The development of endogenous cortisol production and chromaffin cells in larval Lake Sturgeon (*Acipenser fulvescens*)

By

Madison Earhart

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

In partial fulfilment of the requirements of the degree of

Master of Science

Department of Biological Sciences
University of Manitoba
Winnipeg, MB

Copyright © 2018 by Madison Earhart
Abstract

The development of the HPI axis has long lasting effects on behavior, fitness and ultimately survival in teleost fish. Endogenous cortisol production was measured in larval Lake Sturgeon in 2016 to determine the proper development of the hypothalamic-pituitary-interrenal axis. Following the results of 2016, in 2017 baseline cortisol levels were measured again along with stress-induced cortisol levels and metyrapone-treated fish. In 2016 the larvae had a significant increase (P-value=<0.0001) in baseline cortisol prior to exogenous feeding and during the transition from artemia to bloodworm. In 2017 larvae also had an increase (P-value=<0.0001) in baseline levels prior to feeding and metyrapone-treated larvae with lower cortisol never fully transitioned and had developmental abnormalities. These studies indicate a relationship between endogenous cortisol production and dietary shifts. Also in 2017, larvae were sampled for development of chromaffin cells through immunohistochemistry, specifically a tyrosine-hydroxylase (TH) antibody. The TH antibody was reactive at 13 DPF, five days following hatch. Both cortisol production and chromaffin cell proliferation showed a delay in development, only appearing after hatch, suggesting both these important endocrine components to stress undergo a hypo-responsive period during early development in the Lake Sturgeon.
Acknowledgements

I would like to acknowledge my supervisor Dr. Gary Anderson for his support throughout the last few years. The completion of this project would not have been possible without his guidance patience and instruction. I would also like to thank my committee members, Dr. D. Weihrauch, Dr. M. Hanson and Dr. K. Jeffries who gave invaluable insight and direction during my project. I would also like to thank Dr. E. Huebner for his time and allowing use of personal lab equipment. This project would not have been possible without the North-South and Manitoba Hydro team as well as the animal-holding staff at the University of Manitoba.

I would further like to acknowledge the University of Manitoba, NSERC and Manitoba Hydro for the necessary funding to complete the project. Last but not least I would like to acknowledge my family and friends who without their support this would also have not been possible.
# Table of Contents

| List of Figures | 4 |
| List of Tables | 5 |
| Chapter 1: Introduction | 6 |
| 1.1 Introduction to stress | 8 |
| 1.2 Chromaffin Cells | 10 |
| 1.3 Hypothalamic—pituitary—inter-renal axis | 11 |
| 1.4 Development of endogenous cortisol production | 18 |
| Chapter 2: Development of endogenous cortisol production | 28 |
| 2.1 Introduction | 28 |
| 2.2 Methods | 30 |
| 2.2.1 Fish Husbandry | 30 |
| 2.2.2 Sampling for whole body cortisol levels 2016 | 31 |
| 2.2.3 Sampling for whole body cortisol levels 2017 | 32 |
| 2.2.4 Sampling for gene expression | 33 |
| 2.2.5 Cortisol extraction and measurement 2016/2017 | 33 |
| 2.2.6 RNA Extraction | 34 |
| 2.2.7 cDNA Synthesis | 35 |
| 2.2.8 qPCR | 35 |
| 2.2.9 Whole body protein, glucose and triglycerides | 37 |
| 2.2.10 Statistical analysis | 37 |
| 2.3 Results | 38 |
| 2.3.1 Whole body baseline cortisol 2016 | 38 |
| 2.3.2 Whole body baseline cortisol 2017 | 38 |
| 2.3.3 Metyrapone treatment and Whole body measurements | 39 |
| 2.3.4 Molecular expression | 40 |
| 2.4 Discussion | 48 |
| Chapter 3: Development of chromaffin cells | 60 |
| 3.1 Introduction | 60 |
| 3.2 Methods | 63 |
| 3.2.1 Fish Husbandry | 63 |
| 3.2.2 Sampling for Chromaffin Cells | 63 |
| 3.2.3 Tissue processing | 63 |
| 3.2.4 Slide staining | 64 |
| 3.2.5 Imaging | 65 |
| 3.3 Results | 65 |
| 3.3.1 Appearance of tyrosine-hydroxylase | 65 |
| 3.3.3 Positive and negative controls | 66 |
| 3.4 Discussion | 80 |
| Chapter 4: Conclusion | 90 |
List of Figures

Figure 2.1 Timeline of development 41
Figure 2.2 Whole body cortisol 2016 42
Figure 2.3 Whole body cortisol 2017 43
Figure 2.4 Wet mass of control vs. metyrapone treated larvae 44
Figure 2.5 Total body protein of control vs. metyrapone treated larvae 45
Figure 2.6 Total body triglycerides of control larvae 46
Figure 2.7 Total body glucose control vs. metyrapone larvae 47
Figure 2.8 StAR relative fold change shown on line graph 48
Figure 3.1 Micrograph of 8 DPF (hatch day) Lake Sturgeon trunk 67
Figure 3.2 (A) Micrograph of 8 DPF (hatch day) Lake Sturgeon trunk at 4x. (B) 25x 68
Figure 3.3 Micrographs of 10 DPF Lake Sturgeon larvae. 69
Figure 3.4 Micrograph of 13 DPF Lake Sturgeon larvae, first day of positive TH staining 70
Figure 3.5 Micrograph of 18 DPF Lake Sturgeon larvae, anterior end 71
Figure 3.6 Micrograph of 18 DPF Lake Sturgeon larvae 72
Figure 3.7 Micrograph of 18 DPF Lake Sturgeon larvae, length of kidney 73
Figure 3.8 Micrograph of posterior trunk in 3-month-old Lake Sturgeon 74
Figure 3.9 Micrograph of mid-trunk in 3-month-old Lake Sturgeon 75
Figure 3.10 Micrograph of 3-month-old Lake Sturgeon trunk 76
Figure 3.11 Micrograph of 1-year old Lake Sturgeon kidney 77
Figure 3.12 Micrograph of control staining in 1-year old Lake Sturgeon trunk 78
Figure 3.13 Micrograph of 1-year old Lake Sturgeon; negative and positive controls 79
Figure 3.14 Micrograph of 3 positive control in 3-year old Lake Sturgeon 80
Figure 3.15 Kidney shape and posterior cardinal vein in various species 84
List of Tables

2.1 Efficiencies and sequences for primers 37

2.2 Cortisol levels in various species of sturgeon and teleosts during early development 50
Chapter 1: Introduction

Lake Sturgeon, *Acipenser fulvescens*, is listed by IUCN as threatened to critically endangered throughout North America where populations used to be abundant (IUCN red list 2018; Ferguson and Duckworth 1997; Auer 1996; 1999; 2002; 2004). This ancient fish evolved approximately 200 million years ago and is endemic to mesotrophic and oligotrophic freshwater systems (Peterson et al. 2007). Of the three freshwater sturgeon species, the Lake Sturgeon has the largest distribution, living in the Mississippi River, Great Lakes, and Hudson Bay drainage basins. Throughout the past century, Lake Sturgeon populations have plummeted due to extreme overfishing in the early 1900s and more recently, the building of hydroelectric dams disrupting habitat continuity (Auer 1996, 2002; Wilson and McKinley 2004; Peterson et al. 2007). To combat this sharp decline in population numbers, restoration efforts have become widespread throughout the United States and Canada (Peterson et al. 2007). Conservation hatcheries have been releasing both hatchery-raised fry (fall stocked) and fingerling (spring stocked) young-of-the-year (YOY) Lake Sturgeon into their historic range with the goal of restoring historic population numbers but with limited knowledge on the early development of this species (Secor et al. 2002; Barth et al. 2015).

Besides the anthropogenic influences listed above, Lake Sturgeon are at a higher risk of extinction due to slow growth, intermittent spawning, and late maturation usually reaching full maturation at ages 12-15 for males and 18-27 for females (Bruch 1999; Harkness and Dymond 1961). Lake Sturgeon have a large home range including a variety of habitats for nursery, spawning, and growth. They are exclusively bottom feeders, typically preferring shallow lakes and rivers with an abundance of macroinvertebrates such as mayfly nymphs, clams, crayfish,
caddisflies, and chironomid larvae (Baker 1980). Juvenile Lake Sturgeon have been found to prefer sand and clay substrates where food is abundant (Peake 1999). However, different populations and individuals utilize different substrate types depending on developmental stage as reported by Smith and King (2005) where they showed yearling sturgeon to prefer sand substrate and juvenile (> 1 year-old) Sturgeon preferring organic substrate.

Spawning location is system specific, however, fast current to provide large amounts of clean substrate (Baker 1980) such as coarse gravel or cobble (Auer 1996; McKinley et. al 1998) are typical features. Spawning begins in the spring with females broadcast spawning in a variety of water depths and if fertilized, eggs will adhere to substrate (Auer and Baker 2002; Becker 1983). Lake Sturgeon are considered litho-pelagophil because their eggs require substrate to adhere to (Harkness and Dymond 1961). Therefore, substrate type, water flow, temperature, and depth are all critical to egg viability. Harkness and Dymond (1961) observed Lake Sturgeon hatching on the Wolf and Fox rivers at water temperatures between 15-17.5°C, 5-8 days post fertilization and the newly hatched fry were approximately 8 mm in total length. As all energy is allocated to somatic growth and not reproduction, growth is very rapid in the first 10 years of life (Beamish et. al 1996; Baker 1980). That said, fecundity in Lake Sturgeon is related to size such that larger females tend to produce more eggs (Harkness and Dymond 1961; Peterson et. al 2007). Further, natural mortality may be a function of growth and size as by the end of the second growing season, the average length of a Lake Sturgeon is approximately 30.5(+-) centimeters making them too large for most predatory fish (Baker 1980). Thus, the first year of life is the most critical for survival and development, yet this is the period where we have the least understanding of the biology of this species.
1.1 Introduction to stress

Stress is considered as a state of threatened homeostasis that is re-established by a suite of complex physiological responses. Any perceived, physical or chemical stressor can initiate a stress response in fish; the response is considered adaptive as the organism responds to disturbance maintaining homeostasis (Barton 2000). Wendelaar-Bonga (1997) defines stress as a condition where the dynamic equilibrium of an organism is threatened or disturbed as a result of intrinsic or extrinsic stimuli typically referred to as stressors. There are three levels of stress response, primary, secondary and tertiary responses. The primary, or acute stress response changes are largely driven by the release of hormones such as glucocorticoids (eg: cortisol, corticosterone) and catecholamines (eg: epinephrine, norepinephrine) into the circulation. In response to a stressor, mammals increase cortisol causing central nervous system activation, an increase in plasma glucose and an elevation in blood pressure (Bamberger et al. 1996). In fish, elevated plasma cortisol is often used as a primary indicator of stress, rising a few minutes after an exposure to an acute stressor (Wendelaar-Bonga 1997). Similar to mammals, cortisol increases plasma glucose levels after exposure to a stressor as responding to a stressful event comes at a high metabolic cost (Mommsen et al. 1999). To combat this increased energy demand, fuel reserves like glucose and amino acids are released into the circulation following a rise in plasma cortisol (Wendelaar-Bonga 1997; Mommsen et al. 1999). The duration and magnitude of the cortisol response is species and stressor dependent making it challenging to compare baseline and stressed levels across taxa. That being said, once baseline values for each species are established comparing physiological responses to stressful events is feasible. Adding to the convolution of measuring cortisol levels, not all increases may be due to a stressor.

Seasonal and diurnal changes have been reported in many species. Daily peaks in unstressed
individuals are often in the same range as stressed peaks and the highest reported values are usually in wintertime (Wendelaar-Bonga 1997). Further, baseline levels of cortisol in Rainbow Trout, *Oncorhynchus mykiss*, increase during feeding and this increase will persist even when feeding times change (Laidley and Leatherland 1988).

Chromaffin cells or bodies in fish are analogous to the mammalian adrenal medulla and release the majority of circulating epinephrine and norepinephrine in response to a stressor. Typically referred to as the fight or flight response in mammals, an increase in catecholamine levels is immediate, normally peaking within seconds to a minute following exposure to an acute stressor (Wendelaar Bonga 1997; Perry et al. 2004). Acute stress caused by hypoxia, temperature, pH, osmolality, or forced exercise will cause a rapid rise in muscle and plasma lactate and decreased blood pH and oxygen content (Wendelaar Bonga 1997). The release of catecholamines from the chromaffin cells and resultant activation of the adrenergic system increases respiratory function, improving oxygen uptake through increased ventilation rate, stimulation of branchial blood flow and branchial oxygen diffusing capacity, ultimately increasing the oxygen transport capacity of the blood (Wendelaar Bonga 1997). The cells can be scattered throughout the fish, but are typically concentrated near cardinal veins or are found in small clusters in the head kidneys integrated with steroidogenic cells (Wendelaar Bonga 1997; Mommsen et. al 1999). The rate-limiting enzyme in catecholamine synthesis is tyrosine hydroxylase (TH). By examining TH using immunohistochemistry and/or gene expression, the tissues involved in biosynthesis of catecholamines can be examined (Adrio et al. 2002; Pickel et al. 1975; Meek and Joosten 1993; Rink and Wullimann 2001; Forlano et al. 2014). The development of these cells in sturgeon has not been well documented but in zebra fish, *Danio*
rerio, the chromaffin cells begin to develop at 48 hours post fertilization (HPF) and are fully developed by 72 HPF (Nesan and Vijayan 2013).

1.2 Chromaffin Cells

As previously stated, chromaffin cells are responsible for secreting the catecholamines, adrenaline and noradrenaline in response to acute stressors (Wendelaar-Bonga 1997). The chromaffin cells are typically distributed throughout the post-cardinal veins and in small clusters in the head kidneys but the position and development of the cells can vary from species to species (Nandi 1961; Wendelaar-Bonga 1997). In some species, the interrenal tissues and chromaffin cells are highly associated and in others they are not (Nandi 1961). In fish where the interrenal and chromaffin tissue occur together there are many possible arrangements including, chromaffin tissue being dispersed throughout the interrenal tissue, alternating clumps of interrenal and chromaffin tissue or both tissues forming a complex around the lumen of the cardinal veins (Nandi 1961). It is more common for the chromaffin cells to exist on their own in the cardinal veins, adjacent to the endothelium or in the connective tissue of the vein wall with the interrenal tissue external to the wall and chromaffin cells (Nandi 1961).

The release of catecholamines from chromaffin tissue is primarily mediated by preganglionic cholinergic fibers of the sympathetic nervous system (Wendelaar-Bonga 1997). But severing of these nerves does not completely stop catecholamine secretion suggesting a more complex control system in teleosts (Reid and Perry 1994; Wendelaar-Bonga 1997). Once in circulation, catecholamines can target α and/or β-adrenergic receptors found on many tissues in teleosts including the heart, gills, and blood vessels (Randall and Perry 1992; Wendelaar-Bonga 1997). In teleosts, the heart is controlled by both inhibitory and stimulatory β-adrenergic receptor
mechanisms (Randall and Perry 1992). Contrasting, while the vascular control of the gills is mediated by β-adrenergic receptors, the blood vessels are mainly controlled by α-adrenergic receptor mediated innervation (Randall and Perry 1992).

In teleosts, multiple acute stressors have been shown to cause an increase in circulating catecholamines including handling, transport, hypoxia, and temperature changes (Wendelaar-Bonga 1997). As stated earlier, catecholamine release after a stressor is associated with increased oxygen transport (Wendelaar-Bonga 1997). Specifically, in Rainbow Trout, plasma epinephrine increases erythrocyte pH resulting in a more basic cytoplasm and acidified plasma, increasing the affinity of hemoglobin for oxygen (Nikinmaa 2004). Catecholamines also raise hematocrit by enlarging size and increasing number of red blood cells typically caused by a contraction of the spleen (Randall and Perry 1992). Alongside increasing oxygen transport, catecholamines increase glucose release from the liver, resulting in increased blood sugar or hyperglycemia (Wendelaar-Bonga 1997). There is also evidence catecholamines may play a role in mobilizing free fatty acids for increased energy reserves but this varies between species (Wendelaar-Bonga 1997). While both the location of chromaffin tissue and the effects of its contents i.e. catecholamines have been well described in the teleost literature, the development of this important tissue during early life has not been well studied in non-teleosts.

1.3 Hypothalamic – Pituitary – Inter-renal Axis (HPI)

It has been shown in many vertebrates that both the hypothalamus and pituitary gland are in control of releasing corticosteroids by means of corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) (Chrousos and Gold 1992; Ledris et al. 1994; Wendelaar-Bonga 1997; Bernier et al. 2009). This pattern of control is the same for teleosts, CRH neurons
control the stimulation of ACTH release and the role of ACTH in controlling cortisol secretion has been well documented (Donaldson 1990; Sumpter et al. 1994; Wendelaar-Bonga 1997). In mammals, CRH-secreting neurons are distributed throughout the brain with dense concentrations in the hypothalamus (Chrousos and Gold 1992). Excretion of CRH in the brain elicits multiple physiological responses including anxiety, arousal, and the activation of the pituitary-interrenal-axis and sympathetic nervous system (Chrousos and Gold 1992). CRH-like, also called corticotropin-releasing factor (CRF), secreting neurons have been found in homologous parts of the brain in teleosts (Okawara et al. 1988; Lederis et al. 1994; Pepels et al. 2004; Alderman and Bernier 2007). In white suckers, *Catostomus commersoni*, and goldfish, *Carassius carassius*, CRF immunoreactive neurons have been shown to be present in the nucleus preopticus, the nucleus lateralis tuberis in the hypothalamus and occasionally the pituitary (Lederis et al. 1994; Wendelaar-Bonga 1997). In numerous teleosts such as tilapia, *Oreochromis mossambicus*, CRF neurons have also been found in large quantities outside the hypothalamus in the telencephalon and rostral pars distalis (Pepels et al. 2004). In chondrosteans, such as the Sterlet Sturgeon, *Acipenser ruthenus*, immunohistochemistry showed positive staining for CRF in the ventral and dorsal hypothalamus, telencephalon and mesencephalon. Specifically, corticotropin immunoreactive cells were found in the infundibular nucleus and CRF neurons in the tuberal nucleus (Gonzalez et al. 1992). This shows the CRF/CRH role in the brain appeared quite early in evolution and is highly conserved throughout vertebrates highlighting its vital role in the regulation of the endocrine stress response (Bernier et al. 2009).

In mammals, teleosts, and elasmobranchs CRH has been shown to specifically stimulate the precursor molecule, proopiomelanocortin (POMC) which causes the secretion of not only ACTH but also α-MSH. (Lowry et al. 1974; Kawauchi 1983; Tran et al. 1990; Wendelaar-Bonga
It has been reported in many species that α-MSH plasma levels increase post-stressor, suggesting a role in stress adaptation (Sumpter et al. 1985; Lamers et al. 1992; Wendelaar-Bonga 1997; Bernier et al. 2009). In teleosts, both α-MSH and ACTH bind to G-coupled protein receptors, specifically one of the 5 melanocortin receptors (MCRs) (Logan et al. 2003; Klovins et al. 2004; Flik et al. 2006; Bernier et al. 2009; Agulleiro et al. 2013). In fish and mammals, ACTH is the only specific ligand to bind to MC2R, but both ACTH and the other MSHs bind to the other four MCRs (Klovins et al. 2004; Schioth et al. 2005). Unlike mammals, the other four MCRs have a higher affinity for ACTH than MSHs suggesting ACTH the original ligand and increased selectivity of MSHs by the MCRs appeared in tetrapods (Schioth et al. 2005; Bernier et al. 2009). The primary role of ACTH is regulating corticosteroidogenesis in the interrenal cells of the head kidney (Donaldson 1981; Wendelaar-Bonga 1997). Although ACTH is recognized as the main stimulator of cortisol secretion during acute stress (Flik et al. 2006), the response tends to be species specific. In Coho Salmon, Oncorhynchus kisutch, Rainbow Trout, and Brown Trout, Salmo trutta, a rise in plasma ACTH, alongside cortisol, was observed following thermal shock, handling or confinement (Pickering et al. 1986; Sumpter et al. 1986; Balm and Pottinger 1995). However, in Mozambique tilapia, Oreochromis mossambicus, an elevation in cortisol following a handling stressor was not accompanied by an elevation in ACTH (Balm et al. 1994). In White Sturgeon, Acipenser transmontanus, similar to the salmon and trout, ACTH injections triggered dose-dependent secretion of cortisol 15-180 minutes’ post-injection (Belanger et al. 2001).

At a cellular level, once ACTH binds to MC2R it allows for an increased capacity of steroidogenesis (Hagen et al. 2006; Aluru and Vijayan 2008). Specifically increasing StAR, cytochrome p450 side chain cleavage enzyme (P450scc) and 11β-hydroxylase (Hagen et al.
2006; Aluru and Vijayan 2008). StAR protein facilitates the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, a limiting factor to cortisol production, and in general all steroidogenesis (Mommsen et. al 1999; Stocco and Clark 1996). Because cholesterol is hydrophobic, transport is facilitated by StAR protein located in the outer mitochondrial membrane to cross the aqueous intermembrane space (Arakane et al. 1996; Clark and Stocco 1996). StAR is a nuclear encoded mitochondrial protein that is synthesized in the cytoplasm and imported into the mitochondria as a 37-kDa preprotein, the N-terminal mitochondrial import sequence is then cleaved to leave the mature 30kDa StAR protein (Arakane et al. 1996). Further exploration of the StAR protein in mammals showed the removal of the N-terminal sequence did not significantly affect steroidogenesis, but removal of C-terminal amino acids inactivated StAR (Arakane et al. 1996); meaning the residues in the C terminus are essential for steroidogenesis-enhancing activity and StAR acts via this terminal on the outside of the mitochondria (Arakane et al. 1996; Clark and Stocco 1996). This C-terminal is also known as the StAR-related lipid transfer (START) domain and forms a sterol-binding pocket to aid in transport of cholesterol (Tsujishita and Hurley 2000).

StAR has been identified and sequenced in many fish species such as salmonids and White Sturgeon and the gene is considered to be highly conserved among vertebrates (Kuskabe et. al 2009; Kuskabe et al 2002). Kuskabe and colleagues (2009) successfully identified the StAR amino acid sequence in White Sturgeon and identified the tissues where StAR expression was abundant. When examined with real time PCR the yellow corpuscles, kidney, and gonads all showed high expression of StAR. Unlike mammals, the White Sturgeon StAR expression did not increase as cortisol levels increased (Kuskabe et al. 2009). Examining StAR mRNA expression helps create a more complete understanding of an individual’s response to a stressor as it is a
negative feedback response for cortisol production. Other steps in steroidogenesis include P450scc which catalyzes the first step in steroidogenesis converting cholesterol to pregnenolone (Lin et al. 1995) and following that the enzyme 11$\beta$-hydroxylase is the final enzymatic step in the pathway converting 11-deoxycortisol to cortisol.

In mammals glucocorticoids, like cortisol, control hepatic glucose metabolism, stimulating gluconeogenesis and increasing glycogen content in the liver (Goldstein et al. 1992). The two main ways a glucocorticoid stimulates gluconeogenesis are by increasing transcription of phosphoenolpyruvate carboxykinase (PEPCK) and by stimulating the hepatic precursor supply (Granner et al. 1986; Fujiwara et al. 1996). In teleosts, in addition to hydromineral balance cortisol also balances energy by affecting carbohydrate, protein and lipid metabolism (Chrousos and Gold 1992). Specifically, cortisol increases liver glucose production, lipolytic and proteolytic capacity (Aluru and Vijayan 2007). There is a general consensus that cortisol does significantly increase the rate of gluconeogenesis from the liver, as it does in mammals, however, measuring changes in plasma glucose or hepatic glycogen alone is not always reflective of this change (Mommsen et al. 1999). Unlike mammals, fish plasma glucose may not be reflective of an individuals metabolic state. Measurements after corticosteroid exposure in teleosts are extremely variable with most increasing plasma glucose and liver glycogen following exposure but some species such as the American Eel, Anguilla rostrate, and Rainbow Trout responding in the opposite direction (Foster and Moon 1986; Barton et al. 1987; Vijayan et al. 1997). However, measuring enzymatic activity and turnover rates of the metabolite in conjunction with plasma glucose or hepatic glycogen gives insight to cortisol’s effect on metabolism in fish (Mommsen et al. 1999) where cortisol treatment tends to increase activity of
gluconeogenic enzymes such as glucose 6-phosphatase and PEPCK which is indicative of gluconeogenesis (Foster and Moon 1986; Vijayan et al. 1997).

The general consensus, of the effects of cortisol on catabolic activity in fish, is longer term elevations of cortisol leads to protein catabolism in white muscle and liver (Barton et al. 1987; Mommsen et al. 1999). The effects of cortisol on proteins and amino acids is more difficult to measure as even though muscle can exceed 50% of a fish's total body mass, it has a relatively low metabolic rate and any changes in amino acids in the liver or plasma may be below detectable limits (Mommsen et al. 1999). Further, variation between species and experimental design makes for challenging interpretation and comparison across species.

Killifish, *Fundulus heteroclitus*, exposed to prolonged starvation with cortisol administration showed an increase in weight loss compared to starved individuals without cortisol administration (Pickford et al. 1970), and prolonged starvation caused a rise in plasma cortisol coinciding with increased protein catabolism (Pickford et al. 1970; Bar 2014). The protein catabolic effect of cortisol has also been demonstrated in Rainbow Trout where cortisol implants resulted in increased plasma amino acids (Vijayan et al. 1997). Similarly, cortisol administration resulted in a decrease in the hepatosomatic index through increased proteolytic action in juvenile Rainbow Trout (Barton et al. 1987).

Cortisol is also involved in the assimilation of amino acids across the intestine from the diet. In Coho Salmon, *Oncorhynchus kisutch*, chronic elevation over a period of two weeks of cortisol from slow release implants resulted in a significant uptake in L-proline (Collie and Stevens 1985). Furthermore, cortisol may affect the integrity of the intestinal lining, compromising food reabsorption and food conversion (Barton et al. 1987). Most recently, it was reported that Rainbow Trout with lower plasma cortisol had a lower intestinal integrity during
basal conditions but an increased intestinal integrity following a stressful event (Rosengren et al. 2017). While the effects of cortisol on protein metabolism may not be easy to measure, it is clear with the influence of cortisol plasma amino acids increase and are used in key metabolic pathways including glycogenesis, gluconeogenesis and possibly protein synthesis (Mommsen et al. 1999). The net result of chronic elevations in cortisol in teleosts is a decrease in growth rate, however, whether this is due to increased catabolism or reduced rates of protein synthesis is unknown (Barton et al. 1987; Pickering 1990).

In teleosts, cortisol has also been shown to be involved in regulating lipid metabolism, however, the exact role can prove difficult to quantify. In mammals, corticosteroids modulate lipolysis in a number of ways. For example, cortisol administration alters the lipo-protein lipase in human fat tissue increasing enzymatic activity (Ottosson et al. 1994). Conversely, it has also been shown in humans that increases in deposition of adipose tissue is directly related to increased cortisol levels in chronically stressed individuals (Ottosson et al. 1994). In fish, as with the catabolic effect of cortisol on proteins it can be difficult to separate experimental design from species differences but it is generally agreed cortisol has strong peripheral and hepatic lipolytic action causing increases in plasma fatty acids (Mommsen et al. 1999). In the presence of increased cortisol both Channel Catfish, Ictalurus punctatus, and European Eel, Anguilla anguilla, showed an increase in peripheral lipolysis (Davis et al. 1985; Dave et al. 1979). In Coho Salmon cortisol injections increased lipolytic activity in mesenteric fat, liver and red muscle by activating triacylglycerol lipase (Sheridan 1986). Regardless of the specific actions of cortisol it is accepted that exposure to elevated levels of cortisol result in a general metabolic effect and release of energy stores into circulation to support increased oxygen demands (Mommsen et al. 1999).
1.4 Development of endogenous cortisol production

In recent years with the increased need for aquaculture and conservation hatcheries, the development of the stress response in larval fish has become of high interest. Larval and juvenile fish are more sensitive to stressors in general and the proper development of the stress response is vital to survival (Wendelaar Bonga 1997; Nesan and Vijayan 2016). In teleosts and chondrosteans a typical pattern of measureable levels of maternally derived cortisol in eggs is followed by a rapid decline in cortisol during embryogenesis with *de novo* synthesis occurring sometime during the yolk sac or early endogenous feeding phase (De Jesus et al. 1991; Barry et al 1995; Simontacchi et al. 2009; Zubair et al. 2012; Nesan et al. 2012; Tsalafouta et al. 2014). The period of low corticosteroids is known as the hypo-responsive period and has been observed in both mammals and fish (Barry et al. 1995; Sapolsky and Meaney 1986). The delay in steroid synthesis could be for a number of reasons, including the underdevelopment of higher neural centers making it impossible to perceive a stressor (Barry et al. 1995; Bernier 2006). Another explanation could be high levels of corticosteroids during this time are harmful for development having permanent effects on neural origination, so levels are kept consistently low (Barry et al. 1995).

It has also been shown in zebrafish that glucocorticoid receptor (GR) transcripts are maternally deposited, but turn over to zygotically produced mRNA after the mid-blastula transition (Nesan et al. 2012). In zebrafish, GR signaling plays a key role in embryogenesis specifically in muscle development (Nesan et al. 2012). When GR knockdown was successful, the embryos showed defects in somite, tail, cardiac morphogenesis and neurogenesis and rarely survived past hatch (Nesan et al. 2012; Nesan and Vijayan 2013). Growth and developmental rate were also significantly lower in GR-knockdown fish, but when injected with GR mRNA
most of the fish made a full recovery in growth and development (Nesan et al. 2012). In zebrafish, maternally deposited cortisol is also crucial for proper development (Nesan and Vijayan 2016). Embryos injected with either excess cortisol or a cortisol antibody displayed significant developmental abnormalities (Nesan and Vijayan 2016). Embryos injected with the cortisol antibody, (simulating a lack of maternal cortisol input), showed mesoderm malformations such as kinked tails and had heightened stress responses post-hatch. Embryos injected with excess cortisol exhibited cardiac edema, and malformation of the heart, and were unable to mount a stress-mediated cortisol response post hatch (Nesan and Vijayan 2016). Further, modulation of key genes in the HPI axis such as CRF, 11β-hydroxylase, POMC and StAR was seen in both treatment groups, which were upregulated in response to decreased cortisol or downregulated in response to increased cortisol (Nesan and Vijayan 2016). It has been documented in many species that following hatch there is an increase in basal cortisol levels during the transition from yolk-sac to exogenous feeding (De Jesus 1991; Barry et al. 1995; Nesan et al. 2012; Zubair et al. 2012; Tsalafouta et al. 2014). Previously this was attested to the stress associated with initial foraging attempts, however, more recently, research suggests this spike in whole body cortisol during transition from yolk to endogenous feeding may be related to the important role cortisol plays in energy allocation (Tsalafouta et al. 2014).

It is clear that cortisol plays a critical role in early development, however, much of our understanding is based on two species of teleost the Rainbow Trout and zebrafish. The overall objective of this study was to describe the development of steroidogenesis and catecholamine-secreting tissue in a representative chondrostean, the Lake Sturgeon. To do this I measured endogenous cortisol production throughout development across two years in stressed and unstressed individuals i.e. baseline values that were exposed or not exposed to metyrapone, an
inhibitor of 11β-hydroxylase, and therefore steroidogenesis. Alongside cortisol production, mRNA expression levels of StAR protein were also assessed in year two of the study. Development of chromaffin tissue was also examined using immunohistochemical techniques specific for the highly conserved enzyme tyrosine hydroxylase. I hypothesized the development of endogenous cortisol production in Lake Sturgeon would be similar to that of teleosts. I further hypothesized any increases in unstressed individuals or, baseline cortisol, throughout the first year of development would correlate with dietary shifts.
References


Becker GC (1983) Fishes of Wisconsin. The University of Wisconsin Press, Madison, WI, 1052


Collie NL and Stevens JJ (1985) Hormonal effects on L-proline transport in coho salmon (Oncorhynchus kisutch) intestine. Gen Comp Endocrinol 59: 399–409


Harkness WJK and Dymond JR (1961) The lake sturgeon the history of its fishery and problems of conservation. Ontario Department of Lands and Forests, Fish and Wildlife Branch


Rink E and Wullimann MF (2001) The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). Brain Res 889: 316–330


Chapter 2: Development of Endogenous Cortisol Production

2.1 Introduction

Early development in vertebrates is proven to have lasting effects on an individual’s phenotype, and ecological fitness (Alsop and Vijayan 2008; Barry et al. 1995; Mommsen et al. 2009). A particularly important part of early development in fish is the ontogeny of the hypothalamus-pituitary-interrenal (HPI) axis (Nesan and Vijayan 2016; Wendelaar-Bonga 1997) analogous to the hypothalamic-pituitary-adrenal axis in mammals. The HPI axis determines the endocrine stress-response, behavior and daily maintenance in fish (Wendelaar-Bonga 1997; Barton 2000) thus appropriate development is critical to future survival and reproductive success.

In teleosts and chondrosteans (sturgeon and paddlefish) it has been shown that cortisol levels in the eggs prior to fertilization are maternally derived and these levels plummet to negligible levels following fertilization (Barry et al. 1995; Simontacchi et al. 2009; Gessner et al. 2009; Zubair et al. 2012; Falahatkar et al. 2014; Nesan et al. 2012; De Jesus et al. 1991). For example, Rainbow Trout, Oncorhynchus mykiss, were unable to mount a cortisol response following exposure to an acute stressor until 2 weeks after hatching; 6 weeks post fertilization (Barry et al. 1995). Similarly, in Japanese Flounder larvae, Paralichthys olivaceus, cortisol levels were elevated at fertilization; declined within 24 hours post-fertilization, and remained low until 10 days after hatching peaking around 15 days post-hatch (Jesus et al. 1991). Zebrafish, Danio
*rerio*, demonstrate low levels of cortisol until after hatch, 2 DPF, when they exhibit *de novo* synthesis (Nesan et al. 2012). In larval European sea bass, *Dicentrarchus labrax*, an increase in baseline cortisol levels were observed just prior to exogenous feeding similar to Rainbow Trout, Japanese Flounder and zebrafish (Tsalaouta et al. 2014). The authors hypothesize this increase as a reflection of the essential role cortisol plays in carbohydrate and protein catabolism in the transition towards exogenous feeding (Tsalaouta et al. 2014). The delay in cortisol response in fish during early development may be explained by the hypo-responsive period to stress, to maintain constant corticosteroid levels during a critical time when the steroids can have permanent effects on development and neural origination and is similar to what has been reported for corticosterone in rat pups (Barry et al. 1995; Sapolsky and Meaney 1986). The causal factor for the muting of a corticosteroid response during early development in vertebrates is unknown but in fishes is likely the result of an inability to appropriately perceive and communicate a stressor by higher neural centers (Barry et al. 1995; Bernier 2006).

Recent studies have begun to highlight the important roles cortisol plays throughout development and manipulation of endogenous levels of cortisol can significantly affect phenotypic development (Nesan and Vijayan 2016; Tsalaouta et al. 2014; Rosengren 2017). In zebrafish, it has been shown that during embryogenesis and early development, administration of exogenous cortisol, or inhibition of cortisol by use of a specific antibody, influenced organ development and the ability of the fish to mount an appropriate cortisol stress response later in life (Nesan and Vijayan 2012a, 2012b, 2016; Nesan et al. 2012). Comparison of the brain-gut axis activation in Rainbow Trout between individuals with innately low cortisol or high cortisol response showed differences in both brain gene expression and intestinal integrity (Rosengren et al. 2017). Individuals with an innately lower cortisol response showed lower intestinal integrity.
during basal conditions but higher intestinal integrity during stressed conditions i.e. more cortisol. The authors hypothesize the differences are from regulation of tight junction proteins which connect the thin layer of epithelial cells that make up the intestinal barrier (Rosengren et al. 2017).

Lake Sturgeon are a conservation species of high-interest and restoration efforts to restore historic population numbers have become widespread throughout the United States and Canada (Peterson et al. 2007). Conservation hatcheries have been releasing both hatchery-raised fry (fall stocked) and fingerling (spring stocked) young-of-the-year (YOY) Lake Sturgeon into their historic range with the goal of restoring historic population numbers but without much knowledge on the early development of this species (Secor et al. 2002; Barth et al. 2015). The larvae’s survival in the hatcheries is vital to the re-stocking program and high mortality is often seen during developmental changes such as from yolk-sac to exogenous feeding. This is typically around 21 DPF, thus it is crucial to understand the underlying physiology of these shifts around this time to promote survival. In this study we assessed baseline levels of cortisol in developing Lake Sturgeon daily until 30 DPF and determined when Lake Sturgeon were able to mount a cortisol response to a standard stressor. Further, through the addition of metyrapone to the holding tanks we examined the effect of inhibition of cortisol synthesis on morphology and cortisol production over the same developmental period. Whole body cortisol levels are reported alongside mRNA expression levels of StAR protein over a similar time period.

2.2 Methods

2.2.1 Fish Husbandry
In the spring of 2016 and 2017, eggs and sperm from wild-caught spawning female and male Lake Sturgeon were returned to the University of Manitoba for fertilization. Eggs from 2 and 4 females, in 2016 and 2017 respectively, were freely mixed with sperm from at least two males to reduce the potential confounding genetic effects on the resultant hatched larvae. De-adhesed embryos were incubated in McDonald jars at 12°C until hatch in early June. Once larvae hatched they were transferred to sixteen 9L aquariums with flow-through dechlorinated tap water maintained at 15°C ±1. Each tank had bio-balls for substrate until exogenous feeding began when all substrate was removed over a 7-day period (Zubair et al. 2012). A few days prior to yolk-sac absorption live artemia (brine shrimp) were introduced to the tanks. Following exogenously feeding on artemia, the larvae were slowly transitioned to a bloodworm (chronomids) diet over a 25-day period. Tanks were monitored at least twice daily, when mortalities and debris were removed. Highest mortality rates were observed during feeding transitions, about 10% mortality during the yolk-sac to artemia transition and about 20% from artemia to bloodworm transition. All animals used in this study were reared and sampled according to animal use and care guidelines established by the Canadian Council for Animal Care and approved by the Animal Care Committee at the University of Manitoba (Protocol #F15-007).

2.2.2 Sampling for changes in baseline whole body cortisol levels 2016

Individuals were sampled daily from randomly selected tanks from egg (pre-fertilization) to 22 DPF and then every 5 days until 57 DPF (Fig 2.1). To obtain baseline values, fish were immediately transferred to an anesthetic bath containing an overdose of anesthetic (MS-222 0.5g.L⁻¹) buffered with equal volumes of sodium bicarbonate. The minimum detectable limit of
the cortisol assay dictated the number of individuals required to obtain a measurable value for each sampling point. For example, unhatched embryos required combining a minimum of 5 individuals for each point thus for a n value of 8 at that developmental stage a total of 40 individuals were sampled; post-hatch larvae required 2-4 individuals per sampling point. Body mass and total length were recorded for each individual and then samples were immediately frozen in -80°C for later analysis. To avoid introducing bias due to diurnal changes in cortisol (Lankford et al. 2003) sampling was conducted from 11:00am -1:00 pm each day.

2.2.3 Sampling for changes in baseline and peak whole body cortisol levels 2017

Samples were taken daily, from randomly selected tanks, from egg (pre-fertilization) to 30 DPF as described above for baseline levels of whole body cortisol (Fig 2.1).
To determine the onset of endogenous cortisol production following exposure to a stressor, larvae were sampled daily after hatch until 30 DPF. Briefly, larvae were randomly selected from tanks and added to 500 mL glass bowls. To elicit a cortisol stress response larvae were chased for two minutes by gently prodding the caudal tail with a probe. Following the stressor, the larvae were transferred to a second 500ml bowl and after 30 minutes were then placed in an overdose of anesthetic as described above, as it has been shown in previous studies larval and juvenile Lake Sturgeon need at least 20 minutes to reach peak cortisol levels post-stressor (Zubair 2012; Allen 2009). Body mass and total length was then recorded for each individual, and samples were placed in a -80°C freezer for later analysis. In addition, at 13 DPF a subset of larvae were evenly divided between three control tanks and three experimental tanks treated with 10 mg.L⁻¹ metyrapone to inhibit the enzyme 11β-hydroxylase, and presumably endogenous cortisol production. Samples were taken daily from both control and metyrapone treatments for
baseline and peak cortisol responses until 20 DPF then sampled on every-other day, due to excessive mortalities, until all fish were sampled in metyrapone treated fish. Protein and glucose samples were collected from metyrapone treated and control fish at the same time baseline cortisol samples were collected. Samples for triglyceride analysis were only taken from the control fish as 60 larvae per time-point were needed for measurement.

2.2.4 Sampling for gene expression
From 8DPF to 30DPF 10 larvae were randomly selected and placed in an overdose of anesthetic as described. Body mass and total length was recorded for each individual and larvae were then transferred to a vial containing 500 μl of RNAlater (Invitrogen; Thermo Fisher Scientific; Lithuania). Samples were transferred to 4°C for 24h then stored at -80°C until RNA extraction and subsequent measurement of whole body expression of Steroidogenic acute Regulatory protein (StAR).

2.2.5 Cortisol Extraction and Measurement 2016/2017
Whole body samples were homogenized with phosphate-buffered saline (PBS; 0.9% NaCl in PB and pH 7.4) and cortisol was extracted using solid phase extraction using C18-SEP-Pak cartridges (Waters Corporation; Milford, MA, USA) as previously described (Zubair et al. 2012; Deslauriers et al. 2018). Samples were either eluted from the Sep-Pak column using 3 mL of methanol and then dried-down in a Savant Speed Vac (Fisher Scientific) or extracted via diethyl ether. Diethyl ether extractions were conducted as follows, diethyl ether (4x amount of sample
volume) was added to sample and vortexed for 15 seconds and the diethyl ether was transferred into a new tube. This was repeated three times for each sample and all samples were then dried down under a nitrogen stream at room temperature. Samples were then frozen in a -80°C freezer until further use. Each sample was individually reconstituted in 250 μl of radioimmunoassay (RIA) buffer (90 mL Milli-Q water, 10 ml PBS, 0.9 g NaCl and 0.5 g bovine serum albumin) then split into duplicate 100μl samples. 100 μl of titrated cortisol (Perkin Elmer; 5000 disintegrations per minute (DPM)) and 100 μl of cortisol antibody (rabbit anti-cortisol polyclonal antibody; Fitzgerald Industries) was then added to each sample tube. Following vortexing the samples were incubated at room temperature for one hour and then overnight at 4°C. The following day, the assay was terminated by the addition of 100 μl charcoal separation buffer (1.25 g of charcoal and 0.125 g of dextran in 25 mL RIA buffer) to each sample. After vortexing the samples were left on ice for 15 minutes and then centrifuged at 4°C for 30 minutes at 2500 G. The supernatant was then poured into a 6 mL scintillation vial and 4 mL of Ultima Gold scintillation fluid was added (Perkin Elmer). Each sample was counted on a liquid scintillation counter (Perkin Elmer; Tri-Carb 3110 TR) for 5 minutes. Unknown cortisol values were determined by interpolating against a standard curve, generated with each assay. Intra-assay variation and inter-assay variation were 13% and 7% respectively and extraction efficiency was 99.5 ± 1.32%.

2.2.6 RNA Extraction

Whole-body larvae were extracted for total RNA using PureLink RNA Mini Kits (Invitrogen; Ambion Life Technologies; 12183025) following the manufacturers instructions. Briefly, whole larvae were lysed in 500 mL of lysis buffer, for 3 minutes at 30 RPM in a TissueLyser II
(Invitrogen Purelink Mini Kit). 350 µl of 70% ethanol was then added to each sample, vortexed thoroughly, and 700 µl of sample was then added to a spin cartridge with a collection tube. Spin cartridges were centrifuged for one minute at 12,000 x g and after spinning, any liquid in the collection tube was discarded. The cartridge was inserted back into the same collection tube and centrifuged twice more to ensure all flow-through was removed. Then 700 µl of Wash Buffer I was added to each sample and centrifuged for one minute at 12,000 x g and all flow-through was discarded. Lastly, 500 µl Wash Buffer II was added to each sample, centrifuged as described, and the waste was again discarded. The spin cartridge was then inserted into a clean collection tube and centrifuged once more prior to adding 10 µl RNase-free water to the center of the membrane. Once water was added the tubes were incubated at room temperature for 1 minute. To elute the RNA from the membrane, samples were centrifuged for 2 minutes at 16,000 x g. RNA purity and concentration, was assessed for all samples using a Nanodrop 2000c (Thermo Scientific) and tested by gel electrophoresis. RNA samples were then stored in -80°C until further use.

2.2.7 cDNA Synthesis

cDNA was synthesized from 1µg of DNase treated RNA using a qScript cDNA Synthesis Kit following the manufacturers’ instructions (Quantabio 95047-025). Briefly, 1 µg of RNA was added to a 96-well plate, on ice, and then DEPC-treated water was added to make a volume of 8 µl. The plate was sealed and briefly centrifuged before incubating in the thermocycler (SimpliAmp Thermal Cycler; Applied Biosystems) at 25°C for 15 minutes. The plate was then put back on ice and 1 µl 25 mM EDTA was added to all wells to inhibit the DNase I reaction. It was then again sealed and centrifuged before incubating at 65°C for 10 minutes. A master mix of
nuclease free water (5 µl per sample), reaction buffer (4 µl per sample) and reverse transcriptase (1 µl per sample) was made and 10 µl added to each well. A foil plate seal was then tightly adhered, briefly centrifuged for 20 seconds and run in the thermocycler under the following conditions 1 cycle 22°C for 5 minutes, 1 cycle 42°C for 30 minutes, 1 cycle 85°C for 5 minutes and then held at 4°C. cDNA was then stored at -20°C until further use.

2.2.8 qPCR

RT qPCR reactions for each gene were completed with a master mix of Bio-Rad SsoAdvanced Universal SYBR® Green Supermix and nuclease-free water (Bio-Rad; 1725271). StAR primers were based on published primers designed from White Sturgeon and share over 60% homology with other vertebrates (Kuskabee et al. 2009; Table 1.1) Reference genes were created by using a partial ovarian Lake Sturgeon pyrosequenced transcript (Hale et al. 2009). Primer efficiency was validated by using a 1:10 dilution standard curve for each gene. (Table 1.1) All reactions were completed on a 384 well-plate and were run under the same cycling conditions, 2 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C with collection of data. Melting curves were determined by denaturation for 15 seconds at 95°C, a decrease for a minute down to 60°C and followed by a gradual increase of 0.075°C/second to increase back to 95°C, with a continual collection of data. All reactions were done in a QuantStudio 5 (Applied biosystems; Thermo Fischer Scientific).
Table 2.1 List of reference genes and StAR forward and reverse primer sequences. Efficiencies listed as %.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPS6</td>
<td>CTGGCTGGATTCTGATTTGGATG</td>
<td>ATCTGATTATGCCAAGCTGCTG</td>
<td>96.94</td>
</tr>
<tr>
<td>RPL7</td>
<td>GAAGTCCAGGGCGACATAGC</td>
<td>TGAAGATCCTGACCGAGCGA</td>
<td>97.27</td>
</tr>
<tr>
<td>EF1A</td>
<td>GGTTGTCTTCCAGCTTCTTACC</td>
<td>ACTGCTCACATTGCCTGCA</td>
<td>98.76</td>
</tr>
<tr>
<td>StAR</td>
<td>CCCGAGCAAAAAGGCTTCA</td>
<td>TTGGGCGGAAGAACACATACAG</td>
<td>98.71</td>
</tr>
</tbody>
</table>

2.2.9 Whole body protein, glucose and triglycerides

Whole body larvae were homogenized in 50 mM Tris buffer with a TissueLyser II (Invitrogen Purelink Mini Kit) for all three assays. For protein, one larvae was sufficient for readable measurements so both control and metyrapone-treated fish were measured. Total body protein was determined using the Pierce Coomassie (Bradford method) Protein Assay Kit (Thermo Fisher; Catalog No. 23200). To generate a standard curve, varying concentrations of BSA were combined with 150 µl 50 mM Tris buffer. The standards and samples were measured in triplicate in a 96-well plate and absorbance was quantified at 595 nm and 37°C. Absorbance values were measured using a PowerWave XS2 plate reader (BioTek; 236096 Vermont). Glucose levels were measured in both control and metyrapone treated fish using the LabAssay Glucose kit by WAKO (Code No. 298-65, 701). The standard curve was diluted 10x to ensure samples fell in the middle of the curve. All standards and samples were measured in a 96-well plate where absorbance was
measured at 505 nm and 37°C, using the same instrument as above. Whole body triglycerides required 60 larvae per measurement and as such were only measured in control fish. Triglyceride levels were measured using the LabAssay Triglyceride kit by WAKO (Code No. 290-63, 701). All standards and samples were measured in triplicate in a 96-well plate. Absorbance values were obtained at 505 nm and 37°C using the same instrument as above.

2.2.10 Statistical analysis

One-way ANOVA followed by Tukey’s post-hoc tests was used to test for significant differences over time in baseline cortisol values. 2-way ANOVA followed by a Bonferroni’s post-hoc test was used to test for significant differences over time and the magnitude of the cortisol response following a stressor. Depending on the availability of fish, sample size ranged from n values of 6-10 samples. Data are presented as mean±SEM and data was considered significant if p<0.05. Statistical analysis was conducted using Graph Pad Prism 6 software.

2.3 Results

2.3.1 Changes in baseline whole-body cortisol levels 2016

Prior to fertilization, egg cortisol values were 3.74±0.52 ng.egg⁻¹. Following fertilization cortisol values significantly dropped to negligible values (p-value=0.0005; 0.02±0.007 ng.g⁻¹). De novo synthesis of cortisol began at hatch, significantly increasing at 8 DPF from embryo values (p-value<0.0011; 0.631± 0.37 ng.g⁻¹). Baseline cortisol levels significantly increased again prior to exogenous feeding (p-value= 0.0001; 15.83±0.72 ng.g⁻¹) at 16 DPF (Fig 2.2). During the 25-day transition from an artemia based diet to a 100% bloodworm diet baseline cortisol levels increased again and slowly decreased following complete transition. At 22 DPF, when bloodworms were
introduced at a ratio of 1:10 bloodworm:artemia values began to increase (2.75± 1.62 ng.g⁻¹). Values peaked during this transition at day 32 (9.69±1.39 ng.g⁻¹) and back to lower levels by 47 DPF when the food was 90% bloodworm (2.84±0.37 ng.g⁻¹) (Fig 2.2).

2.3.2 Changes in baseline and peak whole body cortisol levels 2017
Prior to fertilization, egg cortisol values were 0.51±0.23 ng.egg⁻¹. Following fertilization cortisol values dropped to negligible values (0.026±0.04 ng.g⁻¹). De novo synthesis of cortisol began at hatch, significantly increasing at 8 DPF (p-value<0.0001; 1.04±0.40 ng.g⁻¹). Baseline cortisol levels significantly increase again prior to exogenous feeding (p-value<0.0001; 4.00±0.63 ng.g⁻¹) at 20 DPF (Fig 2.3). Following exogenous feeding values decreased (0.757±0.14 ng.g⁻¹).
Yolk-sac larvae (8 DPF-21 DPF) were incapable of producing a heightened cortisol response following a chasing stressor. Only following exogenous feeding (21 DPF) were stress-induced cortisol levels higher than baseline cortisol levels. Although not significant at every time point, after exogenously feeding the larvae consistently had higher cortisol levels after 30 minutes, post two minutes of chasing, when compared to baseline levels over all sampling points (mean baseline levels= 0.76±0.14 ng.g⁻¹; mean peak levels= 1.31±0.52 ng.g⁻¹) (Fig 2.3).

2.3.3 Effects of metyrapone treatment on development and whole body cortisol levels
Metyrapone-treated (MT) fish had lower whole-body baseline cortisol every day of measurement. On average metyrapone treated fish had significantly lower cortisol (p-value= 0.0306; 0.157±0.05 ng.g⁻¹) from 15 DPF to the end of the experiment when compared to baseline cortisol in control fish (1.06± 0.34 ng.g⁻¹) (Fig 2.3). In general, developmental rate was slower in metyrapone treated fish, during the course of the experiment metyrapone treated fish never
expunged their anal plugs and never began feeding. Further, developmental abnormalities including large edemas around the heart and abdominal organs and deformed rostrums were frequently observed in the metyrapone treated fish.

When compared to control larvae, metyrapone treated larvae had lower body mass by 18 DPF (p-value=0.02132; Fig. 2.4), whole body protein was also lower (0.613± 0.06 mg.larvae⁻¹; Fig 2.5), compared to control fish (0.779± 0.03 mg.larvae⁻¹) prior to 18 DPF (p-value=0.006). Whole body triglycerides in the control fish increased significantly following exogenous feeding (p-value <0.0001; Fig. 2.6). Whole body glucose levels were not different between the control and MT treated fish (Fig 2.7).

2.3.4 Molecular expression

The relative expression of StAR following hatch remained relatively constant until 19 DPF. The relative expression of StAR increased significantly prior to exogenous feeding at the same time baseline cortisol increased (p-value=0.0049). Following 21 DPF the expression of StAR remains significantly higher at all time points when compared to prior to exogenous feeding (Fig 2.8).
Figure 2.1 Timeline of development for 2016 (top) and 2017 (bottom). Days are expressed as DPF (days post fertilization)
Figure 2.2 Whole body cortisol (ng.g⁻¹ wet mass) pre-and post-fertilization in developing Lake Sturgeon (2016 cohort). Data are expressed as a mean +/- SEM; N=6-10
Figure 2.3. Whole body cortisol (ng.g⁻¹ wet mass) pre-and post-fertilization in developing Lake Sturgeon (2017 cohort). Baseline concentration (black bars), peak (stippled bars) for control and metyrapone treated (grey bars) larvae. Data are expressed as a mean +/- SEM; N=6-10.
Figure 2.4 Wet mass (g) of control (black bars) and metyrapone treated larvae (grey bars) * indicates significant difference between control and treatment (p value <0.05); Data are expressed as a mean +/- SEM; N=10
Figure 2.5 Whole body protein (mg larva\(^{-1}\)) of control (black bars) and metyrapone treated larvae (grey bars). * indicates significant difference between control and treatment (p value <0.05); Data are expressed as a mean +/- SEM; N=10
Figure 2.6 Whole body triglycerides in control fish (ng.g⁻¹); 60 larvae used per sampling point, letters indicate statistical significance from 12 DPF; (p value<0.05) Data are expressed as a mean +/- SEM; N=8.
Figure 2.7 Whole body glucose control and metyrapone treated larvae (mg.\textnormal{larvae}^{-1}); (p value<0.05) Data are expressed as a mean +/- SEM; N=5.
2.4 Discussion

In this study, the overall pattern of endogenous baseline cortisol production in Lake Sturgeon larvae, in both 2016 and 2017, was similar to that of teleosts. Thus, while absolute levels of cortisol may change from species to species, the underlying pattern of production is
consistent, with maternally derived cortisol in the egg decreasing following fertilization and remaining low until hatch when animals are capable of de novo synthesis of cortisol (De Jesus et al. 1991; Gessner et al. 2009; Simontacchi et al. 2009; Nesan and Vijayan 2012; Zubair et al. 2012; Falaharkar et al. 2014; Tsalafouta et al. 2014; Table 1.2). In developing White Sturgeon unfertilized eggs contained approximately 21.5 ng.g⁻¹ with about 6-10 eggs per sample (Simontacchi et al. 2009). By 5 DPF the developing embryos had decreased cortisol concentration to 2.41 ng.g⁻¹. Cortisol dramatically increased around 10 days post hatch (DPH), or the onset of exogenous feeding, suggesting a similar developmental pattern as teleosts, however, when exposed to an acute stressor, White Sturgeon larvae were able to respond with de novo synthesis of cortisol at 3 DPH and beyond (Stimontacchi et al. 2009). Following hatch, Atlantic Sturgeon (Acipenser oxyrinchus) had baseline cortisol levels between 0.02-0.65 ng.g⁻¹ and showed an increase in baseline levels prior to exogenous feeding at 12 DPH (Gessner et al. 2009). Persian Sturgeon did not endogenously produce cortisol until hatch with measurable values from 1.75-8 ng.g⁻¹ (Falahatkar et al. 2014). Although research involving the stress response in larval Lake Sturgeon is extremely limited, Zubair and colleagues (2012) showed an increase of baseline cortisol levels prior to exogenous feeding at around 16 DPF, suggesting de novo synthesis of cortisol prior to exogenous feeding, following hatch. The production of endogenous cortisol following embryogenesis suggests cortisol may be necessary to regulate energy reserves from the yolk-sac.
Table 2.2 Various chondrostean and teleost whole body cortisol levels (ng.g\(^{-1}\)), timing of endogenous production and whether or not baseline increases were observed prior to feeding.

<table>
<thead>
<tr>
<th>Species</th>
<th>Lifestage</th>
<th>Whole Body Cortisol Range (lowest-highest)</th>
<th>Endogenous production</th>
<th>Basal increase observed?</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Sturgeon <em>(A. transmontanus)</em> Simontacchi et al. 2009</td>
<td>Larvae (1 DPH-45 DPH)</td>
<td>1.69-4.32 ng/g</td>
<td>Following acute stressor at 3DPH</td>
<td>Increase seen when switching to exogenous food source (~10 DPH)</td>
</tr>
<tr>
<td>Atlantic Sturgeon <em>(A. oxyrinchus)</em> Gessner et al. 2009</td>
<td>Larvae (1-14 DPH)</td>
<td>0.02-0.65 ng/g</td>
<td>Following acute stressor at 4 DPH</td>
<td>Increase seen when switching to exogenous food source (~12 DPH)</td>
</tr>
<tr>
<td>Lake Sturgeon <em>(A. fulvescens)</em> Zubair et al. 2012</td>
<td>Yolk-sac &amp; Larvae (11 DPF-20 DPF)</td>
<td>5.2-11.2 ng/g</td>
<td>Following acute stressor at 19 DPF</td>
<td>Peak at 16 DPF and exogenous feeding began at 17 DPF</td>
</tr>
<tr>
<td>Persian Sturgeon <em>(A. persicus)</em> Falahatkar et al. 2014</td>
<td>Embryo (1 DPF-10DPF)</td>
<td>1.75-8.0 ng/g</td>
<td>No endogenous production seen prior to hatch</td>
<td>N/A</td>
</tr>
<tr>
<td>Zebrafish <em>(D. rerio)</em> Nesan et al. 2012</td>
<td>Embryo (0 HPF-80 HPF)</td>
<td>0.003-0.005 ng/embryo</td>
<td>De novo synthesis only after hatching; acute response seen at time of exogenous feeding</td>
<td>N/A</td>
</tr>
<tr>
<td>Japanese Flounder <em>(P. olivaceus)</em> De Jesus et al. 1991</td>
<td>Embryo &amp; Larvae (1 DPF-37 DPF)</td>
<td>0.04-2.5 ng/g</td>
<td>Endogenous production seen at hatch (5 DPF)</td>
<td>Basal peak at 18 DPF and at the end of prometamorphosis</td>
</tr>
<tr>
<td>European Sea Bass <em>(D. labrax)</em> Tsalafouta et al. 2014</td>
<td>Embryo &amp; Larvae (0-45 DPF)</td>
<td>0.6-6.8 ng/g</td>
<td>Following hatch; measured at mouth opening</td>
<td>Basal peak at the time of first feeding (9 DPH)</td>
</tr>
<tr>
<td>Rainbow Trout <em>(O. mykiss)</em> Barry et al. 1995</td>
<td>Embryo &amp; Larvae (0-9 weeks post-fertilization)</td>
<td>0.3- 4.9 ng/g</td>
<td>Following hatch; between week 4 and 5</td>
<td>Basal peak prior to exogenous feeding at the end of yolk absorption (6 weeks post fertilization)</td>
</tr>
<tr>
<td>Chum Salmon <em>(O. keta)</em> De Jesus and Hirano 1991</td>
<td>Embryo &amp; Larvae</td>
<td>10-30 ng/g</td>
<td>Endogenous production at hatch (6 weeks post fertilization)</td>
<td>Basal peak at the end of yolk-sac absorption prior to feeding (10 weeks post fertilization)</td>
</tr>
</tbody>
</table>
The relative expression of StAR in the present study coincided with the pattern of \textit{de novo} synthesis of cortisol where measureable levels of expression in StAR were observed at time of hatch and significant increases were observed immediately before exogenous feeding; coincidental with a significant increase in whole-body cortisol at 20 DPH. Importantly, StAR expression remained elevated in comparison to earlier developmental time points following feeding. This could imply an improper stress response prior to feeding is due to a lack of cholesterol, as increases in StAR expression were coincidental with increases in lipids, measured through whole body triglycerides. However, it also implies a role for endogenously produced cortisol in regulating expression of this key component in steroidogenesis. In Rainbow Trout and Zebrafish, StAR expression has been shown to be related to changes in circulating levels of cortisol in adult fish exposed to a stressor (Geslin and Auperin 2004; Aluru et al. 2005; Fuzzen et al. 2011) and in developing zebrafish StAR expression significantly decreased and increased following injection into the embryo of cortisol or a cortisol antibody respectively (Nesan and Vijayan 2016). To our knowledge this is the first study to demonstrate a link between StAR expression and cortisol levels in a developing non-teleost.

Following depletion of their yolk-sac, it is imperative larvae source food for survival. Previously, many studies interpreted the observed increase in cortisol is a typical endocrine stress response to the need for increased foraging effort. However, the observed increase in whole body cortisol could also be a necessary developmental process promoting the switch to exogenous feeding as the animal shifts from a high energy lipid/protein-based yolk diet to an exogenous food source. Interestingly, almost all fishes exhibit an increase in baseline cortisol immediately prior to or at the time of exogenous feeding (De Jesus et al. 1991; Gessner et al. 2009; Simontacchi et al. 2009; Nesan and Vijayan 2012; Zubair et al. 2012; Falaharkar et al. 2014;
Tsalafouta et al. 2014). Cortisol is directly linked to metabolism and the allocation of carbohydrates, proteins and lipids for energy balance (Mommesen et al. 1999). Increases in glucocorticoids, like cortisol, have been directly linked to increased enzymatic activity of enzymes such as glucose-6-phosphotase and PEPCK in gluconeogenesis pathways in teleosts (Foster and Moon 1986; Vijayan et al. 1997). Increases in cortisol have also been linked to increases in plasma amino acids and free fatty acids, which could in turn be used for gluconeogenesis and possibly protein synthesis (Momessen et al. 1999). With the daily role cortisol plays in increasing or decreasing the activity of metabolic pathways, it could also play a pivotal role in developing and triggering these pathways during dietary shifts associated with early development.

In the present study Lake Sturgeon larvae did not elicit a predictable cortisol stress response until after the onset of exogenous feeding, suggesting an inability to synthesize large quantities of steroids as yolk-sac larvae. Previously, 3 days post-exogenous feeding Lake Sturgeon raised at 15°C had significantly higher cortisol levels following a stressor after 20 minutes that returned to baseline levels after 60 minutes (Zubair et al. 2012). This study replicated the larvae’s ability to synthesize cortisol following a stressor post-exogenous feeding. Similarly, the lack of increased cortisol response before feeding in this and previous research (Zubair et al. 2012), could be due to a lack of cholesterol for steroidogenesis, but also could be due to under-developed neural centers and the ability to perceive a stressor (Bernier 2009).

Despite the larvae’s ability to respond appropriately to a stressful event a consistent response in both years of this study and previously published work (Zubair et al., 2012) was an increase in baseline cortisol levels at or near the depletion of the yolk in the larval fish. Because
a fully functional cortisol response was not seen until exogenous feeding the observed increase in baseline levels is suggestive of an alternative endocrine pathway stimulating de novo cortisol production by the fish. Although controversial, it has been reported in several species that α-MSH may also play a role in stress adaptation, as plasma levels increase post-stressor (Sumpter et al. 1985; Lamers et al. 1992; Wendelaar-Bonga 1997; Bernier et al. 2009). In mammals, teleosts, and elasmobranchs CRH has been shown to specifically stimulate the precursor molecule, proopiomelanocortin (POMC) which causes the secretion of not only ACTH but also α-melanocyte stimulating hormone (α-MSH) (Lowry et al. 1974; Kawauchi 1983; Tran et al. 1990; Wendelaar-Bonga 1997). In teleosts, both α-MSH and ACTH bind to one of the 5 expressed melanocortin receptors (MCRs), which belong to a family of G-protein coupled receptors (Logan et al. 2003; Klovins et al. 2004; Flik et al. 2006; Bernier et al. 2009; Agulleiro et al. 2013). In fish and mammals, ACTH is the only specific ligand to bind to MC2R, but both ACTH and the other MSHs bind to the other four MCRs (Klovins et al. 2004; Schioth et al. 2005). The other MCRs also convey intracellular signaling mechanisms specific to the MSH variants. For example, MC4R is known for the regulation of energy balance in fish through modulating feeding behavior (Cerda-reverter et al. 2003; Song and Cone 2007). There have been very few studies linking α-MSH to the stress response during early development despite the fact activation of the HPI-axis stimulates the synthesis of this hormone (Smith and Funder 1988). In a recent study in larval Sea bass, *Dicentrarchus labrax*, whole body α-MSH levels along with cortisol and mRNA expression levels of various MCRs were measured throughout development (Tsalafouta et al. 2014). Here the authors report that as development continued α-MSH levels increased over time in the Sea Bass, with the highest levels of mRNA expression of MC2R and MC4R at the time of first feeding (Tsalafouta et al. 2014). These increases of α-MSH during this
time of development could reflect the hormones involvement in appetite regulation and satiation. Further, following an acute stressor, α-MSH levels responded similarly to whole body cortisol levels, suggesting α-MSH may be also playing a role in regulating the stress response (Tsalafouta et al. 2014). It is possible in the larval Lake Sturgeon that α-MSH is triggering the endogenous cortisol production prior to feeding for energy balance, such that the increase in baseline cortisol seen prior to exogenous feeding may be the result of increasing levels of αMSH due to the need to begin feeding exogenously.

Metyrapone is a strong steroidogenic inhibitor in most vertebrates, preventing the binding of 11-deoxycortisol to 11β-hydroxylase responsible for conversion to cortisol (Bennet and Rhodes 1986). By diminishing the proportion of enzyme molecules that have bound substrate, metyrapone reduces the rate of catalysis (Bennet and Rhodes 1986). The use of metyrapone, administered in different ways, to inhibit de novo cortisol synthesis has been well documented in adult teleosts (Bennet and Rhodes 1986; Bernier and Peter 2001; Milligan 2003; Rodela et al. 2009; 2011; McConnachie et al. 2012). Metyrapone is commonly used in fish to assess the effects of low cortisol on behavior and physiological function such as ion regulation and glycogen metabolism (Milligan 2003; Rodela et al. 2009; McConnachie et al. 2012). Although metyrapone could be inhibiting the synthesis of other steroids as it acts on 11β-hydroxylase, the greatest effect is most likely to be on cortisol as this is the main circulating steroid at this developmental stage. In this study, inhibition of endogenous cortisol production through the addition of metyrapone to treatment tanks had severe effects on exogenous feeding and growth; the treated larvae had developmental abnormalities, slower growth, and feeding was never observed. It is possible without normal baseline levels of circulating cortisol the metyrapone treated larvae were unable to properly utilize proteins and lipids from the yolk-sac for energy
allocation. The larvae are smaller in size and slower in development possibly due to decreased protein synthesis. Cortisol mediates protein catabolism and protein synthesis, and lack of baseline levels in the treated fish could have inhibited these processes (Barton et al. 1987; Mommsen et al. 1999). The proteins are typically the first macromolecule to be used from the yolk-sac (Detlaff et al. 1993) and if the larvae were unable to properly assimilate these proteins, both growth and development would be impaired. If cortisol is indeed triggering, or increasing the rate, of metabolic pathways like gluconeogenesis, the larvae would need to find an exogenous food source to keep up with increased metabolic demands. Similarly, rendering cortisol less available to developing zebrafish embryos by injection with a cortisol-specific antibody led to morphological changes including kinking of the tail, deformed spinal curvature, and decreased length in embryo (Nesan and Vijayan 2016). These results suggest a strong correlation between baseline cortisol and appropriate morphological development, particularly affecting growth and feeding in the developing Lake Sturgeon larvae.

There is high correlation between proper development, yolk-sac assimilation and dietary shifts with baseline cortisol levels. The specific mechanisms cortisol is acting through to affect the development of these processes are still unknown, but the effects of decreasing baseline cortisol levels are having on feeding are obvious. This thesis is the first to explore early development and dietary shifts in larval Lake Sturgeon in relation to baseline cortisol levels and there is still much more to understand. It has been shown in many species that proper development of the HPI axis can affect an individual’s future stress response and ultimately survival. As a conservation aquaculture species, it is imperative that not only Lake Sturgeon properly feed and grow in the hatchery, but also properly develop key endocrine axes for the best chance at survival in the wild once released. And to this end, understanding the effects of cortisol
and the development of endogenous production is vital for proper conservation management techniques.

References


Collie NL and Stevens JJ (1985) Hormonal effects on L-proline transport in coho salmon (Oncorhynchus kisutch) intestine. Gen Comp Endocrinol 59: 399–409


**Development of the chromaffin cells in larval Lake Sturgeon**

3.1 Introduction

Chromaffin cells store catecholamines such as adrenaline and noradrenaline and therefore are a part of the adrenergic system in vertebrates (Reid et al. 1998). Also known as the fight or flight response, a release of catecholamines is typically associated with an acute stressor (Wendelaar-Bonga 1997). Released within seconds to minutes into the bloodstream, catecholamines target multiple tissues to maintain adequate oxygen levels in the blood and selectively increase supply to vital organs (Wendelaar-Bonga 1997; Reid et al. 1998). These hormones have also been shown to mobilize energy reserves to compensate for increased energy demand during the initial physiological response to a stressor (Reid et al. 1998). In addition to storage, catecholamines are synthesized in the chromaffin cells, beginning with the precursor amino acid tyrosine (Blaschko 1939; Reid et al. 1998). The first step in biosynthesis is the hydroxylation of tyrosine by tyrosine hydroxylase (TH) to form L-di-hydroxyphenylalnine (L-DOPA), the rate limiting factor in noradrenaline synthesis. There are a variety of factors that regulate TH in vertebrates including neuronal and hormonal control through positive stimulation negative feedback loops (Johnsson 1929; Reid et al. 1998).

Chromaffin cells are found throughout all vertebrates but exhibit substantial diversity throughout evolution. Mammalian chromaffin cells are concentrated in the adrenal medulla (Coupland 1972); in birds the chromaffin cells are also associated with the adrenal gland but are
distributed with interrenal cells as there is no distinct separation between the adrenal medulla and cortex (Hartman et al. 1947). The distribution of chromaffin cells in lizards and snakes is similar to that of birds (Wright 1955; 1957). In some amphibians chromaffin cells are found in segmented bodies associated with sympathetic nerves on the ventral kidney surface, but in anuran amphibians, the cells are in a mass on top of kidney (Accordi 1982, 1991). In teleosts these cells are found throughout the post-cardinal vein, particularly in the head kidney region (Nandi 1961; Wendelaar-Bonga 1997; Reid et al. 1998). Elasmobranchs contain chromaffin cells in paravertebral autonomic ganglia and are closely associated with the sympathetic nerves (Lutz and Wyman 1927; Shepard et al. 1952; Abrahamsson 1979). Hagfish and lampreys have chromaffin cells distributed throughout the portal and systemic hearts, veins and arteries (Ostuland 1954; Augustinsson 1956; Johnels and Palmgen 1960). Although not many non-teleosts have been studied in this aspect, in Spotted Gar, *Lepisosteus platyrhincus*, chromaffin cells were identified in the walls of the post-cardinal veins (Nilsson 1981). In Beluga Sturgeon, *Huso huso*, chromaffin cells have been located along the entire length of the kidney, localized in the cardinal and caudal veins and their main branches (Gallo et al. 2004).

Chromaffin cells are often observed as single cells, or grouped into several cells and their association with steroidogenic interrenal cells varies amongst teleosts (Nandi 1961; Reid et al. 1998). In fish where the interrenal and chromaffin tissue occur together there are many possible arrangements including, chromaffin tissue being dispersed throughout the interrenal tissue, alternating clumps of interrenal and chromaffin tissue or both tissues forming a complex around the lumen of the cardinal veins (Nandi 1961). It is more common for the chromaffin cells to exist on their own in the cardinal veins, adjacent to the endothelium or in the connective tissue of the vein wall with the interrenal tissue external to the wall and chromaffin cells (Nandi 1961).
While the physiological actions of catecholamines have been extensively studied as well as the location of chromaffin cells, the development of these cells has not been as closely examined in fish. In mammals, chromaffin cells are derived from the neural crest cells, along with sympathetic neurons, initially localized in the embryonic sympathetic chains (Le Douarin and Kalcheim 1999; Kalcheim et al. 2002; Unsicker et al. 2005). These cell types are extremely similar; indeed it has been shown that early rat postnatal adrenal chromaffin cells can develop into neurons following exposure to nerve growth factor (Unisicker et al. 1978). In mammals, it is suggested that when primary sympathetic cells remigrate in a dorsal direction to form the sympathetic ganglia that other cells migrate ventrally to form chromaffin cells in the developing adrenal gland and aorta (Kalchiem et al. 2002). Once the neural crest cells are in the adrenal gland they mix with mesodermal cells that eventually form the adrenal cortex (Kalchiem et al. 2002). In zebrafish, the chromaffin cells develop late compared to other stress related cells (Nesan and Vijayan 2013). These cells are also derived from the neural crest and differentiate at 48 hours post fertilization (HPF) but are not dispersed throughout the steroidogenic cells in the interrenal tissue until 72 HPF (Nesan and Vijayan 2013).

Lake Sturgeon are a conservation species of high-interest and restoration efforts to restore historic population numbers have become widespread throughout the United States and Canada (Peterson et al. 2007). Conservation hatcheries have been releasing both hatchery-raised fry (fingerlings) and young-of-the-year (YOY) Lake Sturgeon into their historic range with the goal of restoring historic population numbers but without much knowledge on the early development of this species (Secor et al. 2002; Barth et al. 2015). The objective of this study was to identify chromaffin cells in larval Lake Sturgeon, examining, timing of development, location and migration of the these cells during early development. Following hatch, yolk-sac larvae, larvae
and 1 year old Lake Sturgeon were examined for development and location of chromaffin cells using immunohistochemical techniques, specifically, a tyrosine-hydroxylase antibody to highlight the precursor in catecholamine synthesis, therefore highlighting the chromaffin cell.

3.2 Methods

3.2.1 Fish Husbandry
In the spring of 2016 and 2017, eggs and sperm were collected from wild-caught spawning female and male Lake Sturgeon and returned to the University of Manitoba for fertilization. Eggs from females were freely mixed with sperm from at least two males to reduce potential confounding genetic effects on the resultant hatched larvae. De-adhesed embryos were incubated in McDonald jars at 12°C until hatch in early June. Once larvae hatched they were transferred to sixteen 9L aquariums with flow-through dechlorinated tap water maintained at 15°C ±1. Each tank had bio-balls for substrate until exogenous feeding began when all substrate was removed over a 7-day period (Zubair et al. 2012). A few days prior to yolk-sac absorption live artemia (brine shrimp) were introduced to the tanks. Following exogenous feeding on artemia, the larvae were slowly transitioned to a bloodworm (chronomids) diet over a 25-day period. Tanks were monitored at least twice daily, when mortalities and debris were removed. All animals used in this study were reared and sampled according to animal use and care guidelines established by the Animal Care Committee at the University of Manitoba (Protocol #F15-007).

3.2.2 Sampling for Chromaffin Cells
Following hatch, 10 individuals were sampled daily from hatch-exogenous feeding, 3 months post fertilization, and 1 year post fertilization in 2017. After randomly selecting from tanks, larvae were over-anesthetized in MS-222 buffered with bicarbonate (0.5g.L⁻¹).

3.2.3 Tissue processing

Whole body larvae were immediately fixed in either 10% neutral buffered formalin (Sigma Aldrich) or Bouin’s fixative (75 ml picric acid; 25 ml formalin; 5 ml acetic acid into 500 ml H20) for three days. Following fixation, tissues were dehydrated in a series of ethanol and cleared with Slidebrite, and embedded in paraffin wax. Paraffin blocks were then sectioned between 5 μm and 7 μm and floated on a 35°C water bath. Sections were then fixed onto albumin-coated glass slides. Slides were then left on a slide warmer at 35°C for at least 24 hours to ensure sections did not fall of during the staining procedure.

3.2.4 Slide staining

The PAP staining protocol previously used by Adrio and colleagues (2002) was adapted to process the sections. First the sections were deparaffinized, cleared and rinsed with phosphate-buffered saline (PBS; 0.9% NaCl in 0.1 mM PB and pH 7.4). All staining was conducted in a humid chamber. The slides were first stained with 10% H₂O₂ for 30 minutes to terminate any endogenous peroxide activity in the cells and then rinsed twice with PBS for 10 minutes each. Next sections were treated with 10% goat serum for 1 hour and then rinsed twice with PBS for 10 minutes each. Then rabbit tyrosine-hydroxylase antibody (dilution 1:1000) was applied overnight and again slides were rinsed with PBS in two 10 minute applications. The secondary
antibody, goat-anti-rabbit Ig (1:100) was then added for an hour and then rinsed twice in 10 minute applications of PBS. The rabbit peroxidase-antiperoxidase (PAP) complex (1:200) was then added to the slides for an hour and then slides were once again rinsed twice with PBS for 10 minutes each. Lastly, the reaction was revealed by immersing slides in 0.05% diaminobenzidine (DAB) and 0.003% H₂O₂ for 15-20 minutes. All dilutions were made in PBS containing 0.2% Triton X-100. The sections were then rinsed in PBS, and counterstained with hematoxylin and eosin. Following counterstaining the slides were fixed with Permount, coverslipped, and heated on a slide warmer for 24 hours.

3.2.5 Imaging

All slides were examined using a light microscope (Olympus; Ref# 2J36670) at multiple magnifications. Pictures were taken using an Infinity 1 camera (Ref# 0195105) and Infinity Capture software.

3.3 Results

3.3.1 Appearance of tyrosine-hydroxylase reactive cells, renal tubules and interrenal cells

Renal tubules appear at time of hatch or at 8 DPF (Fig. 3.1, Fig 3.2). The tubules are first identified in-between the yolk-sac and trunk of body (Fig. 3.1; 3.2). Throughout development the renal tubules migrated from near the yolk-sac to posteriorly down the body (10 DPF; Fig. 3.3). Kidneys formed posteriorly along the length of the body by 18 DPF (Fig. 3.6; Fig 3.7). TH reactive cells, or catecholamine-synthesizing chromaffin cells, were positively stained starting at 13 DPF, 5 days following hatch (Fig 3.4). TH cells first stained near the renal tubules, near the yolk-sac, just anterior to the heart around venous structures (Fig. 3.4). The cell staining
progressively moved down the kidneys, lining the post-cardinal vein (Fig. 3.5; 3.6). Following 13 DPF reactive cells were found at each subsequent time point. Steroidogenic cells can be found in close proximity to both the renal tubules and chromaffin tissue in the 3 month old and 1 year olds (Fig. 3.9; 3.10; 3.11; 3.12). Interrenal cells were identified by deep staining by hematoxylin and the presence of lipid droplets (Humason 1930). As the sturgeon develop, groups of chromaffin cells are found in the head kidney region as well as the posterior cardinal vein (Fig. 3.12).

3.3.3 Positive and negative controls

Positive controls were conducted using juvenile Lake Sturgeon kidney samples (Fig. 3.12; 3.13; 3.14). The juvenile Lake Sturgeon positive control illustrates successful staining in a more developed fish. Two negative controls were conducted for each age group by either not staining with the TH primary antibody or not staining with the secondary antibody (Fig. 3.12; 3.13; 3.14). All negative controls did not show any positive TH staining.
Figure 3.1 Micrograph of 8 DPF (hatch day) Lake Sturgeon trunk at 4x. Notochord (NC), brain (B), skeletal muscle (SM), yolk sac (YS); black arrows point to renal tubules (RT). Counterstained with hematoxylin and eosin.
Figure 3.2 [A] Micrograph of 8 DPF (hatch day) Lake Sturgeon trunk at 4x. Brain (B), muscle (SM), yolk sac (YS) and black arrows point to renal tubules (RT). [B] Renal tubules (RT) and yolk-sac (YS) at 25x. Counterstained with hematoxylin and eosin.
Figure 3.3 Micrographs of 10 DPF Lake Sturgeon larvae. [A] Shows trunk and yolk-sac at 4x, spinal cord (SC), notochord (NC), kidney (K), yolk-sac (YS) and the spiral valve (SV) are labeled. [B] 25x of black square on A; Notochord (NC), Posterior cardinal vein (PCV), and Kidney (K) are labeled. Counterstained with hematoxylin and eosin.
Figure 3.4 Micrograph of 13 DPF Lake Sturgeon larvae, first day of positive TH staining. [A] Renal tubules (RT) and Yolk-sac (YS) are labeled at 10x [B] Renal tubules (RT), Yolk-sac (YS) and skeletal muscle (SM) are labeled. Black arrows indicate positive TH staining in chromaffin cells that are stained black. Counterstained with hematoxylin and eosin.
Figure 3.5 Micrograph of 18 DPF Lake Sturgeon larvae, anterior end. [A] Renal tubules (RT), brain (B), skeletal muscle (SM) and Yolk-sac (YS) are labeled at 4x; [B] Renal tubules (RT), erythrocytes (RBC) Yolk-sac (YS) and skeletal muscle (SM) are labeled at 10x. Black arrows indicate positive TH staining in chromaffin cells that are stained black. Counterstained with hematoxylin and eosin.
Figure 3.6 Micrograph of 18 DPF Lake Sturgeon larvae, caudal end [A] Spiral valve (SV) skeletal muscle (SM) labeled, kidney to the left of ** at 4x [B] More anterior end: Notochord (NC), Spiral valve (SV) and yolk-sac (YS) labeled, kidney to the left of ** at 10x. Black arrows indicate positive TH staining in chromaffin cells that are stained black. Counterstained with hematoxylin and eosin.
Figure 3.7 Micrograph of 18 DPF Lake Sturgeon larvae, skeletal muscle (SM), yolk-sac (YS), posterior cardinal vein (PCV) indicated by black arrows and kidney to the left of ** 4x. Counterstained with hematoxylin and eosin.
Figure 3.8 Micrograph of posterior trunk in 3 month old Lake Sturgeon at 4x. Kidney (K) and posterior cardinal vein (PCV) labeled. Black arrows indicate positive TH staining in chromaffin cells that are stained black. Counterstained with hematoxylin and eosin.
Figure 3.9 Micrograph of mid-trunk 3 month old Lake Sturgeon kidney at [A] 4x. Kidney (K), muscle (SM) and posterior cardinal vein (PCV) labeled. Black arrows indicate positive TH staining in chromaffin cells that are stained black. [B] Micrograph of 3 month old Lake Sturgeon at 25x. Kidney (K), interrenal cells (IRC) and posterior cardinal vein (PCV) labeled. Black arrows indicate positive TH staining in chromaffin cells that are stained black. Counterstained with hematoxylin and eosin
Figure 3.10 Micrograph of 3 month old Lake Sturgeon at 40x. Kidney and interrenal cells. Black arrows indicate positive TH staining in chromaffin cells that are stained black. Counterstained with hematoxylin and eosin.
Figure 3.11 Micrograph of 1 year old Lake Sturgeon kidney at 10x. Kidney and posterior cardinal vein (PCV) and interrenal cells (IRC) labeled. Black arrows indicate positive TH staining in chromaffin cells that are stained black. Counterstained with hematoxylin and eosin.
Figure 3.12 Micrograph of control staining in 1 year old Lake Sturgeon trunk at 4x. Spinal cord, notochord, scutes, posterior cardinal vein (PCV), interrenal cells (IRC) and kidney are labeled. Black circle and arrows indicates positive TH staining for chromaffin cells in head kidney and down the PCV, cells are stained black. Counterstained with hematoxylin and eosin.
Figure 3.13 Micrograph of 1 year old Lake Sturgeon [A] Negative control for TH antibody, kidney and posterior cardinal vein (PCV) labeled 4x. [B] Positive control for TH antibody; Kidney and posterior cardinal vein (PCV) labeled 10x. White arrows indicate positive TH staining in chromaffin cells that are stained black. Counterstained with hematoxylin and eosin.
Figure 3.14 Micrograph of positive control in 3 year old Lake Sturgeon. [A] Chromaffin cells with positive TH stain at 40x; positive TH indicated by black arrows. [B] Kidney (K), interrenal cells (IRC) and posterior cardinal vein (PCV) labeled at 4x. [C] Chromaffin cells with positive TH stain at 40x. [D] Kidney (K), interrenal cells (IRC) and posterior cardinal vein (PCV) labeled at 4x; positive TH indicated by black arrows. All slides counterstained with hematoxylin and eosin.

3.4 Discussion

Catecholamines are involved in a number of physiological processes including stress responses and neurotransmission in the nervous system (Reid et al. 1998; Adrio et al. 2002). They are synthesized in the chromaffin tissues of vertebrates via the Blashcko pathway.
The first step in the pathway involves the hydroxylation of the amino acid tyrosine by TH to form L-DOPA (Blashcko 1939). To function, TH requires both Fe^{2+} and oxygen and there are a variety of factors that regulate its activity (Jonsson and Nilson 1982). Some of these factors in mammals and fish include negative feedback pathways, nervous stimulation of chromaffin cells, and circulating levels of ACTH and glucocorticoids (Muller et al. 1970; Stachowiak et al. 1990). The second step in biosynthesis converts L-DOPA to dopamine via L-aromatic amino acid decarboxylase. Dopamine is then shuttled via secretory vesicles where the enzyme dopamine-B-hydroxylase (DBH) converts it to noradrenaline (Jonsson 1921). In some chromaffin cells, noradrenaline is then transported into the cytoplasm to form adrenaline through methylation by PMNT (Abrahammson et al. 1981). By highlighting one of these enzymes, with the use of an antibody, it allows detection of catecholamine-synthesizing cells from non-synthesizing cells (Adrio 2002). TH in particular is an ideal enzyme to target because it is found in most catecholamine synthesizing cells and is the rate-limiting step in biosynthesis (Smeets and Gonzalez 2000). In this study, highlighting TH shows when the chromaffin cells actively start producing catecholamines and also the location of these cells. But because TH is also involved in the synthesis of dopamine, it is also possible highlighted cells are synthesizing dopamine and not completing the catecholamine pathway.

Various enzymes in the Blashcko pathway have been identified in teleost embryos, larvae (Gallo and Civinini 2001; Ekstrom et al. 2002) and adults (Gallo et al. 2001; Dumbarton et al. 2010). Using antibodies for these enzymes has been shown to be highly specific and useful for identifying chromaffin cells in larval fish. Salmonids, Perciformes, and Cyrinodontiformes have all shown positive reactions for DBH and PMNT activity throughout development (Milano et al. 1997). Specifically, in salmonids activity, of these enzymes was identified close to hatch and in
Perciformes the enzymes were slightly delayed, becoming evident at 20 DPH (Milano et al. 1997). In Cyprinodontiformes the enzymes were not identified until complete absorption of the yolk sac (Milano et al. 1997). In developing Rainbow Trout, enzymatic activity was found at 27 DPF, near the time of hatching (Gallo and Civinni 2005). In the present study, the Lake Sturgeon larvae showed positive staining at 13 DPF which falls in the range of the various species listed above. It is possible the enzymes are not active until days after hatch because the sympathetic nervous system is not yet fully functional. As part of the hypo-responsive stress period during early development, it is more than likely these hormones do not have the fully developed pathways (Barry et al. 1995; Sapolsky and Meaney 1986).

Because of the important role catecholamines play, one would hypothesize the development of chromaffin cells that produce catecholamines would be well understood. But to our knowledge, there are no past published studies on the development of these cells in a non-teleost, and literature of chromaffin cell development in teleosts is limited. In the current study, the TH positive cells were highlighted by the antibody starting at 13 DPF, 5 days after hatch whereas renal tubules can be seen at the time of hatch, 8DPF. Although, it is possible the sensitivity of the antibody could not detect lower levels of TH prior to 13 DPF. In zebrafish, these cells are developing at 48 HPF (hatch) and are dispersed throughout the steroidogenic cells at 3 DPF, later than development of interrenal cells (24-36HPF) and steroidogenesis (28 HPF) (Liu 2007; Nesan and Vijayan 2013). The parallel development of interrenal tissues and chromaffin cells is similar to the formation of the adrenal gland in mammals (Liu 2007).

As in mammals, the cells are derived from the neural crest through multiple subsequent stages of differentiation (An 2002; Unsicker et al. 2005). Specifically, the neural crest gives rise to the dorsal root ganglion, sensory neurons and sympathetic neurons which then all undergo
different rates of differentiation (An 2002). After neuronal differentiation, sympathetic neurons will express adrenergic markers such as DBH and TH (An 2002). A second population of adrenergic non-neuronal cells, located initially within cervical sympathetic neurons, differentiate into chromaffin cells eventually present within the kidneys in more mature fish (An 2002). The zebrafish chromaffin cells highlighted by DBH appear at 2 DPF as separate cells from the steroidogenic primordium but by 3 DPF they are fused into one domain (To et al. 2007). At 5-7 DPF the chromaffin cells move closer to the trunk midline and appear enveloped by the steroidogenic compartment of the kidney (To et al. 2007).

In the current study, if it is assumed the antibody sensitivity can detect levels through all stages, the delayed development of these cells when compared to other endocrine cells related to the stress response, would be similar to that seen in zebrafish (An 2002; Liu 2007; To et al. 2007; Nesan and Vijayan 2013). Similarly, to zebrafish, the Lake Sturgeon larvae develop renal tubules, with chromaffin cells “invading” the tissue approximately 5 days after. Following the differentiation of these cells, the Lake Sturgeon chromaffin cells also begin to move similarly to zebrafish cells. Starting near the renal tubules by the yolk sac, the cells eventually migrate to the midline of the kidneys, grouping around cardinal veins, interspersed with interrenal cells.

As discussed in the introduction, chromaffin cells vary in location throughout vertebrates but are typically associated with the posterior cardinal veins and head kidneys in fish (Wendelaar-Bonga 1997). The cardinal vein and bilateral kidney arrangement vary between species as seen in the figure adapted from Waheed (2011; Fig. 2.15). In the cockscomb prickleback, Anoplarchus purpureascens, the kidneys are fused together immediately following the cephalic end with a single PCV that begins in the right kidney and moves more towards the
midline caudally (Fig. 2.15 A; Youson et al. 1989). In American eel the anterior kidneys are separated until the posterior end where they are fused, they have a short IPCV that supplies a small portion of the cephalic kidney and a rPCV that runs through the rest of the kidney (Fig. 2.15 B). In the starry sturgeon the kidneys are fused at the caudal end and the rPCV supplies the cephalic half of the right kidney while the IPCV crosses to the middle of the kidneys and supplies the posterior region of both kidneys (Fig. 2.15 C). Paddlefish kidneys are completely fused throughout their length and the IPCV supplies the length of the kidneys while the rPCV drains the cephalic end of the kidney (Fig. 2.15 D). The Spotted gar kidneys are fused at the caudal end with a PCV in each side of the kidney (Fig. 2.15 E). The bowfin kidneys are similar to the Spotted gar but they only have one PCV on the right side (Fig 2.16 F; Mok 1981). In this
study there appears to be a main post cardinal vein, with the possibility of a similar arrangement to Starry Sturgeon with the IPCV joining into one PCV.

In 3 month and 1-year old Lake Sturgeon the chromaffin cells were found throughout the length of the kidney, associated closely with the cardinal veins and interrenal tissue. The distribution of the chromaffin cells in the Lake Sturgeon is similar to that seen in other adult sturgeon and Spotted Gar (Balashov et al. 1981; Nilson 1981). In the Spotted Gar, adrenaline and noradrenaline were the highest in the cardinal veins when compared to other organs and the blood plasma (Nilsson 1981). Further, with the use of fluorescent tags, the chromaffin cells were highlighted in the lumen of the posterior cardinal vein with a very similar adrenergic system to teleosts (Nilson 1981). In the Beluga Sturgeon, both adrenaline and noradrenaline levels and fluorescent tags for chromaffin cells were the highest and most prominent, respectively, in the cardinal veins (Balashov et al. 1981). In the Baltic sturgeon, Acipenser sturio, the chromaffin tissue is related to the walls of PCVs (Giacomini 1994).

This study is the first to examine location and timing of developing chromaffin cells in Lake Sturgeon. It is not unexpected that the development of these cells is similar to that of zebrafish as the process seems to be highly conserved throughout vertebrates. As the larvae developed in the juvenile Lake Sturgeon, we identified similar chromaffin cell locations to the Beluga Sturgeon and the Spotted Gar. The ancient fish along with teleosts further illustrate the highly conserved pattern of these cells. The findings in this study are a first step to understanding the development of the adrenergic response in Lake Sturgeon, a species of conservation concern. Through understanding proper timing and development of catecholamine-releasing cells, the effects of raising larvae in a hatchery, on this important endocrine system, can now be studied.
References


Augustinsson KB, Fa`nge R, Johnels A, Ostlund E (1956) Histological, physiological and biochemical studies on the heart of two cyclostomes, hagfish (Myxine) and lamprey (Lampetra). J Physiol. 131:257–76


Chapter 4: Conclusion

The objective of the studies presented in chapters 2 and 3 was to understand the development of both endogenous cortisol production and chromaffin cells. Based on the findings from 2016, in 2017 further experiments were conducted to understand the importance of cortisol throughout development in relationship to dietary shifts. In 2016, there were multiple increases in baseline cortisol throughout development, all associated with diet. The first peak observed once the yolk-sac was depleted and the larvae exogenously feed has also been reported in multiple species (De Jesus et al. 1991; Gessner et al. 2009; Simontacchi et al. 2009; Nesan and Vijayan 2012; Zubair et al. 2012; Falaharkar et al. 2014; Tsalafouta et al. 2014). The second peak occurs during the transition from artemia to bloodworms, another early dietary shift, and a third peak, not discussed in the context of this thesis was also observed during overwintering starvation.

This increase in cortisol has been associated with a stress response to lack of food and the need to find it. But, in this thesis I have presented data to show this increase in baseline cortisol is necessary for proper development and switching diets, not just a response to stress. In 2017, with the addition of metyrapone, causing an overall decline in baseline cortisol, the larvae never expunged their anal plugs and never fed. While these results are only correlative, there was also a decline in total body protein and wet mass in the metyrapone treated fish. This suggests cortisol playing a large role in larval growth, energy assimilation and overall survival. One of the main roles cortisol plays throughout life is maintaining physiological homeostasis and that includes the allocation of resources for energy (Wendelaar-Bonga 1997). It is possible cortisol also helps develop and trigger these pathways during crucial times of development like early dietary shifts.
There need to be more studies directly linking cortisol effect on these pathways, unfortunately the genome has not yet been sequenced nor do full transcriptomes for Lake Sturgeon yet exist, but in the future comparing expression of HPI axis genes, enzymes involved in gluconeogenesis and protein/lipid synthesis to baseline cortisol levels would help define the mechanistic action of cortisol during development.

The timing of cortisol production and proper development of the HPI axis is crucial to an individual’s survival and ultimate reproductive success. An improper development of this axis due to differing cortisol levels has been shown in zebrafish larvae (Nesan and Vijayan 2016). The larvae were unable to respond to stressors later in life, implicating a non-plastic response to early cortisol levels that are long-lasting. This is especially troublesome for an endangered species such as Lake Sturgeon. With many conservation hatcheries releasing larval fish into the wild, it is imperative their HPI axis develop properly so they can survive in the wild. To understand the effects raising larvae in a hatchery has on overall survival requires a definition of proper development. A part of this thesis project was to define the pattern of endogenous cortisol production in larval Lake Sturgeon, so future studies can test how different hatchery parameters alter developmental trajectory. It is important to highlight natural variation of cortisol within each day and overtime so not to assume every increase seen is a response to a stressor. The baseline increases in this study were necessary for survival and feeding, and at first glance could have easily been interpreted as a negative stress response. Also, the decrease in cortisol observed after fertilization is key to proper HPI axis development and is known as the hypo-responsive stress period. Identifying this time period in Lake Sturgeon will assist hatcheries in ensuring low-stress environments throughout this time i.e adequate aeration, consistent temperature and minimal physical disturbance.
The development of the chromaffin cells showed high parallelism to zebrafish chromaffin cell development. Although zebrafish develop at a much faster pace, the timing and movement of the cells is almost identical in Lake Sturgeon. When these cells develop can provide better insight to the development of the fight or flight stress response and the individuals ability to cope with acute stressors. At hatch, the Lake Sturgeon would still be absorbing their yolk-sac in the substrate away from predation so it is possible they may not need a fully developed fight or flight response. The appearance at 13 DPF and the rapid movement of the cells to the midline throughout the kidney by 18 DPF highlights the importance of developing these cells during yolk-sac absorption prior to emergence and foraging for an exogenous food source.

There are many other studies needed to further understand the development of the HPI axis and the stress response and how it will affect Lake Sturgeon once released into the wild. It would be ideal to conduct a catch and release study with wild fish, measure baseline cortisol and stress responses and compare to these fish raised in the hatchery. Another study that would provide further insight to both the development of the HPI axis would be injecting embryos with a specific cortisol antibody and injecting other embryos with excess cortisol. This antibody would ensure cortisol is the only steroid being blocked rather than blocking \(11\beta\)-hydroxylase which can block other steroid production. The embryos injected with more cortisol could mimic increased maternal input into the egg, which would give insight into how a stressed female effects development. Further effects on dietary shifts and development could be studied during embryogenesis and throughout development in both treatments. Enriching the artemia and bloodworms with lipids or proteins could give further insight into the mechanistic action of cortisol during these shifts and give a more realistic view into wild fish. By injecting embryos with genetic florescent tags for chromaffin cells we could further understand the development of
chromaffin cells. This would allow for understanding of chromaffin cell development prior to hatch and would allow for comparisons between structural development and functionality.

Overall, ontogeny of endogenous cortisol production and chromaffin cells during early development is undoubtedly critical to fitness and overall survival. As such, studies like this can impact and change current conservation aquaculture practices to ensure more fit individuals for release. As stated previously, it is imperative more molecular and physiological studies are completed to fully understand the development, especially in an endangered species like Lake Sturgeon.
References


