

Do social interactions or life on land serve as stressors to the bichir, *Polypterus senegalus*?

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Abstract

Polypterus senegalus is a basal ray-finned fish that breathes both air and water, and can tolerate life on land for extended periods. Previous research reported that *P. senegalus* held on land for several months exhibited lower growth rates than counterparts fed equal amounts of food but held under aquatic conditions. Because chronic stress can impair growth, the present research aimed to determine whether *P. senegalus* experience stress when held under terrestrial conditions. *P. senegalus* were held for 1, 2, 4, or 8 weeks in aquatic or terrestrial conditions, following which tissues were sampled for analysis. Growth rates were significantly lower in fish held under terrestrial conditions, but neither plasma cortisol levels nor transcript abundances of genes of the stress axis differed between aquatic and terrestrial fish. Terrestrial fish exhibited lower transcript abundance in liver of the growth regulator insulin-like growth factor 1, and higher transcript abundances in muscle of myostatin, an inhibitor of muscle growth, and muscle RING-finger protein-1, an indicator of muscle breakdown. With knowledge from anecdotal observations that *P. senegalus* tend to be aggressive when housed with a conspecific, *P. senegalus* were held in a second experiment in pairs for 6 d to investigate stress axis function. Clear differences in territorial and aggressive behaviours between the individuals in a pair were consistent with one fish being dominant over the other, subordinate fish. Dominant fish tended to have higher cortisol levels than subordinate fish, although the difference was not significant, and cortisol levels were correlated with the frequency of aggressive and territorial behaviours, regardless of social status. However, transcript abundances of stress axis genes generally did not differ between dominant and subordinate fish. Collectively, these results suggest that *P. senegalus* is unusually tolerant of conditions that serve as stressors in other fish species, including emersion and social interactions.

Résumé

Polypterus senegalus est un poisson basal à nageoires rayonnées qui respire à la fois l'air et l'eau et peut tolérer la vie sur terre pendant de longues périodes. Des recherches antérieures ont indiqué que *P. senegalus* détenu sur terre pendant plusieurs mois présentait des taux de croissance inférieurs à ceux de ses homologues nourris avec des quantités égales de nourriture mais détenus dans des conditions aquatiques. Étant donné que le stress chronique peut nuire à la croissance, la présente recherche visait à déterminer si *P. senegalus* subit un stress lorsqu'il est maintenu dans des conditions terrestres. *P. senegalus* a été détenu pendant 1, 2, 4 ou 8 semaines dans des conditions aquatiques ou terrestres, après quoi les tissus ont été échantillonnés pour analyse. Les taux de croissance étaient significativement plus faibles chez les poissons détenus dans des conditions terrestres, mais ni les niveaux de cortisol plasmatique ni l'abondance des transcrits des gènes de l'axe de stress ne différaient entre les poissons aquatiques et terrestres. Les poissons terrestres présentaient une plus faible abondance de transcrits dans le foie du facteur de croissance analogue à l'insuline du régulateur de croissance 1, et des abondances plus élevées de transcrits dans le muscle de la myostatine, un inhibiteur de la croissance musculaire, et de la protéine RING-finger-1 du muscle, un indicateur de dégradation musculaire. Dans une deuxième expérience, avec la connaissance d'observations anecdotiques selon lesquelles *P. senegalus* a tendance à être agressif lorsqu'il est hébergé avec un congénère, nous avons maintenu *P. senegalus* par paires pendant 6 jours pour étudier la fonction de l'axe de stress. Des différences claires dans les comportements territoriaux et agressifs entre les individus d'une paire étaient cohérentes avec le fait qu'un poisson était dominant sur l'autre poisson subordonné. Les poissons dominants avaient tendance à avoir des niveaux de cortisol plus élevés que les poissons subordonnés, bien que non significatifs, les niveaux de cortisol étant corrélés à la fréquence des

comportements agressifs et territoriaux, quel que soit le statut social. Cependant, les abondances de transcrits de l'axe de stress ne différaient généralement pas entre les poissons dominants et subordonnés. Collectivement, ces résultats suggèrent que *P. senegalus* est exceptionnellement tolérant aux conditions qui servent de facteurs de stress chez d'autres espèces de poissons, y compris l'émersion et les interactions sociales.

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List of abbreviations

ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of Variance
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cDNA	Complementary DNA
CF	Condition factor
CRF	Corticotropin releasing factor
DAPI	4',6-diamidino-2-phenylindole
Dom	Dominant
EIA	Enzyme immunoassay
<i>fasn</i>	Fatty acid synthase
GR	Glucocorticoid receptor
HK	Head kidney
HPI	Hypothalamic-pituitary-interrenal
<i>igf-1</i>	Insulin-like growth factor 1
IHC	Immunohistochemistry
MC2R	Melanocortin 2 receptor
min	Minute
MR	Mineralocorticoid receptor
<i>mstn</i>	Myostatin
<i>myrf</i>	Muscle RING finger protein
<i>myoD</i>	Myoblast determination protein 1

N	Sample Size
P	P-value
PCA	Principal component analysis
PCR	Polymerase chain reaction
POA	Pre-optic area
POMC	Proopiomelanocortin
<i>pygl</i>	Glycogen phosphorylase
P450scc	Cholesterol side chain cleavage enzyme
q-PCR	Quantitative polymerase chain reaction
SEM	Standard error of the mean
SGR	Specific Growth Rate
StAR	Steroidogenic acute regulatory protein
Sub	Subordinate
TH	Tyrosine hydroxylase
3 β HSD	3 β -hydroxysteroid dehydrogenase
11 β -hyd	11 β -Hydroxysteroid dehydrogenase

Chapter 1: General Introduction

The physiological consequences of chronic stress have been extensively studied in many species of fish (reviewed by Schreck and Tort, 2016; Vijayan et al., 2010; Wendelaar Bonga, 1997), but most of these studies have focused on teleost fishes. Much less is known about the stress axis or stress responses of the basal actinopterygians (i.e. the chondrosteans, bichirs and reedfish, sturgeon and paddlefishes; and the holosteans, bowfin and gar) (reviewed by Bouyoucos et al., 2021). The bichir *Polypterus senegalus* is an amphibious, basal ray-finned fish that can tolerate life on land (reviewed by Wright and Turko, 2016; Turko et al., 2019), making it a useful study species from the perspective of investigating whether life on land serves as a stressor for amphibious fishes. Previous work demonstrated lower growth rates in *P. senegalus* housed in a terrestrial environment as opposed to their aquatically-housed counterparts, even when the aquatic and terrestrial fish were fed equal rations of food (T. Du and E.M. Standen, unpublished observations). This difference could be caused by several factors, including greater energy expenditure in a terrestrial environment or chronic stress caused by terrestrial life. Stress hormones stimulate the mobilization of energy reserves to help the animal cope with the short-term consequences of a stressor. However, chronic stressors that cause prolonged elevation of glucocorticoids can deplete energy reserves, negatively impacting the growth rate and potentially the overall fitness of fishes (Wendelaar Bonga, 1997; Schreck et al., 2016). It is unknown, however, whether amphibious fishes experience chronic stress in a terrestrial environment and whether this factor contributes to the lower growth rates reported in terrestrial *P. senegalus*. Thus, the present thesis aimed to investigate this possibility. Specifically, I tested the hypothesis that *P. senegalus* housed in a terrestrial environment experience chronic stress. I predicted that *P. senegalus* housed in terrestrial conditions would exhibit elevated levels of circulating cortisol concentrations as well as elevated transcript abundances of several key genes of the

hypothalamic-pituitary-interrenal (HPI) axis. As a tool to probe stress axis activity, I also tested the hypothesis that *P. senegalus* housed (aquatically) in a social environment experience stress; specifically, that *P. senegalus* confined in pairs would form social hierarchies in which the subordinate fish within a pair would experience chronic social stress. This hypothesis was based on the observation that *P. senegalus* are aggressive towards conspecifics (E.M. Standen, unpublished observations). The general introduction below provides context for the experimental chapters by briefly reviewing current research and the literature focused on the stress response, the stress axis in fishes, and social stress.

P. senegalus is an African freshwater, amphibious, air-breathing fish that can tolerate life on land (Du et al., 2016). The species is characterized by a respiratory system consisting of both gills and air-sac-like structures which are their true lungs (Bagrosky et al., 2003). *P. senegalus* and other bichirs belong to the Polypteridae family which is the last existing family in the order Polypteriformes, and *P. senegalus* is one of two remaining genera (Near et al., 2012). The bichirs are one of the most primitive extant ray-finned fishes, and the ability of *P. senegalus* to survive terrestrial environments is a unique characteristic of bichirs compared to other basal ray-finned fish species that are air-breathing but solely aquatic (Near et al., 2012). By studying the physiology of the stress response in *P. senegalus*, we can learn more about the evolution of the stress response in both ray-finned fishes and vertebrates as a whole. Substantial gaps exist in our understanding of the physiology and behaviour of *P. senegalus* and the effects of stress on its physiological function. In particular, it is unknown whether life on land serves as a stressor for this species (or indeed, for any amphibious species), and the effects of chronic stress on *P. senegalus*' physiological function have not been examined. Although there is evidence that *P. senegalus* often come to the surface to breathe (Magid, 1966), there is little evidence that they

voluntarily choose to be on land (Du et al., 2016). However, at least in a laboratory setting, *P. senegalus* is capable of surviving on land for prolonged periods (Standen et al. 2014). As a basal ray-finned fish species, *P. senegalus* is an excellent model for exploring the evolution of the HPI axis, allowing us to link their stress response with that of other, better-documented fish groups that arose later in the evolutionary tree.

1.1 The stress response

‘Stress’ can be defined in many ways. In a physiological context, stress is often described as a condition in which the dynamic equilibrium of the animal, its homeostasis, is threatened or disturbed as a result of intrinsic or extrinsic stimuli, commonly defined as stressors (Herbarium et al., 2017). The current consensus from the literature is that stress and homeostasis are related, and that exposure to stressors elicits a physiological response, the stress response (Vijayan et al., 2010; Harris, 2020). A stress response is thought to be adaptive in that it allows the fish to overcome the threat at hand (Breuner et al., 2008; Schreck, 2010; Dhabhar, 2018). However, if an animal experiences a prolonged, repeated or chronic stressor, the stress response can lead to inhibition of growth, reproductive failure, and reduced resistance to pathogens (Wedemeyer and McLeay, 1981; Wedemeyer et al., 1990; Wendelaar Bonga, 1997). The magnitude and duration of the stress response depend on the severity and duration of the stressor (Schreck and Li, 1991). There are three main stages to the stress response; alarm (primary), resistance (secondary), and compensation or exhaustion (tertiary) (Schreck and Tort, 2016). Perception of the stressor initiates the primary response, which consists of activation of the endocrine system to increase circulating concentrations of the corticosteroid and catecholamine stress hormones. The stress hormones activate secondary stress responses that allocate resources to vital processes by

increasing circulating glucose levels, enhancing gill vascularization to improve oxygen uptake, and increasing heart rate (Schreck and Tort, 2016). If the stressor is overcome by these compensatory actions, then the homeostatic norm can be re-established, but if the stressor is too great to overcome, then the fish enters the tertiary or exhaustion phase, which can lead to death (Selye, 1950; Schreck, 2000).

Because chronic activation of stress responses can have deleterious effects, questions have been asked about whether chronic stress benefits the animal in terms of adaptability and fitness (Boonstra, 2013). Bioenergetics models may help our understanding of the impacts of chronic stress by examining energy input and output of the animal, and the ways in which this energy is used. A simple bioenergetics model (Deslauriers et al., 2017) partitions consumed energy into metabolism, wastes, and growth. The metabolic demands of the fish involve the energetic cost of maintenance (i.e. standard metabolic rate), energy expenditure due to activity, and the energy required to digest food (Deslauriers et al., 2017). This model then can be used to observe and predict how different environments impact the animal's performance. For example, Boisclair and Leggett (1989) used growth estimates and consumption rates to estimate the quantity of energy allocated to activity in yellow perch (*Perca flavescens*), finding that energy costs increased as food consumption increased. There are several bioenergetic costs associated with chronic stress (Schreck, 1980). For example, during chronic stress, resources are allocated away from processes such as growth, reproduction, and immune function for a significant amount of time, therefore potentially lowering the animal's fitness (Schreck and Tort, 2016). If terrestrial exposure serves as a chronic stressor for *P. senegalus* and causes prolonged elevation of glucocorticoids, this response might result in a depletion of energy reserves that negatively impacts growth rates and potentially the overall fitness of the fish (Wendelaar Bonga, 1997). It is

unknown, however, whether terrestrial exposure serves as a stressor for *P. senegalus*, and this question therefore was the focus of my research. Social interactions serve as a stressor in a variety of fish species (Johnsson et al., 2005), and therefore I also asked whether exposure to a social environment could be used to probe stress axis function in *P. senegalus*.

Fish are an excellent indicator of environmental changes and studies focusing on fish can aid in the development of conservation strategies (Sneddon et al., 2016). They live across a wide range of habitats and are affected by the many variations of climate change and habitat disturbance (Van Vliet et al., 2013). These disturbances can cause elevations in stress levels, negatively impacting the physiology of the animal and moving it away from the homeostatic norm (Glencross and Bermudes, 2012; Schreck and Farrell, 2016). Humans are a large source of the changes that occur within the natural environment, and therefore there is a clear imperative to study the stress response in fishes to better understand the physiological outcomes of human impacts.

1.2 The stress axis in fishes

As previously mentioned, perception of a stressor initiates the primary stress response, which consists of activation of the endocrine system to increase circulating concentrations of the corticosteroid and catecholamine stress hormones. Catecholamine release is fast; it relies on the nervous system and allows the animal to react to a stressor within seconds to minutes (Perry and Bernier, 1999). In teleost fishes, catecholamines are released by chromaffin cells that are innervated by pre-ganglionic neurons of the autonomic nervous system. The chromaffin cells are located in the head kidney region around the posterior cardinal vein (Reid et al., 1998). In *P. senegalus*, chromaffin cells are similarly located within the walls of the posterior cardinal vein

(de Smet, 1970). The adrenergic response to a stressor elevates blood glucose levels and enhances oxygen uptake and delivery to the tissues (Vijayan et al., 2010). The corticosteroid stress hormones, primarily cortisol in teleost fish, are the end product of the hypothalamic-pituitary-interrenal (HPI) axis which is homologous to the HP-adrenal (HPA) axis of mammals (Norris and Hobbs, 2006). Like catecholamines, cortisol levels increase in response to a stressor, but cortisol levels are slower to rise than catecholamines, and elevated levels of cortisol may be maintained whereas catecholamine levels quickly return to baseline levels (Schreck et al., 2016). Upon perception of the stressor, neurons of the preoptic area (POA) of the brain produce corticotropin releasing factor (CRF) (Vijayan et al., 2010; Schreck and Farrell, 2016). The CRF-producing neurons terminate at corticotropes in the anterior pituitary, where CRF stimulates the release of adrenocorticotrophic hormone (ACTH), which is a product of the precursor protein proopiomelanocortin (POMC). The release of ACTH into the circulation allows it to travel to the steroidogenic cells of the head kidney which express melanocortin type 2 receptors (MC2R) (Sapolsky et al., 2000; Sewer and Waterman, 2003; Vijayan et al., 2010). Activation of MC2R by ACTH triggers a cAMP signalling cascade that increases the biosynthesis of cortisol.

Cortisol is produced from its precursor cholesterol with the first step being the movement of cholesterol into the mitochondrion, a step facilitated by steroidogenic acute regulatory protein (StAR) (Stocco, 2000). Cholesterol is then cleaved to pregnenolone, a reaction catalyzed by the enzyme P450 side chain cleavage enzyme (P450_{scc}) (Sewer and Waterman, 2003). These two initial processes are the rate-limiting steps in cortisol production (Aluru and Vijayan, 2006). In subsequent steps, pregnenolone is converted to the steroid hormone progesterone, a reaction catalyzed by 3 β -hydroxysteroid dehydrogenase (3 β HSD) (Vijayan et al., 2010). In the final step of cortisol synthesis, 11 β -hydroxylase (11 β -hyd) catalyzes the 11 β -hydroxylation of

deoxycortisol to cortisol (Vijayan et al., 2010). Because cortisol is synthesized on demand, the time required for a detectable elevation in circulating cortisol levels varies among fish species, but several minutes are usually needed (Lawrence et al., 2018).

The corticosteroid receptors include a glucocorticoid receptor (GR) and a mineralocorticoid receptor (MR) (Sturm et al., 2005; Norris and Hobbs, 2006; Vijayan et al., 2010). In fishes, these receptors are expressed in a wide range of tissues, including gill, kidney, liver, intestine, and brain (Mommsen et al., 1999; Norris and Hobbs, 2006). It is also known that in teleost fishes, the GR gene is duplicated (Prunet et al., 2006; Bury and Sturm, 2007; Bury et al., 2017). In several species, including rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*), one GR isoform (GR2) was more sensitive to cortisol, and may play a more significant role in regulating gene expression at basal circulatory cortisol levels, whereas the other isoform, which was less sensitive to cortisol levels, may be more important in stress responses (Bury et al. 2017). However, the specific roles of GR1, GR2 and MR remain unclear in teleost fishes, although activation of corticosteroid receptors affects a wide variety of physiological processes, including osmoregulation, ionoregulation, and the mobilization and replenishment of energy stores (Mommsen et al., 1999; Faught and Vijayan, 2018).

As discussed above, cortisol is the main circulating glucocorticoid in teleost fish. Although an anecdotal description of corticosteroid measurements in plasma of *P. senegalus* reported that corticosterone rather than cortisol was detected (Kime, 1985), other chondrosteans as well as holostean fishes that have been studied in more detail appear to produce cortisol (Hanson and Fleming, 1979; Youson and Butler, 1985; Barton et al., 1998; reviewed by Bouyoucos et al., 2021). These observations suggest that *P. senegalus* also uses cortisol as its main corticosteroid, although I am not aware of any (recent) measurements of plasma

corticosteroids in polypterid fishes. Despite the apparent similarity in the end-product of the HPI axis between basal ray-finned fishes and teleosts, both morphological and molecular differences are present in the HPI axis itself (reviewed by Gallo and Civinini, 2003; Youson 2007; Bouyoucos et al. 2021).

Anatomically, the steroidogenic cells of the HPI axis are located in “yellow corpuscles” near the posterior cardinal and renal veins in the anterior kidney in *P. palmas* (Youson and Butler, 1985), as well as reedfish (Youson et al. 1988), sturgeon (Idler and Sangalang, 1970; Idler and O’Halloran, 1970), and bowfin (Butler and Youson, 1986) (reviewed by Youson 2007). The yellow corpuscles are spherical and evenly distributed throughout the anterior kidney. By contrast, in teleost fishes the steroidogenic cells are located in the tissue of the head kidney, in chondrichthyan fishes the steroidogenic cells are found in the interrenal gland, and in agnathans, these cells are in the pronephric and mesonephric kidney (reviewed by Bouyoucos et al., 2021). The location of the corticosteroidogenic cells in *P. senegalus* has not been reported although they would be expected to be present in yellow corpuscles, as in *P. palmas* (Youson and Butler, 1985). In addition to differences in the location of the cortisol-producing cells, teleost fish have direct innervation of the anterior pituitary by the CRF-producing neurons of the POA, contrary to basal actinopterygians that exhibit a well-developed portal system with some direct innervation in parts of the pituitary (reviewed by Bouyoucos et al., 2021). In addition, several species of basal actinopterygians such as Dabry’s sturgeon (*Acipenser dabryanus*) and spotted gar (*Lepisosteus oculatus*) have been found to possess several types of CRF peptides and receptors (Qi et al. 2020; Cardoso et al., 2016), suggesting that early-branching ray-finned fishes possess more types of CRF than teleosts (Gorissen and Flik, 2016; Bouyoucos et al., 2021). However, basal ray-finned fishes only express a single GR compared to the two types in teleosts (Bury and

Sturm, 2007). These findings suggest that there have been possibly several losses and duplications of HPI axis genes between basal ray-finned fishes and teleosts, providing context for the differing components of the HPI axis in *P. senegalus* in an evolutionary context. Whether structural differences in the HPI axis between teleost fishes and *P. senegalus* have functional consequences is not yet known. Thus, examination of HPI axis function is warranted, but requires that suitable stressors be identified. Even for fishes that have the capacity to tolerate terrestrial life, prolonged exposure to terrestrial conditions may serve as a stressor, and testing this possibility was the major goal of the present thesis. However, it remained possible that life on land does not serve as a stressor for amphibious fishes, and therefore a second potential stressor, social interaction, also was investigated.

1.3 Social interactions and social stress

Belonging to a group can provide benefits to an animal including cooperation in finding resources to distribute amongst the group, and protection from predation (Johnsson et al., 2005). However, conflict among members of a group can arise over factors such as unequal distribution of resources, including food, shelter, and limited reproductive opportunities (Johnsson et al., 2005). Whether the benefits of group membership outweigh the costs depends on the species as well as the availability of limited resources (Brown & Brown, 1993; Sloman et al., 2000). Individuals may compete for resources within a group but not interact aggressively, therefore lowering the cost of competition. On the other hand, the interactions could be aggressive but the benefit of obtaining the resource could outweigh the cost of the aggressive interaction (Boyd and Silk, 1983; Johnsson et al., 2005). In some fishes that form groups, the establishment of social hierarchies helps reduce conflict amongst group members (Brown & Brown, 1993). High-

ranking group members tend to obtain the most resources whereas lower-ranking members obtain fewer resources. Although this distribution of resources is unequal, it is balanced by the lowering of competition among members in the group. An animal's position in the hierarchy can be established in several ways, such as dominance over other members in the group during social interactions, or monopolizing and defending resources (food, shelter, or mates) from other members (Johnsson et al., 2005). The more successful an animal is in these interactions, the greater the chance an animal has of obtaining a favorable position in the dominance hierarchy (Peters et al., 1980; Abbott and Dill, 1985; Overli et al., 1999b). These interactions can become a primary source of stress to an animal and affect HPI axis activity (Creel et al., 2012). Interactions between conspecifics may involve vigorous aggressive behaviours, but often these decrease in frequency once a dominant-subordinate relationship has been established (Sloman et al., 2000; Winberg et al., 2016). Both dominant and subordinate fish exhibit increased cortisol levels during the initial stages of social interactions (Johnsson et al., 2005). The more aggressive the interaction and the greater the frequency of interactions, the greater the stressor for the member of the group that holds a subordinate position, as demonstrated by elevated plasma cortisol levels (Sloman et al., 2000). For the dominant fish, however, cortisol levels typically return to baseline within hours of establishment of the hierarchy (Winberg and Lepage, 1998; Pottinger and Carrick, 2001), although in some social environments, dominant fish exhibit elevated cortisol levels, likely as a result of the stress of maintaining a dominant social position (Mileva et al., 2009). This maintained elevation of cortisol in subordinate fish can result in decreased growth, reduced reproductive potential, and increased susceptibility to pathogens (Johnsson et al., 2005). There are also several behaviours considered to be indicative of subordinate status including hiding, evading other conspecifics, and reduced activity (Sloman et al., 2000).

Aggressive interactions may also occur with normally solitary individuals that do not live in groups, for example in defending resources such as food and territory from conspecifics (Gomez-Laplaza, 2000). For example, in the weakly electric knifefish *Apteronotus leptorhynchus*, a species that normally is solitary, the number of aggressive behaviours (“chirps”, a specific type of electric organ discharge) increased significantly when an individual was paired with a conspecific (Dunlap et al., 2013). Similarly, *P. senegalus* is a fish that is normally solitary (anecdotal observations). Under laboratory holding conditions, they often behave in an aggressive manner when held with a conspecific (E.M. Standen, unpublished observations) and therefore it seems likely that being housed with a conspecific will constitute a stressor for one or both members of the pair. Indeed, this type of social interaction might be a more significant stressor than terrestrial exposure given that *P. senegalus* is known to tolerate life on land (Standen et al. 2014). If placing *P. senegalus* in an environment where they are forced to interact with a conspecific is an effective approach to elicit a stress response, it could provide an alternative way of studying stress axis function in this species. I was unable to find any literature documenting social interactions or behaviours in *P. senegalus*. Other species of fish use displays, chasing, charging, and nipping in agonistic interactions (Johnsson et al., 2005), and therefore I expected *P. senegalus* to exhibit a similar suite of behaviours.

1.4 Hypothesis and predictions

The present thesis aimed to test the hypothesis that *P. senegalus* housed in a terrestrial environment experience chronic stress. Based on this hypothesis, *P. senegalus* housed in terrestrial conditions would be predicted to exhibit elevated circulating levels of cortisol as well as increased transcript abundances for genes of the HPI axis. I also tested the hypothesis that *P.*

senegalus housed with a conspecific, i.e., in a social environment, experience stress. Based on this hypothesis, I predicted that *P. senegalus* housed in pairs would engage in agonistic interactions, and that the loser of these fights would exhibit elevated circulating concentrations of cortisol as well as increased transcript abundances of stress axis genes. Finally, I expected that subordinate *P. senegalus* would have higher circulating cortisol levels and greater HPI axis activity (transcript abundances) than fish housed under terrestrial conditions.

**Chapter 2: The effects of a terrestrial environment on
stress axis activity in *Polypterus senegalus***

2.1 Introduction

Polypterus senegalus is an amphibious, air-breathing fish that can tolerate life on land (Du and Standen, 2017). Despite their ability to withstand a terrestrial environment, previous observations have detected a reduced growth rate in *P. senegalus* held on land for extended periods of time compared to their aquatically-housed counterparts (T. Du & E.M. Standen, unpublished observations). Fish growth reflects in large part muscle hyperplasia (Mommensen and Moon, 2001) and is a complex process influenced by a variety of factors including food intake and nutrient assimilation, the allocation of energy to different processes, and its utilization (Sadoul and Vijayan, 2016). Bioenergetics models (Fig. 2.1) provide a useful approach for considering the factors that affect growth because they take into account all of the variables that determine energy allocation, partitioning the energy that is available into metabolism, wastes and growth (Deslauriers et al., 2017). Thus, factors that increase metabolic energy expenditure (e.g. increased activity) or that increase energy lost as wastes tend to reduce growth. The Wisconsin model (Fig. 2.1) appears to be a good predictor of consumption and energy output; for example, Karjalainen et al (1997) estimated food consumption of larval and juvenile fish under different experimental conditions, and found that the model was accurate when comparing observed and predicted values. According to the model, an important effect of stress in fish is loss of appetite and therefore a reduction in food consumption (Koojiman, 2010), which lowers the energy available for maintenance and growth of the fish. Another effect of stress is the inhibition of digestive function, which decreases the capacity of the fish to assimilate nutrients, also directly affecting available energy (Barton et al., 1986; Olsen et al., 2002; Olsen et al., 2005). Stress causes proteolysis and structural protein breakdown to allocate energy resources to survival, thereby reducing growth (Barton et al., 1986). There may also be increases in metabolic rate

(Mommsen et al., 1999). Given the impacts of stress on growth in fishes, one hypothesis that may account for low growth rates in terrestrial *P. senegalus* is that life on land is perceived as stressful.

Fish that experience chronic stress typically exhibit a sustained elevation of plasma cortisol concentrations (Mommsen et al., 1999; Schreck et al., 2016). Cortisol is the major corticosteroid produced by the HPI axis in ray-finned fishes (Sadoul and Vijayan, 2016), and chronic activation of the stress axis may contribute to the sustained elevation of cortisol during chronic stress (Balasch and Tort, 2019). Chronic activation of the stress axis would be expected to be accompanied by increases in the transcript abundances of stress axis genes (Faught et al., 2016) (Gorissen and Flik, 2016; Winberg and Lepage, 1998). By contrast, there is evidence of decreased *gr* mRNA abundance in cases of chronic stress (Sapolsky et al., 2000; Jeffrey et al., 2012a). Basal ray-finned fishes have been understudied in the field of stress physiology, and this is particularly true of polypterids (Bouyoucos et al., 2021). Therefore, I embarked upon a limited description of the stress axis in *P. senegalus* prior to quantifying transcript abundances in response to life on land, with the aim of gaining a clearer understanding of the HPI axis in a polypterid fish.

An alternative or additional hypothesis to account for lower growth rates in *P. senegalus* exposed to a terrestrial environment is increased energy expenditure because the metabolic cost of life on land is higher than that of life in water. Life on land may be energetically costly compared with life in an aquatic environment because of differences in activity levels and/or the metabolic cost of different activity types. Preliminary data (M. Tunnel, A.M. Zimmer, M. Mandic & E.M. Standen, unpublished observations) suggested that rates of oxygen consumption

are significantly higher in *P. senegalus* held on land than those for aquatic fish. Therefore, in the present study I quantified time spent on locomotory activity on land versus in water.

To better assess the regulation of growth in aquatic vs terrestrial fish, transcript abundances of a series of metabolic markers were measured. Insulin-like growth factor-1 (*igf-1*) is a major endocrine regulator of growth through its regulation of protein synthesis (Sadoul and Vijayan, 2016; Sadoul and Geffroy, 2019). It is produced in the liver and peripheral tissues (Johnston et al., 2011) and has several receptors and binding proteins located in hepatocytes and myocytes. Increased *igf-1* expression may play a direct or indirect role in muscle growth (McCormick et al., 1992; Wood et al., 2005; Reindl and Sheridan, 2012), and can be inhibited by cortisol production (Kajimura et al., 2003). Liver fatty acid synthase (*fasn*) and glycogen phosphorylase (*pygl-1*) are genes that code for enzymes playing important roles in intermediary metabolism. Fatty acid synthase (*fasn*) is expressed in both liver and muscle tissue, where it is involved in lipid synthesis and adipogenesis in tissues (Salmerón, 2018). Liver glycogen phosphorylase (*pygl-1*) catalyzes the rate-limiting step in glycogen breakdown and transcript abundance is reduced when growth is inhibited (Furukawa et al., 2018). In muscle, myoblast determination protein-1 (*myoD*) plays a major role in regulating differentiation in both hyperplasia and hypertrophy of the muscle tissue, and directly targets muscle gene transcription (Johnston et al., 2011). By contrast, muscle RING finger protein-1 (*murf*) is involved in muscle cell degradation by ubiquitination. Increased levels of this target may reflect a low specific growth rate (SGR) (Johnston et al., 2011). Myostatin (*mstn*) is another strong inhibitor of muscle growth that acts by downregulating muscle fibre number and size (Johnston et al., 2011). Growth in fish largely reflects the accumulation of muscle mass (Johnston et al., 2011; Sadoul and Vijayan, 2016),

making these targets in muscle tissue particularly important in providing insight into differences in growth rate between aquatic and terrestrial fish.

Thus, the present study had several goals. First, I assessed growth rates in aquatic versus terrestrial environments over periods ranging from 1 to 8 weeks and evaluated a suite of factors that could provide insight into the regulation of growth. Second, I tested two possible hypotheses that could account for lower growth rates in *P. senegalus* held under terrestrial conditions, namely that *P. senegalus* housed in a terrestrial environment experience chronic stress, and that *P. senegalus* experience higher energy expenditure in terrestrial versus aquatic environments. I tested the chronic stress hypothesis by measuring circulating cortisol levels as well as relative mRNA abundances of key HPI axis genes. Time spent active was measured to test the elevated energy expenditure hypothesis. Finally, aspects of the stress axis were characterized to better support measurements of stress axis activity in terrestrial versus aquatic fish.

$$C = R + A + SDA + F + U + G$$

Figure 2.1. Wisconsin Bioenergetics model. Energy input (i.e., consumed food, C) is equal to metabolic demands (standard metabolism, R; energy expenditure due to activity, A; and specific dynamic action, SDA, or the energy required to digest food), waste losses due to egestion (F) and excretion (U), and somatic and/or gonadal growth (G); units are typically in joules per day (Deslauriers et al., 2017).

2.2 Materials and Methods

2.2.1 Experimental animals and protocols

Juvenile *P. senegalus* (2.54 – 7.86 g, mass \pm SEM; $N = 54$) obtained from Mirdo Importations Canada (Montreal, QC, Canada) were held at the University of Ottawa Aquatic Facility in tanks supplied with flowing, aerated, dechloraminated city of Ottawa tap water at 26°C (“system” water). Fish of both sexes were used; the sex of individual fish was not known prior to experimentation because secondary sex characteristics are not present in juvenile *P. senegalus* (Du & Standen, 2020). Fish were held in acrylic 37.5 L tanks containing a gravel substrate and supplied with flowing system water. Each holding tank contained eight individual holding areas. The fish were kept on a 12:12 light:dark photoperiod and were fed daily with ground beef heart or fish pellets (New Life Spectrum Cichlid formula, Homestead FL, USA). Fish were acclimated to these holding conditions for at least 2 weeks before being used in experiments. All holding and experimental protocols were approved by an institutional animal care committee (BL-1926) and followed the guidelines of the Canadian Council on Animal Care for animal use in research and teaching.

The experimental protocol consisted of holding fish under standard aquatic conditions (“aquatic”) or in 3 mm of water (“terrestrial”) for 24 h, 1, 2, 4 or 8 weeks ($N = 6$ fish per condition per sampling time). Fish were lightly anesthetized (300 mg L⁻¹ tricaine methanesulphonate MS222, Syndel Canada, Nanaimo, BC, buffered with 300 mg L⁻¹ NaHCO₃), and length and weight were measured. Fish were then allocated to the two treatment groups as size-matched pairs. During the experiment, fish were held individually within isolated chambers in the 37.5 L holding tank. Each chamber contained gravel substrate and was supplied with

flowing system water. A standpipe was used to adjust water depth to 13 cm or 3 mm for tanks holding aquatic versus terrestrial fish, respectively. The water in tanks holding terrestrial fish was raised to 13 cm for 1 h during feeding to allow the fish to eat. Animals were pair-fed three times per week for the experiment duration, with aquatic fish being offered the mass of food (ground beef heart) that had been consumed by their size-matched terrestrial counterpart. Temperature was kept constant between treatments in the aquatic and terrestrial trials.

At the designated end-point, fish were rapidly euthanized by immersion in terminal anaesthetic (a NaHCO₃-buffered solution of 750 mg L⁻¹ MS-222), and mass and length were measured. Blood was collected within ~3 min of disturbing the fish by caudal severance into heparin-coated micro-haematocrit tubes (Thermo Fisher Scientific, Ottawa, ON, Canada). Blood samples were centrifuged for 3 min at 10,000 g and the plasma was removed and stored at -80°C until analysis of plasma cortisol concentrations. Whole brain plus pituitary, liver, anterior kidney including the yellow corpuscles, intestine, heart, gill, and white muscle tissues were collected, flash frozen in LN₂ and stored at -80°C until analysis of transcript abundances.

To assess activity under aquatic versus terrestrial conditions, a separate group of *P. senegalus* was individually housed in aquatic ($N = 6$) and terrestrial ($N = 5$) tanks fitted with a videocamera (Wyze Cam v2, Seattle, WA, USA) on the lid of the tank. The experimental conditions were as previously described except that fish were fed fish pellets (New Life Spectrum Cichlid formula) every other day. Fish were video-recorded continuously for 6 days to measure their spontaneous activity levels.

2.2.2 Analysis of cortisol concentrations

Plasma samples were diluted 200-fold and cortisol concentrations were analyzed by commercial EIA (Neogen; Lansing MI, USA) according to the manufacturer's instructions. Samples were analyzed in duplicate. The inter- and intra-assay coefficients of variation were 4.97 and 5.63 %, respectively.

2.2.3 Visualization of steroidogenic tissue by immunohistochemistry

Prior to collecting tissues for analysis of transcript abundances, immunohistochemical approaches were used to visualize the steroidogenic tissue of *P. senegalus*. Fish (5.67 ± 4.37 g, $N = 3$) were euthanized as described above. The dorsal trunk containing the anterior end of the kidney and the spine was dissected out to maintain the structural integrity of the fragile kidney tissue. This tissue was fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; $0.01 \text{ mol L}^{-1} \text{ Na}_2\text{HPO}_4$, $0.0027 \text{ mol L}^{-1} \text{ KCl}$, $0.137 \text{ mol L}^{-1} \text{ NaCl}$, pH 7.4) overnight at 4°C . The fixed tissue was rinsed 3 x 1 min in 30 mL of PBS and cryoprotected by immersion in a 30% sucrose solution in PBS overnight at 4°C . The cryoprotected tissue was embedded in Cryomatrix (Shandon Cryomatrix, Thermo Fisher Scientific) for sectioning at $20 \mu\text{m}$ using a cryostat (Leica CM3050S; Richmond Hill, ON, Canada). Sections were mounted on Superfrost® Plus microscope slides (Thermo Fisher Scientific) and stored at -20°C until immunohistochemistry was performed.

To perform immunohistochemistry, slides were thawed for 5 min and then incubated for 5 min in boiled 10 mM sodium citrate buffer containing 0.05% Tween-20 (BioShop, Burlington, ON, Canada) for antigen retrieval. Slides were rinsed 3 x 1 min with PBS and then permeabilized for 2 h at room temperature using 1% Triton-X in PBS-Tween (PBST; PBS containing 0.1%

Tween-20). Primary antibodies diluted in PBS were added and the slides were incubated overnight at room temperature in a humidity chamber. Steroidogenic cells were identified using rabbit anti frog-11 β -hydroxylase [11 β -hyd; 1:500; a kind gift of Hiroshi Takemori (Nonaka et al., 1995)], and catecholamine-producing chromaffin cells were identified using mouse anti rat-tyrosine hydroxylase (TH; 1:2000; Immunostar, Hudson, WI, USA); TH is the enzyme that catalyzes the rate-limiting step in catecholamine production. The specificity of these heterologous antibodies was confirmed by western blotting (see below). Omission of primary antibody was used as a negative control to identify non-specific binding. Following incubation with primary antibodies, slides were washed 3 x 1 min with PBS and incubated with fluorescently-labelled secondary antibodies. Goat anti-rabbit (Alexa Fluor 568; 1:400; Life Technologies, Eugene, OR, USA) and goat anti-mouse (Alexa Fluor 488; 1:400; Life Technologies) secondary antibodies diluted in PBS were added and slides were incubated overnight at room temperature in a dark humidity chamber. Slides were washed 3 x 1 min with PBS and mounted with cover slips (Thermo Fisher Scientific) using Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) as a cell nucleus marker (Vector Laboratories, Burlingame, CA, USA). The slides were imaged by confocal microscopy (Olympus FV1000 confocal microscope; Olympus Canada, Toronto, ON, Canada).

To confirm the specificity of the (heterologous) primary antibodies for use in *P. senegalus*, western blotting was carried out. Head kidney tissue was added to 15 volumes of radioimmunoprecipitation assay (RIPA) buffer [150 mmol L⁻¹ NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mmol L⁻¹ Tris-HCl, 1 mmol L⁻¹ ethylenediaminetetraacetic acid, 1 mmol L⁻¹ phenylmethanesulphonyl fluoride] containing protease inhibitor (Roche, Mississauga, ON, Canada), and homogenized through sonication

(Sonic Dismembrator, Model 100; Fisher Scientific, Waltham, MA, USA). The homogenate was centrifuged at 15,000 g for 10 min and the supernatant was collected. A bicinchoninic acid assay (Sigma Aldrich, St. Louis, MO, USA) with bovine serum albumin (BSA) as standards was used to determine the protein concentration of the supernatants. The extracted proteins were combined 1:1 with 2X Laemmli buffer containing 10% 2-mercaptoethanol (Sigma Aldrich) and heated for 10 min at 65°C.

The samples and a protein ladder (Page Ruler Prestained; Thermo Fisher Scientific) were loaded into wells for SDS polyacrylamide gel electrophoresis (SDS-PAGE; 12% resolving gel and 4% stacking gel) at 200 V for 40 min. Separated proteins were transferred from the gel to Immune-blot® low fluorescence polyvinylidene fluoride membranes (LF PVDF; Bio-Rad, Mississauga, ON, Canada) using a Trans-Blot SD transfer system (Bio-Rad) set to 15 V for 1 h. Membranes were incubated in a sealed bag for 1 h at room temperature on a shaker with 5% skim milk powder in Tris-buffered saline containing 0.05% Tween-20 (TBST; 152 mmol L⁻¹ Tris-HCl, 46 mmol L⁻¹ Tris base, 1505 mmol L⁻¹ NaCl, pH 7.5) to block non-specific binding. Membranes were then incubated overnight at 4°C on a shaker with primary antibodies (1:4000 with 2% skim milk powder in TBST). The following day, membranes were washed 3 x 5 min in TBST on a shaker and then incubated for 1 h at room temperature on a shaker with secondary antibody, rabbit-horseradish peroxidase (HRP) or mouse-HRP (Bio-Rad) diluted 1:4000 in 2% skim milk powder in TBST. Membranes were washed 3 x 15 min in TBST with shaking and Luminata Classico™ Western HRP substrate (Millipore, Etobicoke, ON, Canada) was used for band detection. Membranes were visualized using Image lab software 5.2 (Bio-Rad) and a Molecular Imager® Chemi Doc™ XRS+ (Bio-Rad).

2.2.4 Analysis of transcript abundances

Transcript abundances were analyzed for genes of the stress axis and genes indicative of metabolism and/or growth. Primers for these targets (Table 2.1) were developed in two different ways. Primers for *star*, *crf*, *gr* and *mr* were designed prior to publication of the *P. senegalus* genome. Degenerate primers were designed from sequence alignments of the closely related reedfish, *Erpetoichthys calabaricus*, and other fish species, to yield amplicons of 200-1000 base pairs. Following sequencing of these amplicons (G enome Qu ebec; Montr eal, QC, Canada) to confirm their identity, primers for real-time RT-PCR were designed using Primer-BLAST (NCBI; Ye et al. 2012). The primers for *pomc*, *mc2r*, *p450scc*, and metabolic/growth targets (Table 2.1) were designed following publication of the *P. senegalus* genome using CODEHOP (Rose et al. 1998). OligoAnalyzer 3.1 (IDT) was used to assess the quality of the selected primers. To confirm primer specificity, pooled PCR products were sequenced (G enome Qu ebec) and sequences were checked against the *P. senegalus* genome to confirm identity using BLAST (NCBI; Ye et al. 2012).

Total RNA was extracted using 1 mL of TRIzol Reagent (Invitrogen, Burlington, ON, Canada) per 50-100 mg of tissue (that had been thawed on ice) according to the manufacturer's instructions. Samples were homogenized with TRIzol by sonication (Sonic Dismembrator Model 100, Fisher Scientific). The resulting RNA pellet was re-suspended in 20-50  L of RNase-free water (diethylpyrocarbonate-H₂O) by pipetting up and down. The RNA content was quantified using a NanoDrop  (NanoDropTM 2000, Thermo Fisher Scientific). cDNA was synthesized from 0.5  g of RNA using an iScript(tm) Advanced cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions.

Relative abundance of mRNA was determined through semi-quantitative real-time RT-PCR carried out using the SsoAdvanced(tm) Universal SYBR^(R) Green Supermix (Bio-Rad) and a Bio-Rad real-time RT-PCR instrument (c1000 thermal cycler, cfx96). Reactions were carried out according to the manufacturer's instructions, but volumes were scaled to 10 μ L (5 μ L of 2x SYBR Green real-time PCR master mix, 1 μ L of primer pair, 3 μ L of nuclease-free water, and 1 μ L of template). Samples were run in duplicate, together with negative controls (a no-template control in which cDNA was replaced with water, and a no-RT control in which the cDNA synthesis reaction was carried out without reverse transcriptase). Cycling conditions were as follows: 5 min at 95°C, 42 cycles of 10 s at 95°C, 10 s at 58°C and 10 s at 72°C, followed by melt curve analysis, as per the manufacturer's instructions. Standard curves were generated using serial dilutions of pooled samples and relative transcript abundances were calculated according to Pfaffl (2001) using 18S as a housekeeping gene.

2.2.5 Measurement of muscle [protein]

Muscle protein concentrations were measured using a bicinchoninic acid assay (Sigma Aldrich), with BSA as standards, on soluble proteins extracted from muscle tissue. Muscle tissue was added to 400 μ L of RIPA buffer containing a protease inhibitor cocktail (Roche) per 100 mg of tissue. The tissue was homogenized through sonication (Sonic Dismembrator, Model 100), and the homogenate was centrifuged at 15,000 *g* for 10 min. The supernatant was collected, diluted 100-fold, and assayed.

2.2.6 Video analysis and statistical analyses

The SGR was calculated as $[\ln(m_{final}) - \ln(m_{initial})] \times 100 / D$, where m is the mass of the fish in grams and D is the number of days between the measurement of initial and final mass.

Time spent active was determined from video recordings using event-scoring software (BORIS; <http://www.boris.unito.it/>). For both aquatic and terrestrial fish, time spent active was analyzed for 3 days per fish. In both cases, the hours of 5-9 am and 5-9 pm were analyzed to take into account potential differences in activity with and without light. Activity was defined as physical displacement of the fish.

All data are presented as means \pm 1 standard error of the mean (SEM). The distribution of transcript abundances across tissues were analyzed by one-way analysis of variance (ANOVA). All other analyses used two-way ANOVA with the factors being environmental condition (aquatic versus terrestrial) and exposure time (24 h – 8 weeks).

Table 2.1. Primers used for semi-quantitative real-time RT-PCR.

Gene	Primer pair	GenBank accession number	Function/marker	Efficiency (%)	Amplicon size (bp)
<i>18s</i>	F: GCGGGCGTTATTCCCATGACC R: GGTGGTGCCCTTCCGTCAATTC	XR_005632055.1	Housekeeping gene	97-118	177
<i>pomc</i>	F: GCAGAGACTTGTCCACGGAA R: GGAGGCTGCAGATGACCATT	AF465781.1	HPI axis gene	97.9	109
<i>mc2r</i>	F: TGAAGCCCACCTACTGAAGTT TTT R: TCACTTCTTCACAGTCGCTGG	XM_039738597.1	HPI axis gene	103.7	104
<i>p450scc</i>	F: TGATCCGCTCAGTGCCTTTG R: AAAGTGCACGAGAGTACC TGC	XM_039773703.1	HPI axis gene	87.7	143
<i>crf</i>	F: CTCCAGCAGCAGTCTCTTCC R: TGTCGGGAGGCACATTTGAT	XM_028803584.1	HPI axis gene	105.1	100
<i>star</i>	F: GGGAGACTGGAACCCCAATG R: GGAGTCTCGGAGGCAACTTC	XM_028799827.1	HPI axis gene	89.8	98
<i>mr</i>	F: TGAAGTGGTTTATGCGGGCT R: CGCGGATCATCTGCTTCTCT	XR_003716336.1	cortisol receptor	93.9	98
<i>gr</i>	F: AGAAGGAAGAAGTGCCTGC R: GGCATTTGTCTGAACCCCT	XM_028812778.1	cortisol receptor	98.6	114
<i>igf-1</i>	F: ATGCCAAAAGCACAAAAGGAGG R: TCCGGTAGTTTCTGTTGCCTG	XM_039761713.1	Regulates growth	107	72

<i>pygl</i>	F: GCCATCTACCAGCTTGGCTT R: TGCCGTA CT CATAGCGGATG	XM_039741162.1	Glycolytic enzyme	95	163
<i>mstn</i>	F: GATGTCGCCCATCAACATGC R: TTGCCGACGTGAGAAACGTA	XM_039757897.1	Inhibitor of myogenesis	90.8	135
<i>myoD</i>	F: CTGTTCGGACGGAATGATGG R: AAATGCGCTCCACAATGCTG	XM_039738508.1	Regulates myogenesis	104	161
<i>myrf-1</i>	F: GGAGGAGACGCTCAATGTGT R: GAGGTAGACGTCCGACAGGA	XM_039755122.1	Marker of muscle degeneration	110	115
<i>fasn</i>	F: GATGGGCCTGCAACCAAATG R: CCTCCCAGGTTAACCCAACG	XM_039740493.1	Involved in adipogenesis	84.5-112.7	72

An annealing temperature of 58°C was used for all primers.

pomc, proopiomelanocortin; *mc2r*, melanocortin 2 receptor; *p450scc*, P450 side chain cleavage enzyme; *crf*, corticotropin releasing factor; *star*, Steroidogenic acute regulatory protein; *mr*, mineralocorticoid receptor; *gr*, glucocorticoid receptor 1; *igf-1*, insulin-like growth factor 1; *pygl*, glycogen phosphorylase; *mstn*, myostatin; *myoD*, myoblast determination protein; *myrf-1*, muscle RING finger protein 1; *fasn*: fatty acid synthase

2.3 Results

Steroidogenic tissue was localized to the anterior portion of the kidneys through immunohistochemistry using a heterologous antibody against 11 β -hyd (Fig. 2.1). A heterologous antibody against TH was used to identify chromaffin cells. Western blots confirmed that the heterologous antibodies recognized single proteins (Fig. 2.2). The band detected for TH was of the expected size (~52 kDa), but the band detected for 11 β -hyd, at ~28 kDa, was smaller than the expected size (~57 kDa). Steroidogenic cells were located on both sides of the spine with chromaffin cells in the same general area but not in close proximity to the steroidogenic cells.

Real-time RT-PCR was used to assess the tissue distribution of key HPI axis genes and cortisol receptors (Figs. 2.2, 2.3). For *crf*, the highest transcript abundance was observed in muscle, but although a significant difference was detected among tissues (RM ANOVA on ranks, $P < 0.001$), post hoc testing failed to locate the source of this difference. Transcript abundances for *pomc* were variable across tissues, with no significant differences being detected (RM ANOVA, $P = 0.254$). Transcript abundances of *mc2r* (RM ANOVA on ranks, $P = 0.002$), *star* (RM ANOVA, $P < 0.001$) and *p450scc* (RM ANOVA, $P = 0.005$) were highest in anterior kidney tissue that included the yellow corpuscles, with little or no expression in other tissues. The cortisol receptors exhibited a broader tissue distribution, with relatively high transcript abundances in a range of tissues including gill, anterior kidney and muscle for *gr* (RM ANOVA on ranks, $P = 0.004$) and gill for *mr* (RM ANOVA, $P = 0.022$).

Measurement of SGR for fish held under terrestrial versus aquatic conditions revealed that aquatic fish had a significantly higher SGR than terrestrial fish, and that SGR was significantly higher for fish held for 4 or 8 weeks than for fish held for 1 or 2 weeks (Fig. 2.4A; two-way ANOVA, $P_{\text{treatment}} < 0.001$, $P_{\text{time}} = 0.001$, $P_{\text{treatment} \times \text{time}} = 0.394$). The fish did not differ

in mass, length, or condition factor prior to the exposure period (Table 2.2). The sex of fish allocated to each treatment was assessed upon sampling but not included in analyses owing to the number of immature fish (Table 2.2). Aquatic and terrestrial fish were pair fed, and there were no significant differences among treatment exposure durations in the frequency at which food was taken when offered (Fig. 2.4B; ANOVA on ranks, $P = 0.147$). Separate groups of fish held on land or in water for 6 d were video-recorded to assess time spent active under these different environmental conditions (Fig. 2.5). Terrestrial fish spent significantly less time in activity than did aquatic fish, and the activity of the aquatic fish, but not the terrestrial fish, depended on the prevailing light levels. Aquatic fish spent more time active under dark conditions than under light conditions (two-way RM ANOVA, $P_{\text{treatment} \times \text{time}} = 0.007$).

No differences in plasma cortisol levels were detected between aquatic and terrestrial fish, but a significant effect of time on plasma cortisol levels was detected, with cortisol levels after 1 d being significantly lower than those after 1 week (Fig. 2.6; two-way ANOVA, $P_{\text{treatment}} = 0.892$, $P_{\text{time}} = 0.019$, $P_{\text{treatment} \times \text{time}} = 0.440$). Correspondingly, no significant differences between aquatic and terrestrial fish were found in stress axis transcript abundance levels either in the brain (Fig. 2.7) or the anterior kidney and yellow corpuscles (Fig. 2.8). In most cases, however, significant effects of time were detected. In brain, transcript abundances of *crf* ($P_{\text{treatment}} = 0.318$, $P_{\text{time}} = 0.047$, $P_{\text{treatment} \times \text{time}} = 0.222$), *pomc* ($P_{\text{treatment}} = 0.169$, $P_{\text{time}} = 0.007$, $P_{\text{treatment} \times \text{time}} = 0.543$), and *mr* (log transformed; $P_{\text{treatment}} = 0.0659$, $P_{\text{time}} = 0.014$, $P_{\text{treatment} \times \text{time}} = 0.685$), but not *gr* (log transformed; $P_{\text{treatment}} = 0.365$, $P_{\text{time}} = 0.559$, $P_{\text{treatment} \times \text{time}} = 0.426$), were highest at 2 weeks and lowest at 8 weeks. By contrast, transcript abundances of the genes of the steroidogenic tissue, *mc2r* (rank transformed; $P_{\text{treatment}} = 0.134$, $P_{\text{time}} = 0.019$, $P_{\text{treatment} \times \text{time}} = 0.276$), *star* (log transformed; $P_{\text{treatment}} = 0.596$, $P_{\text{time}} = 0.005$, $P_{\text{treatment} \times \text{time}} = 0.329$), and *p450scc*

(log transformed; $P_{\text{treatment}} = 0.578$, $P_{\text{time}} < 0.001$, $P_{\text{treatment} \times \text{time}} = 0.664$), were generally lower at 2 and 4 weeks than at 1 or 8 weeks.

Significant differences were found between aquatic and terrestrial groups for several genes involved in growth and metabolism. In liver, transcript abundance of the growth factor *igf-1* was significantly higher in aquatic than terrestrial fish (Fig. 2.9A; two-way ANOVA on square root-transformed data, $P_{\text{treatment}} < 0.001$, $P_{\text{time}} = 0.048$, $P_{\text{treatment} \times \text{time}} = 0.216$). Although a significant effect of time was detected, the origin of this difference could not be identified by post hoc analysis. Liver transcript abundance of the glycogenolytic gene *pygl* was significantly higher in aquatic than terrestrial fish, with expression decreasing significantly over time (Fig. 2.9B; two-way ANOVA on square root-transformed data, $P_{\text{treatment}} = 0.008$, $P_{\text{time}} = 0.004$, $P_{\text{treatment} \times \text{time}} = 0.816$). The opposite was observed for transcript abundance of *fasn* in the liver, with terrestrial fish exhibiting higher values than aquatic fish. Again, transcript abundance decreased with time (Fig. 2.9C; two-way ANOVA on log-transformed data, $P_{\text{treatment}} < 0.001$, $P_{\text{time}} < 0.001$, $P_{\text{treatment} \times \text{time}} = 0.112$). In muscle, transcript abundance of *mstn*, an inhibitor of muscle growth, was significantly higher in terrestrial than aquatic fish, and was higher at 8 weeks than 2 weeks of exposure (Fig. 2.10A; two-way ANOVA on rank-transformed data, $P_{\text{treatment}} = 0.018$, $P_{\text{time}} = 0.046$, $P_{\text{treatment} \times \text{time}} = 0.192$). Transcript abundance of *myoD*, which plays a role in muscle differentiation, did not differ between aquatic and terrestrial fish but was significantly higher at 8 weeks than at earlier time points (Fig. 2.10B; two-way ANOVA on log-transformed data, $P_{\text{treatment}} = 0.510$, $P_{\text{time}} < 0.001$, $P_{\text{treatment} \times \text{time}} = 0.946$). Transcript abundance of *murf*, a marker of muscle breakdown, was significantly higher in terrestrial than aquatic fish and varied significantly with time (Fig. 2.10C; two-way ANOVA on rank-transformed data, $P_{\text{treatment}} < 0.001$, $P_{\text{time}} < 0.001$, $P_{\text{treatment} \times \text{time}} = 0.931$). For muscle *fasn*, the interaction of holding

condition and time was significant, with terrestrial fish exhibiting significantly higher transcript abundances than aquatic fish at 1 and 2 weeks (Fig. 2.10D; two-way ANOVA on rank-transformed data, $P_{\text{treatment} \times \text{time}} = 0.001$). Finally, no difference in muscle protein concentration was detected, either between treatment groups or as a function of time (Fig. 2.11; two-way ANOVA, $P_{\text{treatment}} = 0.147$, $P_{\text{time}} = 0.391$, $P_{\text{treatment} \times \text{time}} = 0.396$).

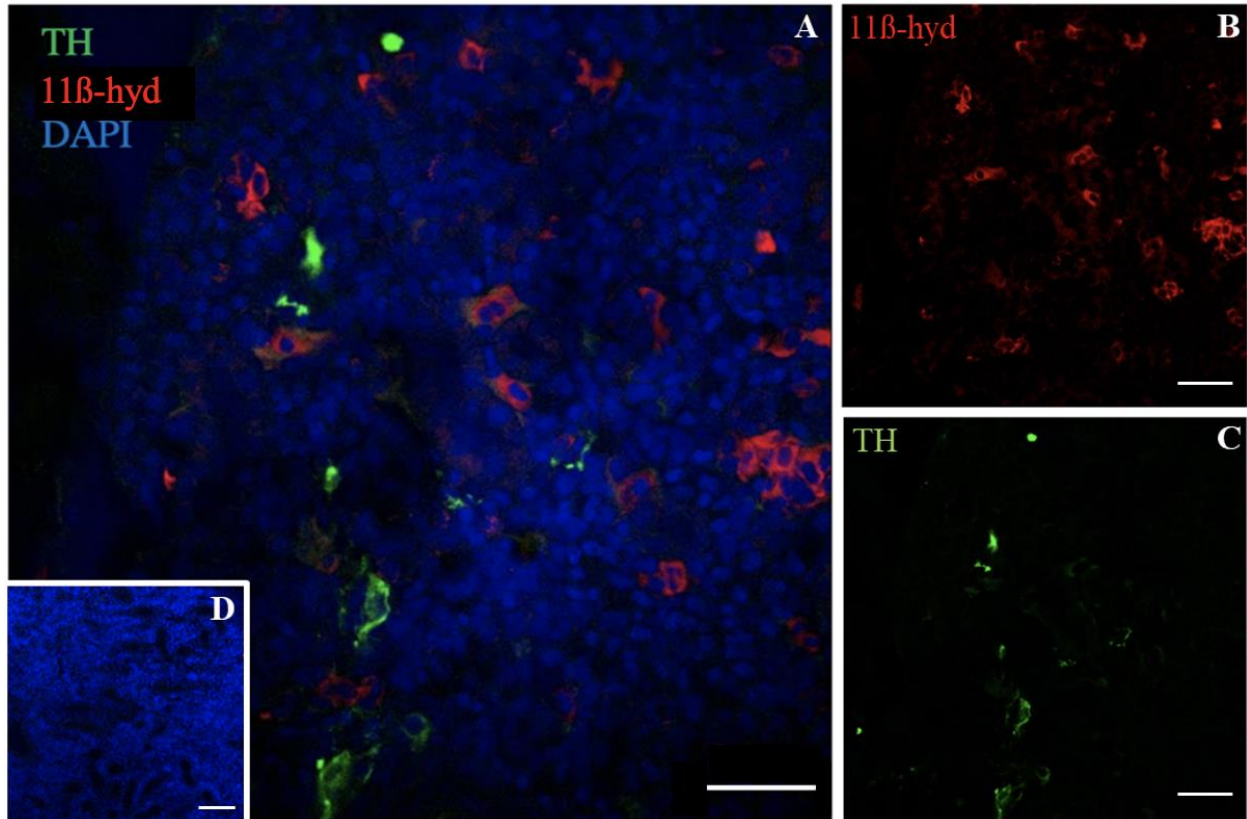


Figure 2.2: Representative transverse sections of *Polypterus senegalus* anterior kidney tissue and yellow corpuscles showing fluorescent immunolabelling of steroidogenic cells (11 β -hyd, in red), chromaffin cells (TH, in green) and cell nuclei (DAPI, in blue). Panel A is an overlay showing all three fluorescent signals; the individual signals for 11 β -hyd (B) and TH (C) are also provided. No 11 β -hyd or TH immunofluorescence was detected when the primary antibodies were omitted (D). Scale bars represent 25 μ m.

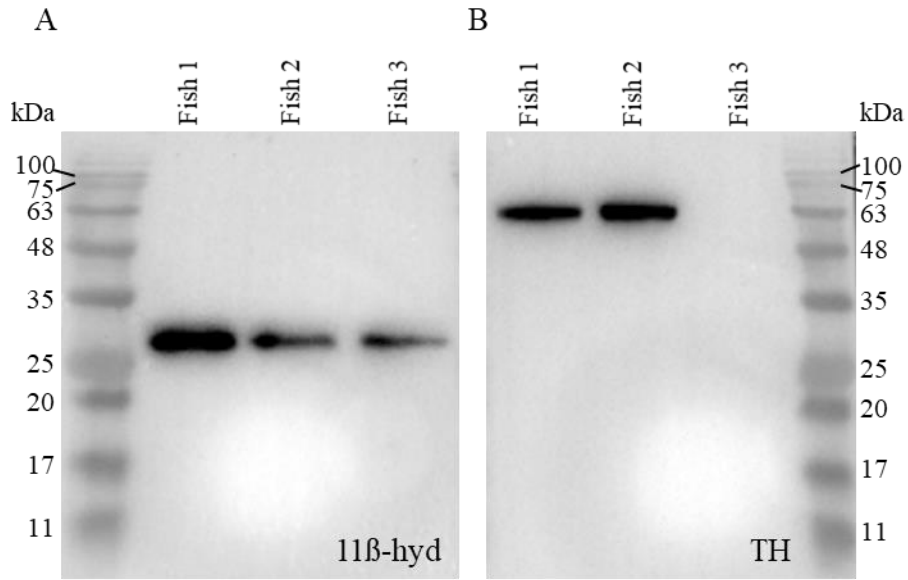


Figure 2.3: Western blots of *Polypterus senegalus* anterior kidney and yellow corpuscle tissue probed with rabbit anti-frog 11 β -hydroxylase (11 β -hyd; A) and mouse anti-rat tyrosine hydroxylase (TH; B).

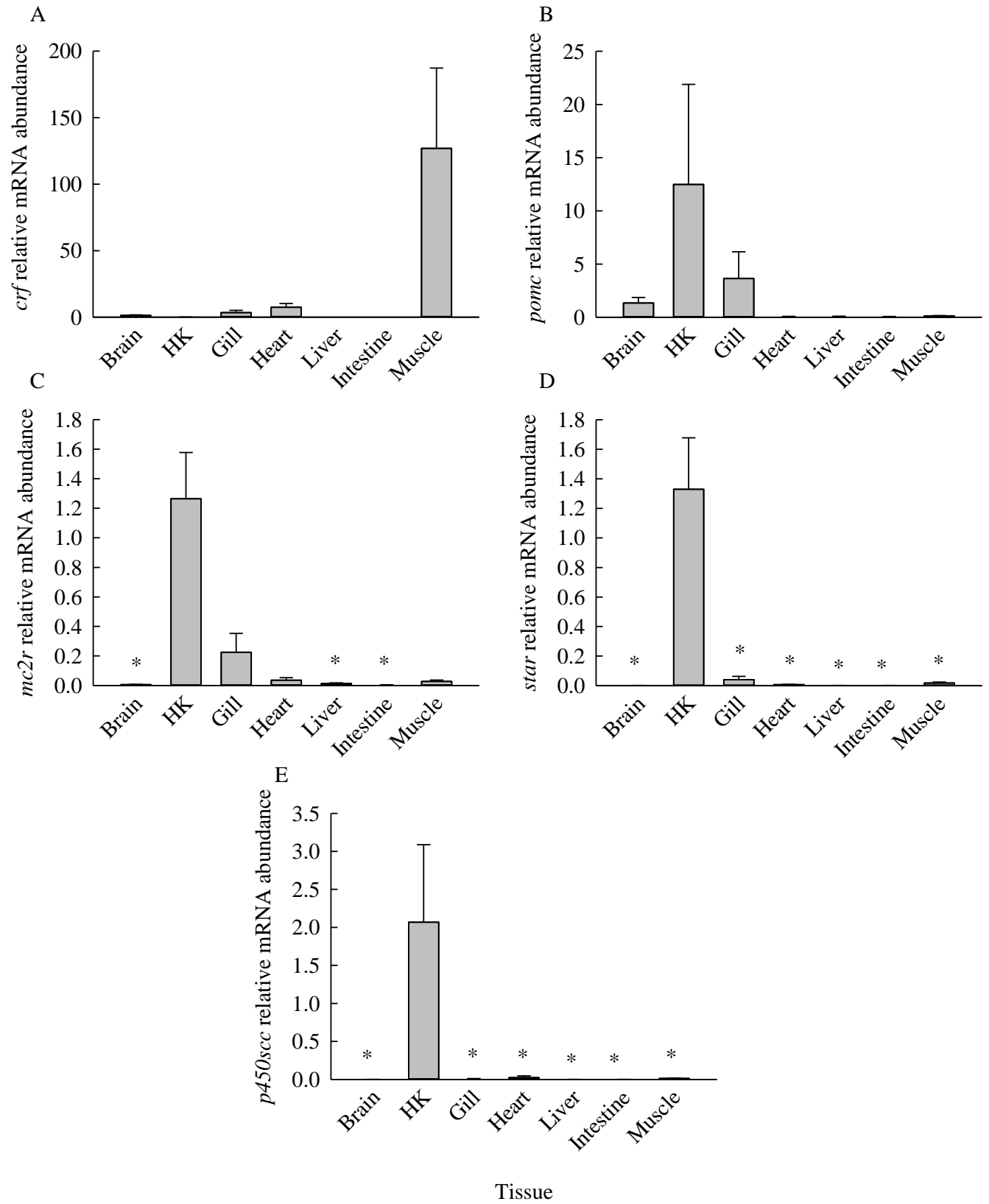


Figure 2.4: Transcript abundances of stress axis genes (A) *crf*, (B) *pomc*, (C) *mc2r*, (D) *star*, and (E) *p450scc* across a range of tissues in *Polypterus senegalus*. Values are means + SEM ($N = 4-6$). Transcript abundances were normalized to the housekeeping gene *18S* and expressed relative to brain (*crf* and *pomc*) or anterior kidney including the yellow corpuscles (HK; *mc2r*, *star* and *p450scc*). An asterisk indicates a significant difference from the tissue to which transcript abundances were normalized (one-way RM ANOVA or one-way RM ANOVA on ranks, $P < 0.001, 0.254, 0.002, <0.001, 0.005$ for panels A to E, respectively). Note that although a significant difference was detected for the tissue distribution of *crf*, post hoc tests failed to identify the source of this difference.

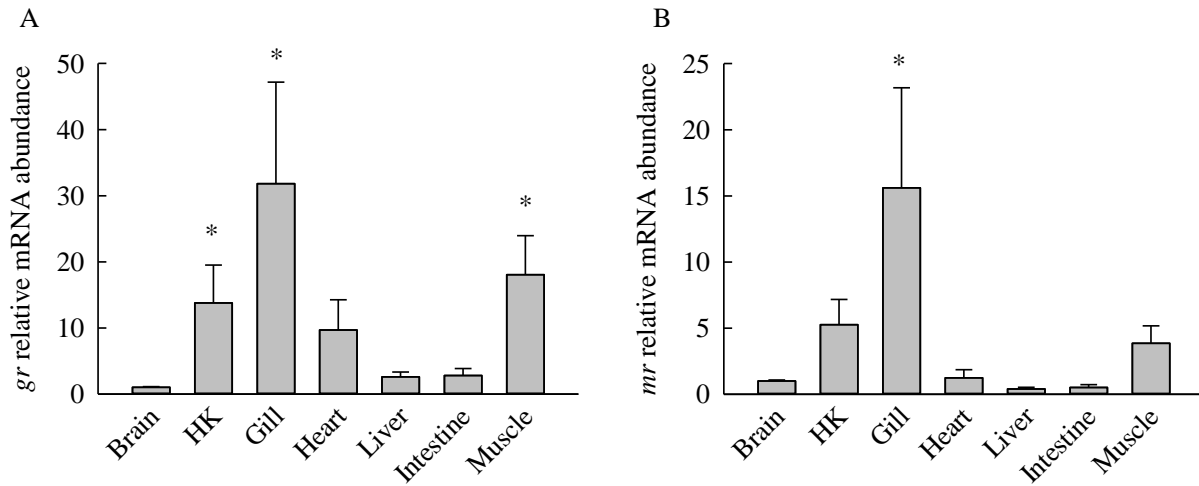


Figure 2.5: Transcript abundances of cortisol receptors (A) *gr*, and (B) *mr* across a range of tissues in *Polypterus senegalus*. Values are means + SEM ($N = 5-6$). Transcript abundances were normalized to the housekeeping gene *18S* and expressed relative to brain. An asterisk indicates a significant difference from the value for brain (one-way RM ANOVA, $P = 0.004$, 0.022 for panels A and B, respectively).

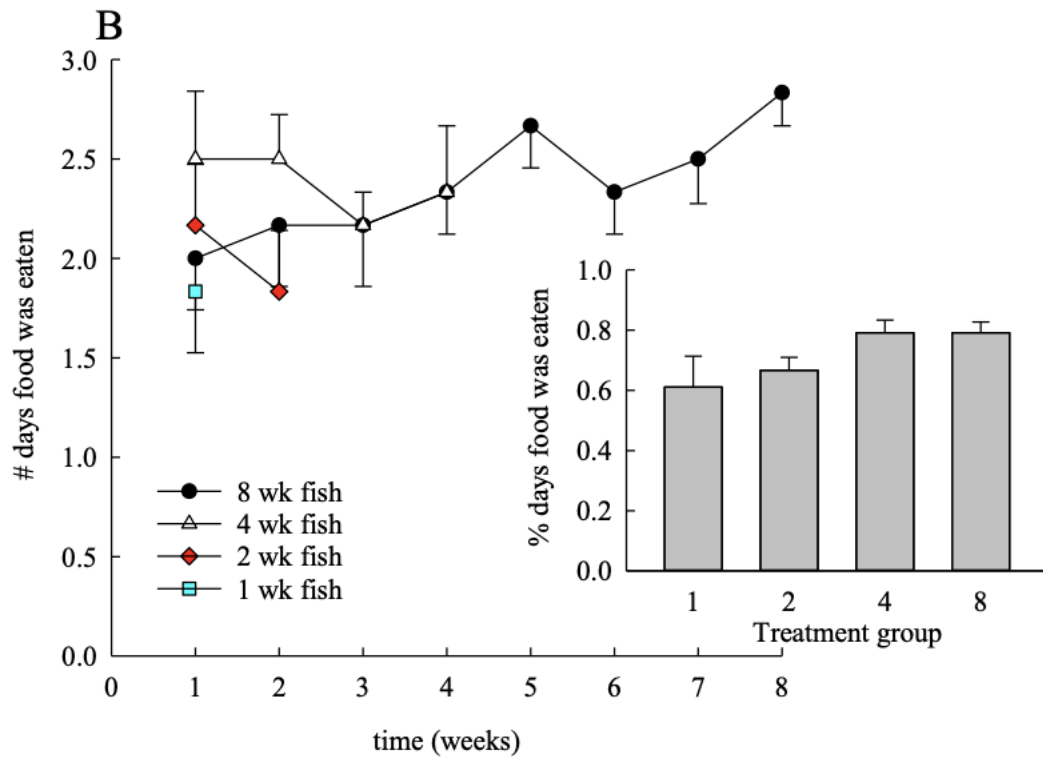
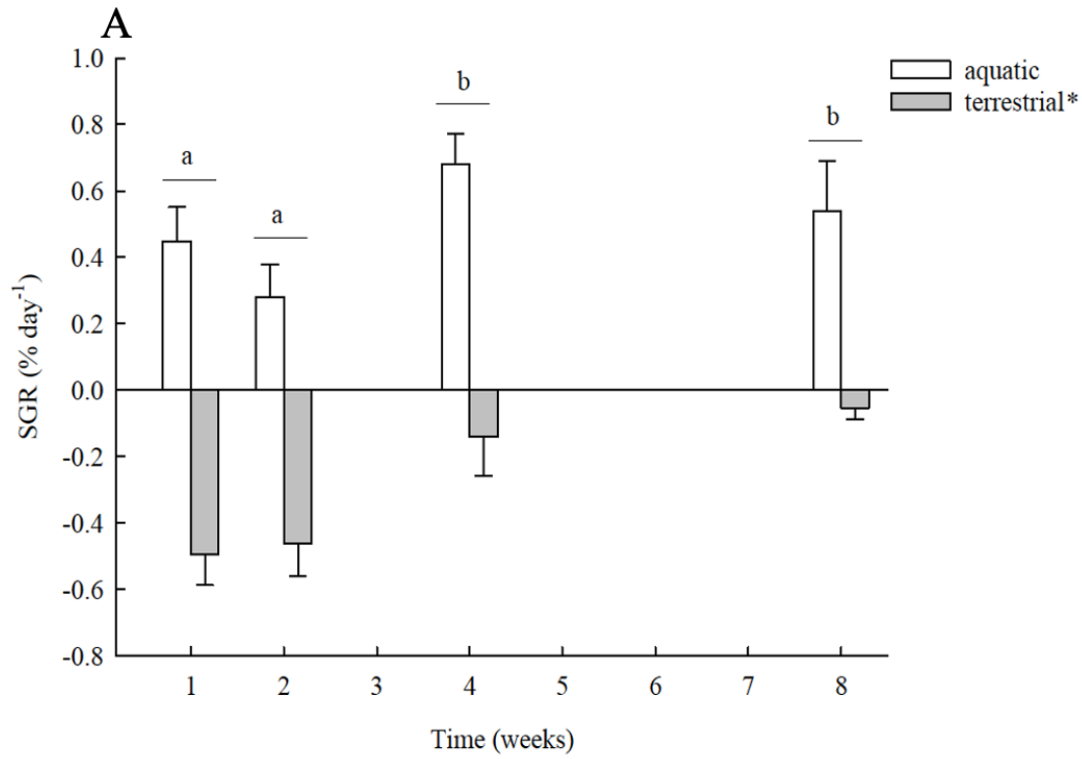


Figure 2.6: Specific growth rate (SGR; A) for *Polypterus senegalus* held under aquatic or terrestrial conditions. Significant main effects of holding condition and time were detected but the interaction of these factors was not significant (two-way ANOVA, $P_{\text{treatment}} < 0.001$, $P_{\text{time}} = 0.001$, $P_{\text{treatment} \times \text{time}} = 0.394$). Pairs of bars that share a letter are not significantly different from one another, and the significant effect of holding condition is indicated by an asterisk in the legend. Fish were pair-fed, and food intake is presented over time and as a percentage of the days on which food was offered (B). No significant differences were detected for food intake as a function of treatment duration (ANOVA on ranks, $P = 0.147$). Values are means \pm SEM; $N = 6$ fish per holding condition per exposure time.

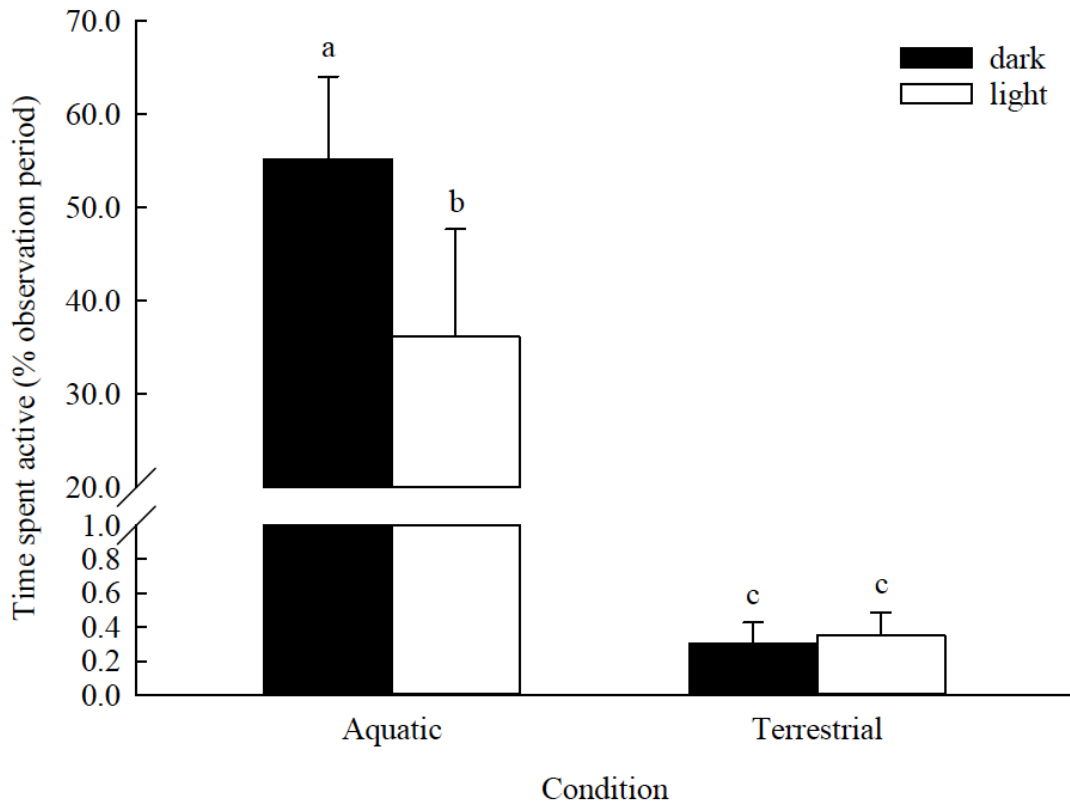


Figure 2.7: Time spent active in aquatic versus terrestrial conditions and as a function of the prevailing light conditions for *Polypterus senegalus*. Values are means + SEM, $N = 6$ (aquatic) and 5 (terrestrial). Bars that share a letter are not significantly different from one-another (two-way RM ANOVA, $P_{\text{treatment} \times \text{time}} = 0.007$).

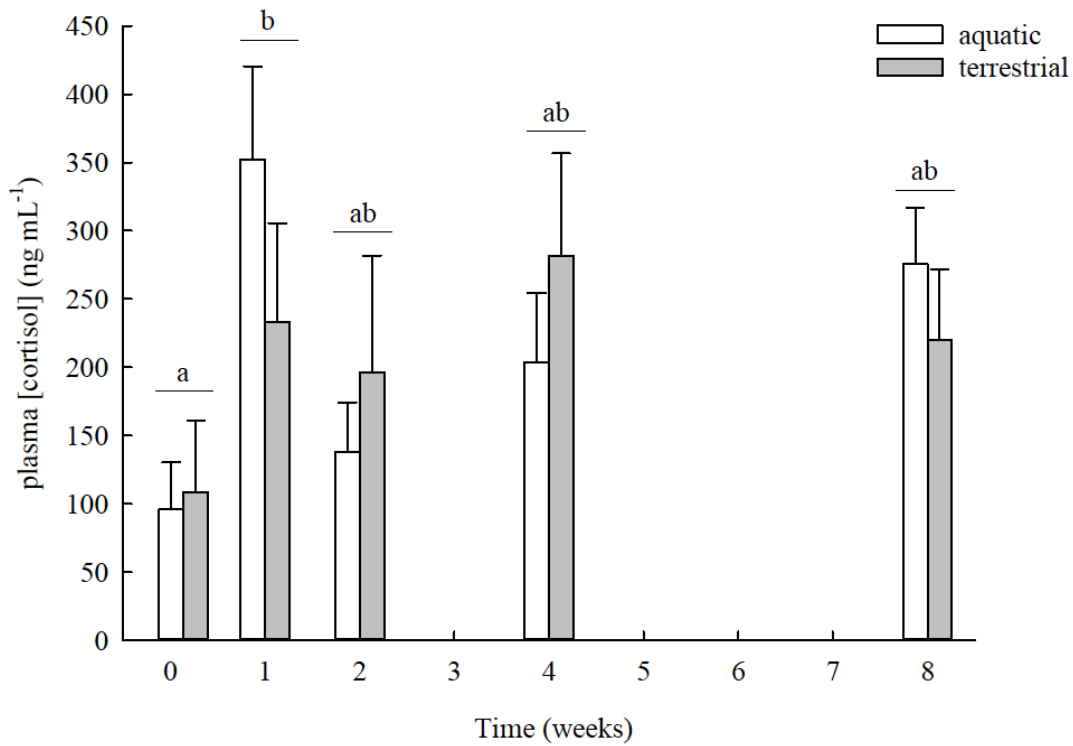


Figure 2.8: Plasma cortisol concentrations for *Polypterus senegalus* held under aquatic or terrestrial conditions. Values are means + SEM, $N = 6$ in all cases. Pairs of bars that share a letter are not significantly different from one another (two-way ANOVA; $P_{\text{treatment}} = 0.892$, $P_{\text{time}} = 0.019$, $P_{\text{treatment} \times \text{time}} = 0.440$).

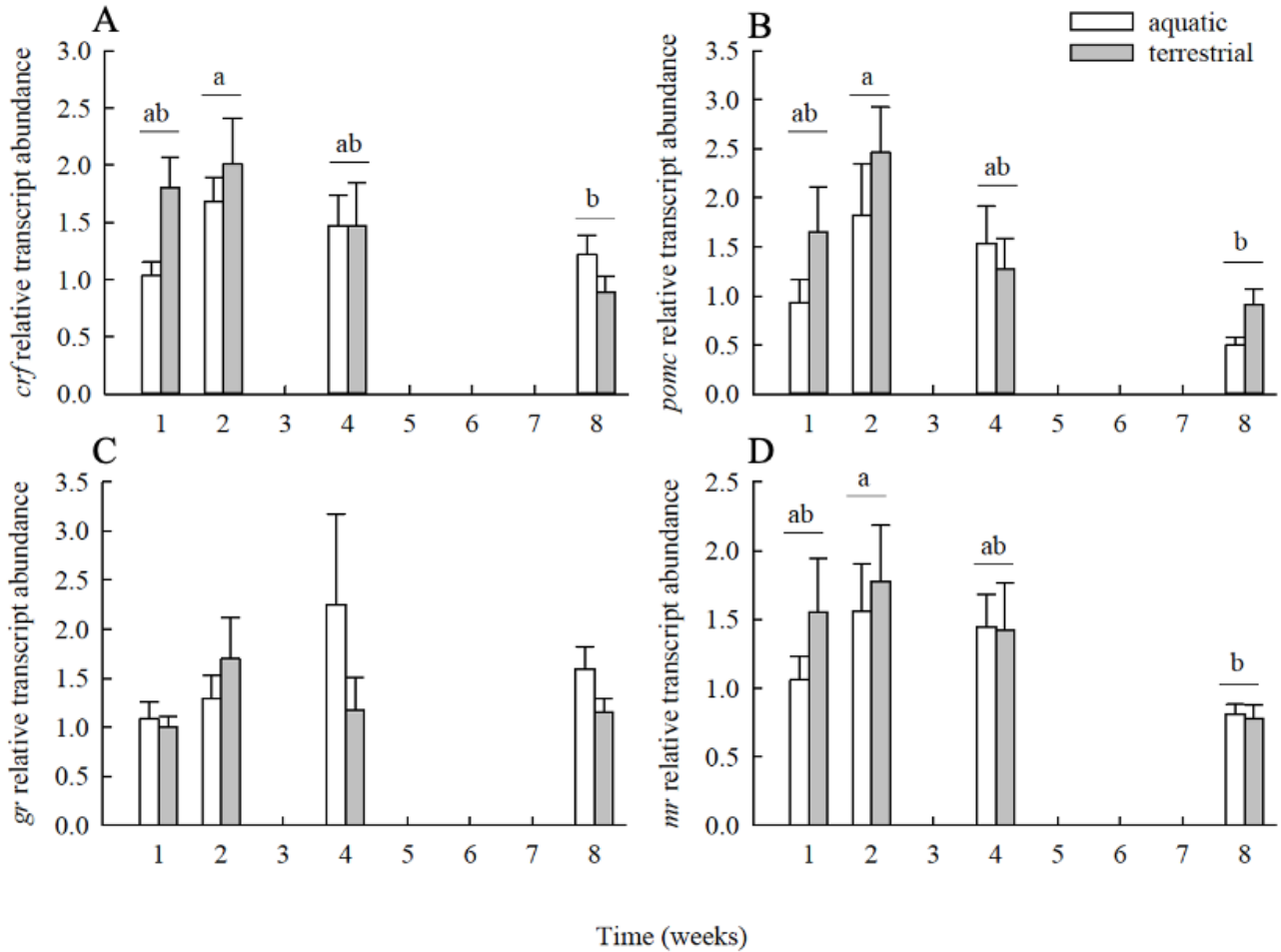


Figure 2.9: Transcript abundances in brain of stress axis genes, (A) *crf*, (B) *pomc*, (C) *gr*, and (D) *mr* for *Polypterus senegalus* held under aquatic or terrestrial conditions. Values are means + SEM, $N = 4-6$. Transcript abundances were normalized to the housekeeping gene *18S* and expressed relative to the value for the 1-week aquatic group. Pairs of bars that share a letter are not significantly different from one another (*crf*, $P_{\text{treatment}} = 0.318$, $P_{\text{time}} = 0.047$, $P_{\text{treatment} \times \text{time}} = 0.222$; *pomc*, $P_{\text{treatment}} = 0.169$, $P_{\text{time}} = 0.007$, $P_{\text{treatment} \times \text{time}} = 0.543$; log-transformed *gr*, $P_{\text{treatment}} = 0.365$, $P_{\text{time}} = 0.559$, $P_{\text{treatment} \times \text{time}} = 0.426$; and log-transformed *mr*, $P_{\text{treatment}} = 0.0659$, $P_{\text{time}} = 0.014$, $P_{\text{treatment} \times \text{time}} = 0.685$).

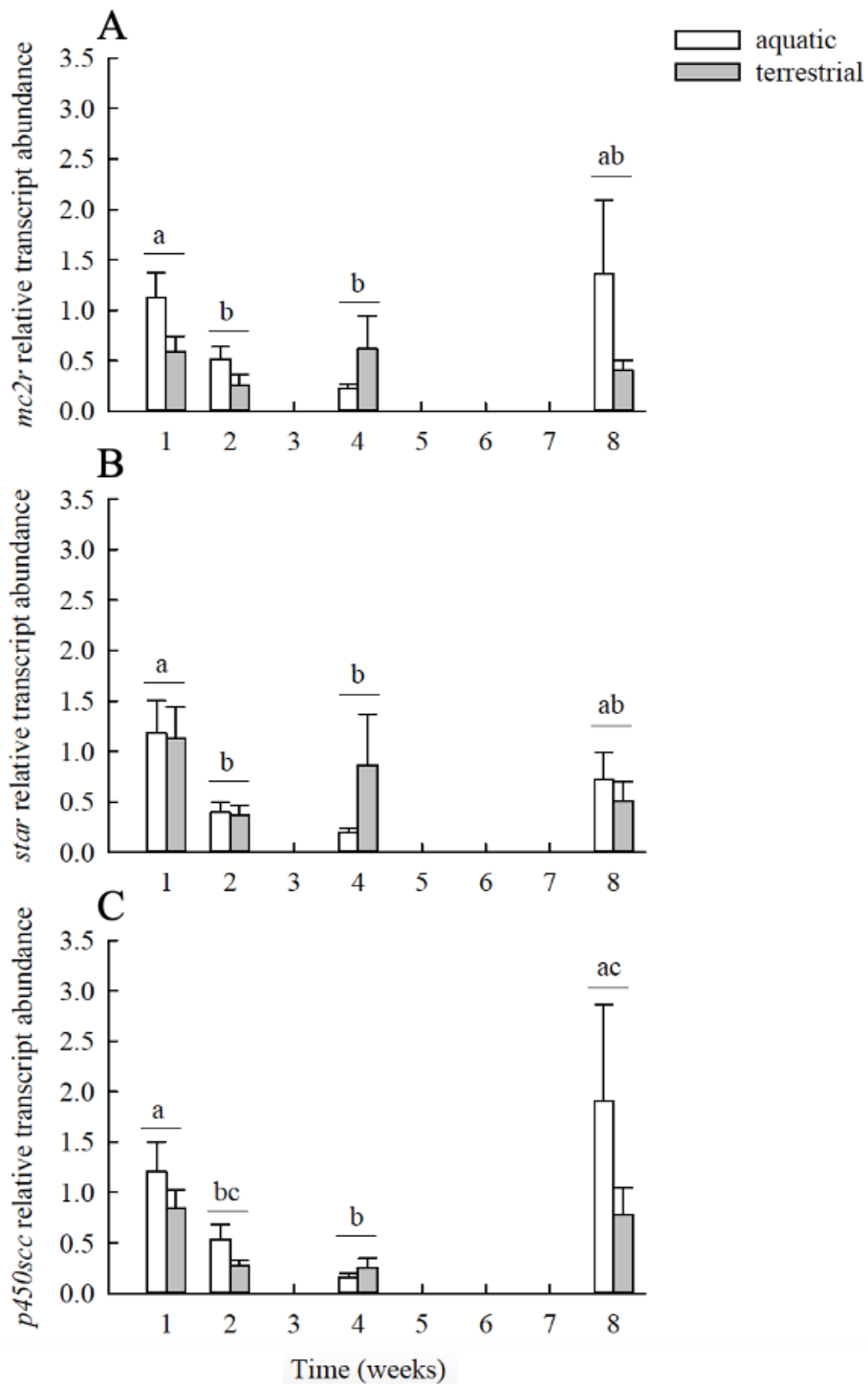


Figure 2.10: Transcript abundances in anterior kidney and yellow corpuscles of stress axis genes (A) *mc2r*, (B) *star*, and (C) *p450scc* for *Polypterus senegalus* held under aquatic or terrestrial conditions. Values are means + SEM, $N = 5-6$. Transcript abundances were normalized to the housekeeping gene *18S* and expressed relative to the value for the 1-week aquatic group. Pairs of bars that share a letter are not significantly different from one another (rank-transformed *mc2r*, $P_{\text{treatment}} = 0.134$, $P_{\text{time}} = 0.019$, $P_{\text{treatment} \times \text{time}} = 0.276$; log-transformed *star*, $P_{\text{treatment}} = 0.596$, $P_{\text{time}} = 0.005$, $P_{\text{treatment} \times \text{time}} = 0.329$; log-transformed *p450scc*, $P_{\text{treatment}} = 0.578$, $P_{\text{time}} < 0.001$, $P_{\text{treatment} \times \text{time}} = 0.664$).

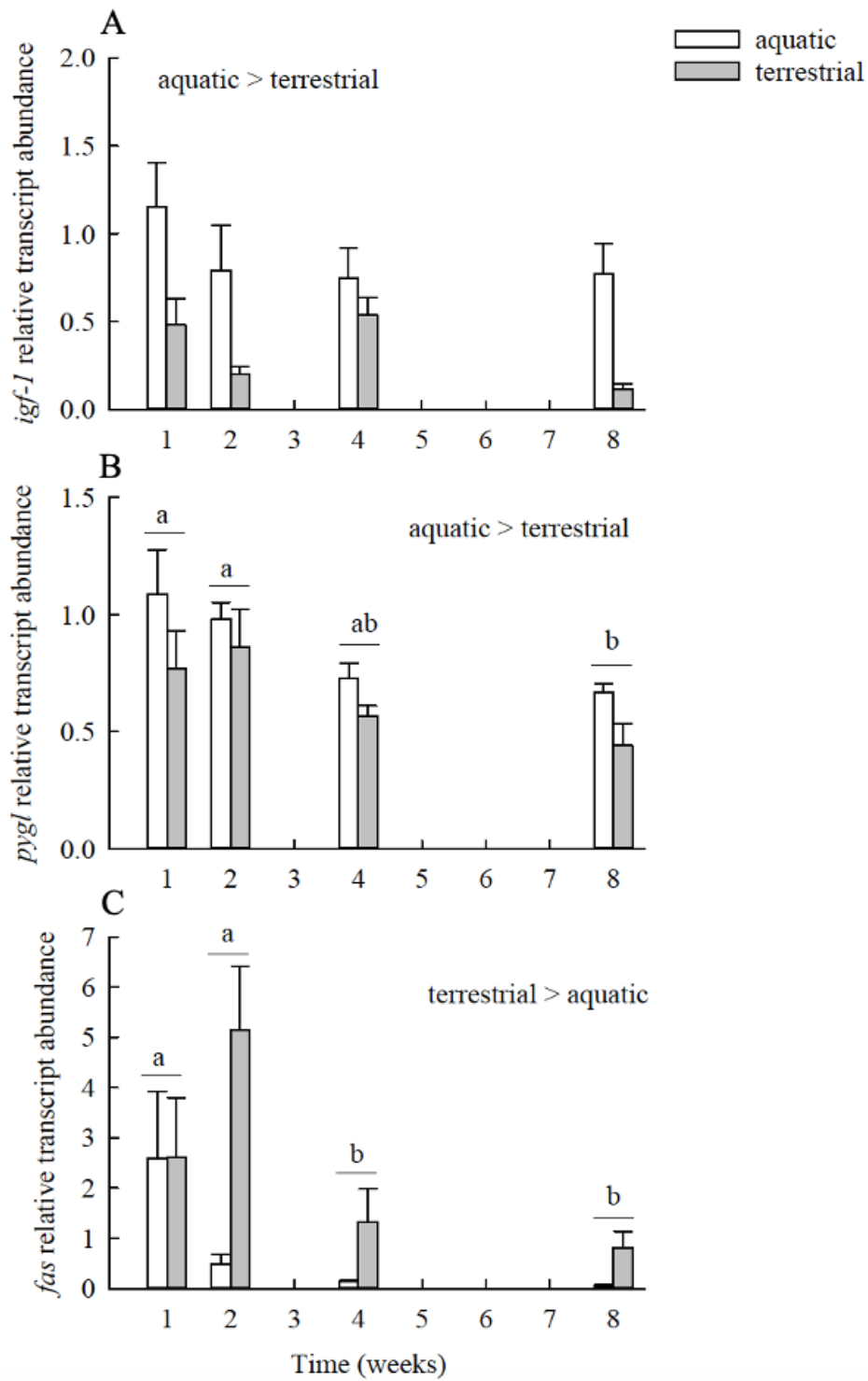


Figure 2.11: Transcript abundances in liver of (A) *igf-1*, (B) *pygl*, and (C) *fasn* for *Polypterus senegalus* held under terrestrial versus aquatic conditions. Values are means \pm SEM, $N = 5-6$. Transcript abundances were normalized to the housekeeping gene *18S* and expressed relative to the value for the 1-week aquatic group. Pairs of bars that share a letter are not significantly different from one another, and significant effects of treatment group are indicated by the text on the figure (two-way ANOVA; on square root-transformed data for *igf-1*, $P_{\text{treatment}} < 0.001$, $P_{\text{time}} = 0.048$, $P_{\text{treatment} \times \text{time}} = 0.216$; on square root-transformed data for *pygl*, $P_{\text{treatment}} = 0.008$, $P_{\text{time}} = 0.004$, $P_{\text{treatment} \times \text{time}} = 0.816$; on log-transformed data for *fasn*, $P_{\text{treatment}} < 0.001$, $P_{\text{time}} < 0.001$, $P_{\text{treatment} \times \text{time}} = 0.112$).

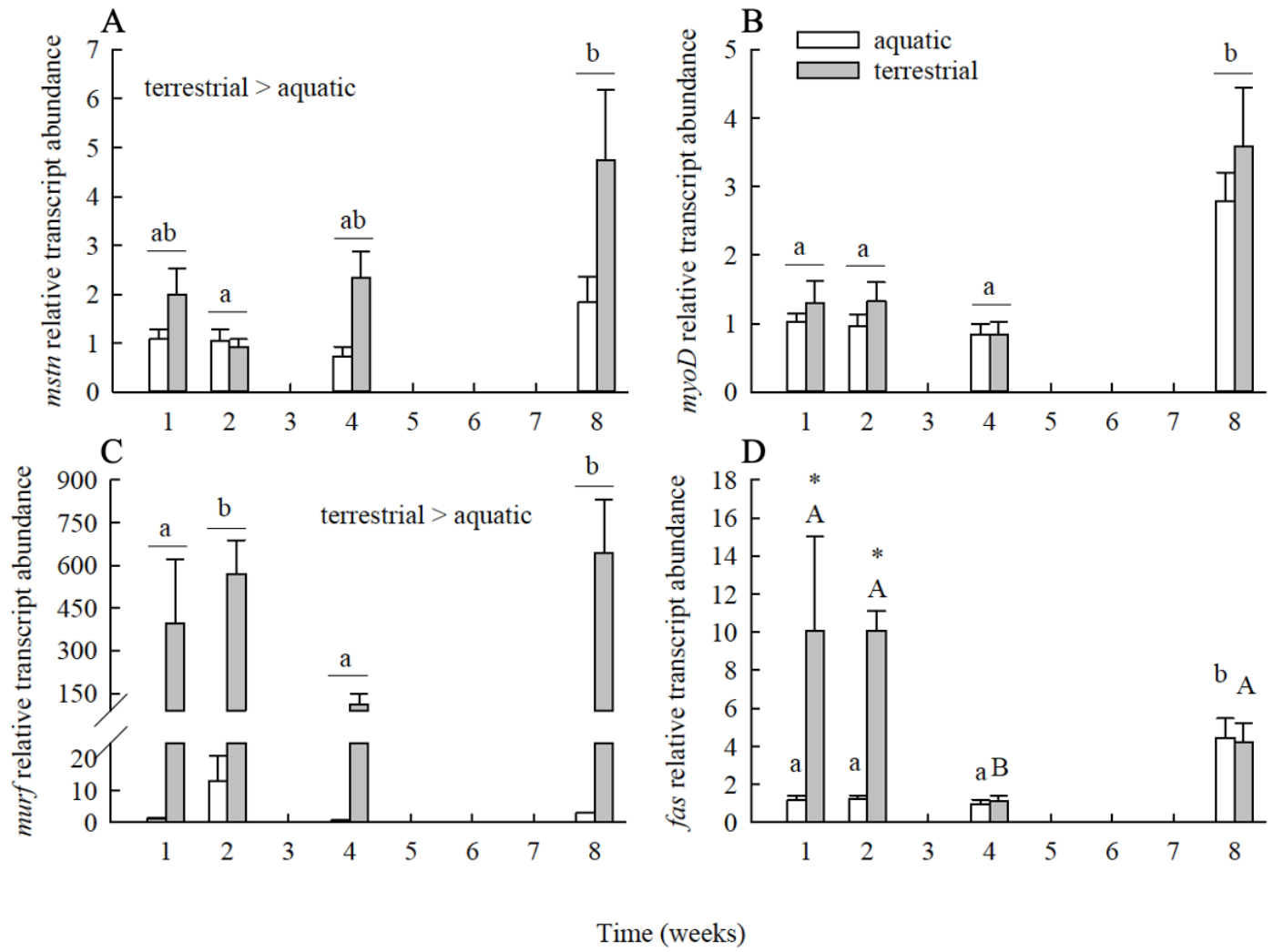


Figure 2.12: Transcript abundances in muscle of (A) *mstn*, (B) *myoD*, (C) *murf*, and (D) *fasn* for *Polypterus senegalus* held under terrestrial or aquatic conditions. Values are means \pm SEM, $N = 5-6$ for all groups. Transcript abundances were normalized to the housekeeping gene *18S* and expressed relative to the value for the 1-week aquatic group. For panels A-C, pairs of bars that share a letter are not significantly different from one another, and significant effects of treatment group are indicated by the text on the figure (two-way ANOVA; on rank-transformed data for *mstn*, $P_{\text{treatment}} = 0.018$, $P_{\text{time}} = 0.046$, $P_{\text{treatment} \times \text{time}} = 0.192$; on log-transformed data for *myoD*, $P_{\text{treatment}} = 0.510$, $P_{\text{time}} < 0.001$, $P_{\text{treatment} \times \text{time}} = 0.946$; on rank-transformed data for *murf*, $P_{\text{treatment}} < 0.001$, $P_{\text{time}} < 0.001$, $P_{\text{treatment} \times \text{time}} = 0.931$). For panel D, bars within a treatment group that share a letter are not significantly different from one another, and asterisks indicate a significant difference between treatment groups at a given time (two-way ANOVA on rank-transformed data, $P_{\text{treatment} \times \text{time}} = 0.001$).

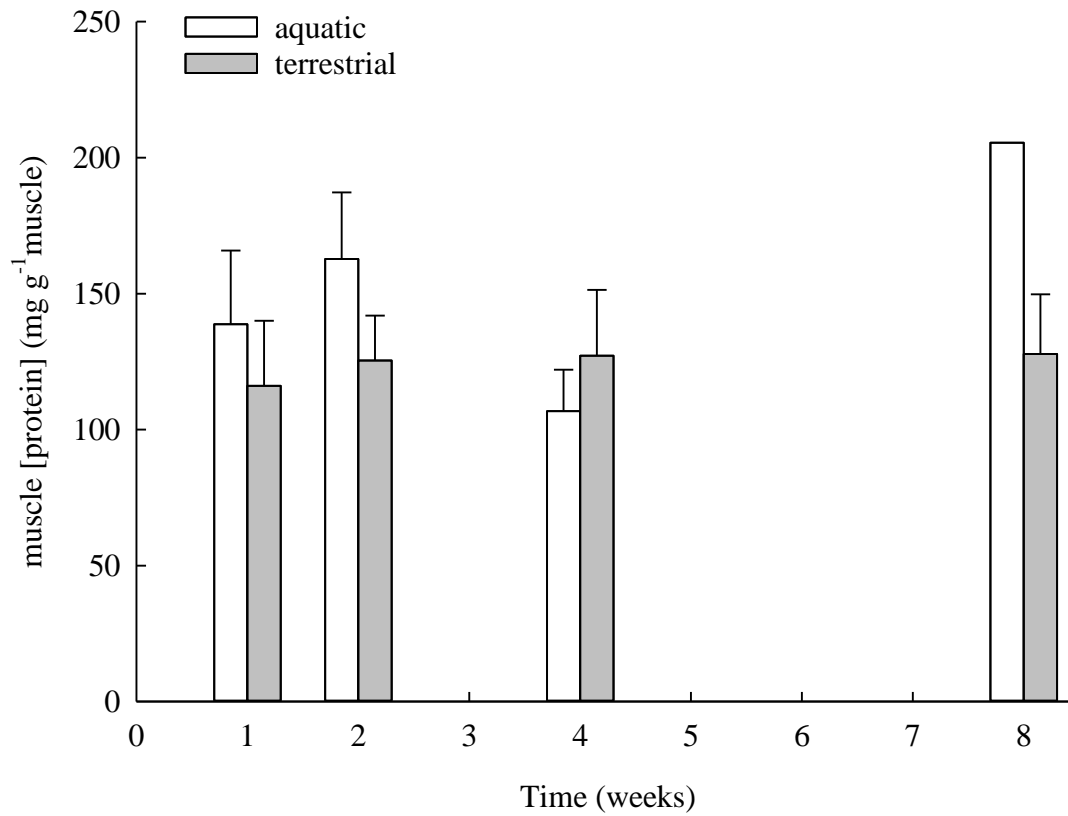


Figure 2.13: Muscle protein concentrations in *Polypterus senegalus* held under aquatic versus terrestrial conditions. Values are means + SEM, $N=5-6$ for all groups except aquatic fish at 8 weeks, where $N = 1$. No significant differences were detected (two-way ANOVA, $P_{\text{treatment}} = 0.147$, $P_{\text{time}} = 0.391$, $P_{\text{treatment} \times \text{time}} = 0.396$).

Table 2.2: Mass, length, and condition factor (CF) for *Polypterus senegalus* allocated to aquatic versus terrestrial treatment groups.

Variable	Aquatic fish ($N = 24$)	Terrestrial fish ($N = 24$)	<i>P</i> value
Mass (g)	5.62 ± 0.25	6.02 ± 0.27	0.214
Length (cm)	97.0 ± 2.1	100.4 ± 1.7	0.168
CF	0.63 ± 0.04	0.59 ± 0.005	0.284
Sex	6 immature male, 9 immature female, 9 unknown	3 immature male, 16 immature female, 5 unknown	

Values are means \pm SEM. Student's *t*-tests were used to compare values for aquatic versus terrestrial fish.

CF, condition factor

2.4 Discussion

The main objective of the present study was to determine whether the lower growth rate of *P. senegalus* held under terrestrial conditions compared to that of fish held in water was because of increased stress axis activity and elevated circulating plasma cortisol levels in terrestrial fish. Given the limited information available on the stress axis in *Polypterus* sp. (reviewed by Bouyoucos et al. 2021), an initial step involved basic characterization of the stress axis in *P. senegalus*. Youson and Butler (1985) localized steroidogenic tissue in *P. palmas* to yellow corpuscles found across the anterior two-thirds of the kidneys. Using immunohistochemistry, a comparable location for both steroidogenic cells and chromaffin cells was confirmed in *P. senegalus*. Moreover, examination of the tissue distribution of transcripts involved in the regulation of cortisol production (*mc2r*) and cortisol biosynthesis (*p450scc*, *star*) revealed that these transcripts were expressed essentially exclusively in the anterior portion of the kidney, as expected (Kobayashi et al., 1998; Aluru and Vijayan, 2006; Jeffrey et al., 2012b). Somewhat unexpectedly, transcript abundances of *crf* (initial step in activation of the HPI axis) and *pomc* (precursor to ACTH) did not show strong expression in the brain. The anterior pituitary, the expected site of *pomc* transcript abundance (Millington, 2007), was collected with brain tissue owing to the small size of the fish used in the present study. The use of whole brain for analysis likely diluted the transcript abundance of *pomc*, making it difficult to detect; notably, critical threshold values for this target were often high. Extra non-target tissue in a sample increases the noise in the sample, which in turn increases the critical threshold values needed to register transcript expression. In addition to the brain, the CRF system is found in peripheral tissues in fishes including the heart, intestine, liver and muscle (Chen and Fernald, 2008; Williams et al., 2017). The present study revealed muscle as a site of high *crf* transcript

abundance in *P. senegalus*. In contrast, transcripts of the glucocorticoid and mineralocorticoid receptors (*gr* and *mr*) in *P. senegalus*, as in teleost fishes, were widely distributed across several tissues (Ducouret et al., 1995), with gill being a site of notably high abundance (Nonaka et al., 1995; Faught and Vijayan, 2018). The *mr* and *gr* expression in target tissues of *P. senegalus* were particularly widely distributed. The broad tissue distribution of these receptors is consistent with the role they play in wide-ranging functions, including growth and metabolism, immune, stress, and osmotic and ionoregulatory function (Vijayan et al., 2010; Faught et al., 2016).

As in previous work (T. Du and E.M. Standen, unpublished observations), *P. senegalus* held under terrestrial conditions exhibited significantly lower SGR than fish held under aquatic conditions. Whereas the previous work used a longer exposure period of 2-6 months, the present study revealed significant negative effects of terrestrial conditions on SGR in as little as one week. Moreover, time had a significant impact on SGR in both terrestrial and aquatic groups, with fish held for 4 or 8 weeks having higher SGR than fish held for 1 or 2 weeks. Although there was no significant interaction between the factors of time and holding condition, the main effect of time appeared to reflect the substantial changes in SGR that occurred in fish held under terrestrial conditions. Differences in SGR between terrestrial and aquatic fish did not reflect differences in food intake, because the amount of food consumed was controlled by size-matching and pair-feeding aquatic and terrestrial fish. In addition, there was no significant impact of experiment duration (1, 2, 4 or 8 weeks) on the percentage of days on which food was consumed, which was used as a proxy for food intake and hence energy available for maintenance, activity, and growth. Thus, differences in SGR over time likely reflected differences in energy expenditure. The main determinants of energy expenditure include costs associated with maintenance of the body, the energetic cost of activity, costs of reproduction, and

costs of growth (Sadoul & Vijayan 2016). The condition of the organism and the environmental situation that it is in will determine the animal's energy budget and where resources are allocated (Kooijman, 2010). For example, costs associated with reproduction were not expected to be significant in the current study because juvenile animals were used.

Given the impact of activity on energy expenditure (Sadoul and Vijayan, 2016), time spent active was investigated as a possible contributor to lower SGR in terrestrial fish. If terrestrial fish are more active than aquatic fish, and/or the metabolic cost of activity is higher on land than in water, then these factors could account for the lower SGR in terrestrial fish. Contrary to this expectation, fish held under terrestrial conditions over a 6-day period spent significantly less time being active (i.e., engaging in locomotion) than did their aquatic counterparts. This observation accords with previous anecdotal observations (T. Du, Lutek. K, unpublished observations) of activity in *P. senegalus* in water versus on land and suggests that activity itself is not the cause of the reduced SGR in terrestrial animals. Interestingly, previous work (M. Tunnell, A. Zimmer, M. Mandic and E.M. Standen, unpublished observations) revealed that *P. senegalus* held under terrestrial conditions exhibited significantly greater rates of oxygen consumption than those held in water; 10.9 ± 2.5 versus 5.8 ± 0.9 $\mu\text{mol g}^{-1} \text{h}^{-1}$ (Student's *t*-test on log transformed data, $P = 0.038$, $N = 6$ for each group). This experiment suggested that energy expenditure in resting animals increases in the terrestrial environment, although the proximate factors underlying this increase were not investigated. Because these studies measured oxygen consumption in fish immediately following transfer from aquatic to terrestrial conditions it is not clear that the observed difference in oxygen consumption would be maintained over one or more weeks. Future experiments measuring oxygen consumption after prolonged terrestrial

acclimation would be useful for determining whether oxygen consumption remains high in terrestrial fish.

There were interesting differences in the effect of diurnal cycle on activity in terrestrial versus aquatic fish. Aquatic *P. senegalus* exhibited light-dependent differences in activity, being more active under dark than light conditions, but this difference did not hold true for the terrestrial condition. *P. senegalus* have poor eyesight, and tend to rely on other sensory modalities, including their lateral line (Znotinas and Standen, 2019). They are not known to possess any adaptations for aerial vision (Kröger et al., 2014), and demonstrate decreased visual acuity in air relative to water (Znotinas, 2018; Znotinas and Standen, 2019). *P. senegalus* are known to be nocturnal ambush predators (Znotinas, 2018; Znotinas and Standen, 2019), which may account for the greater activity under dark conditions in aquatic animals. In terrestrial environments, reduced sensory input coupled with the challenges of locomotion and feeding faced by all amphibious fishes on land (Turko et al., 2021) may mean that their nocturnal predatory behaviour is not triggered. These factors provide a plausible explanation for why there was no difference in activity between light and dark conditions in terrestrial fish.

Measurement of plasma cortisol concentrations and transcript abundances of key genes in the HPI axis did not support the hypothesis that *P. senegalus* held under terrestrial conditions experience chronic stress and that chronic stress contributes to their lower SGR. Plasma cortisol concentrations did not differ between terrestrial and aquatic fish at any time point, nor did transcript abundances of genes of the upper levels of the HPI axis (*crf*, *pomc*), the steroidogenic cells (*mc2r*, *star*, *p450scc*), or the receptors involved in HPI axis regulation (*gr* and *mr* measured in brain tissue). These results suggest that prolonged exposure to a terrestrial environment does not serve as a chronic stressor for *P. senegalus*, contrary to our hypothesis. However, a

significant main effect of time was detected for plasma cortisol levels as well as most transcript abundances in both aquatic and terrestrial groups. Cortisol levels were significantly higher at 1 week than at 24 h. With elevated cortisol levels, a parallel increase in transcript abundances of stress axis genes might be predicted (Faught et al., 2016). By contrast, transcript abundances of *crf* and *pomc* tended to peak at 2 weeks and fall with time, being significantly lower by 8 weeks, and transcript abundances of steroidogenic cells (*mc2r*, *star*, *p450scc*) exhibited their lowest values at 2 and 4 weeks. The lack of concordance among these patterns makes it difficult to discern cause-and-effect relationships. In general, plasma cortisol levels were higher in both aquatic and terrestrial fish than would be expected for unstressed, resting fish, although the lack of data on circulating cortisol levels in basal ray-finned fishes makes such comparisons difficult (Bouyoucos et al. 2021).

To further probe the mechanisms underlying lower SGR in terrestrial *P. senegalus*, muscle protein levels were measured together with transcript abundances of a suite of genes previously reported to play roles in metabolism and growth. Insulin-like growth factor-1 (*igf-1*) is an enzyme located in the liver that plays an important role in regulating growth in fishes and other vertebrates (Vargas-Chacoff et al., 2021). Lowered expression of *igf-1* has been noted in response to diverse factors that lower SGR in fishes. Aksakal and Ekinici (2021) found lower levels of *igf-1* in rainbow trout that were exposed to hypoxia and hyperoxia, and Medeiros et al., (2020) noted lower growth rates and *igf-1* levels in fasted rainbow trout. Aquatic *P. senegalus* exhibited significantly greater *igf-1* transcript abundance than terrestrial fish, a result that is consistent with their higher SGR and suggests an endocrine contribution to the differential regulation of growth in aquatic versus terrestrial conditions. Although the main effect of exposure time also was significant for *igf-1* relative mRNA abundance, post hoc comparisons

between *igf-1* and other results failed to identify the origin of this difference, and therefore it seems unlikely that *igf-1* was a major contributor to time-dependent effects on SGR.

To determine whether differences in SGR were supported by differences in intermediary metabolism, liver fatty acid synthase (*fasn*) and glycogen phosphorylase (*pygl-1*) transcript abundances were assessed. Glycogen breakdown is carried out with *pygl-1*, a rate-limiting enzyme expressed in the liver (Furukawa et al., 2018). Chang et al. (2018) noted increased *pygl-1* levels in a stress-inducing cold exposure in freshwater euryhaline milkfish (*Chanos chanos*) suggesting the use of glycogen breakdown in maintaining energy homeostasis. Differences in *pygl-1* transcript abundance were observed between aquatic and terrestrial *P. senegalus*, with fish from the terrestrial condition having significantly lower transcript levels than aquatic fish. This pattern is consistent with that observed in rainbow trout (*Oncorhynchus mykiss*) experiencing reduced growth owing to chronic social stress, where both transcript abundance (Kostyniuk et al. 2019) and enzyme activity (Gilmour et al. 2012) were lowered. The lower *pygl-1* transcript levels in the terrestrial group suggest an emphasis on the conservation of liver glycogen stores, which are a primary energy reserve for acutely stressful situations (Mommsen et al., 1999; Olsen et al., 2002; Chang et al., 2018). By contrast, hepatic *fasn* transcript abundance was elevated in fish exposed to terrestrial conditions relative to that in aquatic fish. This finding was unexpected because elevated *fasn* transcript abundance has been associated with high energy availability and its storage as triglycerides (e.g., Kostyniuk et al. 2018). It is possible that the mobilization of muscle protein (see below) contributed to the activation of this pathway. Notably, transcript abundances of both *pygl-1* and *fasn* decreased significantly over time, which coincided with the higher SGR observed at 4 and 8 weeks.

Growth in fish in large part consists of the buildup of muscle mass, and consequently it is beneficial to look at markers related to muscle increase and degradation when trying to decipher changes in SGR. Relative to aquatic animals, elevated *mstn* transcript abundances were observed in fish held under terrestrial conditions, which is consistent with the role of myostatin in muscle growth inhibition (Johnston et al., 2011). In addition, *murf* transcript abundance was strongly elevated in the terrestrial group relative to aquatic fish, which is indicative of muscle breakdown (Johnston et al., 2011). Similarly, subordinate trout experiencing impaired growth owing to chronic social stress exhibited elevated muscle expression of *murf* that was accompanied by significant reductions in fractional rates of protein synthesis (Saulnier et al., 2021). Somewhat unexpectedly, however, given the strong apparent induction of *murf*, muscle protein levels in terrestrial fish did not differ from those in aquatic fish. Muscle *fasn* exhibited a large difference in transcript abundance between the aquatic and terrestrial groups at 1 and 2 weeks but not at 4 and 8 weeks. Again, the elevated transcript abundance of *fasn* in fish exposed to terrestrial conditions was unexpected. The significant elevation of *fasn* in terrestrial fish corresponded to the times at which terrestrial fish exhibited strongly negative SGR, suggesting that elevation of *fasn* transcript abundance may have been stimulated by the availability of proteins released from muscle breakdown. The difference in *fasn* transcript abundance between aquatic and terrestrial fish was not present at the longer exposures of 4 and 8 weeks, the times at which SGR was significantly elevated for both groups compared to the first two weeks. Notably, *myoD* transcript abundance was significantly elevated at 8 weeks, which is consistent with higher SGR at this time and the role of *myoD* in muscle differentiation (Salmeron, 2018). However, there was no difference between the terrestrial and aquatic treatments for *myoD* transcript abundance.

In summary, the combination of decreased *igf-1* transcript levels and elevation of *mstn* and *murf* transcript abundances provides insight into the proximate mechanisms responsible for lower SGR among *P. senegalus* held under terrestrial conditions. Although the factors that activate these pathways remain uncertain, chronic stress caused by the terrestrial environment does not appear to be a causal factor based on the lack of evidence from measurements of plasma cortisol levels and transcript abundances of genes critical for HPI axis function.

Chapter 3: Are social interactions stressful for

Polypterus senegalus?

3.1 Introduction

Many animals, including fishes, experience stress caused by social interactions (Overli et al., 1999; Johnsson et al., 2005). In general, competition often occurs over resources that are limited within the environment, with the extent of competition depending on the type of resource, the number of individuals competing for the resource, and the environmental conditions surrounding it (Metcalf et al., 1989; Johnsson et al., 2005). To limit such aggressive encounters, which can be damaging to both individuals involved, many fishes form social hierarchies, with dominant individuals being those that are competitively successful, and subordinates often being excluded from key resources (Abbott et al., 1985, Metcalfe et al., 1989). For example, Metcalfe et al (1989) reported that subordinate Atlantic salmon have lower food intake and therefore lower growth rates than their dominant counterparts. *P. senegalus* is mainly a solitary species of fish and likely does not experience regular social interactions with conspecifics (Znotinas and Standen, 2019). However, individuals of solitary species may still need to defend their resources from conspecific invaders (Gomez-Laplaza, 2000; Sneddon et al., 2016).

Aggressive and territorial behaviours may be employed by individuals to obtain or maintain dominant status within a hierarchy. Aggression includes behaviours such as displays, charging, chasing and nipping (Øverli et al., 1999 a,b; Larson et al., 2006). These types of aggressive behaviours have been observed in a variety of fish species, including *Nannacara anomala*, the stickleback *Gasterosteus aculeatus*, and the rainbow trout *Oncorhynchus mykiss* (Pavlidis et al., 2011), to maintain a dominant position in a social hierarchy. Territoriality is often used to monopolize important resources (Abbott and Dill, 1985; reviewed by Johnsson et al., 2005). For example, juvenile rainbow trout and other salmonids are highly territorial, with

the most competitive fish obtaining the best feeding territories. In a lab setting, this territorial behaviour results in pairs of fish that are introduced into a behaviour chamber (the territory) engaging in agonistic activity until one fish achieves dominance over the other (Jönsson et al., 1998; Winberg and Lepage, 1998; Overli et al., 1999a,b). Whether *P. senegalus* displays aggressive and territorial behaviours when housed with a conspecific is not known but seems likely based on anecdotal observations of aggression observed when *P. senegalus* are held in a tank with a conspecific (E.M. Standen & T. Du; unpublished observations).

Social interactions can cause stress with a resulting elevation of plasma cortisol levels regardless of social status. Dominant individuals in a hierarchy can experience stress from defending their dominant status and/or defending their possession of key resources, whether they be food, shelter, territory, or reproductive opportunities. Stress in dominant individuals is particularly likely to occur when there are periods of social instability (Puckett and Dill, 1985; Abbott and Dill, 1985; Enquist et al., 1990; Saploski, 1992; Sneddon et al., 1999). Individuals that become dominant also experience acute stress when hierarchies are formed, and status is established (Sloman et al., 2001). However, subordinate individuals tend to experience chronic stress at much greater levels, regardless of hierarchy stability or level of aggression (Johnsson et al., 2005). This stress is the result of an environment in which they experience constant threats and a lack of control, and may be exacerbated by receiving aggression from dominant individuals (Winberg et al., 1992; Höglund et al., 2001). For example, plasma cortisol levels are elevated in both the fish that become dominant and those that become subordinate during social hierarchy formation in salmonid fishes (Overli et al., 1999; Sloman et al., 2000). Whereas cortisol levels in dominant fish tend to recover quickly to baseline levels, cortisol levels in subordinate fish remain elevated for days. This pattern occurs in a variety of teleost fishes

including several salmonids (e.g. rainbow trout, coho salmon (*Oncorhynchus kisutch*), Arctic charr (*Salvelinus alpinus*), European eel (*Anguilla anguilla*), and the African cichlid (*Haplochromis burtoni*)) (Johnsson et al., 2005).

Chronic social stress may be associated with physiological impacts. For example, changes in the transcript abundances of key HPI axis genes and glucocorticoid receptors have been reported with social stress (Doyon et al., 2003; Bernier et al., 2004; Jeffrey et al. 2012). At the whole animal level, chronic elevation of cortisol together with reduced food intake in subordinate fish generally appears to lower growth rates, at least in rainbow trout (DiBattista et al. 2006; Culbert & Gilmour 2016; Kostyniuk et al. 2018; Saulnier et al. 2021). Similarly, Nakano (1995) found that within groups of red-spotted masu salmon, dominant fish had higher growth rates than subordinate fish.

In the present study, I tested the hypothesis that *P. senegalus* housed in pairs form dominance hierarchies in which the subordinate fish experiences chronic stress. I predicted that *P. senegalus* housed in pairs would engage in agonistic interactions in which winners and losers could be distinguished on the basis of their behaviour. As a result of the formation of dominance hierarchies, subordinate individuals would be predicted to exhibit elevated circulating levels of cortisol, and increased transcript abundance of stress axis genes.

3.2 Materials and Methods

3.2.1 Experimental animals and protocols

Six pairs of immature bichirs, *P. senegalus* (8.59 ± 2.56 g, mass \pm SEM; $N = 12$ individuals), were acquired from Mirdo Importations Canada, Montreal QC and quarantined for two weeks prior to beginning the experiment. The fish were acclimated alone, with access to a PVC pipe shelter, in transparent, 37.5 L flow-through tanks in the Aquatics Facility at the University of Ottawa. After the acclimation period, the fish were lightly anesthetized by immersion in a buffered solution of MS222 (300 mg L^{-1} MS222, 300 mg L^{-1} NaHCO_3), weight and length were measured, and each fish was marked with an infra-red reflective bead. The bead was tied to the first dorsal fin in one fish and to the third dorsal fin in the other fish, using a surgical needle and thread. Fish were allowed to recover from this procedure for 10 min before being placed with a mass- and length-matched conspecific in an experimental 40 L tank supplied with flowing system water. Water temperature was maintained between 25°C and 26°C over a 6-d experimentation period and fish were fed either frozen beef heart or fish pellets (New Life Spectrum Cichlid formula) every other day. The room was on a 12:12 light-dark cycle. Fish were video-recorded continuously for 6 days using a video camera (Wyze Cam v2) fitted to the lid of the tank. All experimental procedures were carried out according to protocols approved by the University of Ottawa animal care committee (BL-1926) and followed the guidelines of the Canadian Council for Animal Care for the use of animals in research and teaching.

At the end of the 6-day interaction period, fish were rapidly euthanized by immersion in terminal anaesthetic (a NaHCO_3 -buffered solution of 0.75 g L^{-1} MS-222), and weight and length were measured. Blood was collected within ~ 3 min of disturbing the fish by caudal severance

into heparin-coated micro-haematocrit tubes (Thermo Fisher Scientific, Ottawa, ON, Canada). Blood samples were centrifuged for 3 min at 10,000 *g* and the plasma was removed and stored at -80°C until analysis of plasma cortisol concentrations. Plasma samples were diluted 200-fold and cortisol concentrations were analyzed in duplicate by commercial EIA (Neogen) according to the manufacturer's instructions. All samples were measured in a single assay with intra-assay coefficient of variation of 9.04%. Whole brain plus the pituitary, and anterior kidney including the yellow corpuscles were collected, flash frozen in LN_2 and stored at -80°C until analysis of transcript abundances. The procedures used for the analysis of transcript abundances were identical to those described above (see section 2.2.2). Transcript abundances of *crf*, *pomc*, *gr*, and *mr* were measured in whole brain, and the transcript abundance of *star* was measured in anterior kidney.

3.2.2 Video analysis and statistical analyses

To our knowledge, the behaviour of *P. senegalus* during social interactions has not been described previously, and therefore a preliminary experiment was carried out to develop a species-appropriate ethogram. Detailed observations were made of the behaviour of two *P. senegalus* placed together in a tank and video-recorded over at least 24 h under several conditions, including with and without feeding, and with and without the presence of a shelter (a short piece of PVC pipe). Repeated observation of this pair suggested that the fish interacted in an agonistic fashion over acquisition and control of the shelter, but not acquisition of food items. For example, we observed frequent attempts by one fish to displace the other from the shelter, as well as extended periods spent in and around the shelter. As a result of these observations, the ethogram that was developed included the number of times each fish entered the shelter (i.e.,

“enter” being defined as at least the pectoral fins of the fish were within the shelter), and time spent in the shelter. Additional behaviours that were observed appeared to be aggressive because they caused responses such as visibly increased ventilation or displacement. Two of these aggressive behaviours were included in the ethogram. The “snout” behaviour involved one fish approaching the other in a perpendicular direction, causing it to rapidly retreat. “Nips” involved one fish biting the other. Occurrences of these behaviours were counted.

Counts of the number of times each fish entered the shelter and how many times one fish nipped the other were recorded from 24 h of video from the preliminary pair to determine whether there were diurnal patterns of activity. A lack of variation in activity levels across the 24-h period meant that three hours of daylight (4 pm-7 pm) and three hours of darkness (7 pm-10 pm) per day were deemed sufficient for analysis for all subsequent experiments. Thus, for the six pairs used to analyze social interactions, 6 h per day of the 6 d of video recordings were scored for four behaviours: entries into the shelter, time in shelter, number of snouts, and number of nips. Event-scoring software (BORIS; <http://www.boris.unito.it/>) was used to score the behaviours of interest.

Specific growth rate (SGR) was calculated as $[\ln(m_{final}) - \ln(m_{initial})] \times 100 / D$, where m is the mass of the fish in grams and D is the number of days between the measurement of initial and final mass. Data are expressed as mean values \pm standard error of the mean (SEM) and a significance level (α) of 0.05 was used for all tests. Statistical analyses were carried out using R studio (RStudio; <https://rstudio.com/>) and SigmaPlot (<https://www.SigmaPlot.co/>). Paired Student's t -tests were used to assess the statistical significance of differences in morphological traits, physiological variables, transcript abundances, and behaviours between dominant and subordinate *P. senegalus*. Following examination of individual behaviours, a principal

components analysis (PCA) was carried out on time in shelter and number of nips to derive a behaviour score for each fish. The fish within each pair with the higher behaviour score was deemed to be the dominant fish. Regression analysis was used to examine relationships between plasma cortisol levels and behaviour.

3.3 Results

P. senegalus held in experimental tanks in pairs for 6 d formed social hierarchies in which the members of a pair could be distinguished based on two behaviours, percent time spent in the shelter (Fig. 3.1B; paired Student's *t*-test, $P = 0.0248$) and the number of nips directed at the tankmate (Fig. 3.1D; paired Student's *t*-test, $P = 0.0125$). The number of entrances into the shelter (Fig. 3.1A; paired Student's *t*-test, $P = 0.0899$) and the number of snouts directed at the tankmate (Fig. 3.1C; paired Student's *t*-test, $P = 0.0556$) tended to be higher in the more aggressive and territorial fish but did not reach statistical significance. Consequently, a behaviour score was generated through PCA using only time spent in shelter and nips. The fish within a pair with the higher behaviour score was deemed to be the dominant fish, and dominant and subordinate fish differed significantly in behaviour score (Fig. 3.1E; paired Student's *t*-test, $P = 0.040$). Of the six pairs examined, three paired an immature female with an immature male, but in the remaining three pairs, sex was not clear from visual inspection (Table 3.1).

No differences were detected between dominant and subordinate fish for mass, length, or CF prior to social interactions (Table 3.1). At the end of the social interaction period, no significant differences were detected between dominant and subordinate fish in plasma cortisol concentrations (Fig. 3.3A; paired Student's *t*-test, $P = 0.085$) or SGR (Fig. 3.3B; paired Student's *t*-test, $P = 0.0543$), although trends for dominant fish to exhibit higher plasma cortisol and less negative SGR were present. Plasma cortisol levels were significantly correlated with behaviour score (Fig. 3.4A; linear regression, $P = 0.010$). To further explore this relationship, plasma cortisol concentrations were examined as a function of the two variables on which behaviour scores were based. Whereas the relationship between plasma cortisol and the number of nips given was statistically significant (Fig. 3.4C; linear regression, $P = 0.003$), the relationship with

time spent in shelter failed to reach significance (Fig. 3.4B; linear regression, $P = 0.063$).

Transcript abundances for HPI axis genes were measured between dominant and subordinate fish. In brain, neither *crf* (Fig. 3.4A; paired Student's *t*-test, $P = 0.145$) nor *pomc* (Fig. 3.4B; paired Student's *t*-test, $P = 0.182$) transcript abundances differed between dominant and subordinate fish. Although *gr* transcript abundance in brain was significantly lower in subordinate than dominant fish (Fig. 3.4C; paired Student's *t*-test, $P = 0.00971$), no difference in *mr* transcript abundance was detected (Fig. 3.4D; paired Student's *t*-test, $P = 0.552$). Transcript abundance of the steroidogenic gene *star* did not differ in the anterior kidney and yellow corpuscles of dominant versus subordinate fish (Fig. 3.4E; paired Student's *t*-test, $P = 0.622$).

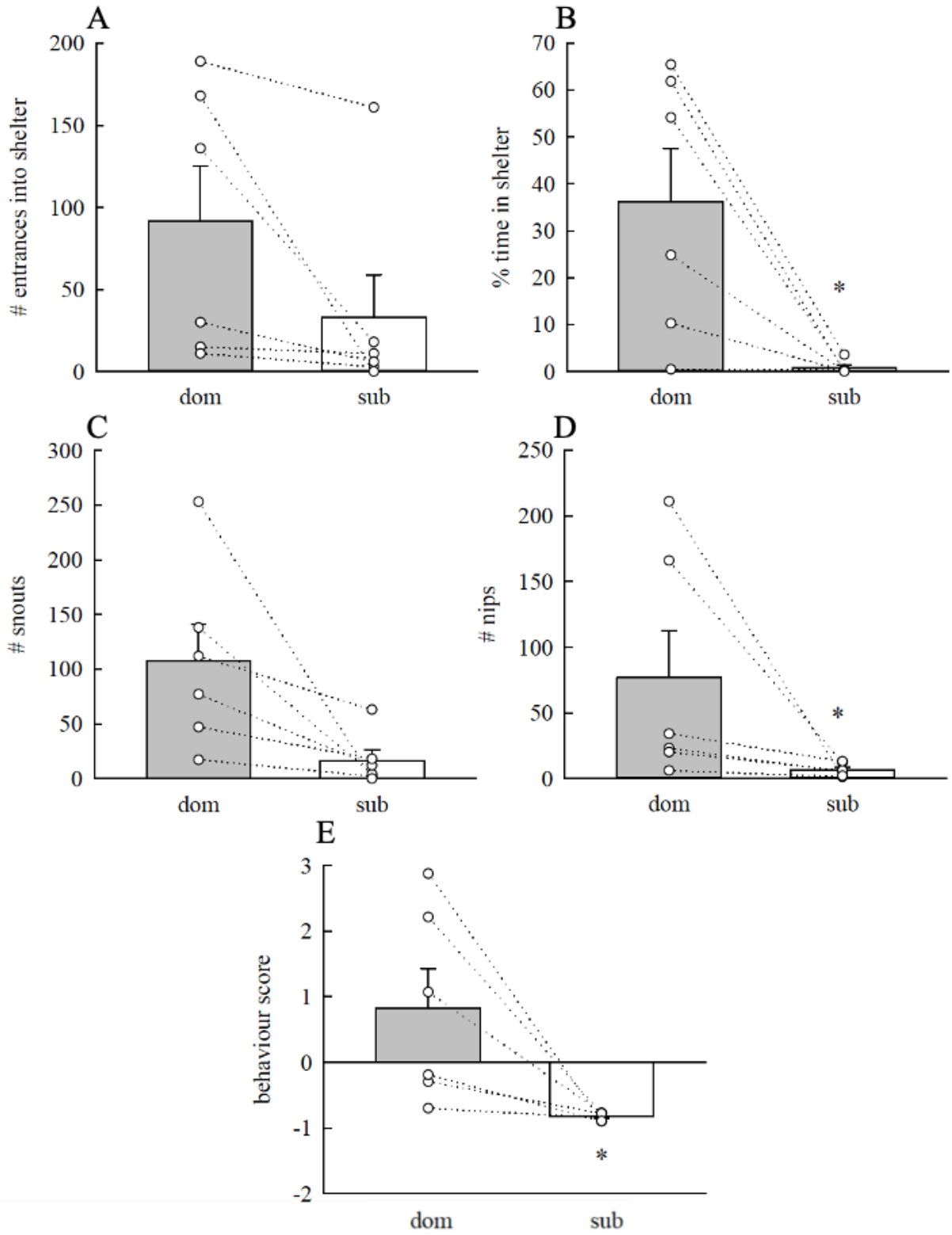


Figure 3.1: The occurrence of territorial (number of entrances in the shelter, A; time spent in the shelter, B) and agonistic (number of snouts given, C; number of nips given, D) behaviours in *Polypterus senegalus* that were identified as dominant (dom) or subordinate (sub) on the basis of a behaviour score (E) calculated from time in shelter and the number of nips given. Bars present means + SEM, $N = 6$ per group, and the symbols are values for individual fish, with the members of a pair joined by a dotted line. An asterisk indicates a significant difference between dominant and subordinate fish (paired Student's t -tests, $P = 0.0899, 0.0248, 0.0556, 0.0125,$ and 0.040 for panels A to E, respectively).

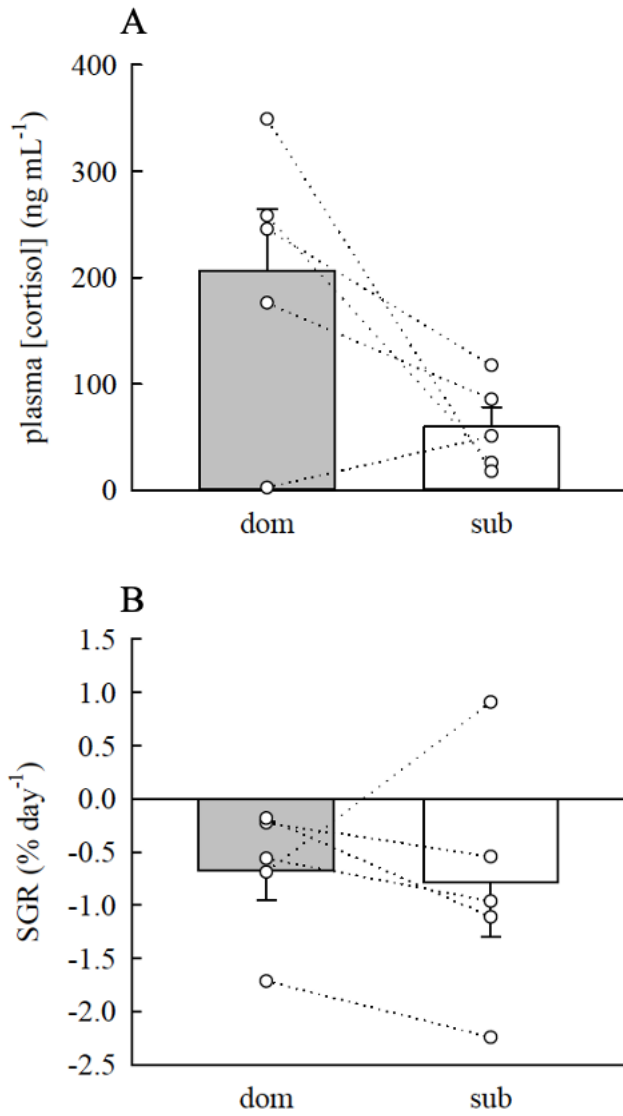


Fig. 3.2: The effects in *Polypterus senegalus* of dominant (dom) or subordinate (sub) social status on (A) plasma cortisol concentrations, and (B) specific growth rate (SGR). Bars present means + SEM, $N = 5$ per group, and the symbols are values for individual fish, with the members of a pair joined by a dotted line. No significant difference was detected between dominant and subordinate fish (paired Student's t -tests, $P = 0.0849$, 0.0543 for panels A and B, respectively).

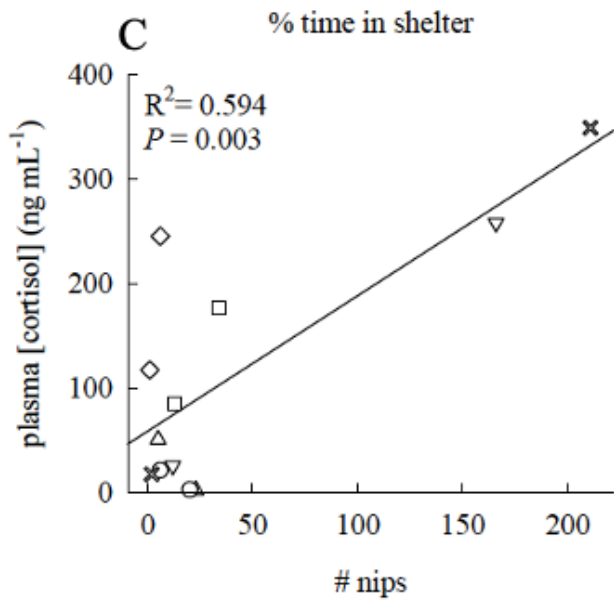
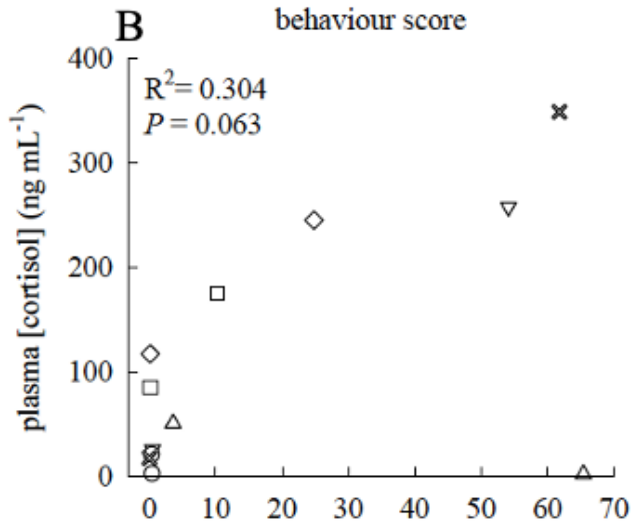
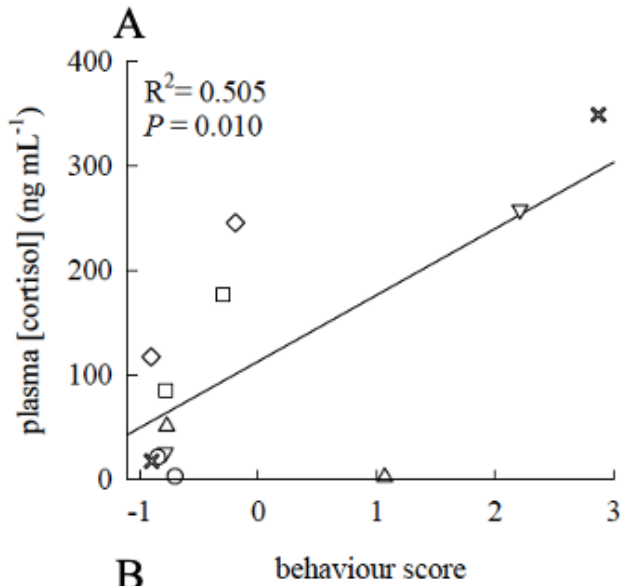


Fig. 3.3: Regression analysis of plasma cortisol concentrations and behaviour score (A), time in shelter (B), and number of nips given (C) for *Polypterus senegalus* held in pairs for 6 d. The plots present values for individual fish ($N = 10$), with the members of a pair represented by the same symbol. Regression lines are included for relationships that were significant (P and R^2 values are included on the individual plots).

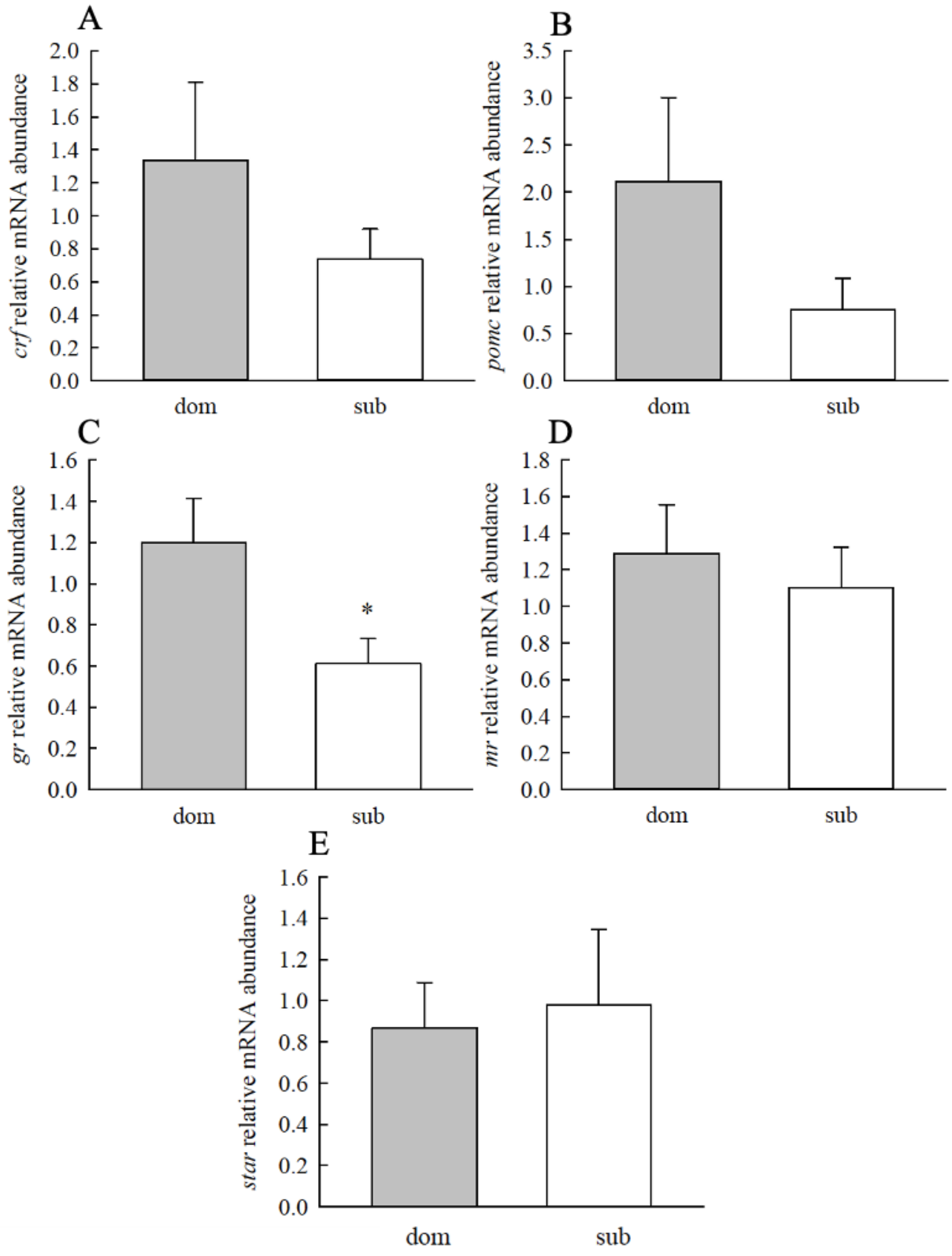


Figure 3.4: Transcript abundances of stress axis genes in the brain (A) *crf*, (B) *pomc*, (C) *gr*, and (D) *mr*, or anterior kidney and yellow corpuscles, (E) *star*, in dominant versus subordinate *Polypterus senegalus* following 6 d of social interaction. Values are means + SEM, $N = 5$ for both groups. Transcript abundances were normalized to the housekeeping gene *18S* and expressed relative to the value for the dominant group. An asterisk indicates a significant difference between the members of a pair (paired Student's *t*-tests, $P = 0.145, 0.182, 0.00971, 0.552,$ and 0.622 for panels A to E, respectively).

Table 3.1: Mass, length, and condition factor for *Polypterus senegalus* that became dominant vs subordinate during a 6-d social interaction period.

Variables	Dominants ($N = 6$)	Subordinates ($N = 6$)	P value
Mass (g)	11.40 ± 0.25	11.35 ± 0.24	0.959
Length (cm)	8.6 ± 0.7	8.5 ± 0.6	0.6419
CF	0.58 ± 0.01	0.58 ± 0.03	0.9315
Sex	3 female; 3 immature	3 male; 3 immature	

Values are means \pm SEM. Comparisons between dominant and subordinate fish were carried out using Student's *t*-tests.

CF, condition factor

3.4 Discussion

The objective of the present study was to determine whether *P. senegalus* held with a conspecific engaged in social interactions that resulted in chronic stress. Because no information on social interactions in *P. senegalus* was found in the literature, an initial step involved the production of a behaviour scoring system or ethogram to differentiate dominant and subordinate fish. A detailed description of the generation of the *P. senegalus* ethogram was provided by C. Corless (University of Ottawa undergraduate honours thesis, 2021). In brief, the ethogram focused on behaviours associated with territoriality and aggression, because the outcome of aggressive encounters is one of the main factors in determining rank in a social hierarchy (Winberg et al., 2016b). We identified four key behaviours associated with dominance: “nips”, “snouts”, “entrances into a shelter”, and “time spent in a shelter”. Nips and snouts can be categorized as aggressive because similar behaviours occur in agonistic encounters in other fish species. For example, dominant male zebrafish exhibit higher occurrences of chasing, sparring, and repelling when compared to their subordinate counterparts (Filby et al., 2010). Similarly, male cichlid fish use overtly aggressive acts like biting and ramming, as well as lateral displays and gill flaring to signal dominance (Desjardins et al., 2012). For *P. senegalus*, “nips” constitute a more aggressive behaviour owing to the overt biting contact, whereas “snouts” are the less aggressive because they are more passive while still maintaining aggression through contact. “Time spent in shelter” and “entrances into a shelter” constitute territorial behaviours because they involve monopolization of the sheltered area, reducing access by the other fish. Statistical analysis of these behaviour observations revealed significant differences between the members of a pair in “nips” and “time spent in shelter” but not “snouts” or “entrances into the shelter”, although clear trends were observed for both of the latter. The greater use of aggressive and

territorial behaviours by one member of a pair allowed us to distinguish between dominant and subordinate fish within a pair, using a principal components analysis. It is worth noting that one pair of fish was eliminated from further analysis because their behaviour scores were too similar to reliably identify a dominant or subordinate individual.

Once fish were identified as dominant or subordinate, plasma cortisol levels and SGR were measured to determine whether there were physiological consequences of social status. Although plasma cortisol tended to be higher in dominant fish and SGR to be lower in subordinate fish, neither trend reached statistical significance. Over the 6-day interaction period, both members of the pair tended to lose mass, possibly resulting from greater-than-normal energy expenditure associated with engaging in social interactions. Similarly negative SGR values have been reported for both dominant and subordinate fish in short-term social interactions in other fish species (e.g., Saulnier et al., 2021). A small sample size ($N = 5$ pairs) likely hampered our ability to detect significant differences, and in this regard, analysis of additional pairs of fish is warranted.

Somewhat unexpectedly, plasma cortisol concentrations tended to be higher among dominant rather than subordinate fish, potentially because it is physiologically stressful to maintain a dominant position in this species. Similar patterns have been observed in a few other species of fish. For example, in the African cichlid *Neolamprologus pulcher*, dominant breeders had higher baseline cortisol concentrations than subordinate helpers (Mileva et al., 2009). Similarly, elevated cortisol was measured in *N. pulcher* that were ascending to a dominant position, likely owing to the stress of establishing dominance (Culbert et al. 2018). Like *P. senegalus*, the brown ghost knifefish *Apteronotus leptorhynchus* is not a social species but may engage in social interactions when a conspecific is encountered. Brown ghost knifefish held in

pairs exhibit higher cortisol levels than solitary fish, with no difference between dominant and subordinate individuals, and increased production of “chirps”, a specific type of electrocommunication that is considered to be an aggressive signal (Dunlap et al., 2002). In paired brown ghost knifefish, plasma cortisol concentrations and the frequency of chirp production are correlated (Dunlap et al., 2002). Our observations of social interactions in *P. senegalus* parallel those in brown ghost knifefish, including in the detection of a significant correlation between plasma cortisol levels and aggressive behaviour, which in the case of *P. senegalus* was nipping. Thus, performing aggressive behaviours may be associated with stress and an increase in cortisol levels in *P. senegalus* as in brown ghost knifefish. Further investigation of possible links between aggressive behaviour and plasma cortisol levels in *P. senegalus* would be helpful in confirming this relationship.

In agreement with the absence of significant differences in plasma cortisol concentrations, no differences were found between dominant and subordinate fish in transcript abundances of HPI axis genes with the exception of brain *gr* transcript abundance, which was significantly lower in subordinate than dominant fish. In a stressful environment, GR protein abundance and *gr* relative mRNA levels may be decreased in response to chronic elevation of plasma cortisol as a mechanism to avoid over-activation of target tissue responses (Schreck and Farrell, 2016). For example, subordinate rainbow trout exhibited lower liver *gr2* relative mRNA abundance and GR protein levels than dominant fish (Jeffrey et al. 2012). However, dominant *P. senegalus* tended to have higher plasma cortisol levels than subordinate fish, which is not consistent with this pattern. Brain *gr* would be expected to play a role in negative feedback regulation of HPI axis activity (Li et al., 2012; Sapolsky et al., 2000), and therefore differences in transcript abundance at 6 d of social interaction may reflect differences in cortisol levels that

occurred earlier in the social interaction period. Plasma cortisol levels in the present study provided only a snapshot for the time at which sampling occurred; patterns of change in cortisol levels with time still require investigation. Other studies have reported increases in plasma cortisol among subordinate fish during hierarchy formation and as early as 24 h after hierarchy formation (Winberg and Lepage, 1998), with elevated cortisol levels being maintained for at least a week (Sloman et al., 2000). Whether *P. senegalus* experienced acute stress during hierarchy formation remains to be determined, but their plasma cortisol levels after 6 days of social interaction suggest that subordinate fish do not experience chronic stress.

From the present study it can be concluded that when *P. senegalus* are confined together, they form social hierarchies, as demonstrated by increased levels of aggressive and territorial behaviours displayed by the dominant fish. However, the formation of a social hierarchy had no significant effect on HPI axis gene expression or circulating cortisol levels at 6 d of social interaction. More work is needed to determine whether plasma cortisol levels are affected by shorter or longer periods of social interaction.

Chapter 4: General Discussion

The current study examined two potential stressors, terrestrial conditions and social interactions, in *P. senegalus*. In general, *P. senegalus* did not experience chronic stress from the environmental challenges that were presented, and therefore chronic stress does not appear to be responsible for the lower SGR observed in both terrestrial and subordinate (where a trend rather than a significant effect was observed) fish. In terrestrial fish, inhibition of muscle growth and increased muscle breakdown seemed to contribute to their lower SGR. Future studies would benefit from analyzing these pathways more in depth, including measuring transcript levels of additional genes. For example, measuring hormone sensitive lipase (*hsl*) would provide insight into whether lipid reserves were mobilized (Salmerón, 2018; Saulnier et al., 2021), and measurement of growth hormone (*gh*) would be useful in elucidating the hormonal regulation of the changes in SGR.

I found that introducing *P. senegalus* to a social environment caused aggressive behaviour by the fish that became dominant but did not provide a method of probing stress axis activity. Neither cortisol levels nor transcript abundances of HPI axis genes differed between dominant and subordinate fish. An exception was a decrease in *gr* transcript abundance detected in brain of subordinate fish. This result was somewhat surprising in that reduced transcript abundance of *gr* usually correlates with an increase in plasma cortisol (Norris and Hobbs, 2006), which was not the case in the current study. However, interpretation of this finding is difficult owing to the use of whole brain. Indeed, a limitation of both experiments in the present study was the use of whole brain for the measurement of transcript abundance, an approach that was necessitated by the small size of the animals available. In future studies it would be better to target specific brain areas for study.

A comparison of cortisol values between the social interaction experiment and the terrestrial experiment revealed similar levels in both, averaging $\sim 100\text{-}250\text{ ng/mL}^{-1}$, substantially higher than what is often considered to be an ‘unstressed’ cortisol concentration in a teleost fish ($<10\text{ ng mL}^{-1}$; (Pottinger et al., 2000)). However, peak cortisol values in the terrestrial experiment were greater than those in the social experiment. This finding suggests that a terrestrial environment may be more stressful than a social environment for *P. senegalus*, although such conclusions must be drawn with caution owing to differences between the experiments, e.g., animal masses and experimental time-courses differed. A terrestrial environment may be more of a physiological stressor than a social environment for *P. senegalus* owing to the need to withstand the effects of gravity on land (Turko et al., 2021). In addition, there is a high potential for evaporative water loss in a terrestrial environment. The absence of water poses other challenges, such as changes in nitrogenous waste excretion, ion and acid-base regulation, and temperature (Turko et al. 2021). These differences could also be potential reasons for greater energy expenditure on land than in water, accounting for the lowering of SGR in terrestrial fish. It would be useful to measure evaporative water loss during the early stages of exposure to terrestrial conditions. *P. senegalus* mitigates the effects of evaporative water loss with gill-remodeling as early as 7 days in a terrestrial environment (Turko et al., 2019), suggesting that water loss may contribute to mass loss and therefore influence SGR early in exposure to terrestrial conditions. In addition, future investigations should assess activity and the cost of transport on land as potential contributors to the lower SGR in terrestrial animals. It is likely that the cost of transport on land is substantially higher than that in water, something that was not captured in measuring time spent in activity in the fish of the present study. Bioenergetics models (Deslauriers et al., 2017) identify energy expenditure due to activity as a

contributor to energy consumption. A lower cost of transport in aquatic fish may compensate for greater time spent in activity, allowing more energy to be allocated to areas such as growth (Jahn and Seebacher, 2019). Therefore, measurements of the cost of transport on land versus in water for *P. senegalus* would be useful.

In a social environment, *P. senegalus* may experience more psychological stress than when held individually on land. The threat of attacks from an aggressive conspecific did not appear to have strong physiological effects on the subordinate fish, but significant behavioural effects were apparent. *P. senegalus* generally lead a solitary life, and therefore territory defence may constitute a stressor. In the present study, the members of a pair were introduced into their tank at the same time, so neither fish had had an opportunity to establish the tank as its territory. Setting up a different type of social interaction, e.g., an intruder-type study, might be useful in determining whether territory defence serves as a stressor. In addition, creating a detailed time course of social interactions and their impact on cortisol levels would shed light on whether shorter-term interactions are more stressful than the 6-d interaction period used in the present study. In general, the present study revealed behavioural effects of a social environment and physiological effects from exposure to a terrestrial environment. In neither case, however, did cortisol levels appear to be elevated. Thus, the findings of the present thesis point to the conclusion that *P. senegalus* is unusually tolerant of conditions that serve as stressors to many other species of fish.

This thesis contributes new knowledge to our understanding of stress physiology and stress axis function in basal ray-finned fishes, and therefore contributes to our understanding of the evolution of the stress axis and the stress response within fishes (Bouyoucos et al 2021). It was also the first study to describe behaviours associated with social hierarchies in *P. senegalus*.

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