky et al., 2013
	R: AGAGTAGACATGAGCCGTGA					
gr2	F: TCGTTCCAACAATGTTATCC	204	POA: 111	60	EF661651	Taborsky et al., 2013
	R: GCAGAGTCATCTGATCATCC					
p450scc	F: AAGTCAGGGAGACTTGCTGG	81	HK: 97	60	XM_006789280	
	R: GGATGCAACCTGAGTGTTTCC					
star	F: TCAGTGTCCGATGTGCCAAG	105	HK: 94	60	XM_006805871	
	R: TTTCCGCTCTGACAACACCC					
mc2r	F: CATATACGCCTTCCGCATCG	89	HK: 97	60	XM_006795993	
	R: CCAGTTAAATCAGCAGAGCTTCC					

18s, 18S ribosomal RNA; *crf*, corticotropin-releasing factor; *gr1*, glucocorticoid receptor 1; *gr2*, glucocorticoid receptor 2; *p450scc*, cytochrome P450 side-chain cleavage enzyme; *star*, steroidogenic acute regulatory protein; *mc2r*, melanocortin 2 receptor

Figure 2.1 Illustrations in the first column depict the behavioural assays used to measure dominance (A); affiliation received (B); workload (C); locomotor activity (D); and defense against an intruder (E). The second column refers to changes in behaviour as a result of ascension. The third column shows the associated differences in behaviours of subordinate, ascending, and dominant males. Behaviours were recorded over 10 minutes. Affiliative acts received, aggressive acts, and locomotor activity (lines crossed) refer to counts of these behaviours over the 10 minute period. Values are means ± SEM. Treatment groups that share a letter are not significantly different from one another. An asterisk indicates a difference with ascension (see text for additional details).

Figure 2.2 Regulation of stress axis activity by social status. In response to a stressor, corticotropin-releasing factor (CRF) from the preoptic area stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary; ACTH circulates to the head kidney and stimulates cortisol production (A). Circulating plasma cortisol levels (B); transcript abundance of *gr1* (C), *gr2* (D), and *crf* (E) in the preoptic area; and transcript abundance of *star* (F), *p450scc* (G), and *mc2r* (H) in the head kidney of subordinate, ascending, and dominant males. Ascending males were sampled 72 h after being provided the opportunity to ascend. Values are means ± SEM. Treatment groups that share a letter are not significantly different from one another (see text for further details). Post hoc analysis failed to identify the source of the significant differences in Panel G.

Figure 2.1

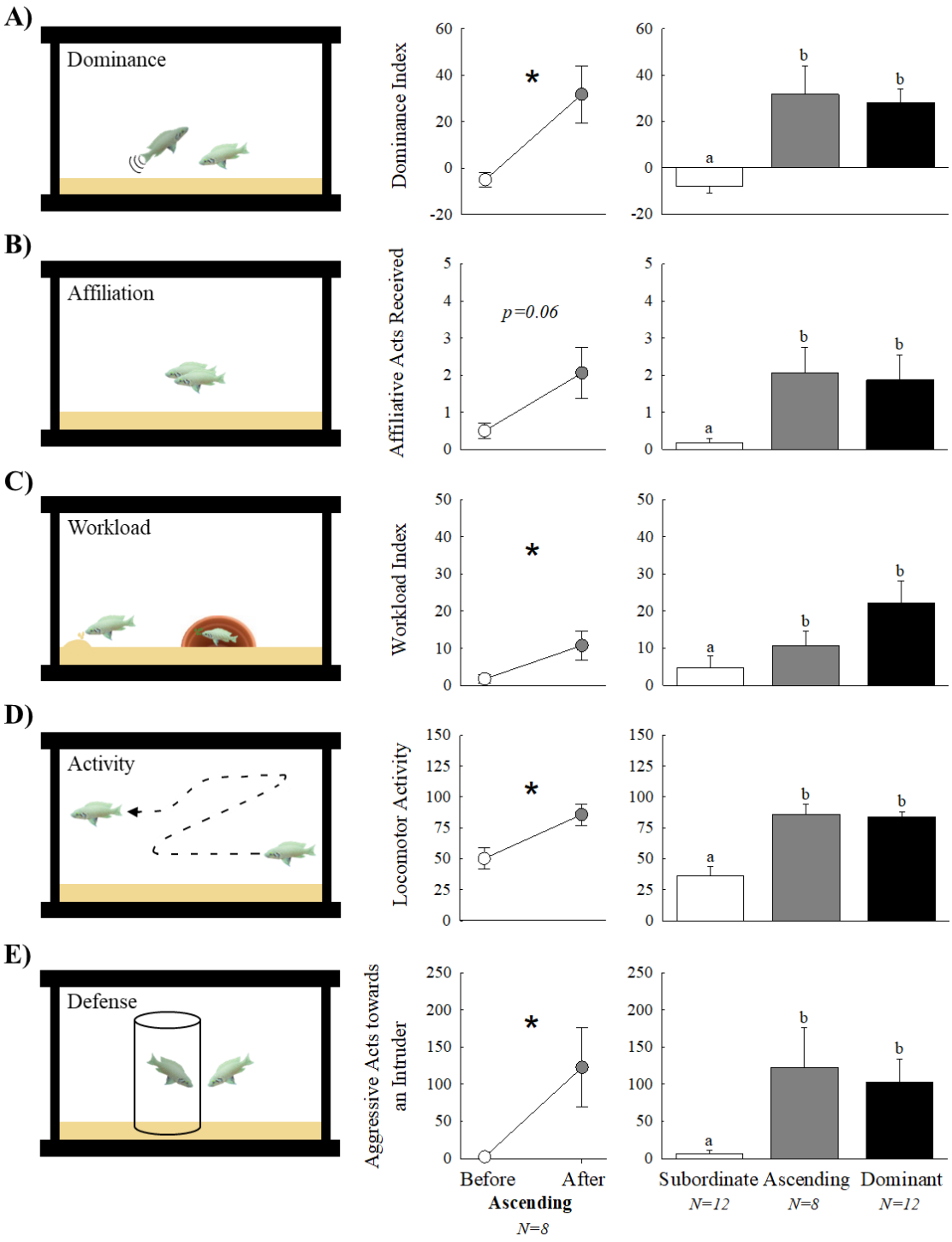
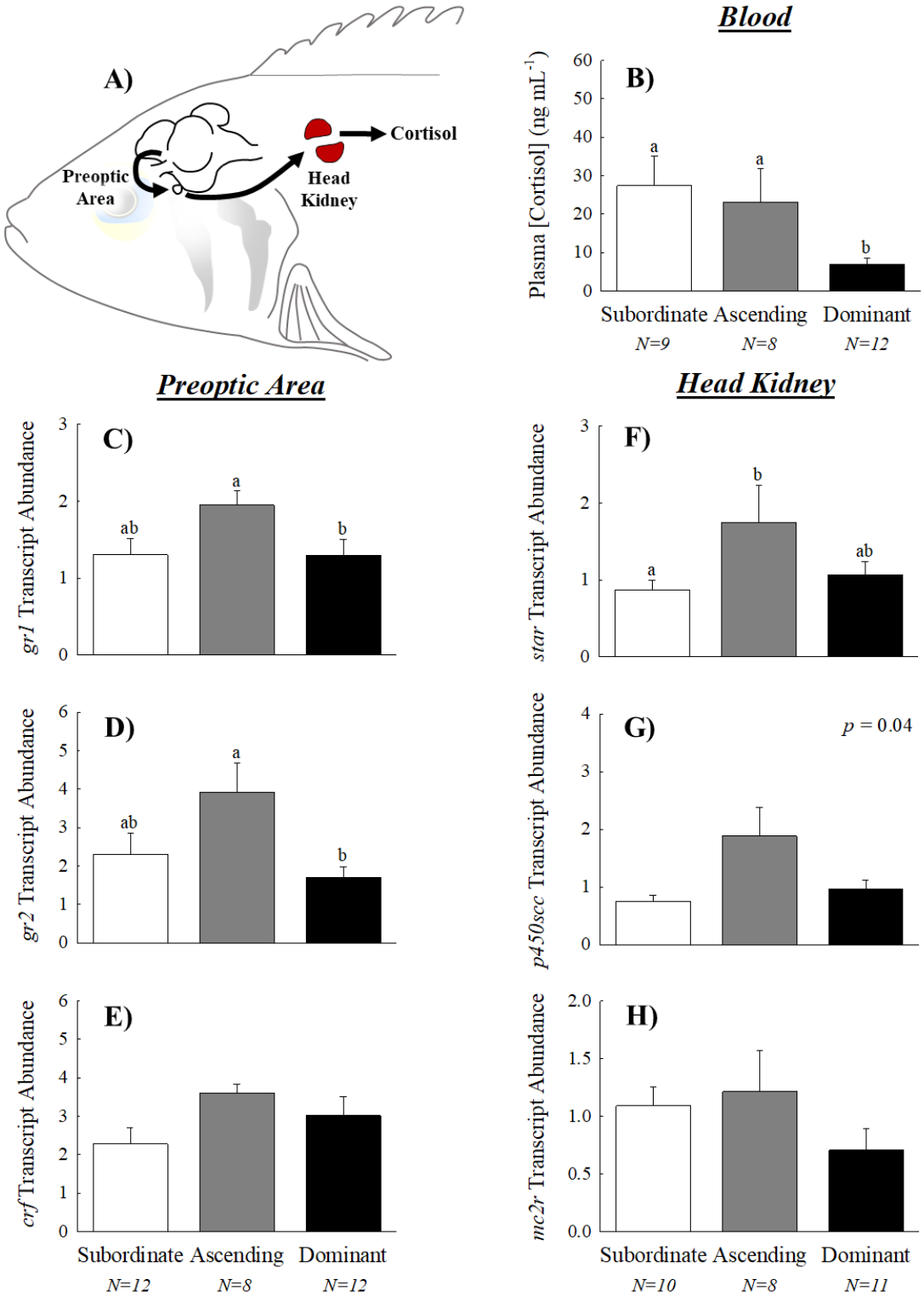

A)
Dominance
Dominance Index
*
a
b
b
B)
Affiliation
Affiliative Acts Received
p=0.06
a
b
b
C)
Workload
Workload Index
*
a
b
b
D)
Activity
Locomotor Activity
*
a
b
b
E)
Defense
Aggressive Acts towards an Intruder
*
a
b
b
Before
After
Ascending
N=8
Subordinate
Ascending
Dominant
N=12
N=8
N=12

Figure 2.2


A)
Cortisol
Preoptic Area
Head Kidney
Blood
B)
Plasma [Cortisol] (ng mL-1)
Subordinate Ascending Dominant
N=9 N=8 N=12
Preoptic Area
C)
gr1 Transcript Abundance
D)
gr2 Transcript Abundance
E)
crf Transcript Abundance
Subordinate Ascending Dominant
N=12 N=8 N=12
Head Kidney
F)
star Transcript Abundance
G)
p = 0.04
p450scc Transcript Abundance
H)
mc2r Transcript Abundance
Subordinate Ascending Dominant
N=10 N=8 N=11

Chapter 3: Physiological regulation of growth during social ascension in a group-living fish

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3.1 Abstract

In social groups, dominant animals typically are larger and have better access to resources than subordinates. When subordinates are given the opportunity to ascend to a dominant position, they will elevate their rates of growth to help secure dominance. The present study investigated the physiological mechanisms facilitating this increased growth. Using the group-living cichlid, *Neolamprologus pulcher*, we investigated whether the insulin-like growth factor (IGF) system—a key regulator of growth—is involved in the regulation of growth during social ascension. We also assessed differences in energy storage and expenditure among dominant, subordinate, and ascending males to determine the energetic costs associated with ascension. Daily growth rates tripled during ascension, and ascending males expended more energy following ascension, owing to higher rates of energetically costly social behaviours, increased locomotor activity, and larger home ranges. Ascenders did not increase food intake to offset increasing energetic costs, but had half the liver glycogen energy stores of subordinates. Together, these results indicate a reliance on stockpiled energy reserves to fuel the high energetic demands associated with ascension. Transcript abundance of insulin-like growth factor binding proteins 1 (*igfbp1*) and 2a (*igfbp2a*) were low in ascenders relative to subordinates, suggesting a higher capacity for growth during ascension through increased bioavailability of circulating IGF-1. Our findings provide clear evidence of the energetic costs of social ascension, and offer novel insight into the physiological mechanisms modulating the social regulation of growth.

3.2 Introduction

Body size is a key predictor of competitive ability, with larger individuals typically attaining dominant positions within social hierarchies (Abbott et al. 1985; Rabeni 1985; Tokarz 1985; Forsyth and Alcock 1990; Haley et al. 1994; Schuett 1997). Dominant animals generally secure better access to limited resources, including food, shelter, and reproductive opportunities (Milinski and Parker 1991; Clutton-Brock and Huchard 2013), and often have higher fitness than subordinates (von Rueden et al. 2011; Wilson et al. 2011; Majolo et al. 2012). In social groups, the ability of subordinates to challenge dominants for their social position is largely dependent on body size (Clutton-Brock et al. 2006; Wong et al. 2007; Reddon et al. 2011), and conflict between social ranks increases as subordinates approach the size of dominants (Wong et al. 2007, 2008; Ang and Manica 2010). To avoid such conflict, subordinates can restrict their growth and remain smaller than dominants (Buston 2003; Heg et al. 2004; Buston and Cant 2006; Dengler-Crish and Catania 2007; Wong et al. 2007; Matthews and Wong 2015), a feat that is often accomplished through a reduction in food intake (Wong et al. 2008; Ang and Manica 2010). However, when a subordinate perceives the opportunity to ascend to a dominant position it can rapidly grow and thus increase its likelihood of becoming dominant (Buston 2003; Russell et al. 2004; Dengler-Crish and Catania 2007; Huchard et al. 2016; Thorley et al. 2018). Such periods of enhanced growth are energetically demanding, and although previous studies have hypothesized that subordinates accumulate energy reserves to meet these demands (Taborsky 1984; Hellmann et al. 2016), energy regulation during periods of social transition has not yet been investigated.

In fact, despite the fitness benefits that are typically associated with a large body size, few studies have investigated the physiological mechanisms by which individuals adjust their growth based on social circumstances.

One of the most important regulators of growth and development is the insulin-like growth factor system (IGF; Fuentes et al. 2013). In particular, IGF-1 plays a central role in mediating somatic growth, and high levels of IGF-1 are associated with elevated rates of growth and metabolism in mammals (Swanson and Dantzer 2014) and fishes (Fuentes et al. 2013). Production of IGF-1 occurs in most tissues (Murphy et al. 1987; Daughaday and Rotwein 1989; Duguay et al. 1992; Wood et al. 2005), however, the majority of circulating IGF-1 is produced in the liver (Sjogren et al. 1999; Yakar et al. 1999; Stratikopoulos et al. 2008; Ohlsson et al. 2009). Biosynthesis of IGF-1 is stimulated by growth hormone (Froesch et al. 1985), and secretion of growth hormone from the pituitary is stimulated and inhibited by the actions of somatocrinin (Ling et al. 1984) and somatostatin (Brazeau et al. 1973), respectively. In *Astatotilapia burtoni*—a cichlid fish where transitions between territorial and non-territorial status frequently occur—faster growth by non-territorial males and males that recently gained a territory likely reflects an increase in IGF-1 production via stimulation of growth hormone secretion, because non-territorial and recently ascended males produce less somatostatin relative to territorial males (Hofmann et al. 1999; Hofmann and Fernald 2000; Trainor and Hofmann 2007). Indeed, production of IGF-1 differs with social rank in several species, with dominant wild baboons (*Papio anubis*; Sapolsky and Spencer 1997) and dominant pudu deer (*Pudu puda*; Bartoš et al. 1998) displaying higher circulating levels

of IGF-1 compared to subordinates. As well, higher hepatic transcript abundance of *igf-1* was associated with higher growth rates in dominant Nile tilapia (*Oreochromis niloticus*; Vera Cruz and Brown 2007). These findings suggest that IGF-1 may play an important role in the social regulation of growth. In addition, bioavailability of circulating IGF-1 is tightly regulated through the combined actions of several binding proteins (IGFBPs; Rajaram et al. 1997; Duan and Xu 2005; Shimizu and Dickhoff 2017). In particular, IGFBP-1 (Lee et al. 1997; Kajimura et al. 2005) and IGFBP-2 (Eckstein et al. 2002; Wheatcroft and Kearney 2009) strongly suppress the growth promoting actions of IGF-1. Here, we tested the hypothesis that IGF signaling regulates growth during social transitions.

To explore how ascension to dominant status influences the regulation of energy storage and growth, we used *Neolamprologus pulcher*—a cooperatively breeding African cichlid that lives in social groups organized in a size-based hierarchy (Wong and Balshine 2011a). Subordinate *N. pulcher* restrict their growth to remain smaller than dominants (Heg et al. 2004), but males grow rapidly when provided the opportunity to ascend within the hierarchy (Hamilton and Heg 2008). To assess the physiological mechanisms regulating growth during ascension, we removed dominant males, creating an opportunity for subordinate males to assume the dominant position within a social group. We predicted that ascension would be energetically demanding, owing to the performance of more social behaviours, increased activity, and elevated growth rates, and that these demands would be met through increased food intake and the utilization of stored energy reserves. Additionally, we predicted that elevated growth rates in ascenders would be

associated with increased activation of the IGF system. Therefore, we quantified transcript abundances of IGF-1 (*igf-1*) and IGFBP-1 and -2 (*igfbp-1, igfbp-2a, and igfbp-2b*) in the livers of dominant, subordinate, and ascending males.

3.3 Materials and methods

3.3.1 Experimental animals

The experiment was conducted between November 2016 and April 2017 using a colony of *Neolamprologus pulcher* housed at McMaster University in Hamilton, Ontario, Canada. All fish used in the experiment were laboratory-reared descendants of wild-caught breeders from Lake Tanganyika, Africa. Social groups (n = 20) consisted of a dominant male-female breeding pair, 1–3 large helpers (standard length (SL) > 4.5 cm), and 1–4 small helpers (SL < 4 cm). Groups were held in 189 litre aquaria filled with carbon-filtered city of Hamilton tap water at 27˚C. All social groups had been together for at least a month and had produced young prior to any experimental manipulation. Each fish was given a unique dorsal fin clip for identification, which does not adversely affect behaviour (Stiver et al. 2004). Each aquarium contained two large sponge filters, a heater, 3 cm of coral sand for substrate, two terracotta flowerpot halves, two mirrors, and two PVC tubes as shelter. A 13L:11D photoperiod was maintained throughout the experiment. Fish were fed 1% combined group body weight daily with NorthFin floating cichlid pellets (1 mm; Canadian Aquatic Feed Inc., Toronto, ON, Canada). All experimental protocols were approved by the Animal Research Ethics Board of McMaster University (Animal Utilization Protocol No. 14-02-05), and were in

compliance with the guidelines of the Canadian Council on Animal Care (CCAC) regarding the use of animals in research and teaching.

3.3.2 Experimental protocols

Thirty-two focal males were targeted in this experiment. At the start of the experiment (Day 0), body mass (to the nearest mg) and standard length (to the nearest 0.1 mm) of all individuals within the social groups of each focal fish were recorded. To ensure precision, all body measurements throughout the study were taken three times and averages of these measures are reported. Each social group was randomly assigned to be either a control (n=8; average of 6.8 ± 0.5 group members) or a treatment group (n=12; average of 6.8 ± 0.4 group members). On the morning of Day 11, dominant males (n=12; mass = 7.45 ± 0.35 g, SL = 6.73 ± 0.18 cm, mean ± SEM) were removed from treatment groups, and subordinate males (n=12; mass = 3.79 ± 0.31 g, SL = 5.23 ± 0.12 cm) were removed from control groups. These fish were euthanized, measured, and dissected (see Section 2.4). The remaining fish in each group were measured and groups were returned to their respective tanks. In treatment groups, dominant removal provided an opportunity for large subordinate males to ascend. On the morning of Day 14, males that had ascended to the dominant position were sampled as above (n=8; mass = 5.21 ± 0.38 g, SL = 5.86 ± 0.17 cm). In four of the twelve treatment groups, a clear dominant male had not emerged by Day 14 and therefore target ascending males were not collected from these groups.

3.3.3 Behavioural analyses

All social groups were video-recorded twice (Days 10 and 11) to assess the behaviour of each focal fish. Additionally, treatment groups were recorded twice following dominant removal (Days 13 and 14) to assess behavioural changes of ascending fish. On Days 11 and 14, groups were recorded immediately prior to sampling. Following placement of a video camera (Canon; VIXIA HF S200) in front of each tank, fish were given 5 minutes to acclimate and then recorded continuously for 10 minutes. Previous studies have used similar methods to assess behaviour in this species (Fitzpatrick et al. 2008; Wong and Balshine 2011b). From the 10 minute video recordings, we scored all aggressive (chases, bites, rams, opercular flares, aggressive postures, and lateral displays), submissive (flees, and submissive postures and displays), and territory maintenance (digs and carries—the act of picking up and moving substrate with their mouths) behaviours performed by each focal fish. Locomotor activity was measured by recording the proportion of time that focal fish were in motion during each video. To assess the size of the home range of focal fish (Werner et al. 2003), tanks were visually split into 12 quadrats using a grid, and the number of unique squares that focal fish entered during the observation period was counted and expressed as a proportion of the total squares. Additionally, the proportion of time that focal fish spent in the upper third of the tank was recorded, because this zone represents a more risky and less preferred area for these substrate-bound cichlids (Konings 2015). Behaviours are reported as averages of the two observation periods (*i.e.* Days 10/11 or Days 13/14).

Food intake of focal fish was measured from 5 minute video-recordings of feedings on Days 5, 7 and 9. To determine whether ascending males adjusted their feeding following dominant removal, treatment groups were also video-recorded on Days 11, 12 and 13. Feeding rates are expressed as the total number of feeding acts performed by each focal fish while food was present. Observations concluded when all pellets had been consumed (average duration = 2.88 ± 0.63 minutes), and feeding rates are reported as averages of the three observation periods (*i.e.* Days 5/7/9 or Days 11/12/13). All groups were fed between 1300 and 1400 h.

3.3.4 Tissue sampling

Fish were rapidly netted and euthanized via terminal anaesthesia (0.5 g L^{-1} ethyl-*p*-aminobenzoate; Sigma-Aldrich, Oakville, ON, Canada) and mass and standard length were recorded. Gonads and livers were removed and weighed, flash frozen and stored at -80°C. Half of each liver was used to measure liver glycogen levels (Keppler and Decker 1974), and the remaining liver tissue was used to measure transcript abundance of IGF system components by semi-quantitative real-time RT-PCR.

3.3.5 Transcript abundance analysis by real-time RT-PCR

Livers were homogenized on ice in TRIzol reagent (Invitrogen, Burlington, ON, Canada) using a sonicator (Sonic Dismembrator Model 100; Thermo-Fisher Scientific) and total RNA was extracted according to the manufacturer's protocol. Extracted RNA was quantified (NanoDrop 2000c UV-Vis Spectrophotometer; Thermo-Fisher Scientific)

and complementary DNA (cDNA) was generated using a QuantiTech Reverse Transcription Kit (Qiagen, Toronto, ON, Canada) following the manufacturer's protocol.

Gene specific primers (Table 1) were used to assess changes in transcript abundance by semi-quantitative real-time RT-PCR. Previously published primers were used for the reference gene *18s*. Primers for target genes in the IGF system (*igf-1, igfbp-1, igfbp-2a,* and *igfbp-2b*) were designed using Primer-BLAST (NCBI; Ye et al., 2012) based on predicted sequences. Pooled PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced (Génome Québec, Montreal, QC, Canada) to confirm primer specificity.

Real-time RT-PCR reactions were performed in duplicate using a Rotor-Gene SYBR Green PCR Kit (Qiagen) and a Rotor-Gene Q real-time PCR system (Qiagen), following the manufacturer's protocol with the exception that reaction volumes were scaled to 10 μL from 25 μL. Each reaction contained 5 μL SYBR 2x PCR mix, 1 μL of combined forward and reverse primers (10 μM of each), 3 μL of RNase/DNase free water, and 1 μL of cDNA template. Cycling parameters consisted of a 5 minute activation step at 95 ºC, followed by 40 cycles consisting of a 5 second denaturation step at 95 °C, and a combined 10 second annealing and extension step. Standard curves were developed for each primer set using serial dilutions (4x) of cDNA pooled from each individual, and conditions were adjusted to optimize the efficiency of each reaction. Negative controls, including no template controls (where cDNA was replaced with water) and no reverse transcriptase controls (where reverse transcriptase was replaced with water in the synthesis of cDNA) were included. Melt curves were performed at the end of each run to

confirm the presence of a single product, as well as the absence of primer dimers. Transcript abundance was calculated relative to the subordinate group using the modified delta-delta Ct method (Pfaffl 2001), normalizing to mRNA abundance of the reference gene *18s*, which did not vary among groups.

3.3.6 Statistical analysis

Specific growth rate (SGR) was calculated as $[\ln(SL_{final}) - \ln(SL_{initial})] * 100 / D$, where SL is the standard length of the fish in centimeters and D is the number of days elapsed between measurements (Ricker 1975).

Statistical analyses were conducted using R (version 3.3.2; R Core Team 2014). All data are presented as means ± 1 standard error of the mean (SEM), and a significance level (α) of 0.05 was used for all tests. Data were tested for normality and homoscedasticity using Shapiro-Wilk and Levene's tests, respectively. Data that did not meet these assumptions were either log or logit (for data presented as proportions) transformed. To investigate differences among dominant, subordinate, and ascending males, general linear mixed models (LMM) were fit using the lmer function in the 'lme4' package (Bates et al. 2015). Group ID was included as a random factor in all models to account for the fact that in four social groups, two subordinate males were sampled. When overall differences were detected using the Anova function in the 'car' package (Fox and Weisberg 2011), Tukey's HSD *post hoc* analysis was performed using the glht function in the 'multcomp' package (Hothorn et al. 2008). Final standard length (taken immediately prior to dissection) was included as a covariate in LMMs of daily growth

(total change in body length divided by number of days between measurements) to account for differences in somatic growth due to body size (Taborsky 1984). Final body mass was included as a covariate in LMMs of gonadal and liver investment, as well as food intake. The residuals of log liver mass against log body mass (determined using least-square linear regression) were included as a covariate in LMMs of hepatic glycogen reserves to account for individual differences in relative liver investment. To assess changes in behaviour and growth of ascending fish prior to and after removal of the dominant male, LMMs were performed including individual id as a random factor.

3.4 Results

3.4.1 Ascenders increased their growth rates

Specific growth rates of ascenders (Fig. 3.1A; LMM, X^2 = 9.99, df = 2, p = 0.007) were higher than those of both dominants (p = 0.005) and subordinates (p = 0.04). Ascenders also had higher absolute growth per day (Fig. 3.1B; LMM, X^2 = 7.55, df = 2, p = 0.02) compared to dominants (p = 0.02), but not relative to subordinates (p = 0.64). Growth increased with body length (X^2 = 8.12, df = 1, p = 0.004).

Following dominant removal, ascending males grew faster in terms specific growth rate (Fig. 3.1A; X^2 = 15.62, df = 1, p < 0.001), as well as absolute growth per day (Fig. 3.1B; X^2 = 4.45, df = 1, p = 0.03). In contrast, non-focal fish (dominant females and non-ascending large helpers) did not increase their growth following dominant removal (Table 2).

3.4.2 Ascenders utilized energy reserves, but did not increase gonadal investment

Ascenders had lower liver glycogen reserves (Fig. 3.2A; X^2 = 42.65, df = 2, p < 0.001) than subordinates (p < 0.001). Glycogen stores did not differ between ascending and dominant males (p = 0.22), and did not vary with residual liver mass (X^2 = 0.09, df = 1, p = 0.76). Liver investment did not differ across social ranks (X^2 = 3.19, df = 2, p = 0.20), but absolute liver mass increased with body mass (X^2 = 6.30, df = 1, p = 0.01). Ascending males did not adjust their gonadal investment (Fig. 3.2B; LMM, X^2 = 12.26, df = 2, p = 0.002), which did not differ from that of subordinates (p = 0.64). Gonadal investment of ascenders was lower than that of dominant males (p = 0.003). Absolute gonad mass increased with body mass (X^2 = 4.55, df = 1, p = 0.03).

3.4.3 Ascenders increased their performance of social and locomotor behaviours

Dominant and ascending males performed more aggressive acts (Fig. 3.3A; LMM, X^2 = 14.84, df = 2, p < 0.001) and fewer submissive acts (Fig. 3.3B; X^2 = 36.66, df = 2, p < 0.001) than subordinates. Ascending males performed levels of territory maintenance (Fig. 3.3C; X^2 = 10.80, df = 2, p = 0.004) intermediate to that of dominants (p = 0.13) and subordinates (p = 0.57). Ascenders and dominants were more active (Fig. 3.4A; X^2 = 48.11, df = 2, p < 0.001) and spent less time in the top third of the tank (Fig. 3.4B; X^2 = 24.59, df = 2, p < 0.001) compared to subordinates. Ascending males had larger home ranges (Fig. 3.4C; X^2 = 44.02, df = 2, p < 0.001) than subordinates (p = 0.003), although the home ranges of ascenders remained smaller than those of dominants (p = 0.02). Feeding rates did not differ across social ranks (Fig. 3.4E; X^2 = 1.58, df = 2, p

= 0.45), but tended to increase with body mass (data not shown; X^2 = 3.42, df = 1, p = 0.06).

Following dominant removal, ascending males became more aggressive (Fig. 3.3A; LMM, X^2 = 16.67, df = 1, p < 0.001) and less submissive (Fig. 3.3B; X^2 = 6.53, df = 1, p = 0.01). No change in territory maintenance behaviours was detected (Fig. 3.3C; X^2 = 1.75, df = 1, p = 0.19). Ascenders became more active (Fig. 3.4A; X^2 = 34.69, df = 1, p < 0.001), spent less of their time in the upper third of the tank (Fig. 3.4B; X^2 = 6.19, df = 1, p = 0.01), and expanded their home ranges (Fig. 3.4C; X^2 = 13.73, df = 1, p < 0.001). However, ascending males did not adjust their feeding rates following dominant removal (Fig. 3.4D; X^2 = 0.01, df = 1, p = 0.92).

3.4.4 Subordinates had higher transcript abundance of igfbps

No differences in hepatic *igf-1* transcript abundance were detected across social ranks (Fig 3.5A; LMM, X^2 = 4.05, df = 2, p = 0.13). However, subordinates had elevated transcript abundance of *igfbp-1* (Fig. 3.5B; X^2 = 6.80, df = 2, p = 0.03) compared to dominants (p = 0.02), and elevated transcript abundance of *igfbp-2a* (Fig. 3.5C; X^2 = 10.55, df = 2, p = 0.005) compared to ascenders (p = 0.01) and dominants (p = 0.01). Subordinates also tended to have higher transcript abundance of *igfbp-2b*, but this difference did not reach significance (Fig. 3.5D; X^2 = 4.84, df = 2, p = 0.08).

3.5 Discussion

Ascending to a dominant position within a social group is generally assumed to be a socially and energetically demanding life-history transition, but few studies have tested this prediction empirically. Previous studies have shown that subordinate *N. pulcher* often have larger livers than dominants relative to their body size (Sopinka et al. 2009; Hellmann et al. 2016), as well as greater muscle energy reserves (Hellmann et al. 2016), suggesting that subordinates may store energy to prepare for energetically demanding events, such as ascension to dominance. In the present study, we found that ascending males had half the liver glycogen stores of subordinates 72 h after ascending to a dominant position, supporting the hypothesis that subordinates stockpile energy reserves in preparation for ascension. Periods of ascension are often associated with elevated glucocorticoid production (Huffman et al. 2015; Culbert et al. 2018), and the catabolic actions of glucocorticoids likely aid in the rapid mobilization of these energy reserves (Mommsen et al. 1999). In rainbow trout (*Oncorhynchus mykiss*), social subordination is associated with elevated cortisol production which, at least in part, results in increased rates of hepatic gluconeogenesis (Gilmour et al. 2012) and beta-oxidation (Kostyniuk et al. 2018), along with depletion of energy reserves, including liver glycogen (Gilmour et al. 2012; Culbert and Gilmour 2016). A similar process may occur during ascension, where increased cortisol production (Culbert et al. 2018) stimulates the mobilization of energy reserves, providing ascenders with the fuel necessary to establish dominance.

During periods of high metabolic demand, many vertebrates will increase their food intake and/or enhance their capacity to absorb nutrients to offset energetic costs (Christiansen et al. 1992; Dykstra and Karasov 1992; Speakman and McQueenie 1996; Hammond and Kristan 2000). However, we did not observe any changes in food intake during ascension. This observation is consistent with recent findings for wild Kalahari meerkats (*Suricata suricatta*), in which food intake was not adjusted during periods of social ascension (Huchard et al. 2016). In the wild, *N. pulcher* forage in the water column above their territories on ephemeral patches of zooplankton, with feeding occurring in large aggregations consisting of members of many groups (Balshine et al. 2001). As such, socialization (on their territories) and feeding (above their territories) usually occur separately. When food availability was experimentally reduced, rates of helping behaviours (territory maintenance/defense and broodcare) decreased because fish had to spend more time foraging (Bruintjes et al. 2010). Therefore, it is possible that ascending males were unable to increase their foraging rates because they were too busy securing their newly acquired social rank through social interactions.

Social interactions are important for group-living animals (Taborsky and Oliveira 2012), but the nature and frequency of specific types of interactions typically vary with social rank (Milinski and Parker 1991; Stockley and Bro-Jørgensen 2011; Clutton-Brock and Huchard 2013). We observed that ascending males became more aggressive and less submissive, while continuing to perform high levels of territory maintenance—characteristics associated with dominance in *N. pulcher* (Wong and Balshine 2011a; Taborsky 2016). Grantner and Taborsky (1998) observed that aggressive, submissive, and

territory maintenance behaviours in *N. pulcher* resulted in 3.9-, 3.3-, and 6.1-fold increases in routine metabolic rate, respectively. To assess how energy consumption owing to the performance of social behaviours changed during ascension, the rates at which behaviours of each class were performed in the present study were multiplied by the relative metabolic cost associated with each behavioural class determined by Granter and Taborsky (1998). Based on these calculations, we estimate that males spent almost three times as much energy on the performance of social behaviours following ascension (Before ascension: 4.79 ± 0.77; After ascension: 13.64 ± 3.93). Additionally, ascending males also became more active and defended a larger home range, further increasing their energy expenditure. Despite spending more energy on locomotion and the performance of costly social behaviours, ascenders managed to achieve high growth rates.

Across animal taxa, body size is one of the most reliable predictors of competitive ability (Rabeni 1985; Haley et al. 1994; Schuett 1997; Reddon et al. 2011), and ascending males in the current study tripled their somatic growth rates following dominant removal. These findings are consistent with previous studies of growth during social ascension in both mammals (Dengler-Crish and Catania 2007; Huchard et al. 2016; Thorley et al. 2018) and fishes (Hofmann et al. 1999; Buston 2003; Bergmüller et al. 2005). Interestingly, this increase in somatic growth occurred in the absence of changes in gonadal investment. Fitzpatrick et al. (2008) observed that by seven days following dominant removal, ascending male *N. pulcher* had greater gonadal investment (by 66%) compared to subordinates, and ascenders with the largest gonads displayed the lowest growth rates. These authors therefore suggested a trade-off between somatic growth and

gonadal investment during periods of social ascension. Our results indicate that the direction of this trade-off varies as a function of time following dominant removal. Early investment in somatic growth (as observed in the current study) may aid in securing the dominant position within a group, followed by a later period of increased gonadal investment (Fitzpatrick et al. 2008) that would be necessary to enhance reproductive capacity once dominance is secured.

Although IGF-1 is an important regulator of growth and development—stimulating growth and cellular proliferation (Froesch et al. 1985; Wood et al. 2005; Duan et al. 2010)—increased growth during ascension did not appear to be mediated by changes in production of IGF-1, because no differences in hepatic *igf-1* transcript levels were detected. This observation contrasts with the situation in dyadic hierarchies of Nile tilapia (*Oreochromis niloticus*), where differences in growth between dominants and subordinates mirrored differences in IGF-1 production (Vera Cruz and Brown 2007). Instead, elevated hepatic transcript levels of *igfbp-1* and *-2a* in subordinates suggest an important role of IGFBPs in the social suppression of growth. IGFBP-1 and -2 bind circulating IGF-1, preventing it from binding to IGF receptors and hence suppressing its growth-stimulating actions (Shimizu and Dickhoff 2017). During ascension, *igfbp* transcript levels fell in ascenders, likely relieving the suppression of IGF-1 activity by IGFBPs, allowing ascenders to increase their growth rates. This mechanism not only explains increased somatic growth during periods of ascension, but is, to our knowledge, the first time IGFBPs have been implicated in the social regulation of growth.

In conclusion, social ascension is an energetically costly event for which subordinates must prepare through the accumulation of energy reserves. These reserves appear to be rapidly utilized during ascension, fuelling increased activity, the performance of costly social behaviours, and rapid growth. Our study also implicated IGFBPs as key regulators of growth during ascension, providing a novel mechanism for the social regulation of growth.

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Table 3.1. Gene-specific primers used for real-time RT-PCR.

Gene	Primer Sequence (5' to 3')	Amplicon Size (bp)	Efficiency (%)	Annealing Temperature (˚C)	Accession Number	Reference
18s	F: ACAAGAAGAGACCTTCACCTGG R: CTCAATCTCGTGTGGCTGAA	146	91	60	AF337051	O'Connor et al., 2013
igf-1	F: ATGGCCGTTCTTAGTTGGTG R: TGCTGGGCATTTGTCCATTT	130	96	60	XM_006780878	
igfbp-1	F: TGGACACCATAGCCACCTCT R: GATGACTCGCACTGCTTGG	109	104	60	XM_006786434	
igfbp-2a	F: GGCTTTGAGTACACCTGGCT R: TTACGGTCATGTCCTTCGGC	104	98	60	XM_006800269	
igfbp-2b	F: TATCTGCCAAGGTGCTCCAC R: GTGTTTAGAGGCGGTCTCCC	194	92	60	XM_006793717	

18s, 18S ribosomal RNA; *igf-1*, insulin-like growth factor 1; *igfbp-1*, insulin-like growth factor binding protein 1; *igfbp-2a*, insulin-like growth factor binding protein 2a; *igfbp-2b*, insulin-like growth factor binding protein 2b

Table 3.2. Specific growth rates (SGR) and absolute growth rates of non-focal fish before and after removal of the dominant male. Values are means ± SEM. No significant differences were detected. See text for a full description of the statistical analysis.

		SGR (% day $^{-1}$)				Growth (mm day $^{-1}$)			
	N	Before	After	X^2	*p*	Before	After	X^2	*p*
Dominant Females	8	0.15 ± 0.04	0.12 ± 0.11	0.09	0.76	0.09 ± 0.02	0.08 ± 0.07	0.03	0.87
Non-Ascending Helpers	9	0.19 ± 0.03	0.14 ± 0.08	0.67	0.41	0.10 ± 0.02	0.06 ± 0.04	0.46	0.50

Figure 3.1 Specific growth rates (A); and daily growth rates (B) of subordinate (*N* = 12), ascending (*N* = 8), and dominant (*N* = 12) male *Neolamprologus pulcher*. Symbols represent individual fish and lines indicate linear trends within each social rank. Significant differences among social ranks are indicated on the figures using letters. The inset panels show growth rates of ascending males before and after ascension (*N* = 8). An asterisk indicates a significant difference as a result of ascension. Values are means ± SEM. See text for a full description of the statistical analysis.

Figure 3.2 Liver glycogen reserves (A); and gonad mass (B) of subordinate (*N* = 12), ascending (*N* = 8), and dominant (*N* = 12) male *Neolamprologus pulcher*. Symbols represent individual fish and lines indicate linear trends within each social rank. Significant differences among groups are indicated on the figures using letters. See text for a full description of the statistical analysis.

Figure 3.3 Rates of aggression (A), submission (B), and territory maintenance (C) performed by subordinate (*N* = 12), ascending (*N* = 8), and dominant (*N* = 12) male *Neolamprologus pulcher*. Treatment groups that share a letter are not significantly different from one another. The inset panels show the behaviour of ascending males before and after ascension (*N* = 8). An asterisk indicates a significant difference as a result of ascension. Behaviours were recorded over 10 minutes and are expressed as the number of acts performed per minute. Values are means ± SEM. See text for a full description of the statistical analysis.

Figure 3.4 Activity levels (A); position in tank (B); size of home range (C); and feeding rates (D) of subordinate (*N* = 12), ascending (*N* = 8), and dominant (*N* = 12) male *Neolamprologus pulcher*. Treatment groups that share a letter are not significantly different from one another. The inset panels show the behaviour of ascending males before and after ascension (*N* = 8). An asterisk indicates a significant difference as a result of ascension. Activity, position in tank, and home range were recorded over 10 minutes. Feeding rates are reported as the number of feeding attempts performed over a period of 5 minutes, or until all food was consumed. Values are means ± SEM. See text for a full description of the statistical analysis.

Figure 3.5 Relative transcript abundance of *igf-1* (A); *igfbp-1* (B); *igfbp-2a* (C); and *igfbp-2b* (D) in the livers of subordinate (*N* = 12), ascending (*N* = 8), and dominant (*N* = 12) male *Neolamprologus pulcher*. Treatment groups that share a letter are not significantly different from one another; in panel D, the *p* value is reported. Values are means ± SEM. See text for a full description of the statistical analysis.

Figure 3.1

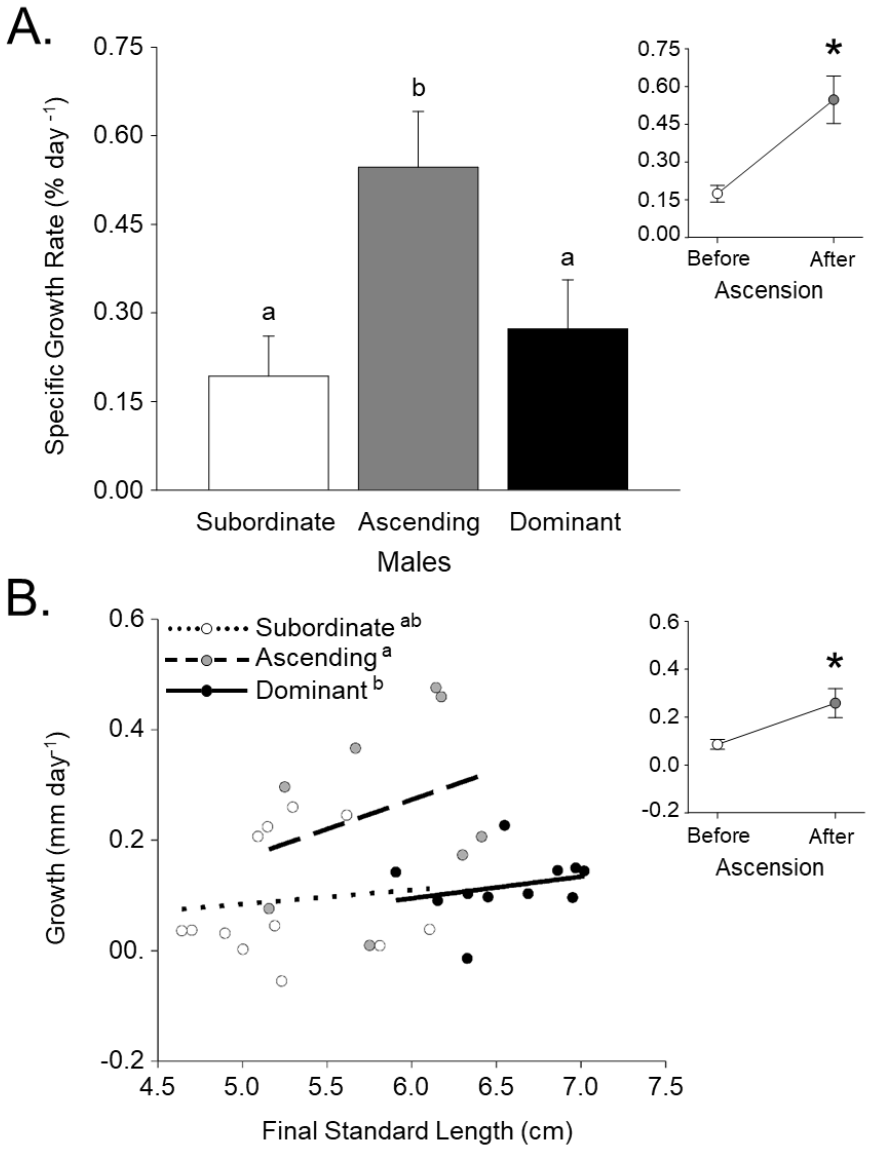

A.
Specific Growth Rate (% day -1)
0.75
0.60
0.45
0.30
0.15
0.00
a
b
a
Subordinate
Ascending
Dominant
Males
0.75
0.60
0.45
0.30
0.15
0.00
*
Before
After
Ascension
B.
Growth (mm day-1)
Subordinate ab
Ascending a
Dominant b
0.6
0.4
0.2
00.
-0.2
4.5
5.0
5.5
6.0
6.5
7.0
7.5
Final Standard Length (cm)
0.6
0.4
0.2
0.0
-0.2
*
Before
After
Ascension

Figure 3.2

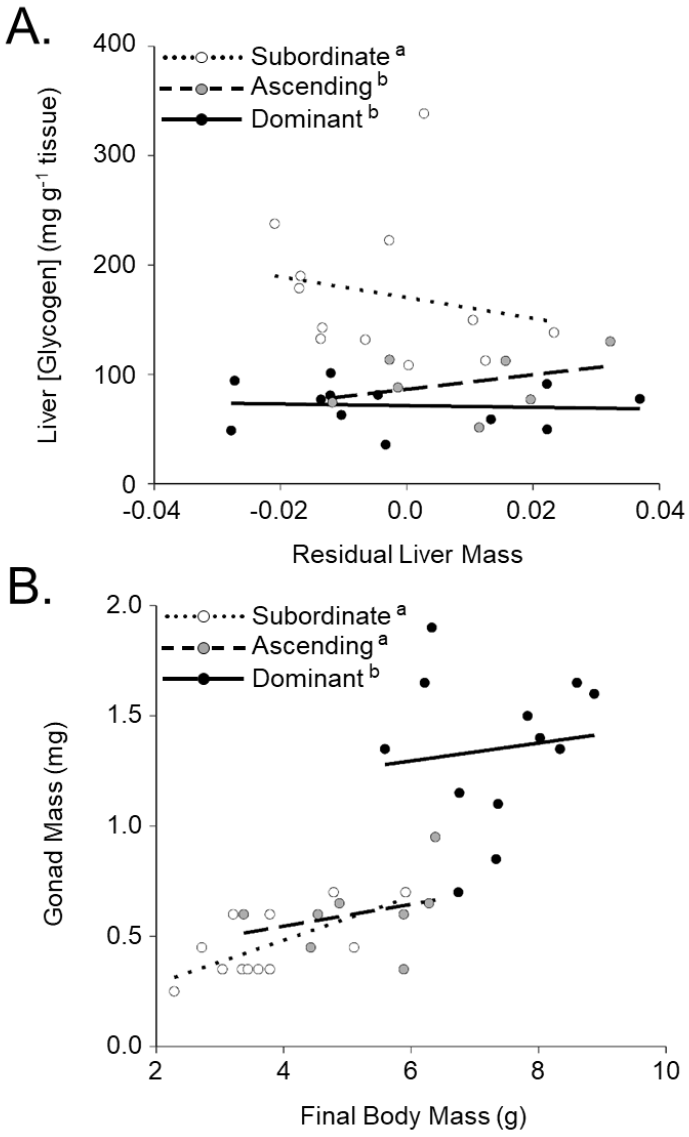

A.
Subordinate a
Ascending b
Dominant b
Liver [Glycogen] (mg g-1 tissue)
400
300
200
100
0
-0.04
-0.02
0.0
0.02
0.04
Residual Liver Mass
B.
Subordinate a
Ascending a
Dominant b
Gonad Mass (mg)
2.0
1.5
1.0
0.5
0.0
2
4
6
8
10
Final Body Mass (g)

Figure 3.3

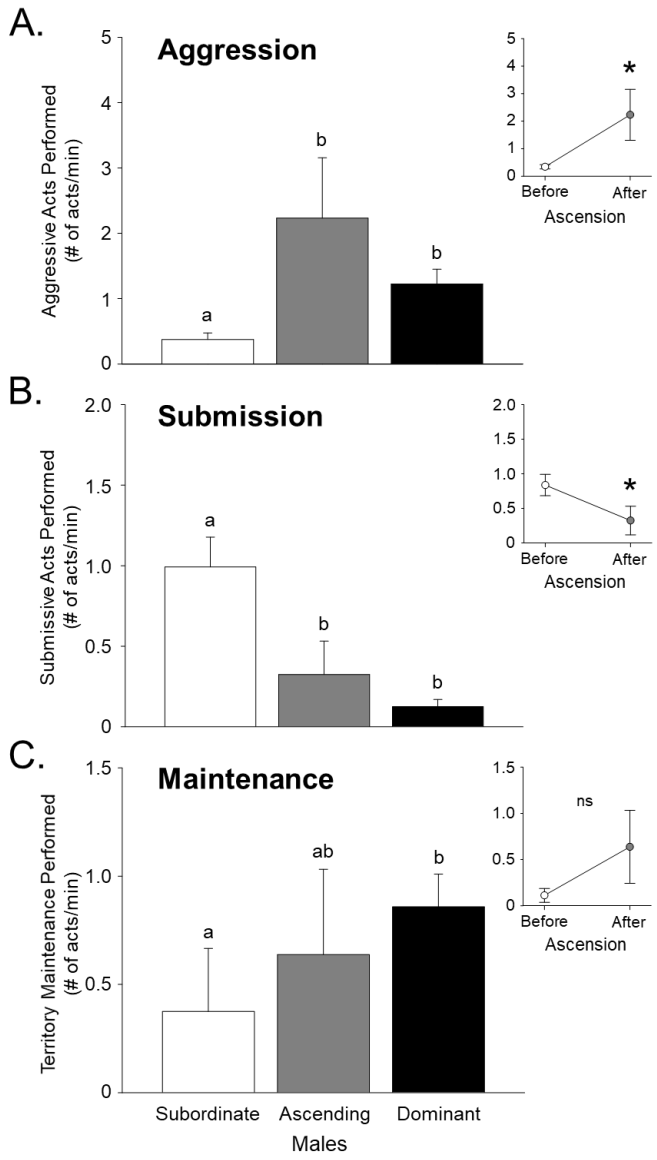

A.
Aggression
Aggressive Acts Performed (# of acts/min)
a
b
b
Before
After
Ascension
*
B.
Submission
Submissive Acts Performed (# of acts/min)
a
b
b
Before
After
Ascension
*
C.
Maintenance
Territory Maintenance Performed (# of acts/min)
a
ab
b
Before
After
Ascension
ns
Subordinate
Ascending
Dominant
Males

Figure 3.4

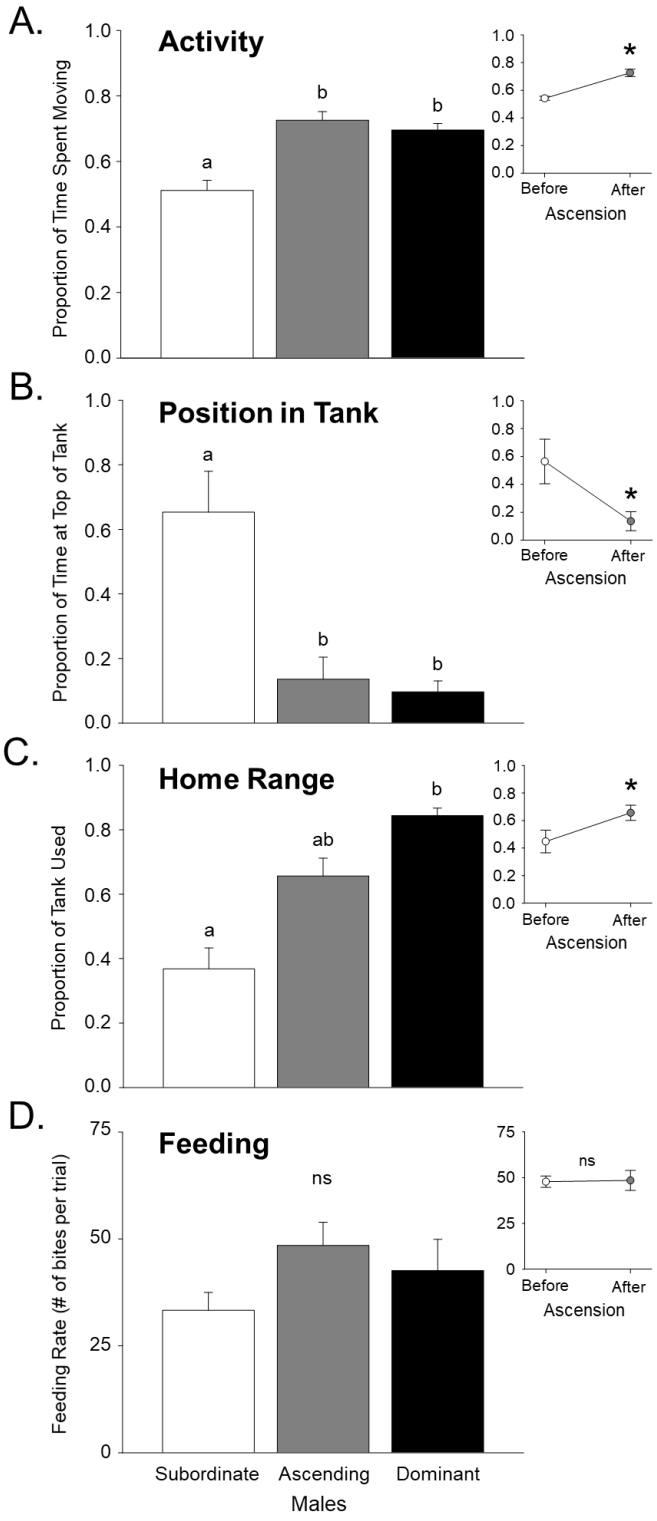
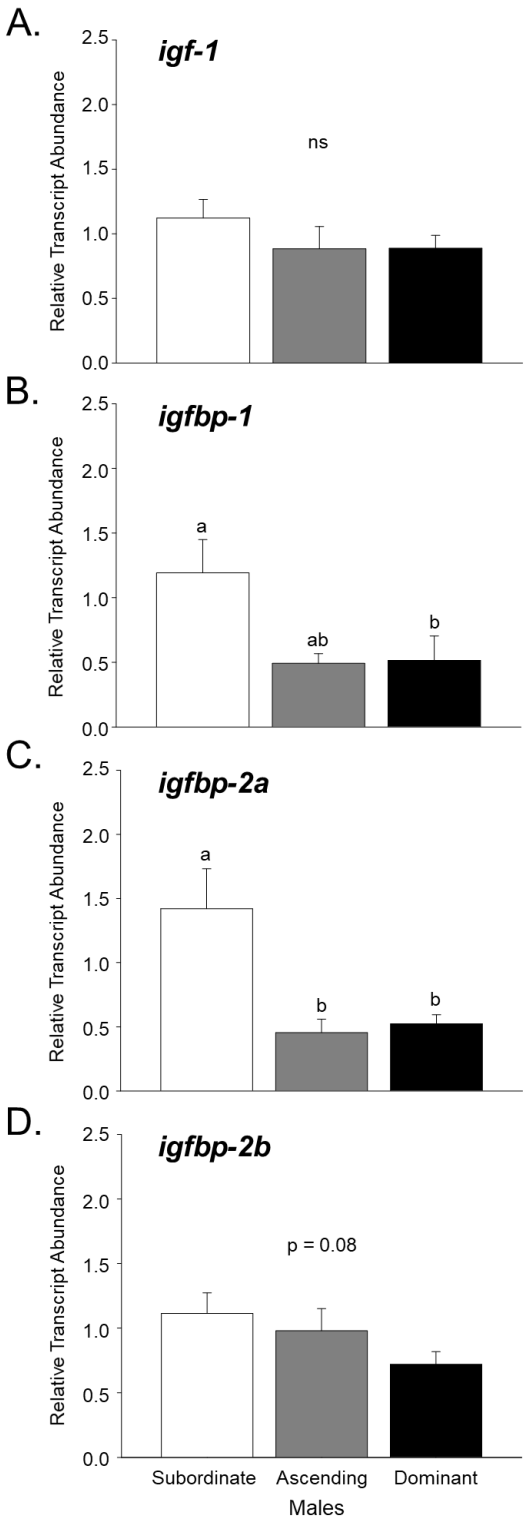

A.
Activity
Proportion of Time Spent Moving
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
*
Before
After
Ascension
B.
Position in Tank
Proportion of Time at Top of Tank
a
b
b
*
C.
Home Range
Proportion of Tank Used
a
ab
b
*
D.
Feeding
Feeding Rate (# of bites per trial)
75
50
25
0
ns
ns
Subordinate
Ascending
Dominant
Males

Figure 3.5


A.
igf-1
ns
B.
igfbp-1
a
ab
b
C.
igfbp-2a
a
b
b
D.
igfbp-2b
p = 0.08
Relative Transcript Abundance
0.0
0.5
1.0
1.5
2.0
2.5
Subordinate
Ascending
Dominant
Males

Chapter 4: General discussion

Brett M. Culbert

4.1 Summary

In this thesis, I investigated how social ascension to dominance influences the physiology and behaviour of male *Neolamprologus pulcher* (Figure 4.1). In particular, I focused on the mechanisms responsible for the regulation of stress and growth. I investigated these topics from an integrative perspective, combining behavioural assays with biochemical, hormonal, and molecular endpoints, providing novel insight into the physiological adjustments that occur during social ascension. In this final discussion chapter, I explore the implications of my results and expand upon how my findings advance our understanding of the proximate mechanisms regulating social ascension. Wherever possible, I also highlight potential future experiments that could be conducted to extend my work.

4.2 Regulation of the stress axis

Chapter 2 investigated the role of the stress axis during ascension to dominance. I found that social ascension had widespread effects, influencing transcript abundance of key components of the stress axis in the preoptic area of the brain and in the head kidney, and, ultimately, circulating cortisol levels. Elevated circulating cortisol levels are typically associated with social stress (Abbott et al., 2003; Creel et al., 2013; Goymann and Wingfield, 2004), and I found that both subordinate and ascending males had higher circulating cortisol levels than dominant males. This suggests that subordinates and ascenders both experience higher levels of stress than dominants, however, I did not

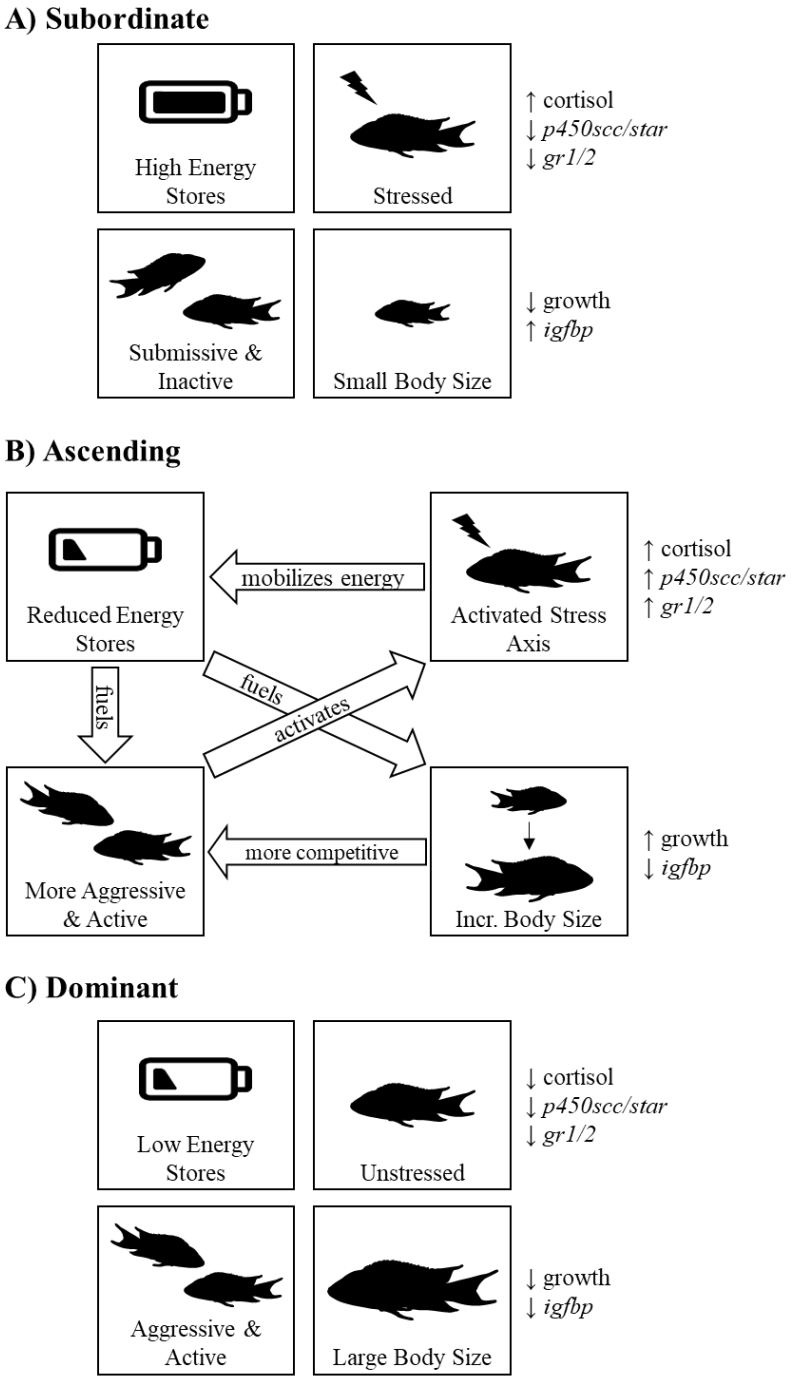


Figure 4.1 Diagram of behaviour, growth, stress, and energy storage in subordinate and dominant male *N. pulcher*, and how these factors are adjusted during periods of social ascension. *gr1/2*, glucocorticoid receptors 1 and 2; *igfbp*, insulin-like growth factor binding protein; *p450scc*, cytochrome p450 cholesterol side-chain cleavage enzyme; *star*, steroidogenic acute regulatory protein.

detect any difference in plasma cortisol levels between subordinates and ascenders. This was somewhat surprising since ascending males were behaviourally distinct of subordinates, having already become behaviourally dominant within their respective social groups (Chapters 2 and 3). Cortisol levels in ascenders seemed to reflect the degree of social stability within groups, because ascenders with the lowest cortisol levels also displayed the least amount of aggression towards their groupmates, implying group cohesion and stability. Overall, this finding suggests that as social order returns, rates of conflict decrease, and cortisol production is reduced.

To confirm the relationship between group stability and cortisol production during social ascension, as well as to determine how stress is regulated during the early-stages of ascension, a high-resolution time course study of cortisol production during ascension would need to be conducted. However, the small body size of *N. pulcher* precludes repeated collection of blood samples, and existing techniques available for repeated cortisol measurements in small bodied fish (e.g., excretion into water; Ellis et al., 2004; Pavlidis et al., 2008; Scott and Ellis, 2007) involve substantial handling and confinement that make the measurement of baseline cortisol levels difficult. Furthermore, these measurements are often performed on isolated fish, which may inherently influence cortisol production in a highly social species like *N. pulcher*. These fish are almost never by themselves, typically remaining in close proximity of their territory and group mates. Additionally, habituation procedures that are frequently used during such protocols appear to blunt the functionality of the stress axis, as cortisol responses associated with the collection protocol typically decrease following repeated collections (e.g., Ligocki et

al., 2015; Pavlidis et al., 2008). Despite these methodological difficulties, Ligocki et al. (2015) found that helpers excrete more cortisol when levels of conflict between dominant males and females are high, raising the question of how social ascension influences non-ascending helpers. Indirect effects of social ascension within groups have been shown to influence glucocorticoid production in non-ascending chacma baboons (*Papio hamadryas ursinus*; Engh et al., 2006), however, the physiological mechanisms regulating glucocorticoid production during periods of social instability have not been investigated in non-ascending animals. Clearly, further work is required to understand the role of the stress axis during periods of social instability, and I believe that *N. pulcher* provides a convenient model to address these questions going forward.

One of the primary functions of cortisol is to regulate metabolism, aiding in the mobilization of energy reserves during times of metabolic demand (Mommsen et al., 1999). As such, elevated levels of circulating cortisol in ascenders compared to dominants may reflect the high metabolic demands associated with ascension. Ascenders had half the liver glycogen levels of stable subordinates only 72 hours after dominant removal (Chapter 3). While liver glycogen is a relatively labile energy pool, this is a short period of time to mobilize such large amounts of energy, and the catabolic actions of cortisol would certainly help increase energy mobilization. Periods of chronic cortisol elevation typically are associated with a reduction of glucocorticoid receptor (GR) expression in energy-rich tissues, including the liver (Jeffrey et al., 2012; Lee et al., 1992; Pottinger, 1990; Sathiyaa and Vijayan, 2003; Svec, 1988; Vijayan et al., 2003). Reduced expression of GR likely serves as a protective mechanism to decrease responsiveness to

cortisol and avoid continued stimulation, preventing energy depletion during periods of chronic stress. While hepatic transcript abundance of *gr1* and *gr2* does not differ between stable dominant and subordinate *N. pulcher* (O'Connor et al., 2013), whether GR expression is adjusted during ascension has not been investigated. I predict that ascending fish will have higher expression of glucocorticoid receptors in energy-rich tissues, such as the liver, which would aid in the mobilization of energy reserves to fuel the metabolic costs associated with ascension.

Subordinates typically exhibit an attenuated glucocorticoid stress response compared to dominants (Jeffrey et al., 2014; Ling et al., 2009; Michopoulos et al., 2012; Sapolsky, 1983, 1982), which appears to be a consequence of altered production of and/or sensitivity to ACTH in subordinate individuals (Jeffrey et al., 2014; Michopoulos et al., 2012; Sapolsky, 1983; Sloman et al., 2002). Additionally, following an acute stressor, glucocorticoid levels in subordinates take longer to return to pre-stress levels than in dominants (Jeffrey et al., 2014; Sapolsky, 1983), which is thought to be a consequence of impaired negative feedback via the corticosteroid receptors in subordinates (Sapolsky, 1983). While ascending male *N. pulcher* had similar levels of circulating cortisol compared to subordinate males, differences in transcript abundance of key components of the stress axis between ascenders and subordinates suggests that ascenders may mount a larger acute stress response compared to subordinates. Higher transcript abundance of *p450scc* and *star* in the head kidney indicates that ascenders may have the steroidogenic machinery in place to mount a faster and/or stronger cortisol response than subordinates. Additionally, higher transcript abundance of *gr1* and *gr2* in

the preoptic area of the brain of ascenders may reflect an enhanced ability to return cortisol levels to pre-stress values following a stressor. Overall, these transcriptional changes support the notion of ascenders having the capacity to mount a stronger stress response that returns to baseline quicker than non-ascending males. However, whether these transcriptional changes do indeed lead to changes in protein abundance that influence the acute stress response of ascenders still needs to be assessed.

4.3 Regulation of growth

Chapter 3 examined how growth rates are adjusted as male *N. pulcher* ascend to dominance. I found that ascenders tripled their growth during the first 72 h of ascension, consistent with previous studies (Buston, 2003; Hamilton and Heg, 2008; Hofmann et al., 1999; Huchard et al., 2016; Russell et al., 2004; Thorley et al., 2018; Wong et al., 2008). Socially-regulated growth, where subordinates restrict their growth to remain smaller than dominants, appears to play an important role in stabilizing social groups (Buston, 2003; Buston and Cant, 2006; Dengler-Crish and Catania, 2007; Heg et al., 2004; Wong et al., 2007). Several studies suggest that in linear, size-based hierarchies, subordinates can grow up to ~90-95% of the size of their immediate dominant, without increasing rates of conflict (Ang and Manica, 2010; Wong et al., 2008, 2007). When subordinates pass this 95% size threshold their ability to overthrow the individual immediately dominant to them increases (Reddon et al., 2011; Wong et al., 2007) and dominants become more aggressive towards subordinates that approach this threshold (Ang and Manica, 2010; Wong et al., 2008, 2007). Indeed, subordinate male *N. pulcher* who are similar in size to

the dominant male within their group are more submissive and stay further away from breeding sites to avoid conflict (Hamilton et al., 2005). Subordinates that supress their growth, remaining below this 95% size threshold, may incur lower energetic costs than subordinates who do not, because the performance of submissive acts is energetically costly (Grantner and Taborsky, 1998). In Chapter 3, I show that energy reserves stored by subordinates are critical, because these reserves provide the fuel necessary to ascend to dominance. As such, limitations on growth, and the associated conservation of energy that otherwise would have been spent on the performance of submissive behaviours, likely are critical to allow subordinates to stockpile the energy reserves necessary to become dominant in the future. Overall, these findings indicate that subordinate growth is likely regulated through a combination of top-down suppression by dominants, as well as self-imposed restriction of feeding and growth.

One mechanism by which social suppression of growth may manifest is through increased glucocorticoid production by subordinates (Chapter 2). The suppressive actions of cortisol on somatic growth are well established (Allen, 1996), largely acting through the insulin-like growth factor (IGF) system (Pierce et al., 2005). Chronic treatment with cortisol results in increased production of low molecular weight IGF binding proteins (IGFBP-1 and -2), which appear to be responsible for reducing growth during chronic stress (Madison et al., 2015; Peterson and Small, 2005). It seems likely that increased levels of conflict between dominants and subordinates when size differences are small (Ang and Manica, 2010; Wong et al., 2008, 2007) cause circulating cortisol levels to rise in subordinates. Indeed, subordinate *N. pulcher* that were more submissive to dominants

(a means of appeasing dominants to reduce conflict) had lower waterborne cortisol levels compared to less submissive subordinates (Bender et al., 2006). Therefore, elevated levels of cortisol in subordinates that challenge dominants may suppress growth via increased production of IGFBPs. This suppression of growth by IGFBPs would continue until size differences between dominants and subordinates are large enough that subordinates would no longer be considered a threat by dominants, causing rates of conflict to decrease, and hence, reduce cortisol production. To test this prediction, subordinates could be treated with metyrapone—an inhibitor of cortisol production—when they approached the body size of dominants. If conflict-induced elevations in cortisol production are indeed responsible for the suppression of subordinate growth, then metyrapone-treated subordinates should continue to grow, despite elevated levels of conflict.

Stored energy reserves appear to play a critical role during life-history transitions. In the field, *N. pulcher* helpers that dispersed and attained dominance in another group decreased their rates of territory defense immediately prior to dispersal (Zöttl et al., 2013). It is likely that this decrease in the performance of metabolically costly helping behaviours serves to conserve energy that can later be used by helpers to successfully disperse. In my work, I found that ascenders had half the liver glycogen stores of stable subordinates. This difference is likely the consequence of elevated energy consumption by ascenders, fuelling increased growth, higher rates of activity, and more frequent performance of social behaviours. Although these changes are necessary to assume dominance, such rates of energy consumption can likely only be supported for a short

period given the apparent metabolic costs (i.e., reduction in energy reserves). As such, it is probable that growth rates decrease shortly after dominance has been secured, such that ascenders do not completely deplete their energy reserves. Interestingly, hepatic glycogen levels of ascenders were consistent with that of dominants, suggesting that dominants are able to maintain their energy reserves at these levels despite continuing to be highly active and perform high rates of social behaviours. Dominant animals often exhibit more effective digestion and nutrient absorption than subordinates (DiBattista et al., 2006; Earley et al., 2004; Olsen and Ringo, 1999), and it seems plausible that dominant *N. pulcher* follow a similar trend. An enhanced digestive capacity would aid in supporting the energetic costs of a dominant lifestyle, especially given that no differences in food intake were found between dominants and subordinates (Chapter 3). No evidence of increased investment in the digestive tract by dominants was detected based on intestosomatic index measures (B. Culbert unpublished data), but this finding does not negate the possibility of more effective intestinal function in dominants.

Although I did not detect any differences in food intake during social ascension, I did find that ascenders had approximately 50% less liver glycogen than subordinates (Chapter 3). This finding suggests that changes in the regulation of food intake and energy status likely play an important role during ascension. Regulation of energy status involves cross-talk among several organs (e.g., stomach, intestine, liver, brain) and many different peptides and hormones, which either act to increase (orexigenic) or decrease (anorexigenic) food intake depending on a variety of factors, including stored energy reserves and overall metabolic status (Rønnestad et al., 2017; Volkoff, 2016). I conducted

a preliminary investigation of hypothalamic feeding peptides (CRF and neuropeptide Y), but did not find differences between social ranks in transcript abundance of either peptide (B. Culbert unpublished data). However, these feeding peptides represent only a small portion of the many physiological signals responsible for regulating feeding and energy status, making it difficult to draw firm conclusions based on this preliminary study. In fact, only a handful of studies have investigated the physiological mechanisms responsible for the social regulation of feeding (Cunningham et al., 2016; Melhorn et al., 2010; Porter et al., 2017; Wu et al., 2014), and our knowledge of the subject is still in its infancy (Fischer and O'Connell, 2017). Recent work suggests that leptin, a potent anorexigenic hormone, is involved in the suppression of feeding by subordinate rainbow trout (*Oncorhynchus mykiss*; Jennings et al., in prep). Leptin plays an important role in the regulation of feeding, relaying information regarding energy status to the brain (Gorissen and Flik, 2014; Park and Ahima, 2015). Although leptin is produced in adipose tissue and functions as a lipostatic signal in mammals, in other vertebrates (including fish), leptin is produced in the liver and appears to exert its metabolic actions by influencing carbohydrate metabolism (Deck et al., 2017). In fish, leptin treatment stimulates carbohydrate metabolism, causing increased rates of hepatic glycogenolysis and hyperglycemia (Aguilar et al., 2010; Baltzegar et al., 2014). During ascension, leptin may aid in the mobilization of hepatic glycogen reserves, helping to provide the fuel required to meet the heightened metabolic demands associated with ascension. Further work is required to better understand how social hierarchies influence feeding and energy balance, and the role that these systems play during social ascension.

4.4 Female ascension

An interesting avenue for future studies will be to investigate how the physiological changes that accompany social ascension differ between sexes. The majority of studies focus on social dominance in males, and, consequently, very few studies investigating ascension have been conducted using females. Given the vast physiological differences that exist between males and females, including differences in hormone production (Handa et al., 1994; Kudielka and Kirschbaum, 2005), brain neurochemistry (Albers, 2015; Terranova et al., 2016), and metabolism (Mauvais-Jarvis, 2015; Varlamov et al., 2015), the physiological mechanisms regulating ascension undoubtedly differ between males and females. In addition to these physiological differences, males and females typically utilize different life-history strategies, further increasing the likelihood of sex-specific mechanisms regulating ascension.

In group-living animals, sex-specific differences in dispersal are common and are thought to reduce inbreeding (Clutton-Brock and Lukas, 2012; Hutchings and Gerber, 2002; Perrin and Mazalov, 2000; Pusey, 1987; Waser, 1985). In *N. pulcher*, males typically gain dominance through dispersion, attaining dominance in a different group from the one in which they were raised, whereas females form a dominance queue and inherit dominance within their own social group (Stiver et al., 2007, 2006, 2004). If female social successors are generally pre-established, then rates of intragroup competition during periods of social opportunity would be predicted to be much lower compared to periods of social opportunity for males. As such, levels of stress experienced

by females during ascension would also be predicted to be lower than the stress experienced by males during ascension. However, I am unaware of any investigations on whether sex-specific behavioural dynamics during social ascension differentially regulate the stress axis. More generally, in species with female social queues, less marked physiological changes may be expected during ascension to dominance by females. Indeed, female *N. pulcher* do not adjust their growth during ascension (Hamilton and Heg, 2008; Heg, 2010). This observation contrasts with findings in cooperatively breeding mammals, because ascension is associated with elevated growth rates in female meerkats (*Suricata suricatta*; Huchard et al., 2016), female naked mole-rats (*Heterocephalus glaber*; Dengler-Crish and Catania, 2007), and female Damaraland mole-rats (*Fukomys damarensis*; Thorley et al., 2018). Clearly, more research is required not only to determine how the physiological mechanisms regulating social ascension differ between sexes, but also why these mechanisms vary between cooperatively breeding species.

4.5 Conclusions

In this thesis, I investigated physiological regulation of social ascension to dominance in the group-living cichlid, *Neolamprologus pulcher*. Specifically, I explored how stress and growth are regulated during ascension. I found that the behaviour of *N. pulcher* exhibits remarkable plasticity, because ascending males became behaviourally dominant within 72 h following removal of the dominant male. However, these ascenders appeared to be under higher levels of stress than dominants, as they exhibited higher

circulating levels of cortisol and higher transcript abundance of key steroidogenic genes in the head kidney. I also found that enhanced growth during ascension appears to be mediated by a reduction in production of insulin-like growth factor binding proteins 1 and 2, resulting in increased bioavailability of the growth stimulating hormone, insulin-like growth factor 1. Finally, I found that ascension is an energetically costly life-history transition, because ascenders had half the liver glycogen stores of stable subordinates. Overall, this work provides insight into the physiological adjustments that occur during social ascension, and helps to elucidate the proximate mechanisms regulating social hierarchies.

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