THE USE OF FISH SCALE HORMONE CONCENTRATIONS AS A NON-LETHAL BIOMONITORING TOOL IN TELEOST FISHES

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ABSTRACT

Teleost fish serve as an essential resource to the human population and thus their conservation is of extreme importance. In an effort to monitor the effects of anthropogenic activity in fishes, the quantification of cortisol as an indicator of increased stress is often employed. Cortisol is a glucocorticoid released by the hypothalamic-pituitary-inter-renal (HPI) axis in fishes in response to a stressor. Cortisol alongside other stress hormones then serves to equip the fish with the resources required to overcome the stressor. However, if this response is repeatedly engaged the state of stress can change from acute to chronic often resulting in adverse effects.

Fish scales are durable calcified structures that can be sampled non-lethally from many fish species. Recently the fish scale has been shown to incorporate and store cortisol for long periods of time allowing it to serve as a medium for long-term stress assessments. This has now been accomplished in eight species of fish and recent studies have provided promising evidence for the use of fish scale cortisol concentration in the evaluation of a variety of chronic stressors. However, cortisol quantification alone is limited in its ability to diagnose chronic stress and thus my thesis research sought to investigate additional stress related hormones within the scale. The first two experiments quantified scale cortisol, cortisone and DHEA after a 14-day randomized stress protocol. In goldfish this resulted in significantly elevated circulating cortisol and cortisone and while these hormones were only somewhat elevated in the scales the scale cortisol:DHEA ratio was significantly elevated in stressed goldfish. By contrast, the same stress protocol significantly elevated scale but not serum concentrations of all three hormones in rainbow trout in the second experiment.

The third experiment included cortisol, progesterone, testosterone and 11-ketotestosterone to allow for the evaluation of both chronic stress and associated effects on reproduction. Artificial elevation of cortisol, progesterone and testosterone using coconut oil implants produced many interactions both among different hormones and among the two sample media: serum and scale. It was found that injected progesterone and testosterone were converted to 11-ketotestosterone, the primary androgen in teleost fishes and that well-studied negative interactions between cortisol and androgens are also relevant to scale hormone analyses. While the roles and relationships between the hormones explored in these experiments as well as their partitioning from blood to scale still requires further investigation, fish scale hormone concentrations appear to provide a unique and useful tool for future biomonitoring efforts.

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Figure 4.2 Scale hormone concentrations in control (C), vehicle control (VC), cortisol injected (FI), progesterone injected (P4I), testosterone injected (TI) and mix injected (MI) fish: (A) cortisol, (B) progesterone, (C) testosterone and (D) 11-ketotestosterone concentrations presented as the mean with error bars representing the standard deviation. All injected groups were compared to control and vehicle control only. Stars indicate significant difference from control, asterisks indicate significant difference from vehicle control ($p < 0.05$; n = 51 fish).

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AR	Androgen receptor
C	Control
CYP enzyme	Cytochrome P450 enzyme
CYP11b	Cytochrome P450 enzyme 11 b
CYP17a1	Cytochrome P450 enzyme 17a1
DHEA	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone-sulfate
DHP	17,20b-dihydroxy-4-pregnen-3-one
DHT	Dihydrotestosterone
E2	17β-estradiol
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
F	Cortisol
FI	Cortisol injected
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
GR	Glucocorticoid receptor
HPA axis	Hypothalamic-pituitary-adrenal axis
HPI axis	Hypothalamic-pituitary-inter-renal axis
HPG axis	Hypothalamic-pituitary-gonadal axis

LH	Luteinizing hormone
LOD	Limit of detection
MIS	Maturation inducing steroids
MI	Mix injected
MR	Mineralocorticoid receptor
P4	Progesterone
P4I	Progesterone injected
StAR	Steroidogenic acute regulatory protein
SC axis	Sympathetic-Chromaffin axis
T	Testosterone
TI	Testosterone injected
VC	Vehicle control
11KT	11-Ketotestosterone
11β-HSD (1,2 and 3)	11 beta-hydroxysteroid dehydrogenase (1, 2 and 3)
20βS	17,20b,21- trihydroxy-4-pregnen-3-one

CHAPTER 1

1.0 GENERAL INTRODUCTION

1.1 Stress in teleost fishes

1.1.1 Cortisol and the HPI axis

Stress, defined by Schreck (2000) as "The physiological cascade of events that occurs when the organism is attempting to resist death or re-establish homeostatic norms in the face of insult," is induced in fish upon the perception of a stressor. Immediately following perception, primary responses to stress trigger the release of select compounds into the blood stream via the sympathetic-chromaffin (SC) and hypothalamic-pituitary-inter-renal (HPI) axes (Pankhurst, 2011; Reid et al., 1998; Wendelaar Bonga, 1997). Within seconds, catecholamines such as epinephrine and norepinephrine are secreted by chromaffin cells into circulation where they act to modulate cardiovascular and respiratory activity in order to maintain adequate oxygen levels in the blood (Gorissen and Flik, 2016; Reid et al., 1998). This is followed closely by the release of glucocorticoids by inter-renal cells (Gorissen and Flik, 2016; Wendelaar Bonga, 1997). Glucocorticoids are steroid hormones important in a wide variety of processes across vertebrate species. Cortisol, the primary glucocorticoid in fish is produced by the steroidogenic cells of the inter-renal gland via 3β-hydroxysteroid dehydrogenase and a variety of cytochrome P450 (CYP) enzymes (Fig. 1.1) (Butler, 1973; Mommsen et al., 1999). Similar to the hypothalamic-pituitary-adrenal (HPA) axis in mammals, cortisol secretion via the HPI axis begins with the secretion of corticotropin releasing hormone (CRH) from the hypothalamus (Mommsen et al., 1999; Pankhurst, 2011; Wendelaar Bonga, 1997). This triggers the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary which then acts on the steroidogenic cells of the inter-renal gland (Wendelaar Bonga, 1997). Equivalent to the formation of all steroid hormones, cortisol synthesis begins with cholesterol which is transported into the mitochondria via

steroidogenic acute regulatory protein (StAR) (Rajakumar and Senthilkumaran, 2020). This process involves numerous enzymes and intermediates, but in most teleosts the final step is carried out via CYP11b, converting 11-deoxycortisol into cortisol (Rajakumar and Senthilkumaran, 2020). Upon release into circulation cortisol can act both genomically, via binding to the glucocorticoid receptor (GR), a nuclear transcription factor, and non-genomically via binding to a variety of membrane bound receptors (Groeneweg et al., 2012; Sadoul and Vijayan, 2016; Takahashi and Sakamoto, 2013; Thomas, 2003). In addition, cortisol has a high affinity for the mineralocorticoid receptor (MR). It is suggested that cortisol acts as a surrogate for aldosterone, an agonist of the MR present in mammals but not in fish (McCormick and Bradshaw, 2006; Takahashi and Sakamoto, 2013). However, although cortisol is capable of activating both receptors, defined roles for either interaction have yet to be fully characterized in teleost fish (Cruz et al., 2013; Takahashi and Sakamoto, 2013). Primarily, cortisol acts to regulate energy usage, maintaining proper carbohydrate, lipid and protein metabolism during normal functioning and reallocating energy to mitigate stress when required (Mommsen et al., 1999). Due to the high protein requirements of muscle tissue formation, cortisol has implications in fish growth as well (Mommsen et al., 1999; Sadoul and Vijayan, 2016; Wendelaar Bonga, 1997). In addition, the GR as well as other cortisol targets are widely distributed throughout the body allowing cortisol to act in other processes such as osmoregulation, immunity and reproduction (Mommsen et al., 1999; Takahashi and Sakamoto, 2013; Wendelaar Bonga, 1997). Free circulating cortisol is eliminated from the blood stream via diffusion into surrounding tissues (Sadoul and Geffroy, 2019). Cortisol metabolism is then carried out within the liver and head kidney by a variety of enzymes including reductases and hydroxysteroid dehydrogenases (Mommsen et al., 1999). Ultimately this results in the production of a suite of metabolites,

including tetrahydrocortisone, tetrahydrocortisol, 20β-cortolone, 5β-dihydrocortisone and cortisone, which are subsequently excreted as water soluble conjugates (Mommsen et al., 1999; Pottinger et al., 1992; Truscott, 1979)

1.1.2 Other key steroid hormones

While cortisol plays a key role in the physiological stress response in fish, the total state of stress within an organism is defined by many interactive components. As the mitigation of stress in vertebrates has numerous benefits for overall health, mechanisms for such purposes are abundant. One such mechanism involves the relationship between cortisol and two other steroids: cortisone and dehydroepiandrosterone (DHEA). DHEA is a steroid hormone categorized as a precursor steroid and androgen that has been shown to have anti-glucocorticoid properties in humans and other mammals (Hu et al., 2000; Kalimi et al., 1994). Generally, during periods of stress, the steroidogenic pathways shift away from DHEA production towards glucocorticoid production (Kalimi et al., 1994). This reduction in DHEA and increase in glucocorticoids can then contribute to the observed adverse effects of stress as DHEA has been shown to negate the effects of cortisol on the immune system, cardiac system, and metabolic function in mammals (Azevedo et al., 2020; Hu et al., 2000; Kalimi et al., 1994; Maninger et al., 2009). Actual mechanisms of action behind the effects of DHEA have yet to be wholly confirmed but current investigations surrounding this hormone have revealed numerous receptor possibilities (Clark et al., 2018). DHEA can interact with steroid hormone receptors such as the androgen receptor (AR) as well as hepatic nuclear receptors such as the peroxisome proliferator alpha and pregnane x receptors (Clark et al., 2018; Prough et al., 2016). Interactions with the estrogen receptors ERα and ERβ (Miller et al., 2012) as well as the MR (Lindschau et al., 2011) and GR (Cardounel et al., 1999; Hu et al., 2000) are also being investigated; still, direct

contributions of these interactions to stress mitigation remain somewhat unclear (Clark et al., 2018). One possible link between DHEA and reductions in circulating cortisol is the interaction between DHEA and the 11 beta-hydroxysteroid dehydrogenases (11β-HSD). The conversion of cortisol to its inactive metabolite cortisone is carried out by 11β-HSD 2, an enzyme present in both fish and mammals (Balazs et al., 2008; Gilchriest et al., 2000). The reverse reaction can then be carried out by 11β-HSD 1 (Tomlinson et al., 2004). DHEA has been shown to increase both the transcription and activity of 11β-HSD 2 likely leading to an increase in cortisol inactivation (Balazs et al., 2008; Maninger et al., 2009). In addition, in mammals DHEA has been shown to downregulate the transcription of 11β-HSD 1 (Maninger et al., 2009). The 11β-HSD 1 enzyme has yet to be identified in fish however the 11β-HSD 3 enzyme, now identified in multiple fish species, is likely to carry out the same functions and thus alterations to this enzymatic pathway could also come into play (Baker, 2004). Although cortisone is not an active component of the stress response, its quantitation can be used in estimations of total cortisol releases and thus HPI axis activity (Raul et al., 2004; Schreck and Tort, 2016). Increases in 11β-HSD 2 transcription have also been correlated with periods of increased stress in both rainbow trout *(Oncorhynchus mykiss)* and European sea bass *(Dicentrarchus labrax)* (Goikoetxea et al., 2021; Kusakabe et al., 2003b, 2003a). Thus, in addition to cortisol, cortisone and DHEA likely play a role in the state of chronic stress in fishes (Kamin and Kertes, 2017; Maninger et al., 2009).

1.1.3 Acute vs. chronic stress

The stress response in vertebrates is extremely complex and plays an important role in both healthy physiology and the initiation of adverse effects. As such the stress response is generally divided into three levels: primary, secondary, and tertiary (Sopinka et al., 2016).

Primary responses are triggered immediately following the perception of a stressor and result in an increase in circulating hormones that serve to enact secondary responses (Barton, 2002; Schreck and Tort, 2016). This includes an increase in heart rate, increased gill permeability as well as glycogenolysis all serving to properly equip the organism to overcome the stressor (Barton, 2002). This is usually followed by homeostatic restoration, allowing the organism resume normal functioning (Schreck and Tort, 2016). Primary and secondary stress responses are therefore considered adaptive and necessary for the mitigation of acute or short-term stressors such as predatory encounters (Schreck and Tort, 2016; Wendelaar Bonga, 1997). However, as primary, and secondary stress responses lead to increased metabolism and energy consumption, the constant triggering of the stress response can rob other physiological processes of the energy needed to carry out important tasks (Wendelaar Bonga, 1997). Tertiary stress responses are thus characterized by a wide variety of adverse effects including reduced immune function (Small and Bilodeau, 2005; Tort, 2011), problems with osmoregulation (Laiz-carrión et al., 2003), stunted growth (Pickering, 1993; Sadoul and Vijayan, 2016) and perhaps most problematic from an ecological perspective, reproductive inhibition (Schreck, 2010a; Schreck et al., 2001). These tertiary responses can thus be the manifestation of a state of chronic or long-term stress and of extreme concern with regards to fish well-being.

1.1.4 Stress and reproduction

Akin to the stress response, reproductive processes in fish involve interplay between the pituitary gland and the hypothalamus. This begins with the release of gonadotropin releasing hormone (GnRH) from the hypothalamus, triggering the activation of the hypothalamic-pituitary-gonadal (HPG) axis by stimulating the gonadotrophs of the pituitary to release gonadotropins (Zohar et al., 2010). Follicle stimulating hormone (FSH) and luteinizing hormone

(LH), the major gonadotropins in teleost fish, then act on the gonads resulting in the synthesis and secretion of a variety of steroid hormones (Pankhurst, 2016). Produced by the thecal and granulosa cells of the ovary and the Leydig as well as Sertoli cells of the testes, reproductive steroids can be generally subdivided into three categories: estrogens, androgens and progestogens (Rajakumar and Senthilkumaran, 2020). The main estrogen in fish, 17β-estradiol (E2), has many roles in reproduction, but perhaps most important is its involvement in the synthesis of proteins crucial for proper egg formation such as vitellogenin, the yolk precursor protein (Nagahama and Yamashita, 2008; Tokarz et al., 2015). Unlike mammals, the most prominent androgen in teleost fish is 11-ketotestosterone (11KT), produced from testosterone by 11β-HSD 2, the same enzyme that converts cortisol to cortisone (Fig. 1.1) (Tokarz et al., 2015). Unlike in mammals 11KT is a more potent agonist of the AR than testosterone and dihydrotestosterone (DHT) in fish and therefore highly important in the development of secondary sex characteristics, spermatogenesis and other male-specific processes (Borg, 1994; Olsson et al., 2005; Tokarz et al., 2015). Lastly, one of the most abundantly produced progestogens in teleost fish is 17-α-OH progesterone, created from progesterone (P4) by CYP17a1 (Fig. 1.1) (Rajakumar and Senthilkumaran, 2020; Tokarz et al., 2015). This compound is then converted to one of two more potent progestogens, produced by both sexes, and often referred to as the maturation inducing steroids (MIS): 17,20b-dihydroxy-4-pregnen-3-one (DHP) or 17,20b,21- trihydroxy-4-pregnen-3-one (20βS) (Tokarz et al., 2015). Notably, the production of either DHP or 20βS is usually species specific (Nagahama and Yamashita, 2008; Tokarz et al., 2015). The progestogens, important in both males and females, play crucial roles in spermatocyte/oocyte maturation, the production of seminal fluid and ovulation (Lubzens et al., 2010; Nagahama and Yamashita, 2008; Scott et al., 2010).

Many reproductive events in fishes are highly energetically costly. As mentioned previously this presents an opportunity for chronic or long-term stress to hinder reproductive processes. Additionally, due to their common origin the interplay between stress and reproductive related steroid hormones presents an opportunity for stress-reproduction interactions (McQuillan et al., 2011; Pottinger et al., 1996). For example, progesterone formation occurs very early in the steroidogenesis pathway, making progesterone a precursor for many hormones, including cortisol. Progesterone has also been proposed as a ligand for the MR in shark and ray finned fishes, possibly providing additional opportunity for cortisol-progesterone interactivity, however these concepts are still under investigation and are likely limited to these families of fishes (Baker and Katsu, 2020). Likewise, both testosterone and 11KT have been shown to interact with the HPI axis, suppressing cortisol production (Mommsen et al., 1999; Pottinger et al., 1996; Young et al., 1996). By contrast, E2 has been shown to increase HPI responsiveness to stress and thus cortisol secretion in salmonids (Lerner et al., 2007; Pottinger et al., 1996). However, these findings were not reflected in a study by Fuzzen et al. 2011 that found the exact opposite relationships between cortisol and 11KT and cortisol and E2 in zebrafish suggesting that there are other factors influencing these interactions. This complicated intertwining of the stress and reproductive axes lends substantial opportunity for adverse outcomes upon increases in stress. Consequently, the negative impacts of elevated stress on reproduction have occupied researchers of numerous disciplines for many years. Decades of research have demonstrated the negative effects of cortisol on reproduction including reduced gamete quality (Campbell et al., 1994; Valdebenito et al., 2013), decreases in testosterone and 11KT in male rainbow trout and reduced vitellogenin in females (Campbell et al., 1994; Pickering et al., 1987). Some research even suggests that increases in cortisol and subsequent

adverse effects can be transferred to offspring (Stratholt et al., 1997). McCormick (1998) It has been shown that cortisol concentrations in females were highly correlated with those found in eggs as well as with decreases in larval length (Mccormick, 1998). Following studies have outlined a variety of adverse effects in the progeny of stressed females including behavioural alterations (Sloman, 2010), morphological and growth abnormalities and ultimately survival (Eriksen et al., 2007, 2006). Subsequent reviews have provided additional insight into the negative effects of stress on reproduction (Pankhurst, 2016; Schreck, 2010a; Schreck et al., 2001), many of which include changes in circulating sex steroids in fish (Barkataki et al., 2011; Carragher and Sumpter, 1990; Clearwater and Pankhurst, 1997; Foo and Lam, 1993a; Jardine et al., 1996; Pankhurst and Dedualj, 1994; Van Der Kraak et al., 1992). Due to their role in essential reproductive processes, such as gonadal development, sex determination and pheromone signaling, alterations in sex steroid secretion in relation to external stressors could be detrimental to a variety of fish species.

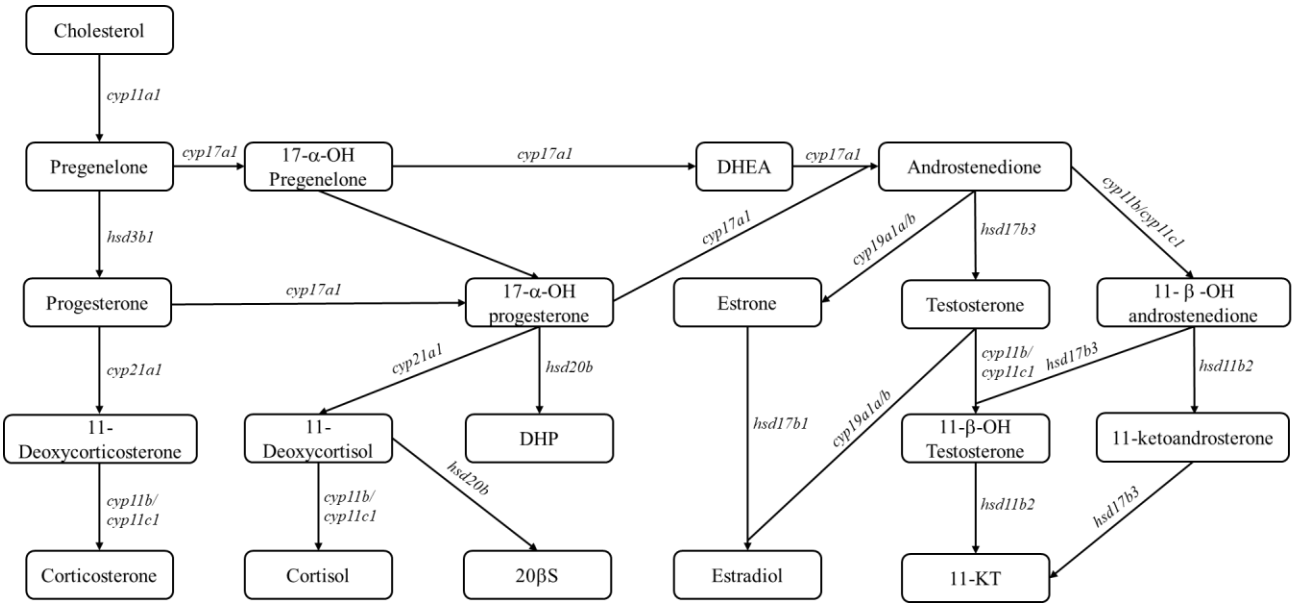

Figure 1.1 Proposed pathway of steroidogenesis in teleost fishes.

1.2 Current threats to fish populations

As fish serve as an essential resource to the human race, their exploitation has led to long-term stressors abundant both in magnitude and variety (Huntingford et al., 2006). These stressors can be physical in nature, for example Wysocki et al. 2006 exposed common carp (*Cyprinus carpio*), gudgeon (*Gobio gobio)* and European perch (*Perca fluviatilis*) to increased noise, simulating the increase in sound pollution in aquatic environments occurring as a result of increased aquatic transport and recreation. These exposures resulted in increased circulating cortisol secretion across all three species (Wysocki et al., 2006). However, these stressors can also come in the form of chemical contaminants. For example, aquatic contaminants such as selenite (Miller et al., 2007), crude oil (Kennedy and Farrell, 2005), copper (Pelgrom et al., 1995), PCBs (Vijayan et al., 1997) and other aryl hydrocarbon receptor (AHR) agonists (Aluru and Vijayan, 2004; Gesto et al., 2008) can stimulate the stress response, increasing circulating cortisol concentrations. Additionally, just as wild fish populations are subject to increases in stress, growing demands for food production is resulting in additional stressors in aquaculture environments as well. Aquacultural malpractice in the form of overstocking and reduced water quality has been shown to induce stress in fishes (Hanke et al., 2020; Sundh et al., 2019). For example, Hanke et al. 2020 compared four different aquaculture sites and found that the site with the highest stocking density had the highest scale cortisol. Other contributing factors may have included differences in feed quantities and compositions (Hanke et al., 2020). This is an additionally important consideration from a research perspective as ethical considerations are crucial in the proper conduct of research in fish and maintenance of lab stocks. Acting as a stressor across sectors, increased water temperatures induced by climate change is also leading to increased stress in fish species. Positive correlations between water temperatures and cortisol

have been found in many species suggesting that climate change will have a large impact on chronic stress in fishes as the situation worsens (Arends et al., 1998; Goikoetxea et al., 2021; Hanke et al., 2019; Madaro et al., 2018; Metz et al., 2003).

While the aforementioned examples relate directly to the stress response and could thus involve reproductive alterations and changes to circulating sex steroids, a variety of contaminants have also been shown to directly modify this endpoint (Haddy and Pankhurst, 1999; Jardine et al., 1996). Endocrine disrupting compounds, particularly agonists of the estrogen receptor, are becoming increasingly prevalent in aquatic environments (Kidd et al., 2007; Martinović et al., 2007). Pollutants with known estrogenic activity such as bleached kraft pulp mill effluents (Jardine et al., 1996; Munkittrick et al., 1991), pharmaceutical estrogens (Martinović et al., 2007; Trudeau et al., 1993), organophosphate pesticides (Singh and Singh, 1992), organochlorine pesticides (Singh and Singh, 1992), crude petroleum (Truscott et al., 1983) as well as other contaminants and even decreases in environmental pH (Freeman et al., 1983) have all been shown to lower circulating androgens in fish, in some cases manifesting in readily observable reproductive abnormalities (Martinović et al., 2007; Truscott et al., 1983). Similar effects have also been demonstrated with regards to circulating estrogens and progestogens, some of which induce increases (Chang and Lin, 1998; Tilton et al., 2002) while others result in decreases (Munkittrick et al., 1991; Singh and Singh, 1992; Tintos et al., 2007). As evidenced by these examples the consequences of increased anthropogenic activity could be detrimental to fish populations. Monitoring changes in steroid hormones secreted by essential physiological processes such as the HPI and HPG axes could thus provide crucial information required for the assessment of stress and other endocrine disruptions in teleost fishes.

1.3 Non-lethal biomonitoring

1.3.1 Current practices in non-lethal biomonitoring

As anthropogenic activities continue to impinge upon the surrounding environment, the need to ensure the successful coexistence of humans and other animals becomes more and more urgent. To this end, the development of monitoring techniques that are in themselves as non-invasive and as non-stressful as possible is required. The use of circulating cortisol concentrations for the quantification of stress in vertebrates has been carried out for decades. While useful in some contexts, blood samples can be invasive to collect and provide only a snapshot of an organism's overall physiology and wellbeing (Carbajal et al., 2018; Ellis et al., 2013; Pankhurst, 2011; Spagnoli et al., 2016). Research has also indicated that plasma cortisol may fall back within the normal physiological range during chronic stress as a result of various coping mechanisms (Ilan and Yaron, 1983; Mommsen et al., 1999; Vijayan et al., 1997; Wendelaar Bonga, 1997). Additionally, as the act of sampling blood is stressful by nature, particularly in fish, the acute stress response induced during sample collection can then confound the information provided by said sample. In an effort to better assess long-term conditions investigation into the use of integumentary structures such as hair, feathers and scales that incorporate and store steroid hormones for longer periods of time than blood has commenced (Ellis et al., 2013; Sadoul and Geffroy, 2019; Sheriff et al., 2011). The accumulation of hormones within the external structures outlined above can provide more accurate information on the cumulative activity of stress and reproductive responses over relatively long periods of time as has been suggested by a variety of studies (Aerts et al., 2015; Bechshøft et al., 2012; Carbajal et al., 2019b; Sheriff et al., 2011; Will et al., 2014).

In addition to their applications in the assessment of long-term conditions, the ease of sampling of integumentary structures such as hair adds a degree of practicality with regards to their use in animal conservation. One could argue that in comparison to blood sampling, the removal of hair or feathers is only marginally less intrusive. However, while other, perhaps less invasive techniques have been attempted such as the sampling of urine, feces and mucus, these media are still only capable of providing short-term information regarding an organism's overall health (Carbajal et al., 2019a; Ellis et al., 2013; Sadoul and Geffroy, 2019; Sheriff et al., 2011). The retained hormone content of external structures such as fish scales provide an integration of multiple instances of HPI/HPA or HPG axis activity, reducing the required frequency of sampling and thus the invasiveness of the method (Aerts et al., 2015; Carbajal et al., 2019a, 2018; Ellis et al., 2013). The sampling of these external structures for the purpose of monitoring changes in HPI/HPA activity over long periods of time can thus provide pertinent information on the well-being of resident organisms whilst avoiding animal sacrifice (Bechshøft et al., 2012, 2011; Dettmer et al., 2015). The information contained within such structures is also not limited to steroid hormones. The quantitation of toxicants and stable isotope analysis has also been achieved in hair, feathers and scales (Beaudin et al., 2010; Bechshøft et al., 2015; Jaspers et al., 2010; Paruk et al., 2018; Sergiel et al., 2017). Altogether, the sampling of external structures as a means of monitoring wild populations has numerous applications in the realm of biomonitoring and overall animal conservation.

1.3.2 The fish scale as a medium for non-lethal biomonitoring

Modern fish scales can be classified into four groups: placoid, ganoid, cosmoid and elasmoid (Kawasaki, 2016; Sire and Akimenko, 2004). The elasmoid category, which can be further divided into cycloid and ctenoid scales, is most commonly found among teleost fish

(Kawasaki, 2016; Le Guellec et al., 2004; Sire and Akimenko, 2004). Elasmoid scales are comprised of three layers, the basal plate containing elasmodine, a compound characterized by its plywood-like arrangement of collagen fibrils, the external layer and the limiting layer which is highly mineralized and devoid of collagen (Sire and Akimenko, 2004; Sire and Huysseune, 2003). Scale growth is initiated relatively late in development, approximately one month after fertilization and begins in the highly vascularized upper layer of the dermis near the lateral line (Le Guellec et al., 2004; Sire and Akimenko, 2004). From there, squamation occurs in all directions adding rows of scales in a shingle-like pattern (Rasmussen et al., 2018; Sire and Akimenko, 2004). Perhaps not as highly vascularized relative to hair and feathers, numerous studies have indicated that cortisol incorporation into the fish scale is sufficiently high for quantitation via both enzyme-linked immunoassays and mass spectrometry (Aerts et al., 2015; Carbajal et al., 2019a, 2019b, 2018; Hanke et al., 2020). Similar to hair and feathers, the lifespan of a scale is relatively long allowing the incorporation of hormones over long periods of time; however due do their proximity with skin and mucus hormones within the fish scale fluctuate more readily (Laberge et al., 2019). Regardless, fish scale cortisol has now been quantified in eight fish species and correlated with a variety of chronic stressors including overstocking and other changes in aquaculture practices (Hanke et al., 2020), fin injuries (Weirup et al., 2021), increases in water temperature (Hanke et al., 2019) as well as general long-term stress (Aerts et al., 2015; Laberge et al., 2019) demonstrating its potential as a diagnostic tool. Periodically shed throughout the fish's life, the sampling of a small subset of scales can also be considered a moderately invasive procedure (Shackleton, 1988). A study performed in cichlid fish demonstrated that the small wound induced by scale removal is healed within a very short period of time (3-6 hours), limiting the opportunity for infection and additional harm (Sire, 1989).

Preliminary work conducted in our lab indicated that only a small subset of scales (10-15) from large-bodied fish is required for the analysis of cortisol content (Kennedy and Janz, unpublished data). This number may also be reduced upon utilization/development of more sensitive hormone quantitation techniques. Successful studies inducing similar minute injuries in fish, such as that conducted by Baker (2004), strengthen the argument that the sampling of scales could be possible without affecting long term survival. Ultimately, the fish scale appears to provide a practical means for the non-lethal monitoring of stress and related reproductive alterations in large-scaled teleostean fish.

1.4 Purpose of research

The conservation of wild species in an effort to maintain the rapidly dwindling biodiversity of our aquatic ecosystems is essential in the 21st century (Hoffmann et al., 2010). As previously mentioned, the sampling of integumentary structures presents a promising means of non-lethally monitoring changes in steroid hormone secretions and thus the health and fitness of wild and captive populations of mammals and birds (Bechshøft et al., 2013, 2011; Fairhurst et al., 2013; Sheriff et al., 2011; Will et al., 2014). Many studies exploring the use of fish scales for such purposes show promising results (Aerts et al., 2015; Carbajal et al., 2019b, 2019a, 2018; Hanke et al., 2020, 2019). However, in comparison to the volume of research surrounding the use of similar media such as hair and feather there is much to be done with regards to fish scale hormone quantification prior to the application of such practices in the regular monitoring of teleost fish species. Unlike the studies performed to date, my research involves the detection of novel hormones/hormone metabolites within the fish scale, allowing this sampling method to provide much more information on the health of teleost fish than is currently possible. This is additionally important as it is often the case that circulating hormone concentrations will return

to unstressed levels as chronic stressors persist. Therefore, in addition to cortisol, we chose to quantify scale cortisone and DHEA as well in an effort to better assess chronic stress in fishes.

Already accomplished in both hair and feathers, the measurement of sex steroids for the purpose of assessing reproductive fitness could also be tremendously useful in the monitoring of teleost fish (Adámková et al., 2019; Dettmer et al., 2015; Snoj et al., 2012; Ventrella et al., 2018). In mammals, most often this includes the quantitation of E2, testosterone and progesterone. In order to provide synonymous information on reproductive capacity in fish, the chosen sex steroids differed slightly. In addition to testosterone, 11KT, the most potent agonist of the androgen receptor in teleost fish, was also included and progesterone, an important precursor steroid and reproductive mediator. Our intent was to also include E2 and 17-α-OH progesterone, but preliminary work has indicated that these hormones do not partition into scales at concentrations that can be reliably quantified non-lethally. Thus, the total suite of hormones to be quantified throughout the following experiments is: cortisol, cortisone, DHEA, progesterone, testosterone and 11KT. The development of methods for the quantification of these hormones within the fish scale will provide a unique and useful tool, invaluable for the biomonitoring of teleost fishes and is thus the purpose of my research.

1.4.1 Research question

Is fish scale hormone concentration a plausible means of monitoring long-term stress and other hormonal alterations in teleost fish?

This question was addressed by three experiments:

A) A pilot experiment during which cortisol, cortisone and DHEA were quantified in goldfish scale and serum after being repeatedly stressed for a period of two weeks to confirm that increases in stress alters concentrations of these hormones found in scales.

B) A follow-up experiment during which cortisol, cortisone and DHEA were quantified in rainbow trout scale and serum after being repeatedly stressed for a period of two weeks to confirm that these methods are applicable to multiple species.

C) A final experiment during which cortisol, progesterone, testosterone and 11KT levels were quantified in adult rainbow trout scales after following an injection of either cortisol, testosterone, or progesterone to confirm the positive relationship between circulating and scale hormone concentrations as well as ensure gonadal steroids are also quantifiable via these methods.

1.4.2 Objectives

Overall, the objective of this study was to further develop fish scale hormone concentrations as a non-lethal biomonitoring tool. As cortisol was the only hormone to be quantified within the scale, this included the use of additional scale hormones in the assessment of long-term stress and downstream effects on reproduction. More specifically my objectives were:

1. To evaluate the use of fish scale cortisone and DHEA in addition to cortisol in the assessment of long-term stress in two species of teleost fish: goldfish and rainbow trout.
2. To evaluate the use of fish scale cortisol, progesterone, testosterone and 11KT in the assessment of downstream adverse effects of chronic stress on reproduction in rainbow trout via artificial elevation of these hormones in circulation.

1.4.3 Hypotheses

$H0_1$: Long-term stressing in both goldfish and rainbow trout will not result in changes to scale cortisol, cortisone, and DHEA.

$H0_2$: Artificial elevation of circulating cortisol, progesterone, testosterone and 11KT will not result in elevated scale concentrations of said hormones.

$H0_3$: Negative interactions between scale cortisol and scale reproductive related hormones (progesterone, testosterone and 11KT) will not be apparent.

CHAPTER 2

2.0 FIRST LOOK INTO THE USE OF FISH SCALES AS A MEDIUM FOR MULTI-HORMONE STRESS ANALYSES

Preface

To our knowledge this is the first reporting of scale DHEA and cortisone concentrations. The purpose of this research was therefore to validate methods and generate preliminary data to be used in following experiments. This included the validation of a scale washing protocol for the detection of scale cortisol, DHEA and cortisone as well as an investigation into the effects of long-term stress on scale concentrations of said hormones.

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The author contributions to chapter 2 of this thesis were as follows:

Emily K. C. Kennedy (University of Saskatchewan) collected, processed, and analyzed all samples, performed all statistical analyses, and drafted the manuscript.

David M. Janz (University of Saskatchewan) provided scientific input and guidance; reviewed and revised the manuscript, providing comments and corrections; procured and provided funding required to conduct the research.

2.1 Abstract

Recent efforts have provided convincing evidence for the use of fish scale cortisol concentration in the assessment of long-term stress in fishes. However, cortisol alone is not sufficient to fully describe this state of long-term stress. Dehydroepiandrosterone (DHEA) is an androgen with actions that oppose those of cortisol. The means by which DHEA negates the effects of cortisol can occur via changes in the metabolism of cortisol to cortisone. The quantitation of cortisol, DHEA and cortisone could therefore provide a more comprehensive assessment of the overall status of physiological stress. As DHEA and cortisone have yet to be quantified within the fish scale our first objective was to ensure our sample processing protocol for cortisol was applicable to cortisone and DHEA. Following this, we induced a state of long-term stress in goldfish (*Carassius auratus*). Some degree of elevation in all hormones were observed in the stressed fish scales. Additionally, cortisol and cortisone were significantly elevated in the stressed fish serum in comparison to controls while DHEA was undetectable in either group. Overall, these results suggest that fish scales provide an appropriate medium for the assessment of long-term stress in fishes via the quantitation of relevant steroid hormones.

2.2 Introduction

The quantification of cortisol in an effort to assess the state of stress in fish has been carried out in myriad media. Serum or plasma is most common, but others include feces, urine, mucus and surrounding water (Ellis et al., 2013; Fernández-Alacid et al., 2018; Sadoul and Geffroy, 2019; Sheriff et al., 2011). While many of these methods have proven successful, the use of fish scale hormone concentrations in the assessment of stress in fish presents some unique and useful features (Aerts et al., 2015). Similar in concept to hair or feathers which are already used for hormone quantification purposes, scales have been shown to incorporate cortisol over

long periods of time (Aerts et al., 2015; Carbajal et al., 2019b, 2018; Hanke et al., 2020, 2019; Laberge et al., 2019). The cortisol concentration of a scale sample is thus an integration of hormones secreted by the hypothalamic-pituitary-inter-renal (HPI) axis over weeks and perhaps even months rather than a single time point as is characteristic of other media (Aerts et al., 2015; Laberge et al., 2019). Additionally, the cortisol concentration of the scale has been shown to be unaffected by brief increases in stress incurred upon capture, a problem often encountered when sampling blood or mucus (Laberge et al., 2019). This is also relevant when considering diurnal hormone fluctuations. Unlike blood samples, the time of day at which scale samples are collected is unlikely to have a significant effect on the hormone concentration of the scales (Lorenzi et al., 2008). Thus, along with their relative ease of collection, scales appear to provide a useful and convenient means of assessing long-term stress in teleost fishes.

Cortisol is the primary glucocorticoid in fishes and a crucial mediator of the physiological stress response (Faught et al., 2016; Gorissen and Flik, 2016). As such, scale cortisol concentration has been shown to increase as a result of external injuries (Weirup et al., 2021), increased water temperature (Hanke et al., 2019), high stocking densities and changes in feeding strategies (Hanke et al., 2020) as well as general long-term stress (Aerts et al., 2015; Laberge et al., 2019) providing promising evidence for its use as a non-lethal biomarker of long-term stress in both wild and aquacultural fish populations. However, while the quantitation of secreted cortisol provides some indication of the state of stress within an organism, the use of multi-hormone analyses may be capable of uncovering further information (Azevedo et al., 2020; Laberge et al., 2019; Sollberger and Ehlert, 2016). Dehydroepiandrosterone (DHEA) is an androgen and precursor steroid with actions that oppose those of cortisol in mammals (Hu et al., 2000; Kamin and Kertes, 2017; Maninger et al., 2009). DHEA circulates in the blood in its

inactive sulfated form DHEA-S and can later be de-sulfated in order to carry out its function (Kalimi et al., 1994; Mueller et al., 2015). Currently, exploration into the involvement of DHEA in the stress response in fishes is lacking. However, in humans and other vertebrates, high ratios of cortisol to DHEA have been considered indicative of chronic stress and an increased allostatic load (Azevedo et al., 2020; Sollberger and Ehlert, 2016; Wolkowitz et al., 2001). Although not fully understood, the means in which DHEA negates the effects of cortisol in mammals has been shown to occur in part via changes in cortisol metabolism (Balazs et al., 2008). This can result from a variety of mechanisms including increases in the transcription and activity of 11 beta-hydroxysteroid dehydrogenase 2 (11βHSD 2) which converts cortisol to its inactive metabolite cortisone (Chen et al., 2013; Maninger et al., 2009; Ozaki et al., 2006). During periods of stress the conversion of cortisol to cortisone via the 11βHSD 2 enzyme can be enacted in order to protect sensitive organs from cortisol surges (Balazs et al., 2008; Kusakabe et al., 2003a). Reports of increases in rainbow trout 11βHSD 2 equivalent (11rtHSD 2) activity associated with stress in rainbow trout (*Oncorhynchus mykiss*) suggest that an increased cortisone: cortisol ratio could also be an indicator of elevated stress in fishes (Kusakabe et al., 2003a). Additionally, some studies report cortisol + cortisone concentration to better assess total cortisol secretion (Davison et al., 2019; Musana et al., 2020; Raul et al., 2004). Kapoor et al. 2018 demonstrated that circulating cortisol is incorporated into the hair shaft as cortisone by injecting rhesus monkeys (*Macaca mulatta*) with radio-labelled cortisol. Thus, the scale cortisol + cortisone value is likely a better estimate of the total magnitude of the stress response than either hormone alone. The quantification of scale cortisol in addition to scale DHEA and cortisone should therefore provide a more complete picture of the overall state of stress in teleost fish (Laberge et al., 2019).

To our knowledge DHEA and cortisone have not previously been quantified in fish scales. The first objective of this study was thus to ensure that our sample processing protocol previously used for scale cortisol quantification was applicable to these additional hormones. Following this, we induced a state of long-term stress in goldfish, a commonly used teleost for endocrinological research, in order to analyze changes in scale and circulating cortisol, cortisone and DHEA. Upon completion of the stress protocol both scale and serum were collected and analyzed for cortisol, cortisone and DHEA concentration as well as DHEA-S in the case of serum. These values were then used to generate multi-hormone values and assess the state of stress in the goldfish.

2.3 Methods

2.3.1 Wash protocol validation

Our preliminary studies have demonstrated that methanol was effective in removing external cortisol contamination from scales. However, to ensure the efficacy of methanol as a wash solvent for the removal of external cortisol, cortisone and DHEA from goldfish scales, n = 3 replicates of a subset of six scale samples were washed one to six times and the cortisol, cortisone and DHEA content of all six wash solutions as well as their matching scale samples was measured. Scale samples of 200 mg were placed into a 5 ml plastic tube with 4 ml methanol and vortexed for two and a half minutes. These masses were deemed appropriate by analyzing four sub-samples ranging from 25 to 100 mg in order to determine how much dry scale mass was required to create a sample extract sufficiently concentrated to fall within the linear section of the standard curve created with each ELISA. Between each wash, methanol was decanted, scales were blotted dry and any visible debris (skin, etc.) was removed with forceps. Wash tubes were also rinsed between each wash and a fresh aliquot of methanol was used for each successive

wash. After the respective number of washes, the final wash solution for each scale sample was collected into a glass culture tube and scales were placed in a filter paper-lined petri dish with the lid off-set for air flow and allowed to dry on the bench top for 24 h. Collected wash solutions were dried at 38 °C under a gentle stream of nitrogen gas. These tubes were then rinsed four times (1 ml, 0.4 ml, 0.2 ml, and 0.15 ml) alongside the matching scale sample extracts as described in the extraction process outlined in following sections.

2.3.2 Stressor exposure

Goldfish were in the regressed stage (April) and approximately 5 cm in length. Fifty-six goldfish were subdivided into two groups of n = 28, one of which was subjected once daily to a stressor for 14 days and one of which served as a control. The repetitive application of acute stressors can be used to bring about a state of chronic stress (Sopinka et al., 2016). As such, one of three different stressors previously shown to generate an acute stress response in fish by Laberge et al. 2019 was randomly applied to the stressed group: (1) holding above water for two min, (2) chasing for 10 min with a net, or (3) holding in a bucket with an insufficient amount of water for five min . The stressor was also applied at a randomly assigned time of day (9 am, 12 pm or 3 pm) in an effort to maintain the unpredictability of the stressor.

2.3.3 Sample collection

Fish were anesthetized using buffered MS-222 (100 mg/l). A sample of blood was then collected into hematocrit tubes via caudal severance and dispensed into Eppendorf tubes. Blood was left to clot for 3 h on ice and then centrifuged to allow the collection of serum, which was stored at −20 °C until further analyses. Prior to scale collection fish were euthanized via cervical severance and wiped down to remove excess mucus. Scales were then collected by scraping the

length of the body towards the tail with a metal spatula and stored at −20 °C until further analyses.

2.3.4 Hormone extraction and quantitation

Based on preliminary work conducted in goldfish scales, the analysis of each hormone concentration requires 50 mg of dry powdered scale. Due the small size of the fish used in this experiment, the scales of two fish were necessary to meet this requirement. Thus, to ensure the proper analysis of cortisol to DHEA hormone ratios, the powdered scale from four goldfish was pooled and divided into two 50 mg subsamples: one for cortisol analysis and one for DHEA analysis. Any remaining scale mass was used for cortisone analysis.

To remove external contaminants and ensure accuracy of internal scale cortisol concentrations, all scale samples were briefly washed three times with methanol as described above. Washed and dried scales were then ground to a fine powder using a Retsch ball mixer mill. Samples were ground in a 10 ml grinding jar with a 12 mm stainless steel grinding ball for 0.045 s per mg of scale at 30 Hz. Subsamples of 50 mg were then combined with 1 ml of methanol and vortexed briefly for 15 s. Tubes were then placed in a rotator and left for 18 h to extract at room temperature. Extracted samples were centrifuged for 15 min at 4500 rpm and 20 °C and extracts were collected into 5 ml borosilicate glass tubes and dried at 38 °C under a gentle stream of nitrogen gas. A second 1 ml aliquot of methanol was added back to the powdered samples and vortexed for 40 s, then centrifuged, collected, and evaporated as above. These steps were repeated twice for a total of three collections. To concentrate extracted cortisol at the bottom of each tube the sides were rinsed four times with decreasing volumes of methanol (1 ml, 0.4 ml, 0.2 ml, 0.15 ml). Between each rinse extracts were dried at 38 °C under nitrogen gas. Final extracts were then reconstituted in 200 µl of buffer supplied by their respective EIA

kits: Cortisol EIA kit (Oxford Biomedical), Salivary DHEA Enzyme Immunoassay Kit (Salimetrics®) or DetectX® Cortisone Enzyme Immunoassay Kit (Arbor-Assays®). Serum collected from individual fish was pooled in the same manner as the scale samples in order to create matching pairs. They were prepared for analysis using the protocols outlined in the appropriate ELISA kit. In addition to serum DHEA, we also attempted to quantify serum DHEA-S using the DHEA-S Enzyme Immunoassay Kit (Arbor-Assays®). Finally, all samples were run in triplicate following the kit protocols in a Molecular Devices Spectra Max 190 microplate spectrophotometer.

Extracts from multiple samples were pooled for intra-assay variation (n = 5) and inter-assay variation (n = 10), determined as the percent coefficient of variation (%CV, SD/mean). Intra- and inter-assay variation for scale cortisol concentration was 3.9% and 11.9%, respectively, and 6.7% and 8.8% for serum cortisol, respectively. Intra- and inter-assay variation for scale cortisone concentration was 3.7% and 11.8%, respectively, and 2.7% and 8.2% for serum cortisone, respectively. For scale DHEA concentration, intra- and inter-assay variation was 6.5% and 10.1%, respectively. Parallelism between extracted samples and the kit standard curve was determined using a serial dilution of the pooled extract run in triplicate. Parallelism was observed between all standard curves and serially diluted extracts generated from both scale and serum samples. This validation excluded serum DHEA as it was undetectable. Limits of detection (LOD) for cortisol, cortisone and DHEA kits were 5.10 pg/ml, 28.5 pg/ml, and 1.27 pg/ml respectively. Any extracts with a hormone concentration below the limit of detection were assigned the limit of detection concentration. While the LOD for cortisone was higher than LODs for cortisol and DHEA, no scale hormone concentrations in either the control or stressed group were below detection for any hormone; this only occurred in the wash protocol validation.

2.3.5 Statistical analyses

Prior to any statistical testing all data were tested for normality and homoscedasticity using the Shapiro-Wilk test and Bartlett's test, respectively, as well as a visual inspection of the residuals. If parametric, comparisons of wash solution and scale extract hormone concentrations in the wash dynamics study were assessed using one-way ANOVA and multiple comparisons were assessed using a Tukey test. If non-parametric a Kruskal-Wallis test was employed followed by a Dunn's test for multiple comparisons. In the case of scale and blood hormone concentrations, comparisons between control and stressed groups were performed using an unpaired T-test if data sets were parametric. If non-parametric, a Mann-Whitney test was employed. Differences between groups were deemed significant at $p < 0.05$.

2.4. Results

2.4.1 Wash protocol validation

The cortisol concentration of the first wash solution (10.8 ng/ml) was significantly greater than all subsequent wash solutions ($p < 0.05$); however, the cortisol concentration among washes two to six did not differ significantly (Figure 1). Cortisone and DHEA followed the same pattern with concentrations in the first wash solutions (5.00 ng/mL and 0.0454 ng/ml, respectively) being significantly greater when compared to all other wash solutions (Figure 1; $p < 0.05$). In the case of cortisol and DHEA the hormone concentrations among scale samples one to six were not significantly different (Figure 1). In the case of cortisone, the hormone concentration of scale sample one was significantly greater than scale samples three to six ($p < 0.05$); however, cortisone concentration among samples two to six did not differ significantly.

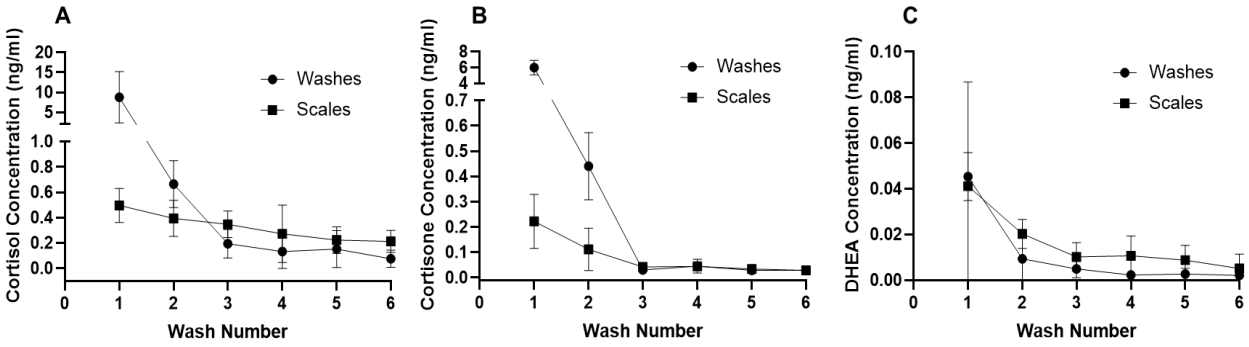

Figure 2.1 Validation of scale washing procedure: (A) cortisol, (B) cortisone and (C) DHEA concentrations in goldfish scale sample extracts washed 1-6 times are presented alongside their matching wash solution extracts. Error bars represent standard deviations, see text for further description of statistical analyses (n = 3 scale/wash extract pairs).

2.4.2 Scale hormone concentrations

Cortisol, cortisone and DHEA concentrations were all somewhat elevated in scales collected from stressed goldfish when compared to the control goldfish scales (Figure 2). While none of these elevations were statistically significant, the comparison of scale cortisol and cortisone concentrations between control and stressed goldfish produced notable p-values of 0.052 and 0.071, respectively. However, the cortisol: DHEA ratio was significantly elevated in the stressed group in comparison to control ($p < 0.05$, Figure 3).

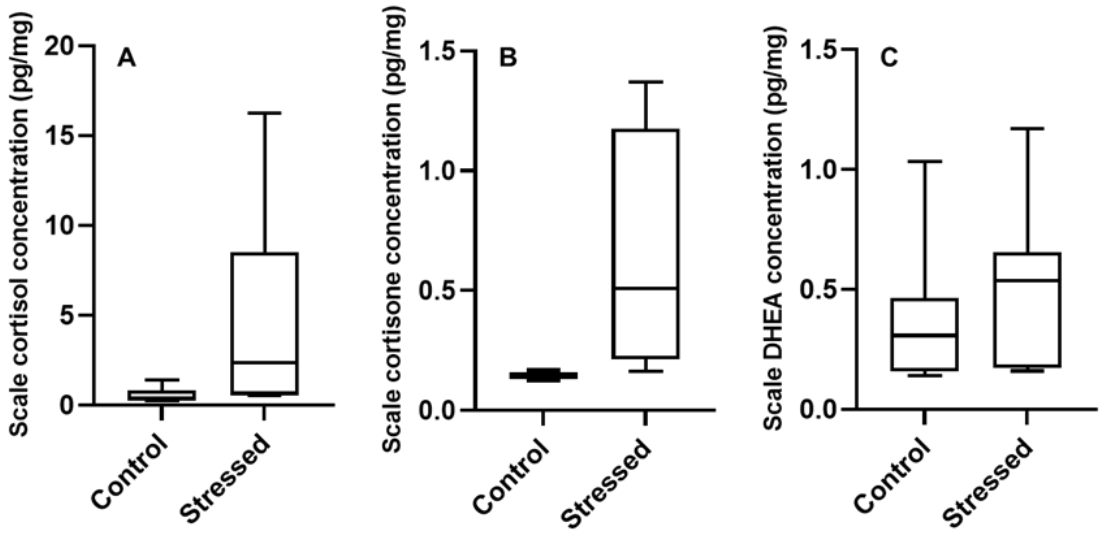

Figure 2.2 Scale hormone concentrations in control and stressed goldfish: (A) cortisol, (B) cortisone, and (C) DHEA concentrations presented as the median (middle line), surrounded by the 95% confidence interval (rectangle) with whiskers extending to the full range of the data. Differences between control and stressed goldfish scale hormone concentrations were not significant ($p > 0.05$; n = 8–14 scale samples).

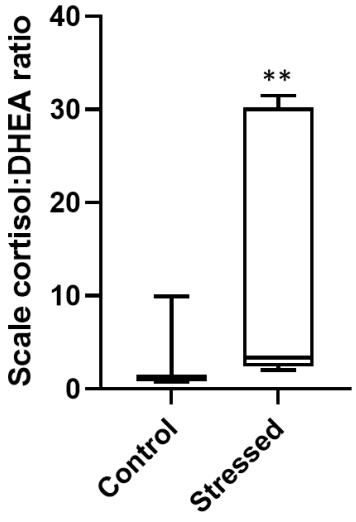

Figure 2.3 Scale cortisol: DHEA ratio in control and stressed goldfish presented as the median (middle line), surrounded by the 95% confidence interval (rectangle) with whiskers extending to

the full range of the data. Asterisks indicates significant difference from control ($p < 0.01$; n =14 scale samples).

2.4.3 Serum hormone concentrations

Serum hormone concentrations are presented in Figure 4. Cortisol and cortisone concentrations were significantly elevated in the stressed goldfish serum ($p < 0.05$). DHEA and DHEA-S were undetectable in serum collected from either the control or stressed group. While the cortisol + cortisone value was significantly elevated in stressed goldfish serum ($p < 0.05$) the cortisone: cortisol ratio was not significantly different between the two groups (Figure 5).

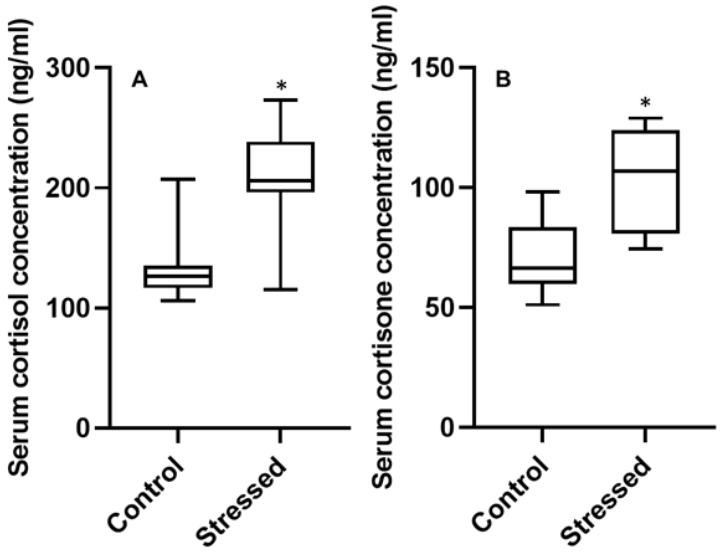

Figure 2.4 Serum hormone concentrations in control and stressed goldfish: (A) cortisol and (B) cortisone concentration presented as the median (middle line), surrounded by the 95% confidence interval (rectangle) with whiskers extending to the full range of the data. Asterisks denote significant differences from control ($p < 0.05$; n = 14 serum samples).

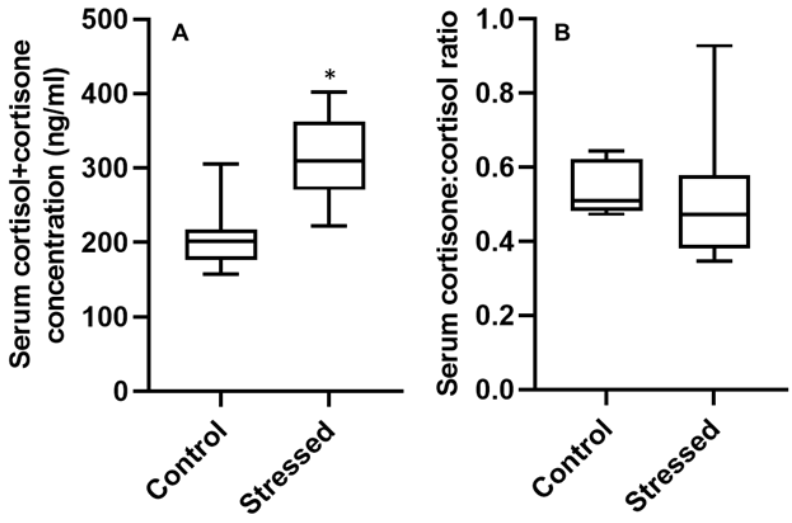

Figure 2.5 Multi-hormone values calculated from goldfish serum hormone concentrations: (A) serum cortisol + cortisone concentration and (B) serum cortisone: cortisol ratio in control and stressed goldfish presented as the median (middle line), surrounded by the 95% confidence interval (rectangle) with whiskers extending to the full range of the data. Asterisk indicates a significant difference from control ($p < 0.05$; $n = 14$ serum samples).

2.5 Discussion

To our knowledge this is the first study quantifying cortisone and DHEA in fish scales. As our results demonstrate, these hormones were sufficiently removed from the scale surface by washing with methanol and were capable of being extracted from powdered scale in the same manner as cortisol. In all cases, two washes appeared to be sufficient in the removal of external contaminants. However, in some cases scale hormone concentrations fell below detection limits after three washes. This indicates that there is potential for leaching of internal hormone content when more than three washes are employed. We thus recommend a minimum of two and a limit of three washes with methanol per scale sample. With these methods we are now able to generate

multi-hormone data useful in the assessment of long-term stress in fish, as was also accomplished during this study.

In order for scale hormone concentrations to serve as a means of assessing stress in teleost fishes, hormones must partition proportionally from blood to scale; however, the relationship between circulating and scale hormone concentrations has yet to be fully elucidated. While concurrent changes in both scale and serum collected from the same organism could aid in confirming their proportionality, due to the rapid and frequent changes in circulating hormone concentrations this may not always be the case (Carbajal et al., 2019b). Neither DHEA nor DHEA-S were detectable in any of the goldfish serum samples yet scale samples collected from both stressed and unstressed fish contained measurable concentrations of DHEA. As DHEA-S is known to circulate in relatively low concentrations in many fish species, this could aid in confirming that fish scales are gradually accumulating DHEA and likely other steroid hormones over time (Rege et al., 2019). By contrast, the significant increases in stressed goldfish serum cortisol and cortisone were not wholly reflected in scale samples; nevertheless, notable elevations in stressed fish scale cortisol and cortisone were observed. Results presented by other groups suggest that this could be due to a lag in the transfer of hormone from blood to scale (Aerts et al., 2015; Laberge et al., 2019). However, there are other phenomenon capable of disrupting blood-scale proportionalities. The 11-βHSD 2 enzyme responsible for the conversion of cortisol to cortisone can be found within fish skin (Gauberg et al., 2017). Similar to the peripheral hypothalamic pituitary adrenal axis present within hair follicles in mammals, this raises concerns regarding preferential deposition of locally generated metabolites within the scale as this could interfere with blood-scale proportionalities (Gauberg et al., 2017; Ito et al., 2005; Laberge et al., 2019). Additionally, as is the case in other cumulative media such as hair

and feathers, the partitioning of steroid hormones from blood to scale likely occurs via passive diffusion (Hein et al., 2021; Kapoor et al., 2018a). Slight variances in the chemical properties of different steroid hormones could therefore increase their degree and depth of incorporation into the scale, as has been reported in hair (Azevedo et al., 2020). Likewise, the rate of hormone clearance from both media must also be considered. Scale composition is not wholly static as scales participate in select physiological processes such as calcium homeostasis (Mugiya and Watabe, 1977; Pinto et al., 2009). As such, many factors could influence the concentration of hormone residing in the scale at the time of sampling including a redistribution of hormone from scale to blood. In depth mechanistic studies exploring scale steroid hormone incorporation and elimination are essential in answering these questions.

Unlike individual scale hormone concentrations, the scale cortisol: DHEA ratio was significantly elevated in stressed fish, suggesting a potentially more robust marker of chronic stress (Azevedo et al., 2020; Sollberger and Ehlert, 2016; Wolkowitz et al., 2001). As cortisol and DHEA have been shown to counteract one another in mammals it is also likely that their net activity, represented by the cortisol: DHEA ratio, better describes the state of stress experienced by an organism than either hormone alone. Other multi-hormone analyses potentially useful in the assessment of long-term stress include the cortisol + cortisone concentration and the cortisone: cortisol ratio. The conversion of cortisol to inactive cortisone is thought to be enacted during stressful periods in order to protect sensitive organs such as the gonads (Kusakabe et al., 2003a; Ozaki et al., 2006; Tokarz et al., 2012). Unfortunately, these two values were difficult to calculate using our scale hormone data as we lacked sufficient powdered scale to measure matching cortisol and cortisone concentrations as were measured for cortisol and DHEA. Our sample size for scale cortisone concentration was also lesser than those of the other two

hormones. Still, the average cortisol + cortisone was greater in the stressed group (5.96 pg/mg) when compared to control group (0.713 pg/mg). By contrast, the average scale cortisone:cortisol ratio was lesser in the stressed group (0.123) than in the control group (0.256). As we were able to collect a sufficient volume of serum to measure matching cortisol and cortisone concentrations, multi-hormone serum comparisons were more easily generated. Similar to the scale cortisone:cortisol ratio the serum cortisone:cortisol ratio was slightly lesser in stressed goldfish however this difference was not statistically significant. As the conversion of cortisol to cortisone has been shown to increase with increased stress this decrease in scale and serum cortisone:cortisol ratios were unexpected; however, the concurrent decrease in both media could add evidence that these hormones are depositing within the scale proportional to circulating concentrations. The conversion of cortisol to cortisone is also particularly relevant to reproduction as previously mentioned (Kusakabe et al., 2003a, 2003b). As our fish were in the regressed stage, this value may be less relevant to the state of stress in goldfish used in the present study.

Similar to the scale cortisol + cortisone concentration, serum cortisol + cortisone was significantly elevated in the stressed fish. Although cortisone is an inactive compound that no longer participates in the stress response, the relationship between cortisol and cortisone via the 11-βHSD 2 enzyme maintains cortisone's relevance in the assessment of stress. Thus, the combined cortisol + cortisone value likely provides a better estimation of the total glucocorticoid release and HPI axis activity than cortisol alone (Kapoor et al., 2018a; Staufenbiel et al., 2015). Alongside the cortisol: DHEA ratio this amplification of the stress response created by the use of hormone ratios and other multi-hormone values is of potential benefit to this area of research (Azevedo et al., 2020). Ultimately, our goal in the development of these non-lethal measures of

long-term stress is to conserve and protect fish populations. Stress responsiveness varies greatly both inter- and intra-specifically making statistical comparisons between control and stressed groups difficult to analyze (Barton, 2002; Sopinka et al., 2016). Power analyses suggest doubling or tripling our sample size would be necessary to conduct more robust statistical analyses. However, by using hormone ratios and increasing our ability to detect meaningful differences between stressed and unstressed organisms we may be able to reduce the required sample size, decreasing negative impacts on future study populations.

2.6 Conclusions

This study validated a laboratory technique to quantify cortisol, DHEA and cortisone in scales collected from goldfish, then applied the measurement of these steroid hormones to the assessment of long-term stress in fishes. Although there are many knowledge gaps left to be filled, the results offer evidence of the practicality of scale hormone concentrations in the assessment of long-term stress in teleost fish. While the sample sizes used in this experiment were not sufficient to provide concrete conclusions, our results suggest that multi-hormone analyses could be more revealing of the state of stress in fish than single hormone values. Altogether the use of scale multi-hormone values in the monitoring of stress in teleost fish has potential as an important tool for the conservation of teleost fishes.

CHAPTER 3

3.0 CHRONIC STRESS CAUSES CORTISOL, CORTISONE AND DHEA ELEVATIONS IN SCALES BUT NOT SERUM IN RAINBOW TROUT

Preface

The research in this chapter was designed to expand upon previously explored topics in chapter 2. Like chapter 2 scale concentrations of cortisol, cortisone and DHEA were measured following a long-term stressing protocol; however, as rainbow trout provide much more scale mass than the goldfish used in chapter 2 a better understanding of scale hormones and hormone ratios as well as an increase in sample size was achieved. The rainbow trout is also a highly relevant species to both research and the environment. This chapter was submitted to the journal Comparative Biochemistry and Physiology: Part A and is currently pending revisions. The anticipated citation is:

Kennedy, E.K.C., Janz, D.M., 2022. Chronic stress causes cortisol, cortisone and DHEA elevations in scales but not serum in rainbow trout. Comparative Biochemistry and Physiology: Part A, (in revision CBPA-D-22-00219).

The author contributions to chapter 3 of this thesis were as follows:

Emily K. C. Kennedy (University of Saskatchewan) collected, processed, and analyzed all samples, performed all statistical analyses, and drafted the manuscript.

David M. Janz (University of Saskatchewan) provided scientific input and guidance; reviewed and revised the manuscript, providing comments and corrections; procured and provided funding required to conduct the research.

3.1 Abstract

Fish scales have been shown to incorporate cortisol over long periods of time and thus provide a promising means of assessing long-term stress in many species of teleost fish. However, the quantification of other stress related hormones has only been accomplished in our previous study conducted in goldfish. DHEA is a precursory androgen with anti-stress effects used alongside cortisol to diagnose chronic stress via the cortisol:DHEA ratio in mammals. Included in DHEA's anti-stress mechanisms are changes in the metabolism of cortisol to its inactive metabolite cortisone suggesting the relationships between cortisol, DHEA and cortisone may be additionally informative in the assessment of long-term stress. Therefore, to further explore these concepts we implemented a 14-day stress protocol in adult rainbow trout and quantified resulting scale and serum cortisol, cortisone, and DHEA concentrations. Elevations in scale concentrations of all hormones were observed in stressed trout compared to controls but were not reflected in serum samples. Significant differences in the cortisol:DHEA and cortisone:cortisol ratios were also found between control and stressed group scales but not serum. These results suggest not only that scales provide a superior medium for the assessment of long-term stress but also that the addition of scale cortisone and DHEA may provide additional relevant information for such assessments.

3.2 Introduction

Pressures imposed by the rapidly developing human population are leading to increases in stress in fish species worldwide. When exposed to a stressor fish initiate a response via the hypothalamic-pituitary-inter-renal (HPI) axis, analogous to the hypothalamic-pituitary-adrenal (HPA) axis in mammals (Wendelaar Bonga, 1997). The resultant glucocorticoid release then acts to re-allocate energy and restore homeostasis (Balasch and Tort, 2019; Mommsen et al., 1999;

Sadoul and Vijayan, 2016). In an instance of short-term stress this response is adaptive as it served to equip the fish with energy and other resources necessary to mitigate the stressor (Schreck, 2010a; Sneddon et al., 2016). However, if repeatedly stimulated, the stress response can become destructive to an organism's health and adverse effects often arise (Wendelaar Bonga, 1997). These effects, including immune suppression (Small and Bilodeau, 2005; Tort, 2011), osmoregulatory problems (Laiz-carrión et al., 2003), halted growth (Pickering, 1993; Sadoul and Vijayan, 2016) and reproductive suppression (Schreck, 2010a; Schreck et al., 2001) can then lead to reduced fitness.

In an attempt to quantify the relationship between anthropogenic stressors and resultant adverse effects in animals, the monitoring of glucocorticoids released in response to stress is often employed (Sadoul and Geffroy, 2019). Primarily, this is accomplished via their extraction from blood (Ellis et al., 2013; Sadoul and Geffroy, 2019; Sheriff et al., 2011). However, the glucocorticoid content of a blood sample provides only a snapshot of the immediate state of stress within an organism and is thus more appropriate for acute stress assessment. In an effort to evaluate the impacts of chronic stressors a movement towards sample media capable of hormone accumulation and storage is currently underway (Ellis et al., 2013; Sheriff et al., 2011; Vašek et al., 2017). Traditionally this has included hair and feathers; however, in more recent years, the fish scale has been investigated regarding its use as a medium for long-term stress assessment in fishes (Aerts et al., 2015; Carbajal et al., 2019b, 2018; Culbert et al., 2021; Hanke et al., 2020, 2019; Kennedy and Janz, 2022; Laberge et al., 2019; Roque d'orbcastel et al., 2021; Weirup et al., 2021). Similar to hair or feathers the fish scale has been shown to incorporate and store cortisol for longer periods of time than blood (Aerts et al., 2015; Hanke et al., 2019; Laberge et al., 2019). As such, scale cortisol concentration has been shown to increase in response to a

number of long-term stressors in both wild and captive settings (Aerts et al., 2015; Carbajal et al., 2019b; Goikoetxea et al., 2021; Hanke et al., 2019; Weirup et al., 2021). These integumentary structures are additionally practical as they not only provide a means of long-term stress assessment but can often be sampled non-lethally allowing for more conservation-focused monitoring regimens (Ellis et al., 2013; Sadoul and Geffroy, 2019; Sheriff et al., 2011).

Still, while cortisol plays a major role in the mediation of the physiological stress response in fish, cortisol alone may also be more relevant to acute stress than chronic. As has been demonstrated in many studies, subjecting fish to repeated, chronic stressors often results in circulating cortisol concentrations that are not significantly elevated above baseline (Aerts et al., 2015; Carbajal et al., 2019a; Madaro et al., 2015; Samaras et al., 2021). The monitoring of additional scale steroids could therefore provide an additional degree of confidence in diagnosing chronic stress in fishes. For example, during stressful life events, increases in the transcription of 11 beta-hydroxysteroid dehydrogenase 2 (11βHSD 2) has been reported in rainbow trout *(Oncorhynchus mykiss)* ovary and testes (Kusakabe et al., 2003a, 2003b). In concurrence with a chronic temperature stressor, elevated expression of the 11βHSD 2 gene has also been reported in European sea bass *(Dicentrarchus labrax)* (Goikoetxea et al., 2021). This enzyme is responsible for the inactivation of cortisol via its conversion to cortisone and thus this phenomenon is thought to occur as means of protecting sensitive tissues from the damaging effects of increased cortisol (Baker, 2004; Kusakabe et al., 2003a, 2003b). Increases in circulating cortisone have also been shown to surpass those of cortisol both in concentration and response time during chronic confinement stress in rainbow trout (Pottinger and Moran, 1993). The addition of cortisone alone as well as in multi-hormone analyses such as the cortisone:cortisol ratio could thus add useful information to stress assessments with regards to

the degree of cortisol inactivation underway. Additionally, injected cortisol has been shown to be incorporated into hair in the form of cortisone (Kapoor et al., 2018a; Stubsjøen et al., 2015). Therefore, the reporting of scale cortisol + cortisone concentration could be equally as informative, as it likely provides a better estimation of total HPI axis activity and the resulting state of stress than either hormone alone (Raul et al., 2004).

In addition to cortisone, dehydroepiandrosterone (DHEA) or its sulfated circulating form DHEA-S also play an important role in stress physiology (Hu et al., 2000; Kalimi et al., 1994). In mammals DHEA(S) has been shown to have anti-glucocorticoid properties, acting in opposition to cortisol in many physiological pathways and exhibiting antioxidant, neuroprotective and immuno-protective characteristics (Hu et al., 2000; Kalimi et al., 1994; Kamin and Kertes, 2017; Maninger et al., 2009). Mechanisms of action responsible for these anti-stress effects could involve the 11βHSD 2 enzyme as well as its opposing partner 11βHSD 1, capable of converting cortisone back to cortisol (Tomlinson et al., 2004). In humans and other mammals DHEA(S) has been shown to decrease 11βHSD 1 transcription as well as increase 11βHSD 2 transcription and activity (Balazs et al., 2008; Maninger et al., 2009). While the 11βHSD 1 enzyme does not appear to exist in teleost fishes (Baker, 2004; Kusakabe et al., 2003b, 2003a), the 11βHSD 3, thought to be the ancestor of the 11βHSD 1 has been found in zebrafish and fathead minnows and is likely to carry out similar functions (Baker, 2004). Although these concepts are clearly less understood in fish, a higher ratio of cortisol to DHEA has been used as an indicator of chronic stress in mammals and suggested to be a more robust biomarker than either hormone alone (Azevedo et al., 2020; Sollberger and Ehlert, 2016; Wolkowitz et al., 2001). Thus, the quantitation of DHEA and cortisone in addition to cortisol will likely provide a more complete picture of HPI axis activity, particularly with regard to chronic stress.

Our previous study conducted in goldfish (*Carassius auratus*) provided promising evidence for the use of scales as a medium for multi-hormone analyses in the assessment of chronic stress (Kennedy and Janz, 2022). However, due the small size of the goldfish this study required the pooling of scales from multiple fish and was limited in its ability to present concrete conclusions regarding scale hormone ratios and relationships between blood and scale hormones. Here we utilize rainbow trout, a much larger fish species, allowing for the quantification of all three hormones in the scales and serum of individual fish as well as an increase in sample size. The major objective of this study was therefore to further examine the relationship between chronic stress and scale concentrations of cortisol, cortisone, and DHEA as well as the partitioning of these hormones from blood to scale. Additionally, as scale cortisone and DHEA have only been quantified in goldish scales this study also sought to ensure the applicability of these methods to rainbow trout, a commercially, environmentally, and recreationally relevant species.

3.3 Methods

3.3.1 Stressor exposure

Thirty adult rainbow trout with an average mass of 156 g were subdivided into two groups of n=15 one of which was subjected once daily to a stressor for 14 days and one of which served as a control. Each treatment group was held in a 700 l rectangular tank at 12 °C. Fish were fed each morning at 8 am and monitored to ensure food intake remained constant throughout the experiment. Ammonia, pH, chlorine, nitrite, and nitrate were monitored at least once per week and a 25% water change was performed each day.

One of three different stressors were randomly applied to the stressed group once a day for 14 days: 1) holding above water for two minutes, 2) chasing for 10 minutes with a net, and 3)

holding in a bucket with an insufficient amount of water for five minutes. The stressor was applied at a randomly assigned time of day (9 am, 12 pm or 3 pm) but the final stressor on day 14 was applied at 9 am. These specific stressors have been shown to mount an acute stress response in fish and the administration of repetitive, randomized acute stressors is generally considered to induce a state of chronic or long-term stress (Aerts et al., 2015; Kennedy and Janz, 2022; Laberge et al., 2019; Sopinka et al., 2016). The control group was left undisturbed.

3.3.2 Serum collection

Twenty-four hours after the final stressor was applied fish were anesthetized two at a time using a solution of 100 mg/l of MS-222 to allow for the collection of blood. Control fish were sampled first followed by stressed fish and all blood sampling was completed within 2 hours. Blood was collected from the caudal vein using a syringe and ejected into a 5 ml Eppendorf snap-cap tube. Following blood sampling fish were euthanized via cervical severance and put on ice. Blood was then left to clot for 3 hours at 4 °C. Next blood samples were centrifuged to separate to allow the collection of serum which was transferred to a 1.5 ml Eppendorf snap-cap tube and stored at -20 °C until further analyses.

3.3.3 Scale collection

As previously described, prior to scale collection fish were euthanized via cervical severance and wiped down to remove excess mucus. Scales from the entire body of the fish were then collected by scraping the length of the body towards the tail with a metal spatula. Scales were transferred to 5 ml snap cap tubes and stored at -20 °C for a maximum of 3 days.

3.3.4 Scale hormone extraction and quantitation

If possible, scales from individual fish were analyzed for all three hormones cortisol, cortisone and DHEA. In some cases, whole body scale mass was not sufficient for the

quantification of all three hormones resulting in $n = 30$ for cortisol, $n = 21$ for cortisone and $n = 28$ for DHEA. Prior to hormone extraction fish scales were washed and ground as validated and described in (Kennedy and Janz, 2022). Briefly, scales samples of approximately 200 mg were washed three times with methanol for 2.5 minutes. This scale washing protocol was chosen as our previous study demonstrated that three brief washes with methanol was sufficient in removing external cortisol, cortisone, and DHEA without significant leaching of internal hormone content (Kennedy and Janz, 2022). Between each wash, methanol was decanted, scales were blotted dry and any visible debris (skin, etc.) was removed with forceps. Wash tubes were also rinsed between each wash and a fresh aliquot of methanol was used for each successive wash. Washed and dried scales were then ground to a fine powder using a Retsch ball mixer mill MM 400.

To extract the desired hormone subsamples of 50 mg of powdered scale were transferred to a 1.5 ml microcentrifuge tube. Extraction of cortisol, cortisone and DHEA was performed by adding 1 ml of HPLC grade methanol/50 mg of sample, then vortexing briefly for 10-15 seconds. Samples were then placed in a rotator and left for 18 hours to extract. Following this, samples were centrifuged for 15 minutes, and the supernatant was collected and transferred into glass culture tubes. Next, the extracts were dried at 38 °C under a gentle stream of nitrogen. One ml of HPLC grade methanol was then added back to the powdered samples followed by a 40 second vortex, the tubes were then centrifuged, collected, and dried as above. These steps were repeated twice for a total of three collections. To concentrate the desired analyte at the bottom of the tube, the sides were rinsed 4 times with successively lower volumes of HPLC grade methanol. Between each rinse, extracts were dried at 38 °C under a gentle stream of nitrogen gas. Extracts were then reconstituted in 200 µl of extraction buffer supplied by their respective EIA kits:

Cortisol EIA kit (Oxford Biomedical), DetectX® Cortisone Enzyme Immunoassay Kit (Arbor-Assays) or Salivary DHEA Enzyme Immunoassay Kit (Salimetrics). Next, the sample tubes were gently vortexed and incubated for 12 hours at 4°C. After 12 hours, they were removed from the fridge, vortexed again, and the entire sample was transferred into a 0.6 ml microcentrifuge tube. Finally, the samples were centrifuged for 5 minutes to remove any remaining powdered scale. The supernatant was then collected and transferred to a clean 0.6 ml tube. Samples were run in triplicate following the kit protocols in a Molecular Devices Spectra Max 190 microplate spectrophotometer.

3.3.5 Serum hormone extraction and quantitation

Serum collected from individual fish was analyzed for all three hormones cortisol, cortisone, and DHEA. A subsample of 100 µl of serum for each hormone was transferred to a borosilicate glass test tube. In a fume hood 1 ml of ether was added to the tube followed by a vigorous 40 second vortex. The tubes were then left to stand for five minutes to allow the ether and aqueous phase to separate. To collect the ether, layer the tubes were flash frozen in liquid nitrogen and the upper ether layer was transferred to a borosilicate glass test tube and evaporated under a gentle stream of nitrogen gas at 50 °C. Once the aqueous phase thawed the above steps were repeated for a total of three collections. The sides of the glass tube were then rinsed three times with decreasing volumes of ether (1ml > 0.4ml > 0.2ml), drying the tube in between each rinse. Finally, the sample was reconstituted in 250 µl of EIA buffer (from the kit to be used for analysis), vortexed gently for 40 s and incubated in the fridge overnight. The following day the sample was vortexed again for 40 s, transferred to a 0.6 ml Eppendorf snap-cap tube and stored at -20 °C until analysis using the respective ELISA kit. The quantification of serum DHEA-S was also attempted using the DHEA-S Enzyme Immunoassay Kit (Arbor-Assays®).

3.3.6 Assay validation

Intra- and inter-assay variation as well as parallelism were determined to ensure the accuracy, precision, and specificity of the hormone quantification methods. Extracts from multiple samples were pooled for intra-assay variation (n = 6) and inter-assay variation (n = 12), determined as the percent coefficient of variation (%CV, SD/mean) and a concentrated sample was developed by collecting the extracts of multiple samples into a single glass tube to test parallelism. Intra- and inter-assay variation for scale cortisol concentration was 7.9% and 11.2%, respectively, and 5.1% and 6.9% for serum cortisol, respectively. Intra- and inter-assay variation for scale cortisone concentration was 4.8% and 8.3%, respectively, and 5.6% and 8.0% for serum cortisone, respectively. For scale DHEA concentration, intra- and inter-assay variation was 2.5% and 3.7%, respectively. Parallelism between extracted samples and the kit standard curve was determined using a serial dilution of the pooled extract run in triplicate. Parallelism was observed between all standard curves and serially diluted extracts generated from both scale and serum samples. This validation excluded serum DHEA(S) as it was undetectable. All validation steps were performed for all hormones. The limits of detection (LOD) for the cortisol, cortisone and DHEA assays were 5.10 pg/ml, 28.5 pg/ml and 1.27 pg/ml respectively. However, no scale or serum hormone concentrations in either the control or stressed group were below detection for any hormone.

3.3.7 Statistical analyses

Prior to any statistical testing all data sets were tested for normality using the Shapiro-Wilk test as well as a visual inspection of the q-q plot. If parametric, comparisons of scale and blood hormone content collected from control and stressed groups were performed using an unpaired T-test. If non-parametric, a Mann-Whitney test was employed. In the case of serum-

scale correlations parametric datasets were analyzed using a Pearson correlation test and non-parametric datasets were analyzed using a Spearman correlation test. Differences between groups were deemed significant at $p < 0.05$.

3.2 Results

3.4.1 Scale hormone concentrations

Scale cortisol, cortisone and DHEA were all significantly elevated in stressed rainbow trout compared to controls ($p < 0.05$, Fig. 1). The scale cortisol:DHEA ratio was significantly decreased in stressed trout ($p < 0.05$, Fig. 2). By contrast the scale cortisone:cortisol ratio and the cortisol + cortisone concentration were both significantly elevated in stressed trout ($p < 0.05$, Fig. 2).

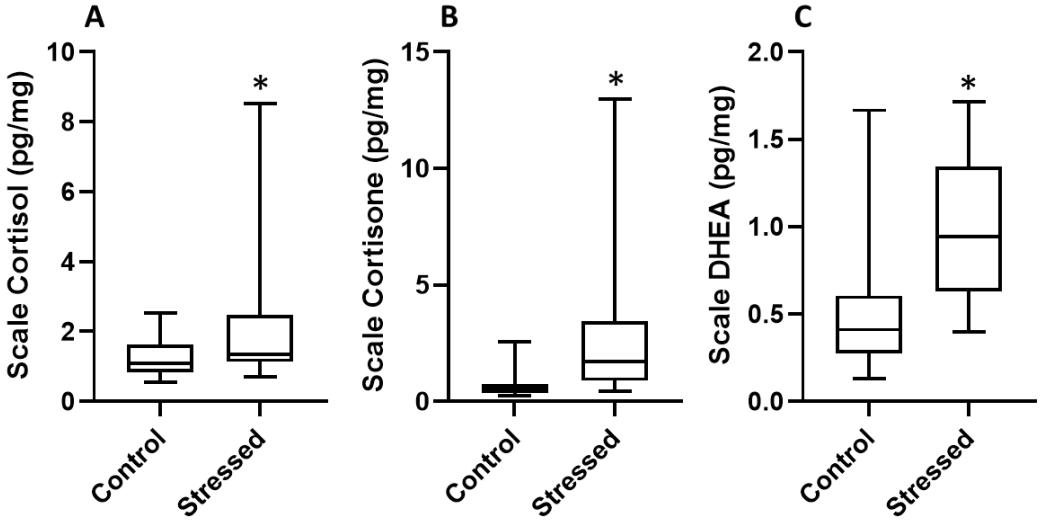

Figure 3.1 Scale hormone concentrations in control and stressed rainbow trout: (A) cortisol, (B) cortisone, and (C) DHEA concentrations presented as the median (middle line), surrounded by the 95% confidence interval (rectangle) with whiskers extending to the full range of the data. All hormones were significantly elevated in stressed fish scales ($p < 0.05$; $n = 21–30$ fish).

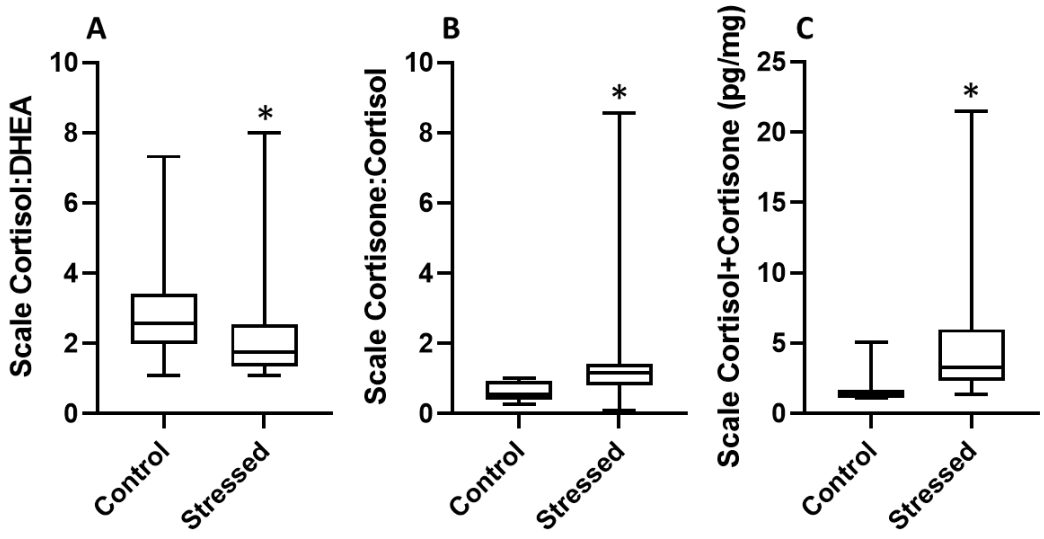

Figure 3.2 Scale multi-hormone analyses in control and stressed rainbow trout: (A) cortisol:DHEA ratio, (B) cortisone:cortisol ratio and (C) cortisol + cortisone concentrations presented as the median (middle line), surrounded by the 95% confidence interval (rectangle) with whiskers extending to the full range of the data. All comparisons were statistically significant ($p < 0.05$; n = 21-30 fish).

3.4.2 Serum hormone concentrations

No significant differences in serum cortisol or cortisone concentrations were observed between control and stressed groups ($p > 0.05$, Fig. 3). Neither DHEA nor DHEA-S was detectable in either control or stressed trout serum. There were also no significant differences

detected in the serum cortisone:cortisol ratio or the cortisol + cortisone concentration between control and stressed trout ($p > 0.05$, Fig. 4).

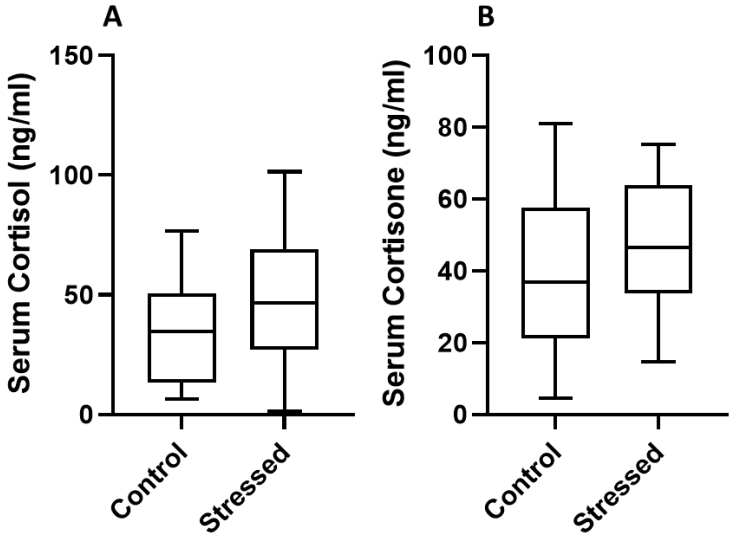

Figure 3.3 Serum hormone concentrations in control and stressed rainbow trout: (A) cortisol and (B) cortisone concentration presented as the median (middle line), surrounded by the 95% confidence interval (rectangle) with whiskers extending to the full range of the data. No comparisons were statistically significant ($p > 0.05$; n = 21-30 fish).

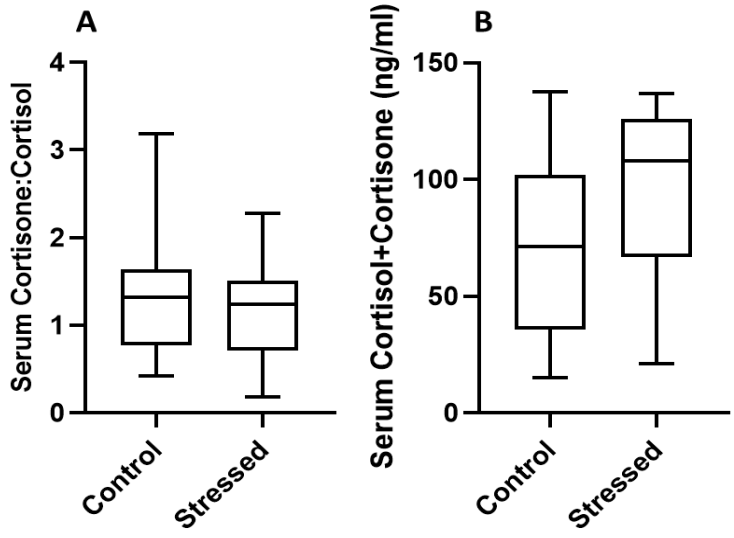

Figure 3.4 Serum multi-hormone analyses in control and stressed rainbow trout: (A) serum cortisone:cortisol ratio and (B) serum cortisol + cortisone concentration in control and stressed rainbow trout presented as the median (middle line), surrounded by the 95% confidence interval (rectangle) with whiskers extending to the full range of the data. No comparisons were statistically significant ($p > 0.05$; n = 21-30 fish).

3.4.3 Serum-scale hormone correlations

No significant correlations between serum and scale hormone concentrations were observed in either control or stressed fish (p >0.05, Fig. 5).

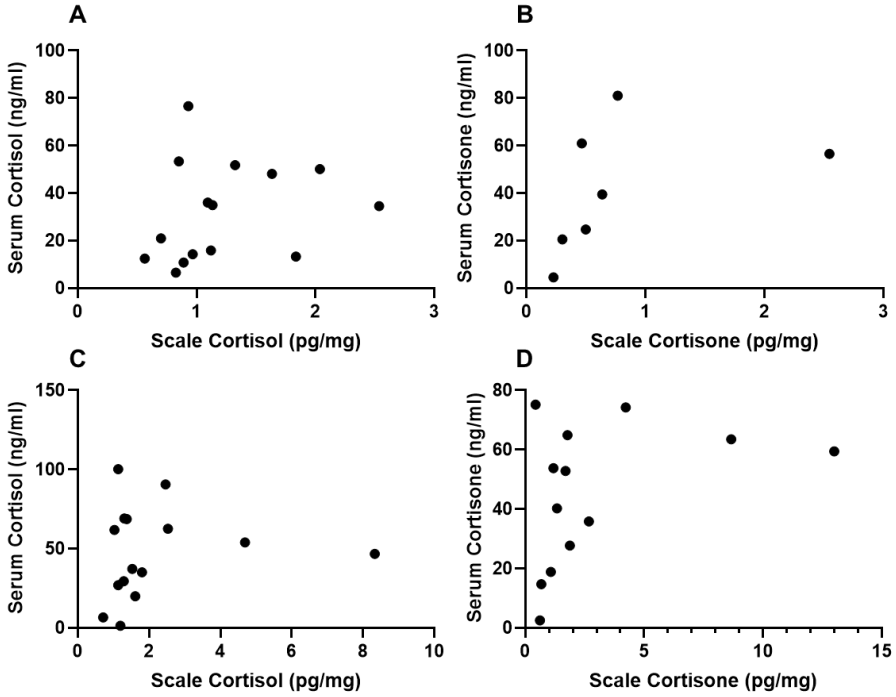

Figure 3.5 Serum-scale hormone concentration correlations in control and stressed rainbow trout: (A) Control cortisol, (B) control cortisone, (C) stressed cortisol and (D) stressed cortisone correlations between serum and scale. No correlations were statistically significant ($p > 0.05$; n = 14-30 fish).

3.5 Discussion

In order to successfully implement fish scale hormone concentrations as a method for long-term stress assessment in teleost fish a species-specific understanding of the changes in scale hormone concentrations in response to stress is of extreme importance. Scale cortisol concentrations reported in this study were similar to concentrations recorded in goldfish (Carbajal et al., 2018; Kennedy and Janz, 2022; Laberge et al., 2019), European sea bass (Samaras et al., 2021), two species of tuna *(Katsuwonus pelamis* and *Thunnus albacares)* (Roque d'orbcastel et al., 2021) and a study that also utilized rainbow trout (Carbajal et al., 2019a). Lower concentrations have been reported in milkfish *(Chanos chanos)* (Hanke et al., 2020, 2019) and higher concentrations have been reported in Catalan chub *(Squalius laietanus)* (Carbajal et al., 2019b) and in a second study conducted using European sea bass (Goikoetxea et al., 2021). As previously mentioned, we recently conducted a similar study using goldfish in which cortisone and DHEA were also measured in addition to cortisol (Kennedy and Janz, 2022). Scale concentrations of all three hormones were similar in both studies however, in direct contrast to the goldfish, we found no significant increases in cortisol or cortisone in stressed rainbow trout serum. Yet, scale cortisol, cortisone and DHEA were all significantly elevated in response to stress. As circulating hormone concentrations change both frequently and rapidly concerns regarding the use of blood hormone concentrations for the assessment of stress have been raised as the act of sampling blood can be acutely stressful (Ellis et al., 2013; Foo and Lam, 1993b; Pickering et al., 1982). If both control and stressed trout mounted an acute stress response during blood sampling, this could explain the lack of significant difference in circulating cortisol and cortisone observed between the two groups. However, our results are more likely explained by reduced stress responsiveness over time as a consequence of chronic HPI axis stimulation

(Mommsen et al., 1999; Sadoul and Geffroy, 2019). As cortisol regulates its own production, chronically elevated cortisol can eventually lead to a reduction in circulating concentrations (Barton, 2002; Evanson et al., 2010). This has been specifically observed in rainbow trout where both cortisol and cortisone levels returned to near baseline concentrations in blood after nine days of confinement stressing in two separate strains (Pottinger and Moran, 1993). These findings are also reflected in other scale cortisol analysis studies and in other species emphasizing the applicability of a cumulative media such as the scale for glucocorticoid quantification in long-term stress assessment (Aerts et al., 2015; Carbajal et al., 2019a; Madaro et al., 2015; Samaras et al., 2021). Finally, a third possible explanation for the lack of significantly elevated cortisol in stressed fish is a return to baseline concentrations following an acute stressor. While acute cortisol responses vary markedly both inter- and intra-specifically, cortisol concentrations following an acute stressor generally return to baseline concentrations within 24 hrs (Faught et al., 2016). If the daily stress protocol used in this study resulted in a pulsatile-type stress response, circulating cortisol concentrations in the stressed group may have returned to baseline following the final stressor on day 14. Regardless, the scales provide a unique means of capturing these pulses allowing for better analysis of repetitive stressing in comparison to blood as evidenced by the results of this and many other scale hormone studies.

The cortisol:DHEA ratio is used in humans and other mammals as an indicator of chronic stress (Azevedo et al., 2020; Sollberger and Ehlert, 2016). Notably, while elevated concentrations of scale cortisol and cortisone reported in this study appear to confirm a state of chronic stress, the scale cortisol:DHEA ratio was significantly decreased. Generally, increases in the cortisol:DHEA ratio during chronic stress are driven primarily by a decrease in DHEA concentrations as cortisol often shows minimal change (Maninger et al., 2009). The increased

scale DHEA concentration and decreased cortisol:DHEA ratio observed here could therefore suggest a different role for DHEA in stress responsiveness in this species especially when compared to our previous study in goldfish where this value was significantly increased in response to stress (Kennedy and Janz, 2022). Again, in contrast to our previous study in goldfish, the ratio of cortisone:cortisol, was significantly elevated in stressed trout scales suggesting an increase in cortisol inactivation via 11bHSD 2. Studies performed by Kusakabe et al. 2003a and b reported that patterns of 11bHSD 2 transcription upregulation in rainbow trout gonads correlated with periods of increased stress. This is further supported by rapid increases in circulating cortisone that surpassed that of cortisol in rainbow trout in response to stress (Pottinger and Moran, 1993). Therefore, although the decreased cortisol:DHEA ratio suggests a decrease in chronic stress, the significantly increased cortisol and cortisone as well as the cortisone:cortisol ratio appear to confirm the contrary. This further highlights the need include multiple hormones in the assessment of long-term stress not only for the sake of stress response amplification as was outlined in our previous study but also to ensure the applicability of these methods across a variety of species (Kennedy and Janz, 2022).

As was the case in our goldfish study, neither DHEA nor DHEA-S were detectable in rainbow trout serum, yet scale concentrations were within similar ranges in both species (Kennedy and Janz, 2022). This is unsurprising as DHEA(S) is known to circulate in relatively low concentrations in fish in comparison to mammals (Rege et al., 2019). While the inability to compare circulating and scale concentrations of DHEA(S) is somewhat problematic, these results do suggest that speculations regarding the retention of steroid hormones within the scale for longer periods of time than blood are correct (Kennedy and Janz, 2022). Similarly, high variation in stressed fish scale cortisol and cortisone in comparison to controls suggests that these

concentrations are a reflection of true HPI axis activity as cortisol responses differ quite significantly intra-specifically (Winberg et al., 2016). However, we found no significant correlations between serum and scale concentrations of cortisol and cortisone. While this suggests there may be complications when attempting to evaluate HPI axis activity using scale hormone concentrations it is important to consider that as sample media, blood, and scale provide different information. As the scale appears to incorporate and retain hormone secretions for multiple days and perhaps even weeks it is likely more appropriate to compare scale concentrations to an average of concentrations measured in multiple blood samples collected over the duration of the experiment (Laberge et al., 2019). This was better accomplished in a recent study Carbajal et al. 2019a who reported strong correlations between the cortisol concentrations measured in scale and plasma samples collected from rainbow trout during the latter half of their 30-day stress protocol which encompassed two blood sampling timepoints. Still, the mechanisms behind hormone incorporation into the scale remain largely unknown. In hair, hormone incorporation has been proposed to occur via passive diffusion thus favouring the incorporation of less-polar compounds (Kapoor et al., 2018). This is thought to be the reasoning behind high concentrations of hair cortisone in comparison to cortisol reported in some studies (Kapoor et al., 2018). Notably, cortisone concentrations were near identical to cortisol concentrations in rainbow trout scales collected in this study and one degree of magnitude lower than cortisol concentrations in the goldfish scales collected in our previous study (Kennedy and Janz, 2022). DHEA is also a non-polar compound and in a recent study Azevedo et al. 2020 DHEA was more abundant than either cortisol or cortisone in lynx (*Lynx pardinus*) hair samples. This is again in direct contrast to our studies as DHEA was the least abundant steroid by mass in both rainbow trout and goldfish scales (Kennedy and Janz, 2022). While hair and scales are

composed of two different proteins, keratin, and collagen respectively, no obvious differences in chemical properties between the two compounds appear to be attributable to these differences in concentration. Thus, further analyses of blood scale relationships are still required in order to properly implement these methods in biomonitoring regimes.

3.6 Conclusions

The conservation of fishes is important now more than ever. While other health monitoring strategies do exist, the scale provides a unique sample media capable of capturing HPI axis activity for long periods of times. This coupled with the addition of multiple hormones allows the scale to provide a reliable means of assessing long-term stress in fish. While these methods still require further investigation, current evidence suggests fish scale hormone concentrations provide a promising biomonitoring tool in both wild and captive teleost fishes.

CHAPTER 4

4.0 THE USE OF FISH SCALE HORMONE CONCENTRATIONS IN THE ASSESSMENT OF LONG-TERM STRESS AND ASSOCIATED ADVERSE EFFECTS ON REPRODUCTIVE ENDOCRINOLOGY

Preface

The purpose of the research in this chapter was to expand upon the information that we can currently glean from the fish scale. This involved the quantification of three new hormones: progesterone, testosterone and 11-ketotestosterone alongside cortisol in order to assess both chronic stress as well as related effects on reproductive endocrinology. This experiment also further examined relationships between blood and scale hormone concentrations This chapter will be submitted to the journal General and Comparative Endocrinology. The anticipated citation is:

Kennedy, E.K.C., Janz, D.M., 2022. The use of fish scale steroid hormone concentrations in the assessment of long-term stress and associated adverse effects on reproductive endocrinology. (in preparation).

The author contributions to chapter 4 of this thesis were as follows:

Emily K. C. Kennedy (University of Saskatchewan) collected, processed, and analyzed all samples, performed all statistical analyses, and drafted the manuscript.

David M. Janz (University of Saskatchewan) provided scientific input and guidance; reviewed and revised the manuscript, providing comments and corrections; procured and provided funding required to conduct the research.

4.1 Abstract

Investigation of the use of fish scales as a medium for non-lethal biomonitoring has recently commenced. Fish scales have been shown to incorporate cortisol over longer periods of time than blood and thus provide a promising means of assessing long-term stress in many species of teleost fish. However, while cortisol is a major mediator of the stress response in fishes, downstream effects of chronic stress on reproduction can involve gonadal steroids such as progesterone and testosterone. The quantification of these additional hormones alongside cortisol could therefore allow for the assessment of both stress and consequential reproductive alterations. To investigate these concepts, we artificially elevated circulating cortisol, progesterone and testosterone in rainbow trout using coconut oil implants for three weeks. Following this we quantified these three hormones as well as 11-ketotestosterone, a potent androgen in fishes. In all cases serum samples reflected a significant increase in the injected hormone confirming the efficacy of this method; however, this did not result in significantly elevated scale concentrations of the same hormone in all cases. As the stress and reproductive axes are closely integrated, these findings are likely a result of interactions along the steroidogenic pathway. Thus, while these methods still require further investigation, fish scale hormone concentrations appear to provide a novel and informative tool in the assessment of long-term stress and resulting effects on reproductive endocrinology in teleost fishes.

4.2 Introduction

As human populations continue to expand, the frequency and duration of stressors applied to fishes in both wild and captive settings is on the rise. The stress response in teleost fishes is mediated largely by the hypothalamic-pituitary-inter-renal (HPI) axis. This begins with the secretion of corticotropin releasing hormone (CRH) from the hypothalamus which acts on the

pituitary to trigger the release of adrenocorticotropic hormone (ACTH) (Mommsen et al., 1999). ACTH then stimulates the steroidogenic cells of the inter-renal tissue resulting in the production of cortisol (Rajakumar and Senthilkumaran, 2020; Wendelaar Bonga, 1997). Cortisol is a glucocorticoid steroid hormone that serves to equip the fish with sufficient energy to overcome the perceived stressor and eventually resume normal functioning (Mommsen et al., 1999). However, if the duration of the stressor is prolonged and homeostasis is not restored the state of stress becomes chronic and this often results in adverse effects to an organism's health (Balasch and Tort, 2019; Schreck and Tort, 2016).

While chronic stress has been shown to interfere with many physiological processes (Laiz-carrión et al., 2003; Pickering, 1993; Sadoul and Vijayan, 2016; Small and Bilodeau, 2005), perhaps most concerning are reproductive alterations due to their potential impact on both present and future populations (Schreck, 2010b; Schreck et al., 2001). As many reproductive events are energetically costly, the dwindling of energy resources in an effort to mitigate prolonged stressors has the potential to dampen reproductive activity as trade-offs between reproductive success and growth or survival are then required (Schreck et al., 2001). This competition between stress and reproduction is highly studied and involves multiple interactions between hormones produced by the HPI and hypothalamic-pituitary-gonadal (HPG) axes. To quantify this competition comparisons between glucocorticoid and androgen concentrations are often employed (Sollberger and Ehlert, 2016). For example, negative correlations between cortisol and both testosterone and 11-ketotestosterone (11KT) concentrations have been observed in fishes (Mommsen et al., 1999; Pottinger et al., 1996; Young et al., 1996). 11KT is a steroid hormone that has a higher affinity for the androgen receptor than testosterone in teleost fishes and is thus considered their primary androgen. Teleost fishes also produce two unique

progestogens often referred to as the maturation inducing steroids (MIS): 17,20b-dihydroxy-4-pregnen-3-one (DHP) and 17,20b,21- trihydroxy-4-pregnen-3-one (20βS) (Tokarz et al., 2015). These hormones are produced from the highly abundant progestogen 17-α-OH progesterone and play an important role in spermatocyte and oocyte maturation, the production of seminal fluid and ovulation (Lubzens et al., 2010; Nagahama and Yamashita, 2008; Scott et al., 2010). Both the MIS and tightly regulated concentrations of cortisol are required for proper egg maturation providing additional opportunity for stress to interfere with reproduction. While relationships between stressors and the progestogens are somewhat less clear than with androgens, stress induced increases in cortisol and 17-α-OH progesterone were reported in zebrafish in response to a stressor (Sedigh et al., 2019). Relationships between cortisol and estrogen are equally studied and like cortisol-androgen relationships, increases in cortisol have been shown to decrease circulating estradiol and its receptor (Faught and Vijayan, 2018). These impacts of chronic stress can then lead to more concerning effects such as reduced vitellogenin production in females (Campbell et al., 1994), reduced gamete quality in both sexes (Campbell et al., 1994; Valdebenito et al., 2013) and even adverse outcomes to progeny (Eriksen et al., 2007, 2006; Mccormick, 1998; Sloman, 2010; Stratholt et al., 1997).

Recent success with the use of fish scales as a medium for steroid hormone quantification in long-term stress assessment has been reported by several groups (Aerts et al., 2015; Carbajal et al., 2019b, 2019a, 2018; Culbert et al., 2021; Goikoetxea et al., 2021; Hanke et al., 2020, 2019; Kennedy and Janz, 2022; Laberge et al., 2019; Roque d'orbcastel et al., 2021; Samaras et al., 2021). This includes cortisol and now more recently cortisone and DHEA as was reported in our recent studies in goldfish and rainbow trout (Kennedy and Janz, 2022). In an effort to further expand this area of research the following study sought to incorporate both stress and

reproductive related steroids into long-term stress assessments using the fish scale. This was accomplished by artificially elevating circulating cortisol, progesterone and testosterone in adult rainbow trout using intraperitoneally injected hormone dissolved in coconut oil.

4.3 Methods

4.3.1 Preliminary hormone assays

Rainbow trout have relatively small scales and thus provide approximately 200 mg of dry scale per fish. This limits the number of hormones that can be quantified per fish. As our previous studies have indicated that scale glucocorticoid analysis generally requires 50 mg of powdered scale per hormone, we performed a preliminary analysis of five gonadal steroids to determine which hormones would be used in the present study. Table 1 outlines the amount of powdered scale required for the analysis of estradiol, 17α-OH progesterone, progesterone, testosterone and 11KT. As estradiol and 17α-OH progesterone require more than 100 mg of powdered scale for reliable analysis we chose to focus our study on cortisol, progesterone, testosterone and 11KT to best use the limited scale mass obtained from each fish.

Table 1. Mass of dry scale required for reliable quantification of hormone via ELISA

Hormone	Dry mass required (mg)
Estradiol	100+
17-α-OH progesterone	100+
Progesterone	50-100
Testosterone	50-100
11-Ketotestosterone	50-100

4.3.2 Treatment groups

A pool of 60 adult rainbow trout with an average mass of 306.5 +/- 76.1 g were divided into six treatment groups. The control group received no treatment, and all other groups received a weekly intraperitoneal injection of 5 µl of coconut oil per g of body mass with or without dissolved hormone for three weeks. The vehicle control group received untreated coconut oil, the cortisol injected group received coconut oil with 8 mg/ml of hydrocortisone, the progesterone and testosterone injected groups received coconut oil with 4 mg/ml of either hormone and finally the mix injected group received coconut oil with all three hormones at the previously mentioned concentrations. This resulted in a weekly dosage of 40 mg/kg of cortisol and/or 20 mg/kg of progesterone and testosterone. These dosages were chosen based on a previous study (Gamperl et al., 1994).

Each treatment group was held in one half of a 700 l rectangular tank at 12 °C separated by a divider and kept under a 14h light:10h dark schedule. Fish were fed each morning at 8 am and monitored to ensure food intake remained constant throughout the experiment. Ammonia, pH, chlorine, nitrite, and nitrate were monitored at least once per week and a 25% water change was performed each day.

4.3.3 Serum collection

Fish were sampled for blood on day 22 of the experiment, 7 days after the final coconut oil injection. Prior to blood collection trout were anesthetized two at a time using a solution of 100 mg/l of MS-222. A sample of blood was then collected from the caudal artery using a syringe, ejected into a 5 ml plastic tube and then left to clot for 3 hours at 4 °C. Next, blood samples were centrifuged to separate to allow the collection of serum which was transferred to a

1.5 ml plastic tube and stored at -20 °C until further analyses. All blood sampling was completed within 2 hours.

4.3.4 Scale collection

Cervical severance was used to euthanize anesthetized fish following blood collection and prior to scale collection. Trout were then wiped down to remove excess mucus and the entire body of scales were collected by scraping the length of the body towards the tail with a metal spatula. Scales were then transferred to 5 ml plastic tubes and stored at -20 °C for a maximum of 5 days.

4.3.5 Scale hormone extraction and quantitation

Scales from individual fish were analyzed for four hormones: cortisol, progesterone, testosterone and 11KT. Although fish were not directly treated with 11-ketotestosterone, this potent androgen in fishes arises from testosterone and was thus included to examine the relationship between these two hormones as well as the other injected steroids. Prior to hormone extraction fish scales were washed and ground as described in Kennedy and Janz 2022. Briefly, scales samples of approximately 200 mg were briefly washed three times with methanol. After each wash, methanol was decanted, scales were blotted dry and any visible debris (skin, etc.) was removed with forceps. Wash tubes were also rinsed with methanol between each wash and a fresh aliquot of methanol was used for each successive wash. Washed and dried scales were then ground using a Retsch ball mixer mill MM 400 until a fine powder was achieved.

To extract the desired hormone subsamples of 50 mg of powdered scale were transferred to a 1.5 ml microcentrifuge tube. The extraction process was the same for each hormone beginning with the addition of 1 ml of HPLC grade methanol/50 mg of sample, then vortexing briefly for 10-15 seconds. Samples were then placed in a rotator and left for 18 hours to extract.

Following this, samples were centrifuged for 15 minutes and the supernatant was collected and transferred into glass culture tubes. Next, the extracts were dried at 38 °C under a gentle stream of nitrogen. One ml of HPLC grade methanol was then added back to the powdered samples followed by a 40 second vortex, the tubes were then centrifuged, collected, and dried as above. These steps were repeated twice for a total of three collections. To concentrate the desired analyte at the bottom of the tube, the sides were rinsed 4 times with successively lower volumes of HPLC grade methanol. Between each rinse, extracts were dried at 38 °C under a gentle stream of nitrogen gas. Extracts were then reconstituted in 200-350 µl of extraction buffer supplied by their respective EIA kits: Cortisol EIA kit (Oxford Biomedical), Progesterone ELISA kit (Enzo), Testosterone ELISA kit (Enzo) and 11-Ketotestosterone ELISA kit (Cayman Chemical). Next, the sample tubes were gently vortexed and incubated for 12 hours at 4°C. After 12 hours, they were removed from the fridge, vortexed again, and the entire sample was transferred into a 0.6 ml microcentrifuge tube. Finally, the samples were centrifuged for 5 minutes to remove any remaining powdered scale. The supernatant was then collected and transferred to a clean 0.6 ml tube. Samples were run in triplicate following the kit protocols in a Molecular Devices Spectra Max 190 microplate spectrophotometer.

4.3.6 Serum hormone extraction and quantitation

Serum collected from individual fish was also analyzed for four hormones: cortisol, progesterone, testosterone and 11KT. A subsample of 100 µl of serum for each hormone was transferred to a glass test tube. One ml of diethyl ether was then added to the tube followed by a 40 second vortex. To allow the ether and aqueous phase to separate the tubes were then left to stand for five minutes. The ether layer was collected by flash freezing the tubes in liquid nitrogen for 7-10 s to allow the upper ether layer to be poured into a borosilicate glass test tube. The ether

was then evaporated under a gentle stream of nitrogen gas at 50 °C. Once the aqueous phase thawed the above steps were repeated for a total of three collections. The sides of the glass tube were then rinsed three times with decreasing volumes of ether (1ml > 0.4ml > 0.2ml), drying the tube in between each rinse. Finally, the sample was reconstituted in 250-350 µl of EIA buffer from the kit to be used for analysis, vortexed gently for 40 s and incubated in the fridge overnight. The following day the sample was vortexed again for 40 s, transferred to a 0.6 ml plastic tube and stored at -20 °C until analysis using the respective ELISA kit.

4.3.7 Assay validation

Intra- and inter-assay variation as well as parallelism were determined to ensure the accuracy, precision, and specificity of the hormone quantification methods. Extracts from multiple samples were pooled for intra-assay variation (n = 6) and inter-assay variation (n = 12), determined as the percent coefficient of variation (%CV, SD/mean). A concentrated sample was developed by collecting the extracts of multiple samples into a single glass tube to test parallelism by comparing the slopes of the standard curve and a serial dilution of the concentrated scale extract. Intra- and inter-assay variation for scale cortisol concentration was 7.9% and 11.2%, respectively, and 5.1% and 6.9% for serum cortisol, respectively. Intra- and inter-assay variation for scale progesterone concentration was 5.7% and 9.7%, respectively, and 6.4% and 14.7% for serum progesterone, respectively. For scale testosterone concentration, intra- and inter-assay variation was 9.4% and 10.5%, respectively, and 7.5% and 7.0% for serum testosterone, respectively. For scale 11KT concentration, intra- and inter-assay variation was 4.5% and 5.4%, respectively, and 6.0% and 9.6% for serum 11KT, respectively. Parallelism between extracted samples and the kit standard curve was determined using a serial dilution of the pooled extract run in triplicate. Parallelism was observed between all standard curves and

serially diluted extracts generated from both scale and serum samples. All validation steps were performed for all hormones. The limits of detection (LOD) for the cortisol, progesterone, testosterone and 11KT assays were 0.00510 ng ml^{-1}, 0.00104 ng ml^{-1}, 0.00602 ng ml^{-1} and 0.000615 ng ml^{-1} respectively. Any extract with a hormone concentration below the LOD was assigned the value of the LOD. This was the case for the testosterone concentration of three serum samples and three scale samples.

4.3.8 Statistical analyses

Prior to any statistical testing all data sets were tested for normality using the Shapiro-Wilk test as well as a visual inspection of the residuals. If parametric, comparisons of scale and blood hormone content collected from control and stressed groups were performed using a one-way ANOVA. If non-parametric, a Kruskal Wallis test was employed. Multiple comparisons were performed between each hormone injection group and both the control and vehicle control only. The control group was also compared to the vehicle control. Differences between groups were deemed significant at $p < 0.05$.

4.4 Results

4.4.1 Serum hormone concentrations

4.4.1.1 Cortisol

Serum cortisol concentrations were significantly elevated in the vehicle control, cortisol injected, progesterone injected and mix injected groups when compared to the control group (Figure 1A, $p < 0.05$). No other comparisons were statistically significant (Figure 1A, $p > 0.05$).

4.4.1.2 Progesterone

Serum progesterone concentrations were significantly elevated in the progesterone and mix injected groups when compared to both the control and vehicle control groups (Figure 1B, $p < 0.05$). No other comparisons were statistically significant (Figure 1B, $p > 0.05$).

4.4.1.3 Testosterone

Serum testosterone concentration was significantly elevated in the testosterone injected group when compared to both the control and vehicle control groups (Figure 1C, $p < 0.05$). No other comparisons were statistically significant (Figure 1C, $p > 0.05$).

4.4.1.4 11KT

Serum 11KT was significantly elevated in the testosterone and mix injected group when compared to both control and vehicle control groups (Figure 1D, $p < 0.05$). No other comparisons were statistically significant (Figure 1D, $p > 0.05$).

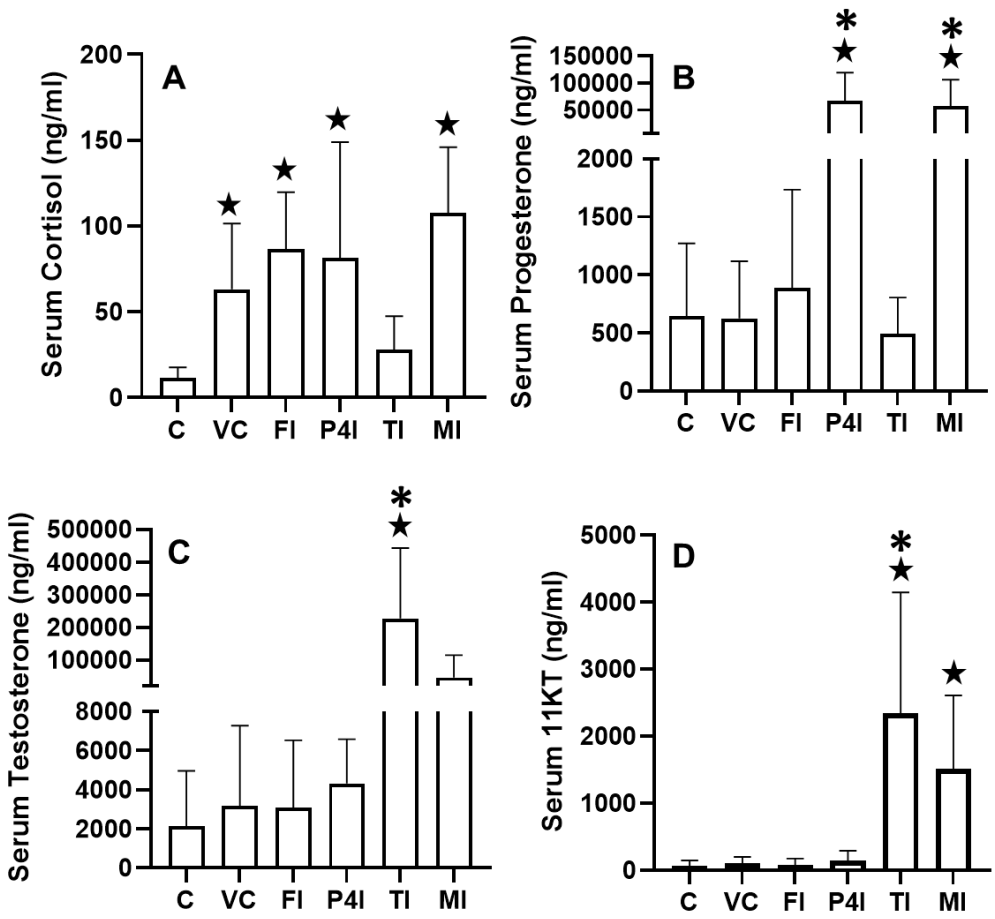

Figure 4.1 Serum hormone concentrations in control (C), vehicle control (VC), cortisol injected (FI), progesterone injected (P4I), testosterone injected (TI) and mix injected (MI) fish: (A) cortisol, (B) progesterone, (C) testosterone and (D) 11KT concentrations presented as the mean with error bars representing the standard deviation. All injected groups were compared to control and vehicle control only. Stars indicate significant difference from control, and asterisks indicate significant difference from vehicle control ($p < 0.05$; $n = 49$ fish).

4.4.2 Scale hormone concentrations

4.4.2.1 Cortisol

Scale cortisol concentration in the testosterone injected group was significantly lower than the vehicle control (Figure 2A, $p < 0.05$). Notably, the p-value obtained when comparing the scale cortisol concentration in the vehicle control to that of the progesterone injected group was near significant $p = 0.0576$. No other comparisons were statistically significant (Figure 2A, $p > 0.05$).

4.4.2.2 Progesterone

Scale progesterone concentration was significantly elevated in the mix injected group when compared to the control group (Figure 2B, $p < 0.05$). No other comparisons were statistically significant (Figure 2B, $p > 0.05$).

4.4.2.3. Testosterone

Scale testosterone concentrations were significantly lower in the cortisol and progesterone injected groups when compared to both the control and vehicle control groups (Figure 2C, $p < 0.05$). No other comparisons were statistically significant (Figure 2C, $p > 0.05$)

4.4.2.4. 11KT

Scale 11KT concentrations were significantly elevated in the progesterone, testosterone and mix injected groups when compared to the control group (Figure 2D, $p < 0.05$). Scale 11KT was also significantly elevated in the testosterone and mix injected groups when compared to the vehicle control group (Figure 2D, $p < 0.05$). The p-value generated when comparing the progesterone injected group to the vehicle control group also verged on significance ($p = 0.0711$). No other comparisons were statistically significant (Figure 2D, $p > 0.05$).

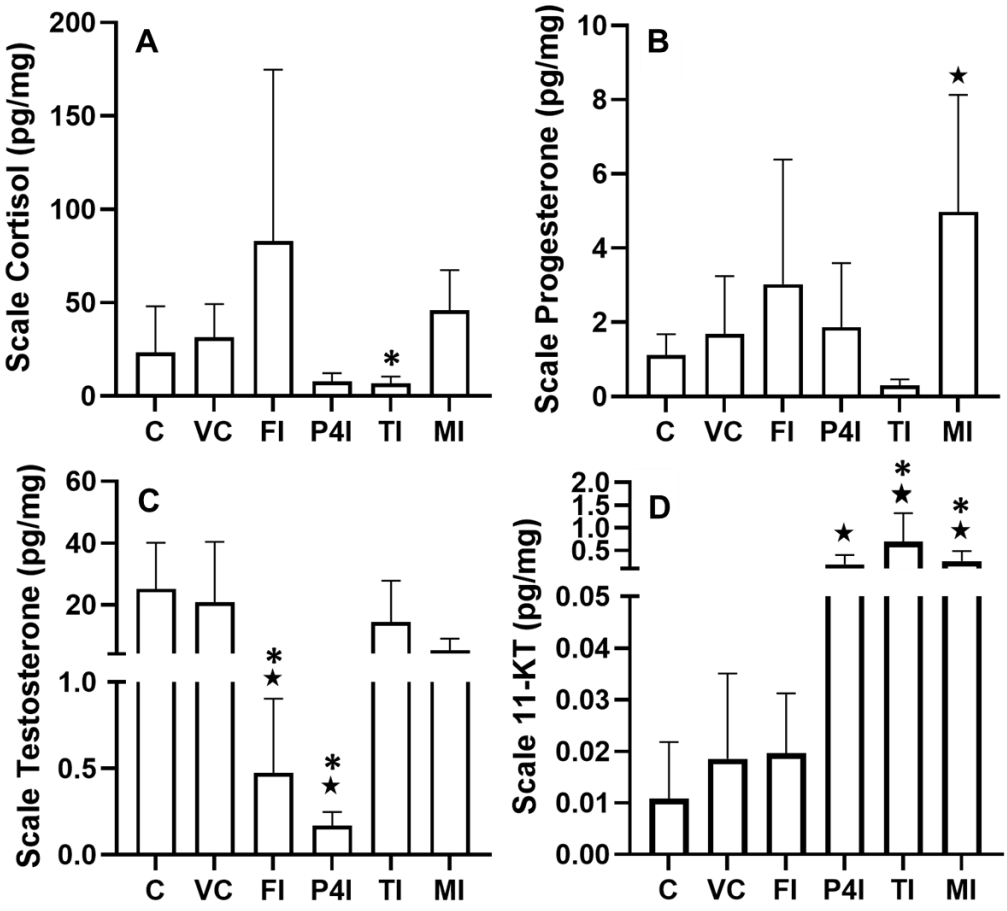

Figure 4.2 Scale hormone concentrations in control (C), vehicle control (VC), cortisol injected (FI), progesterone injected (P4I), testosterone injected (TI) and mix injected (MI) fish: (A) cortisol, (B) progesterone, (C) testosterone and (D) 11KT concentrations presented as the mean with error bars representing the standard deviation. All injected groups were compared to control and vehicle control only. Stars indicate significant difference from control, and asterisks indicate significant difference from vehicle control ($p < 0.05$; $n = 51$ fish).

4.5 Discussion

In combination with our previous studies, we have now quantified eight different steroid hormones within the fish scale (Kennedy and Janz, 2022). This confirms that like hair and feathers, scales incorporate and retain steroid hormones for a longer period than blood allowing them to serve as a biomonitoring medium in a wide variety of species and contexts. While estradiol and 17-α-OH progesterone were not used in this study due to the high scale mass required for their quantification, we recommend further investigation of these hormones in other studies and other species of fish as these hormones are particularly relevant to fish reproduction.

In all cases single-hormone injections were successful in significantly elevating serum hormone concentrations in comparison to controls. However, in addition to being elevated in the cortisol and mix injected groups when compared to the control group serum cortisol was significantly elevated in the vehicle control and progesterone injected group. As the vehicle control and cortisol, progesterone and mix injected groups all had significantly elevated serum cortisol it is likely that the injections acted as a stressor to the fish however, the duration of said stressor is difficult to determine. The serum cortisol elevations were not reflected in scale samples suggesting that the serum cortisol elevations are a result of a relatively short period of stress induced by the accumulation of coconut oil within the intraperitoneal cavity near the end of the experiment. As evidenced by the growing body of evidence pertaining to chronic stress and scale cortisol concentration, if the coconut oil injections had induced a state of chronic stress we would have seen significant elevations in scale cortisol as well (Aerts et al., 2015; Carbajal et al., 2019b, 2019a, 2018; Culbert et al., 2021; Goikoetxea et al., 2021; Hanke et al., 2020, 2019; Kennedy and Janz, 2022; Laberge et al., 2019; Roque d'orbcastel et al., 2021; Samaras et al.,

2021). This also supports the notion that scale cortisol concentration is relatively unaffected by acute stressors as demonstrated by Laberge et al. 2019.

While serum cortisol concentration was significantly elevated in almost all groups this effect was not observed in the testosterone injected group likely due to previously mentioned negative interactions often observed between cortisol and testosterone. For example Pottinger et al. 1996 found that testosterone impregnated cocoa butter injections resulted in a reduced cortisol response after a 1 hour confinement stress in rainbow trout. This interaction was also apparent in scales as scale cortisol was significantly lower than vehicle controls in the testosterone injected group suggesting that scale cortisol and testosterone relationships will play an important role in monitoring chronic stress and reproduction in fishes as it does in mammals (Sollberger and Ehlert, 2016).

Interestingly, although the change only bordered on significance a suppression in scale cortisol was also produced by progesterone injections. Although stress and reproduction are generally considered competitive processes, effects on scale cortisol produced by progesterone injections in this study are more difficult to explain. As progesterone acts as a precursor for most steroid hormones an increase in scale cortisol upon progesterone injection could be expected (Rajakumar and Senthilkumaran, 2020). Cortisol regulates its own production at all steps along the HPI axis and even at the level of 11β-HSD2, as reported by Alderman and Vijayan 2012 who discovered glucocorticoid response elements in the promotor of the 11β-HSD2 gene. Increased transcription of the 11β-HSD2 gene and thus the conversion of cortisol to cortisone could therefore be behind our observed decreases in scale cortisol in the progesterone injected group. Other possible means of 11β-HSD2 increases could come from the relationship between cortisol and the MIS. As previously mentioned, progesterone can be converted to one of two MIS:

17,20b-dihydroxy-4-pregnen-3-one (DHP) or 17,20b,21- trihydroxy-4-pregnen-3-one (20βS) via the highly abundant intermediate 17-α-OH progesterone (Tokarz et al., 2015). In rainbow trout, cortisol and DHP have both been shown to be involved in oogenesis (Faught and Vijayan, 2018; Milla et al., 2009, 2006). As cortisol's effects on reproductive processes such as oogenesis can become negative, concentrations of cortisol are highly regulated during this period (Faught and Vijayan, 2018). Upregulation of 11β-HSD2 during reproduction has thus been proposed as a means of protecting the ovaries from damaging effects of increased cortisol. This suppression of scale cortisol induced by progesterone injection observed here could therefore be a result of 11β-HSD2 upregulation induced by progestogens.

As was intended, progesterone injections were successful in elevating serum progesterone in both the progesterone injected and mix injected groups when compared to the controls. However, this was not wholly reflected in scales as scale progesterone was only significantly elevated in the mix injected group. As outlined above this could be a result of progesterone's conversion to other steroid hormones such as cortisol or DHP prior to incorporation into the scale. Notably, while progesterone injections resulted in a decrease in scale cortisol, cortisol injections produced the opposite effect and resulted in a three-fold increase in scale progesterone when compared to controls. While this increase was not statistically significant, stress-induced cortisol increases have been shown to result in increased 17-α-OH progesterone in zebrafish suggesting a possible positive correlation between cortisol and progestogens (Sedigh et al., 2019). This could also contribute to the significantly elevated scale progesterone observed in the mix group as cortisol was injected alongside progesterone and testosterone in this treatment. Other notable changes in scale progesterone include the visible although not significant decrease in the testosterone injected group. Specific inhibitory effects of

testosterone on progesterone production are unlikely however, as all steroid hormones arise from cholesterol, an increase in any steroid hormone could provide negative feedback that results in a decrease in the conversion of cholesterol to pregnenolone and thus the production of other steroids (Yaron and Levavi-Sivan, 2011).

Like the other injected groups, testosterone injections were successful in elevating serum testosterone in the testosterone injected group when compared to the controls. This however was not the case for the mix injected group likely due to the previously outlined negative interactions between cortisol and testosterone. By contrast, testosterone concentrations measured in the scale showed largely different results with scale testosterone in both the testosterone and mix injected groups not significantly elevated in comparison to controls. Testosterone acts as a precursor for 11KT, which is considered to be the primary androgen in teleost fishes. Therefore, these results are likely a consequence of testosterone conversion to 11KT. This is supported by both the serum and scale 11KT data which showed significant or near significant elevations in the testosterone and mix injected groups when compared to controls. This could also relate to the previously discussed potential increase in 11β-HSD 2 triggered by DHP as 11KT formation from testosterone involves this same enzyme (Rajakumar and Senthilkumaran, 2020). In addition, the progesterone injected group showed significantly elevated scale 11KT suggesting that progesterone is also acting as a precursor for 11KT. If the injected progesterone was converted to 11KT this would also explain the near significant decrease in scale cortisol in the progesterone injected group. This could also aid in explaining the negative testosterone-progesterone interactions observed in both the testosterone and progesterone injected groups, however this likely involves complex interactions and feedback loops along to steroidogenic pathway unable to be discerned by the data collected in this study.

As has been previously outlined, in order for these methods to be successful in diagnosing physiological change, a better understanding of blood-scale partitioning is required. In most cases, treatment groups that were injected with a particular hormone showed significantly elevated circulating concentrations of said hormone confirming that the hormone injections were successful. This however did not produce consistent elevations in the scale concentrations of the same hormone. While further investigation is required, scale cortisol and 11KT appear to provide a means of assessing chronic stress and related effects on reproduction. Although progesterone was quantifiable within the scale, interactions between progesterone and the other steroid hormones were less clear. Relationships between circulating and scale progesterone were also less discernable as progesterone appears to have been converted to 11KT prior to scale incorporation. However, these effects may have resulted from the artificial elevation of our injected hormones and thus these concepts need further exploration in natural settings.

4.6 Conclusions

This study introduced three new steroid hormones to be utilized in long-term stress assessments that incorporate fish scales as a sample media. While fish scale cortisol concentration shows promise as a chronic stress monitoring tool these additional hormones now allow for an extended evaluation of chronic stress that includes alteration to reproductive endocrinology. Although these concepts require further investigation, their implementation as a tool for the biomonitoring of fishes has the potential to contribute to conservation efforts.

CHAPTER 5

5.0 GENERAL DISCUSSION

5.1. Summary of Objectives

Scales are suitable for biomonitoring in fishes as they (1) accumulate and store steroid hormones for longer periods of time than blood, (2) are less susceptible to acute hormonal fluctuations than blood, and (3) can be collected non-lethally. Studies conducted in a variety of different species have demonstrated that fish scale cortisol is increased in response to different forms of long-term stress. This includes stressors relevant to wild populations such as increased water temperatures as well as those related to captive populations like overstocking and fin injuries incurred in flow-through systems. Thus, scales appear to provide a promising medium for conservation focused biomonitoring regimes in teleost fishes; however, this area of research has only just commenced.

The objective of my research was to expand on these methods and explore whether there is more information to be gleaned from the fish scale. Many more hormones in addition to cortisol can be quantified in hair and feathers, warranting similar investigations in scales. I therefore sought to quantify cortisol, cortisone, DHEA, progesterone, 17-α-OH progesterone, estradiol, testosterone and 11KT to determine which hormones would be most practical for biomonitoring in teleost fishes. In the case of 17-α-OH progesterone and estradiol, concentrations in fish scales were too low for reliable scale analysis with a reasonable amount of dry powdered scale. These hormones were thus excluded which led to three experiments in two different species of fish and the quantification of a total of six steroid hormones. My first experiment was a pilot scale experiment conducted in goldfish with a goal of validating sample processing methods and conducting a primary investigation on the use of two new hormones,

cortisone, and DHEA alongside cortisol in chronic stress assessment in fishes. My second experiment expanded on these concepts and further investigated the use of the scale concentrations of these three hormones as bioindicators of chronic stress as well as the use of the cortisol:DHEA and cortisone:cortisol ratios. Finally, my last experiment incorporated both stress and reproduction-related hormones to provide a means of monitoring both long-term stress and the downstream effects of stressors on reproduction. This was accomplished by artificially elevating circulating cortisol, progesterone and/or testosterone to observe the relationships between these hormones as well as their partitioning from blood to scale.

5.2 Overall findings and limitations

5.2.1 Experiment 1

While the first experiment of this research was a pilot scale study it provided crucial information for the following two experiments. This study determined that three washes with methanol was sufficient in the removal of external hormone from the fish scale, a key step in scale hormone analysis. This study also determined that approximately 50 mg of powdered scale was required to create a hormone containing extract sufficiently concentrated to fall within the linear portion of the standard curve created with each hormone ELISA kit. This is essential to ensure the accurate quantitation of each hormone. Results from this study demonstrated that long term stress resulted in significantly elevated serum cortisol and cortisone in goldfish but that there is likely a lag in the transfer of these hormones from blood to scale as has been previously suggested. Notably, this was the first ever quantification of scale DHEA and cortisone as well as the scale cortisol:DHEA ratio. The utilization of the cortisol:DHEA ratio in this study was then able to demonstrate the power of hormone ratios in amplifying differences between control and stressed organisms.

The fish used in this study were small goldfish donated by the college of veterinary medicine that would have otherwise been euthanized and unused. In accordance with the three R's of humane animal experimentation and animal conservation as is the overall goal of this research we utilized these goldfish in our experiment. Unfortunately, the goldfish were not sufficiently large to provide sufficient scale mass for the quantification of all three hormones. The pooling of scales from multiple fish and the prioritizing of certain hormones was thus necessary in order to meet these requirements. This limited the amount of information gleaned from this study as it reduced our sample size as well as our statistical power. This was particularly true for cortisone analyses as we prioritized cortisol and DHEA in order to generate a cortisol:DHEA ratio as this value is more commonly used in chronic stress analyses than our other two multi-hormone values: cortisol + cortisone and the cortisone:cortisol ratio. This also limited our ability to correlate blood and scale hormone concentrations as matching cortisone concentrations could not be calculated in both media. This was additionally limited by our inability to detect DHEA or DHEA-S in serum samples so blood-scale correlations were not examined. Thus, while this experiment was limited in its ability to generate concrete conclusions due to the small sample size and missing scale cortisone and serum DHEA data it provided key information for the following experiments included in my research.

5.2.2 Experiment 2

The second experiment of this research was intended to expand on the first experiment and further explore scale cortisol, cortisone, and DHEA in the assessment of chronic stress in teleost fishes. Using the methods developed in experiment 1, I quantified scale cortisol, cortisone and DHEA in adult rainbow trout, a much larger-bodied species of fish. This allowed me to quantify all three hormones in individual fish as well as generate three multi-hormone values:

cortisol + cortisone, cortisol:DHEA and cortisone:cortisol. Findings from this study emphasize that stress responsiveness differs interspecifically as we found that the same long term stress protocol that elevated serum cortisol and cortisone in goldfish did not do so in rainbow trout. Again in contrast to the goldfish experiment, scale concentrations of all hormones and all multi-hormone values were significantly elevated in stressed fish with the exception of the cortisol:DHEA ratio. Contrary to the goldfish experiment and to what would be expected to occur with chronic stress, this value was significantly lower in stressed trout further emphasizing interspecies differences and the importance of including multiple metrics of stress assessment. This study also examined blood-scale correlations for cortisol and cortisone but found no significant relationships.

Like the goldfish experiment this experiment was somewhat limited by available scale mass as some rainbow trout had experienced significant scale loss and thus did not have enough scales for the analysis of all three hormones. Whether this was a result of the experiment or previous housing conditions cannot be confirmed however it is unlikely that the stressing protocol used in this experiment caused significant scale loss. Husbandry effects potentially provided additional uncertainty to this experiment as fungal infections were highly common in this population of rainbow trout. This did not appear to correlate with any hormonal alterations in either serum or scale but this was not examined in depth.

In contrast to typical cortisol:DHEA ratio trends in mammalian studies with regards to chronic stress, the scale cortisol:DHEA ratio was significantly decreased in stressed trout in this experiment. This was driven in part by the significantly elevated scale DHEA, another finding that opposes general trends in the mammalian literature. DHEA has been shown to negate some of the adverse effects of cortisol and is therefore thought to act in the mitigation of stress in

humans and other mammals. A reduction in DHEA in response to chronic stress would thus be expected, suggesting a different role for DHEA in this species and perhaps in teleost fish in general. However, I was limited in my ability to make concrete conclusions surrounding this finding as research into the role of DHEA in fishes is currently lacking. Akin to the goldfish experiment I was also unable to detect DHEA in serum samples further limiting the information available with regards to DHEA and chronic stress in these species. Nevertheless, the cortisone:cortisol ratio was significantly elevated in stressed trout suggesting an increase in cortisol metabolism which has been correlated with increases in stress in rainbow trout. Unfortunately, this value is much less studied than the cortisol:DHEA ratio and thus interspecies comparisons are difficult to generate.

In order to correlate scale hormone concentrations to actual HPI axis activity an understanding of the partitioning of hormone from blood to scale is required. In mammals, the hair follicle possesses the ability to generate local cortisol in addition to that secreted by the adrenal gland. This has raised concern regarding the preferential deposition of locally generated cortisol within the hair shaft that would interfere with correlations between hair cortisol and true HPA axis activity. In an effort to examine these relationships in scales I calculated serum-scale correlations for cortisol and cortisone. As I could not detect DHEA or DHEA-S in serum, similar correlations could not be made for this hormone. I found no significant correlations between serum and scale for either cortisol or cortisone; however, as has been previously outlined blood and scale steroid concentrations are very different with regards to the amount of time they represent. As scales incorporate hormones over longer periods of time correlations between scales and multiple blood samples collected over the duration of the experiment would have been more appropriate. This would likely have demonstrated an initial rise in serum cortisol and

cortisone and then a gradual decline resulting in the unelevated serum cortisol and cortisone concentrations reported in the stressed trout collected on day 14 of this experiment. The average serum cortisol and cortisone concentrations from the multiple samples would also likely show better correlations with scale concentrations.

5.2.3 Experiment 3

Akin to the first two experiments the final experiment of this research sought to explore relationships between multiple scale hormones and in doing so expand upon the use of fish scale hormone concentrations as a biomonitoring tool. This again included cortisol but in place of cortisone and DHEA, scale concentrations of progesterone, testosterone and 11KT were also quantified. This was done to evaluate the applicability of these methods in the assessment of reproductive alterations as well chronic stress.

One possible complication with this experiment involves the intraperitoneal injections with hormone impregnated coconut oil used to artificially elevated circulating hormone concentrations. While similar methods are commonly used for such purposes, repeated injections over the three-week period resulted in a noticeable build-up of coconut oil within the peritoneal cavity of the trout upon final dissections. While the scale cortisol data suggests that this did not act as a chronic stressor to the fish, significantly elevated serum cortisol in three of four injected groups suggests that the oil build-up may have resulted in a short-term stressor near the end of the experiment. Based on a study by Laberge et al. 2019 this short-term stressor is unlikely to have affected scale cortisol concentrations; however, the effects on the scale concentrations of the other three hormones are unknown. At what point this oil build-up presented as a stressor to the fish is also impossible to identify and thus the duration of the stressor cannot be confirmed.

Additional uncertainty with regards to the hormone concentration of the scale comes from the two possible sources of hormone: injected and biologically produced. Hormone quantification in this experiment was done via ELISA. While this method of hormone quantification is extremely sensitive and likely best suited for this type of study, the use of liquid chromatography mass spectrometry (LCMS) for such purposes could allow for hormone source identification via mass labelling. Injection with mass labeled hormones would provide information on the dispersion of the hormone from the coconut oil injection into the blood and then the scale. This could also aid in determining hormone metabolism prior to scale incorporation for example the conversion of testosterone and progesterone to 11KT as was observed in this study. A study by Kapoor et al. 2018 accomplished similar goals by injecting rhesus monkeys with radio-labelled cortisol and using this label to examine cortisol's distribution. This included the amount excreted in urine and feces, contaminating the outside of the hair shaft and the concentration of a variety of cortisol metabolites present in the hair shaft.

5.3 Suggestions for future research

Research surrounding the use of non-lethal biomonitoring for animal conservation is well underway; however, the use of fish scale hormone concentrations for such purposes has only recently commenced. Therefore, in order for fish scale hormone concentrations to serve as a biomonitoring tool there are many knowledge gaps that must first be filled. While this includes many aspects of scale hormone research the previously described studies have emphasized three major areas in need of additional investigation: methods for scale processing, blood-scale relationships and real-world applications.

5.3.1 Methods for scale processing

As scale hormone analysis has only recently been explored as a means of non-lethal biomonitoring, methods for scale processing prior to hormone extraction still require further validation. For example, as has been demonstrated by (Laberge et al., 2019) there is spatial heterogeneity with regards to the cortisol content of scales sampled from different regions of the fish. This is irrelevant if whole body scales are being removed and homogenized as was done in this research; however, if these methods are to be implemented in non-lethal biomonitoring regimes a standardized location for scale sampling should be outlined. Further investigation identifying whether these spatial patterns exist in other species as well as the driving factors behind this patterns is also of extreme importance. Following scale collection scales must be stored prior to further analyses. Current methods of storage vary among groups including immediate flash freezing and storage at - 80 °C as well as storage at - 20 °C and 4 °C. However, whether method and duration of storage has any effect on final hormone concentrations still needs to be determined. The next key step in scale processing is the removal of surface hormones and other contaminants. In our experiments this was done by three short washes with methanol however, this is not standard practice across groups. Currently, there are three solvents being used to wash scales prior to further analyses: ultrapure water, isopropanol and methanol. The ideal wash solvent will solubilize steroid hormones allowing for their removal but not penetrate the scale to a degree that results in the leaching of internal hormone during the short wash periods. (Carbajal et al., 2018) compared ultrapure water and isopropanol to determine which solvent was more appropriate for scale washing and suggested that water may be leaching internal cortisol from the scale and that isopropanol may not be sufficient in the removal of external cortisol. Our first experiment then demonstrated that methanol is effective in removing

surface contamination but a comparison between all three solvents is crucial in standardizing these methods across groups. The final step that may require further investigation with regards to best practices in scale sampling processing is reducing the size of the scales prior to hormone extraction in order to increase surface area. In my experiments I used a Retsch 400 mixer mill to grind scales to a powder, yet some groups mince the scales with scissors. While (Laberge et al., 2019) has suggested that this step may be unnecessary as they found no difference between the amount of hormone extracted from minced versus intact scales, the inclusion of ground scales to such comparisons is still required as this substantial increase in surface area is likely to increase extraction efficiency.

5.3.2 Blood-scale relationships

In order for scale hormone concentrations to serve as a means of assessing HPI and HPG activity a better understanding of the partitioning of these hormones from blood to scale is required. Following secretion steroid hormones likely enter the scale via passive diffusion as is the case in hair. As steroid hormones are relatively non-polar compounds this should result in a partitioning from blood to scale in proportional concentrations. However, there is concern for the preferential deposition of locally generated hormones as fish skin expresses many steroid metabolizing enzymes such as 11β-HSD 2. Slight differences in chemical properties may also lead to different degrees of incorporation into the scale. As was demonstrated in my third experiment the conversion of one hormone to another prior to its incorporation into the scale is also a possibility adding another degree of uncertainty to these relationships. However, the possibility that steroid hormones are being actively incorporated into the scale is also important to consider. The estrogen receptor has been identified within fish scales as estrogen signaling is key in calcium homeostasis in fishes suggesting that active estrogen incorporation into the scale

could be occurring (Pinto et al., 2009). Still, all steroid hormones arise from cholesterol their compositions bear considerable likeness. Cholesterol is a component of cell membranes and thus the possibility that steroid hormones are acting as cholesterol mimics and being actively incorporated into the scale is a possibility. Therefore, in order to confirm that the concentrations and ratios of hormones within the scale reflect true HPI and HPG activity further research in this area is necessary.

Equally important as scale hormone incorporation is scale hormone removal. Elevated scale hormone concentrations have been shown to remain significantly elevated for up to 9 days and fully return to baseline after approximately four weeks upon cessation of the stressor (Laberge et al., 2019). Unlike hair which appears to permanently incorporate circulating hormones allowing for it to be sectioned to provide a log of past stressors, scale hormone concentration is more dynamic. Whether this is the result of a redistribution to surrounding tissues, a leaching into the surrounding water or breakdown within the scale is unclear. Steps to be taken in answering this question could involve the collection of multiple blood samples to be compared to scales during a chronic stressing protocol to better examine blood-scale correlations. This could also include the detection of hormones as well as their metabolites within the scale following artificial elevation of said hormone in the blood or analyzing the timeline of scale and water hormone concentrations when left in water for an extended period of time.

5.3.3 Real world applications

While the use of scale cortisol concentration as an indicator of chronic stress has been shown to be relevant to a variety of real-world stressors such as elevated water temperature or an increased incidence of fin injuries, our eventual goal in the development of these methods is the

non-lethal biomonitoring of wild populations. Some studies have indeed been performed in wild-caught species however, none of said studies were carried out non-lethally (Carbajal et al., 2019b; Culbert et al., 2021; Roque d'orbcastel et al., 2021). In addition, while some groups have successfully collected scales non-lethally these studies were performed in-lab (Aerts et al., 2015; Hanke et al., 2019). Thus, in order to facilitate these biomonitoring regimes there are many knowledge gaps that must be filled. This would include a development of baseline scale concentrations for a variety of steroid hormones in a variety of species in order to facilitate future comparisons. This would also include developing a better understanding of how scale cortisol varies with life-stage and sex to ensure that differences detected between populations can be attributed to a stressor and not simply a difference in age or sex ratios within the population. Furthermore, this would involve an investigation into the feasibility of scale sampling as a non-lethal practice and a standardization of such methods. As previously mentioned, this has been accomplished in-lab however, captive fish encounter vastly different stressors and challenges than wild populations. Scales are shed to some degree throughout a fish's life, but the quantity required for reliable scale hormone analysis is likely much greater than that which is naturally shed. Scales provide protection from both physical and biological threats to fish health and thus their removal could have direct effects on survival. Nevertheless, the small wound induced upon scale removal has been shown to heal quite rapidly limiting the opportunity for infection (Sire, 1989). A study that induced similar small wounds via biopsy methods also demonstrated that this did not affect fish survival for up to a year post-sampling (Baker et al., 2004). Regardless, these methods require further validation to ensure that non-lethal biomonitoring regimes are in fact plausible.

In addition to cortisol the previously described experiments quantified cortisone, DHEA, progesterone, testosterone and 11KT. This new suite of hormones allows not only for the assessment of chronic stress but also some of the associated effects on reproduction. Even further, the addition of gonadal steroids could allow for the assessment of the effects of endocrine disrupting compounds in fishes. We therefore suggest that scale hormone concentrations be further analyzed with regards to their potential as biomonitoring tools in as many contexts as possible. This could include chemical stressors such as crude oil or pesticides or physical stressors such as increased noise and activity on increasingly impacted bodies of water. Temperature increases, which have already been shown to elevate scale cortisol concentrations in juvenile milkfish could also be further explored particularly as this relates to sex ratios in some populations. For example, the Japanese flounder *(Paralichthys olivaceus)* if reared at 18 °C will produce female offspring however if reared at 27 °C the offspring will be male (Yamaguchi et al., 2010). In this same study cortisol was shown to induce female to male sex reversal (Yamaguchi et al., 2010). Lastly, we also recommend further investigation into scale estradiol and 17-α-OH progesterone. While these hormones were not easily quantified via my methods in our species of choice these hormones are highly relevant to fish reproduction and thus warrant inclusion in biomonitoring efforts if possible.

5.4 Final conclusions

As human populations continue to expand the conservation of aquatic species becomes increasingly important. In order to accomplish this goal biomonitoring regimes that are in themselves as non-destructive as possible are required. While other biomonitoring strategies do exist, scale hormone concentrations provide a unique means of evaluating long-term conditions that other sample media are not able to capture. The addition of multiple hormones now allows

the scale to provide information not only related to chronic stress but also to reproductive function. Although there are many knowledge gaps left to be filled, the results presented here suggest fish scale hormone concentrations provide a promising biomonitoring tool in both wild and captive teleost fishes.

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