Ontogeny of the cortisol stress response in lake sturgeon *Acipenser fulvescens* as influenced by substrate and temperature during early development

by

Sadaf Naeem Zubair

A Thesis submitted to the Faculty of Graduate Studies of

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Ontogeny of the Cortisol Stress Response in Lake Sturgeon *Acipenser fulvescens* as Influenced by Substrate and Temperature During Early Development

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Of

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Abstract

Lake sturgeon eggs were hatched on gravel or slate or in hatchery jars in 2008 to determine the impact of substrate on the ontogeny of the hypothalamic-pituitary-interrenal stress axis. The experiment was repeated in 2009, along with the impact of temperature on the HPI axis. Larvae were unable to respond to a stressor via an increase in cortisol concentrations until the onset of exogenous feeding. Substrate studies indicate that larvae hatched on gravel or slate or in hatchery jars were capable of increasing cortisol levels when stressed. Resting concentrations of cortisol were higher in the slate treatment (17.59± 3.49 $ng.g^{-1}$) as compared to gravel (5.67±0.41 $ng.g^{-1}$). When stressed, the magnitude of response was equivalent for substrates (73.05±8.11 $ng.g^{-1}$ and 62.65±6.98 $ng.g^{-1}$ for slate and gravel, respectively). The duration of response varied, with gravel-treatment larvae exhibiting an extended response. Results from temperature studies indicate that development and yolk sac absorption rates were most rapid at 15 °C. Larvae reared at 9 and 15°C had elevated resting whole-body cortisol concentrations as compared to fish reared at 12°C (38.6 ±3.1 $ng.ml^{-1}$ at 9°C, 5.67±0.41$ng.ml^{-1}$ at 12°C, and 25.38 ±2.84$ng.ml^{-1}$ at 15°C). When stressed, larvae reared at 9°C were incapable of increasing cortisol concentrations. Larvae reared at 12 and 15°C did not differ in the magnitude of response when stressed (62.65±6.98 $ng.g^{-1}$ and 69.05±6.48 $ng.g^{-1}$, respectively). As with the substrate experiments, the duration of responses did vary, with larvae reared in 12°C exhibiting an extended response.

Complementary catecholamine data in response to a stressor indicate higher concentrations of norepinephrine as compared to epinephrine concentrations at rest. Rapid increase in norepinephrine and epinephrine occurred upon stressing, with a greater increase noted for epinephrine within 1 minute post-stress.

Chapter 1: Introduction

1.1: The stress response in fish

Organisms living in the natural world inevitably experience stress. Stress can be described as any entity or circumstance resulting in an imbalance of the internal homeostasis of an organism. The term stress is generally viewed as having negative implications on the physiology and fitness of individuals (Korte et al. 2005). While chronic stressful events are known to cause physical damage (Barton et al 1987, Lankford et al. 2006, Pottinger and Mosuwe 1994), acute stress can confer several positive benefits. The adaptive nature of the stress response allows organisms to cope with a changing environment or situation by maintaining homeostasis (Barton 2002) via neural and endocrine mechanisms (Greenberg et al. 2002).

Allostasis, a process of achieving stability by physiological change, works through multiple layers of feedback mechanisms. Chronic stress causes mediators of allostasis, including adrenal hormones, neurotransmitters, and immune-cytokines, to be released too frequently (McEwen 1998, McEwen 2003). Such events can eventually lead to decreased reproductive fecundity, growth abnormalities, decreased disease resistance, and have negative impacts on cardiovascular physiology, respiratory physiology, and thermoregulation (Barton 2002, Greenberg et al. 2002, Moberg 1992, Ramsay et al. 2009). Similar to mammals, blood plasma concentrations of endocrine substances such as cortisol, epinephrine, and norepinephrine can be used to assess stress responses in

fish (Cataldi et al. 1998). Exposure to a stressor can elicit primary and secondary responses.

A primary response in fish constitutes a change in plasma levels of corticosteroids and catecholamines (Greenberg et al. 2002). Cortisosteriods present in fish blood include cortisol, cortisone, 11-deoxycortisol, and corticosterone, with cortisol being the primary corticosteroid in actinopterygian fishes (Barton et al. 1998, Barton 2002, Hanson and Fleming 1979, Milla et al. 2009, Mills et al. 2009, Mommsen et al. 1999, Sanglang et al. 1971, Webb et al, 2007). An increase in circulating cortisol is accompanied by mobilization of energy substrates such as glucose and free fatty acids in plasma (Kloas et al. 1997, Lays et al. 2009, Nematollahi et al.2009, Webb et al. 2007). Release of catecholamines similarly induces an increase in circulating levels of glucose through a variety of mechanisms including the conversion of glycogen to glucose (Mazeaud et al. 1977). There are many published examples of abiotic and biotic environmental variables or stressors including pollutants, oxygen availability, ion levels, temperature, and substrate. For example exposure to sublethal doses of lead nitrate induced an increase in plasma cortisol in the common carp, *Cyprinus carpio* (Ramesh et al. 2009) and hypoxia and physical disturbance led to increases in plasma cortisol in the spotted wolfish, *Anarhichas mino* (Lays et al. 2009). It is important to note that the intensity of the cortisol response can depend upon the nature of the stressor, as well as the lifestyle of the organism. Lays et al. (2009) noted a weak cortisol response in spotted wolffish, which they hypothesize may be due to the sedentary lifestyle of the species.

Catecholamines are released from chromaffin tissue upon stimulation of cholinergic receptors (Reid et al. 1998). Catecholamines are also believed to be released by a complex interaction of plasma catecholamine levels, cortisol, and plasma concentrations of K^+ and CO_2 (Wendelaar Bonga 1997). At a primary level, catecholamine release contributes to an increase in oxygen uptake via higher ventilation rate, higher cardiac output, and hyperglycemia in different fish species (Wendelaar Bonga 1997).

Prolonged release of cortisol and catecholamines (norepinephrine and epinephrine) results in secondary responses that constitute larger scale changes, such as altered functions of metabolism, cardiovascular, respiratory, immune and reproductive systems (Barton 2002, Milla et al. 2009, Wendelaar Bonga 1997). A web of responses to stressful events is depicted in figure 1.1.

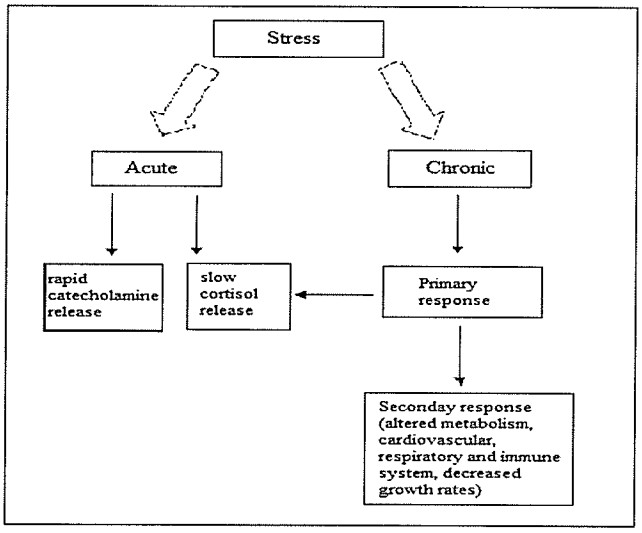


Figure 1.1. The effects of stressors on fish physiology at primary and secondary levels. Figure adapted from Barton 2002 and Mommsen et al. 1999.

Cortisol is released as the end product of the hypothalamic-pituitary-interrenal (HPI) axis in fish (Belanger et al. 2001, Mommsen et al. 1999). In mammals, cortisol is the end product of the hypothalamus-pituitary-adrenal axis (HPA). Following exposure to a stressor in mammals the hypothalamus releases corticotrophin-releasing hormone (CRH) which in turn targets corticotroph cells in the pituitary to release adrenocorticotropin hormone (ACTH) which then targets the adrenal cortex to stimulate the release of cortisol as the principal corticosteroid. Unlike mammals, fish do not have an adrenal gland. Instead steriodogenic cells are located in interrenal tissue which function to release cortisol upon stimulation by ACTH (Lankford et al. 2006). The biosynthesis of cortisol from cholesterol is similar in mammals and fish, with the involvement of numerous microsomal enzymatic pathways (Mommsen et al. 1999). Briefly, the process begins in the cytosolic compartment with the precursor for all steroid synthesis, cholesterol, entering the mitochondria. Steriodogenic acute regulatory protein (StAR) is involved with transporting cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, where cholesterol is converted to pregnenolone with the aid of the cleavage enzyme cytochrome P450scc (Nematollahi et al. 2009). Hydroxylation of this substance by cytochrome P450c17 converts it to 17-OH-pregnenolone. The product is converted with the aid of 3β-hydroxysteroid dehydrogenase (3βHSD) to 17-OH-progestrone. Cytochrome P450c21 then acts to convert it to 11-deoxycortisol. In the final stage, the 11-deoxycortisol is converted to cortisol with the aid of cytochrome P450c11. In comparison to the rapid release of catecholamines, this process has a lag time, thus allowing for more accurate

data on resting levels in plasma corticosteroid concentration (Barton 2002, Lays et al. 2009).

Recent molecular studies have examined mRNA expression levels of proteins involved in the steroidogenic pathway of cortisol and attempted to correlate changes with increases in plasma cortisol concentration in fish. In the rainbow trout, *Oncorhynchus mykiss*, expression of StAR and P450scc proteins in response to stress may be dependent on the severity of stress, with increased mRNA expression only following exposure to high stress levels (Geslin and Auperin 2004, Hagen et al. 2006). *C. carpio* exposed to a severe acute stressor exhibited a corresponding increase and decrease in mRNA levels of 11βHSD2 and cortisol levels respectively, while mRNA levels of StAR and P450c21 were unaffected (Nematollahi et al. 2009). Similar work by Alderman and Bernier (2009) explored the corticotrophin-releasing factor (CRF) system and stress in zebrafish, *Danio rerio*. CRF (analogous to CRH) is responsible for stimulating the release of ACTH from the pituitary, which in turn binds to melanocortin type 2 (MC2R) receptors to initiate cortisol production from cholesterol (Alderman and Bernier 2009).

ACTH stimulation of cortisol release in fish is also evident and has been demonstrated in a sturgeon species, the white sturgeon, *Acipenser transmontanous*, where ACTH caused a dose dependant increase in circulating levels of cortisol (Belanger et al. 2001). Other hormones have been shown to induce increases in circulating levels of cortisol in fish, such as angiotensin II, urotensin I (U I), urotensin II (U II) in the flounder, *Platichthys flesus* (Arnold-Reed and Balment 1994), and 11-keto-testosterone

in rainbow trout, *Oncorhynchus mykiss* (Young et al 1996). While the influence of angiotensin and 11 keto-testosterone on the stress response may be marginal the actions of the urotensins are more pronounced. U I, the G-protein coupled receptors CRF R1 and CRF R2, and a CRF BP binding protein are all involved in the regulation of cortisol secretion through the CRF system (Alderman and Bernier 2009). Interestingly, measurable levels of CRF system component genes have been detected in unfertilized zebra fish eggs (Alderman and Bernier 2009).

Upon cortisol production, fish differ from mammals in that there appears to be a less important role for corticosteroid-binding globulins. However, Mommsen et al (1999) argue that this noted difference may simply be due to a lack of sufficient research in fish. There is a gradual uptake of lipophilic cortisol by passive diffusion or active transport via low-affinity carrier proteins into cells, and subsequent binding to corticosteroid receptors or catabolism of the steroid (Mommsen et al 1999).

Bound cortisol acts as a transcription factor, impacting gene regulation via a direct signal transduction system in fish (Pratt 1993). Blood glucose levels are also impacted, with cortisol causing an increase in gluconeogenesis in fish livers via increase in activities of enzymes, including glucose 6-phosphatase and fructose1,6-biphosphatase (Mommsen et al. 1999). Proteins can be catabolised by the prolonged persistence of plasma cortisol (Barton et al 1987), as indicated by amino acid concentrations in fish plasma (Vijayan et al 1997). Such a process may result in decreased growth rates (Mommsen et al 1999). Other effects of high cortisol levels include central nervous system activation, higher mean blood pressure (Bamberger et al 1996) and enhanced

osmoregulation in saltwater fish species (Mommsen et al 1999). In order to verify the cost of stress and cortisol, Lankford et al. (2005) measured cortisol levels to quantify metabolic scope for activity (MSA) and swimming velocity in chronically stressed green sturgeon, *Acipenser medirostris*. The results indicate a significant decrease in MSA but no effect on swimming velocity. Furthermore, the sturgeon displayed a drastic decrease in liver glycogen, suggesting a considerable cost of the chronic stress (Lankford et al. 2005).

1.2: The endocrinology of stress in juvenile and larval fish

The development of the stress axis during early life stages is key to how an organism will cope with stress throughout its lifetime. Cortisol is found in eggs and larvae of many fish species including Japanese flounder, *Paralichthys olivaceus*; chum salmon, *Oncorhynchus keta*; Mozambique tilapia, *Oreochromis mossambicus*; rainbow trout, *O. mykiss*; Asian sea bass, *Lates calcarifer*; and common carp, *Cyprinus carpio*; with timing of activation of the HPI axis differing between species (Mommsen et al. 1999). A selection of studies finding elevated cortisol levels in larvae and juvenile fish are listed in table 1.1.

Table 1.1 Studies exploring the stress response in egg, larval and juvenile fish species

Species	Age	Methods	Results	Researchers
White sturgeon (*Acipenser transmontanus*)	Juvenile	Exposure to hypoxic and hypercapnic stress	Increase of catecholamines and cortisol plasma for hypercapnic stress.	Cech, J. and Crocker, C. (2002)
Atlantic cod (*Gadus morhua*)	8 day-old larvae	Exposure to acute stressors.	Significant levels of cortisol observed.	King et al. (2006)
Sterlet sturgeon (*Acipenser ruthenus*), Siberian sturgeon (*A. baerii*), Russian sturgeon (*A. gueldenstaedtii*) and starred sturgeon (*A. stellatus*)	2 year-old juveniles	Transfer from freshwater to brackish water. Blood cortisol levels and ATPase activity in gills and kidneys measured.	Greatest increase of plasma cortisol in juvenile starlet, followed by Siberian sturgeon, and Russian sturgeon. No change in Starred sturgeon.	Krayushkina et al. (2006)
Green sturgeon (*Acipenser medirostris*)	Young of the year	Time of day and temperature tested.	Higher cortisol plasma levels at night, with longer responses at cooler temperatures.	Lankford et al. (2003)
Gilthead seabream (*Sparus aurata*)	Juvenile	Chasing and overcrowding stressors	Decreased average growth and survival with chronic overcrowding. Cortisol elevation noted in chased fish.	Montero et al. (2001)
Red drum (*Sciaenops ocellatus*)	Larvae	Diet, temperature and dissolved oxygen cycle fluctuations (environment) stressors tested to study larval endocrine development.	Interrenal axis active during yolk-sac phase. Cortisol levels decreased during larval stage.	Perez-Dominguez and Holt (2006)
Rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*)	Juvenile	Physical disturbance and confinement (acute).	Activity of the HPI axis observed in fish as young as 5 weeks.	Pottinger and Mosuwe (1994)
Turbot (*Scophthalmus maximus*)	23-day old	Crude oil exposure (high levels/acute).	Elevation of whole-body cortisol.	Stephens et al. (1997)
Common carp (*Cyprinus carpio*)	Eggs and larvae	Mechanical pressure (eggs) and netting (larvae).	Elevation of whole-body cortisol 50 hours post-fertilization. Pituitary-interrenal axis is functional at hatching.	Stouthart et al. (1998)

Basal levels of cortisol can be initially detected at various stages of development, depending upon the species. *D. rerio* larvae have low resting cortisol levels upon hatch, which continue to decrease as yolk sac resource is utilized, suggesting cortisol is maternally derived during initial development. Once larvae commence exogenous feeding, endogenous production leads to a rise in resting cortisol levels. Alderman and Bernier (2009) suggest the resting levels of cortisol may be determined by genetic or environmental variables, or both. Interestingly a study by Barry et al. (1995) tested resting levels of cortisol in *O. mykiss* and found high levels of cortisol in fertilized eggs that decreased until hatch, after which they gradually increased. Similar findings have been noted in *D. rerio*, with maternal cortisol depleted during embryogenesis (Alsop and Vijayan 2008, 2009). Jentoft et al. (2002) found initial high levels of cortisol that decreased significantly before levelling off until hatch in *P. flavescens*. Detected cortisol was likely maternally derived, as studies have shown cortisol concentration in unfertilised eggs correspond with levels of cortisol in the maternal system near spawning (Stratholt et al 1997). With eggs stressed during early development exhibiting a reduction in stress sensitivity, having corresponding maternal and egg cortisol levels may be adaptive as it could potentially decrease the stress response of developing embryos exposed to similar environmental stressors (Auperin and Geslin 2008).

Activation of a cortisol stress response can occur at various stages of early development, ranging from prior to hatch to weeks post-hatch and despite all components present for a functional HPI axis, several species are unable to exhibit a cortisol response when stressed during early development (Alderman and Bernier 2009,

Alsop and Vijayan 2008, Auperin and Geslin 2008, Barry et al. 1995, Stouthart et al. 1998). In brown trout, *Salmo trutta*, stress via exposure to hypoxia during the egg phase resulted in decreased survival, delayed emergence from gravel, and decreased swimming activity in the presence of a predator (Roussel 2007). Barry and colleagues (1995) tested the appearance of a cortisol response in *O. mykiss*, and noted a significant delay of 5 weeks post fertilization prior to a response when stressed via air exposure and thermal shock. The authors proposed the delay in a cortisol stress response may be an adaptive mechanism to maintain consistently low levels of corticosteroids that may impair essential neural development during this life stage. More recently, Auperin and Geslin (2008) showed O. *mykiss* larvae had measurable cortisol levels beginning at the eyed stage of development but failed to increase cortisol levels when stressed prior to 9 days post hatch. Furthermore, Auperin and Geslin (2008) demonstrated that stress during critical early life stages can reduce the cortisol response to stress later in life. Finally, yellow perch, *Perca flavescens*, have been shown to respond to stress a week post-hatch, with the stress response increasing in magnitude in the following weeks (Jentoft et al. 2002). The lack of response when stressed prior to hatch may be due to a non-responsive pituitary or to the hypothalamus unable to process the stressor with incomplete development of peripheral and central neural inputs (Alsop and Vijayan 2009, Auperin and Geslin 2008). Molecular work on *D. rerio* suggests a critical final step of activation of sensory inputs in the brain that result in adequate ACTH release to initiate the cortisol stress response (Alsop and Vijayan 2008).

Stress research on larvae and juvenile lake sturgeon is essential because studies indicate fish may be able to respond to stressors at these stages (Barton 2002). Furthermore, and perhaps more importantly, it has been shown that physical and chemical environment may have a bearing on the stress response in later life and fish may have an increased stress response during early stages (Barton 2002, McCormick 1998) that if activated could have a significant impact on development.

1.3: Lake sturgeon biology

Lake sturgeon, *Acipenser fulvescens*, a cartilaginous benthic fish is one of the largest freshwater species in the Great Lakes (Peterson et al. 2007). As one of the remaining 27 species of sturgeon today, there is great interest in their life history (Auer 1996, Hay-Chmielewski and Whelan 1997). Lake sturgeon are the only species endemic to the Great Lakes basin (Auer 1996), with present range including the Mississippi, the Great Lakes, and the Hudson Bay drainages (Priegel and Wirth 1971). Belonging to the family Acipenseridae from the upper Cretaceous period (Hay-Chmielewski and Whelan 1997), lake sturgeon are considered 'living fossils' and several characteristics considered primitive have been retained, including external bony plates, a cellular swim bladder, and a heterocercal tail (Harkness and Dymond 1961).

Most sturgeon species today are considered endangered or threatened (Birstein 1993), with an estimated 1% of past populations surviving in 1974 (Tody 1974). Historically sturgeon were viewed as nuisance bycatch species, providing no real benefit

to humans (Tody 1974). After 1860, European demand for lake sturgeon caviar increased, resulting in the opening of fisheries specifically targeting these fish. As a consequence, lake sturgeon were heavily harvested for caviar, meat, isinglass, and paint additives (Priegel and Wirth 1971, Scott and Crossman 1973).

Slow growth, intermittent spawning cycles (Auer 1996), and late age to maturation, with increasing fecundity at larger sizes, make this sturgeon species highly vulnerable to overfishing (Becker 1983, Boreman 1997, Harkness and Dymond 1961, Hay-Chmielewski and Whelan 1997, Scott and Crossman 1973). Male sturgeon spawn every 1-3 years, while females tend to spawn once every 4-9 years (Peterson et al. 2007). In addition to overfishing (Auer 1999, Cataldi et al. 1998, Wang et al. 1985), habitat degradation (Choudhury et al. 1996, LaHaye et al. 1992) is another factor that has contributed to the decrease in lake sturgeon populations. Habitat fragmentation, through dam construction, has isolated populations from historic spawning sites thereby potentially further reducing healthy fish numbers (Choudhury et al. 1996, Wilson and McKinley 2004). Another cause for declining populations includes degradation of water quality of river systems (Baxter 1977, Jager et al. 2001, Rochard et al. 1990). A key aspect of unsuccessful restoration programs is a lack of knowledge of life stages (Hay-Chmielewski and Whelan 1997, LaHaye et al. 1992, Peterson et al. 2007) particularly critical stages during early development.

1.4: Objectives and Hypothesis

Given the described impact of the environment during development this thesis explores the impact of two important environmental variables, namely, temperature and substrate on the development of the stress axis in juvenile lake sturgeon. I hypothesise that cortisol will be present in the developing eggs, however, this will be maternally derived and developing larvae will show no cortisol stress response. Secondly I hypothesise that both temperature and substrate will influence the cortisol stress response in these developing fish and the stress response, post yolk sac absorption will differ between treatments with a heightened stress response being evident in those fish raised over unnatural substrate and higher temperatures. Specific objectives of this thesis include:

1) Examination of the effects of substrate and temperature on morphological development in larval lake sturgeon, *A. fulvescens.*

2) Examination of the effects of substrate and temperature on the development of the stress axis, in particular timing of the appearance of a cortisol stress response and magnitude and duration of the stress response in larval *A. fulvescens*.

3) Examination of the acute stress response, specifically, epinephrine and nor-epinephrine in juvenile *A. Fulvescens*

Chapter 2: Morphological development

2.1: Introduction

In order to determine when stress responsiveness is activated, an understanding of development of the species is required. Morphological parameters of an organism may provide indicators of internal physiological changes. Studies on fish development have explored yolk absorption rates for larvae as a means to understand growth and metabolic rates, and general development (Kamler 2008). Shortnose, *A. brevirostrum* and Atlantic sturgeon, *A. oxyrinchus* exhibit more rapid yolk absorption at higher temperatures, demonstrating the influence of environmental variables upon development (Hardy and Litvak 2004). Most sturgeon species, including green sturgeon, *A. medirostris*, are incompletely differentiated upon hatching, and are thereby greatly influenced by the environment during their larval phase (Gisbert and Dorochov 2006). Limited data exist on sturgeon morphology and development (Colombo et al 2007, Dettlaff et al. 1993), with little to no data on lake sturgeon, *A. fulvescens*, particularly with respect to genetic and environmental variables.

Morphological changes of lake sturgeon occur at a rapid rate during early life development, particularly the first 2 weeks (Harkness and Dymond 1961). Depending on water temperature, fertilized eggs hatch approximately 8-14 days post fertilization (Kempinger 1988). Upon hatching, the prolarvae are 9-11mm, feeding from their attached yolk sac (Peterson et al. 2007). Development of discernable anatomical

features such as gills and fins, as well as dark pigmentation of body, occurs later when the organism is 17-18mm. At this stage (13-19 days post hatch), the prolarvae disperse several kilometres downstream of their spawning site at night and resettle at the river bottom (Peterson et al. 2007). When the prolarvae are approximately 22mm and have almost completely absorbed their yolk sac, they begin exogenous feeding, thereby initiating the larvae life stage (Harkness and Dymond 1961, Peterson et al. 2007). Lake sturgeon are considered juveniles when they are approximately 250 - 400 mm total length (Peterson et al. 2007). In regard to preferred diet, there are limited data on juvenile lake sturgeon (Auer 1996). However, it is known that their diet is similar to that of adults, focusing primarily on benthic macroinvertebrates such as small crustaceans (Chiasson et al. 1997). During all life stages, from eggs (for attachment), to larvae (for shelter), to juveniles and adults (for feeding with jaws specialized for benthic prey), the nature of substrate at river and lake bottoms plays an important role.

Abiotic and biotic environmental conditions during embryonic and larval stages can influence development, growth and morphology of fish species (LaHaye et al. 1992). Work on Chinese sturgeon, *Acipenser sinensis*, tilapia, *Oreochromis spp.*, and Pacific Herring, *Clupea pallasi*, indicate impaired development due to environmental pollutants (Hu et al. 2009, Incardona et al. 2009, Jezierska et al. 2009, Sun et al. 2009). A study on water hardness in juvenile fathead minnows (Blanksma et al. 2009) found that calcium levels in the water during the juvenile phase impacted skeletal development and whole-body mass.

Based upon work completed by Dettlaff et al. (1993), it is hypothesized that lake sturgeon larvae, like other sturgeon larvae, will grow in size, thereby increasing in total area over time, as well as absorb and utilize the yolk sac, thereby decreasing the yolk sac area leading to the onset of exogenous feeding. Furthermore, it is hypothesised that temperature and substrate will have a significant impact on the developmental rate of the larval lake sturgeon.

2.2: Materials and Methods

The study encompassed two sampling periods in spring 2008 and spring 2009. Eggs and milt were obtained from 6 females and 16 males in 2008 captured by gill netting from the Slave falls area of the Winnipeg River. Unfortunately weights were not recorded at the time of capture. Similarly, eggs and milt were obtained from 5 females (31.3 ± 7.28 kg) and 17 males (14.7 ± 1.8 kg) in spring 2009 approximately 11km upstream of the Slave Falls in the Pointe Du Bois area of the Winnipeg River. Gametes from both sexes were thoroughly mixed to broaden the genetic diversity prior to fertilization.

In 2008 approximately 600 eggs, determined by volume (12.5ml), were measured and placed in a Petri dish. Milt was added in a dropwise fashion to each dish before being gently mixed. Following a 1.5 minute incubation period, 15ml of river water at 10°C was added to promote even distribution of milt and adherence of eggs to substrate. Samples were gently manually shaken for 1.5 minutes before the contents of each Petri dish were evenly scattered within experimental tanks. The fertilization protocol was similar for spring 2009, with the exception that a greater number of fertilized eggs (~700) was added to each experimental tank which were of an identical size to the tanks used in 2008. Despite the greater density in 2009, there was still ample surface area for all eggs in the experimental tanks.

2.2.1: 2008 experimental setup

Fertilization, hatching, rearing, and stress experiments were conducted in a specially designed facility in Pinawa, Manitoba. Experimental tanks were supplied with recirculating river water at 13.8±2.5°C under ambient photoperiod. The setup consisted of 6 flumes divided into 12 equally sized experimental tanks, designed such that water flow was as uniform as possible across all tanks (fig 2.1). All 72 individual tanks were lined with gravel substrate (particle size ≤ 3cm). Half of the experimental tanks were randomly assigned slate substrate, while the remaining 36 tanks were gravel substrate (fig 2.2 a & b). Slate tanks had an additional sheet of grey perspex placed above the gravel substrate to mimic bedrock on a river bed.

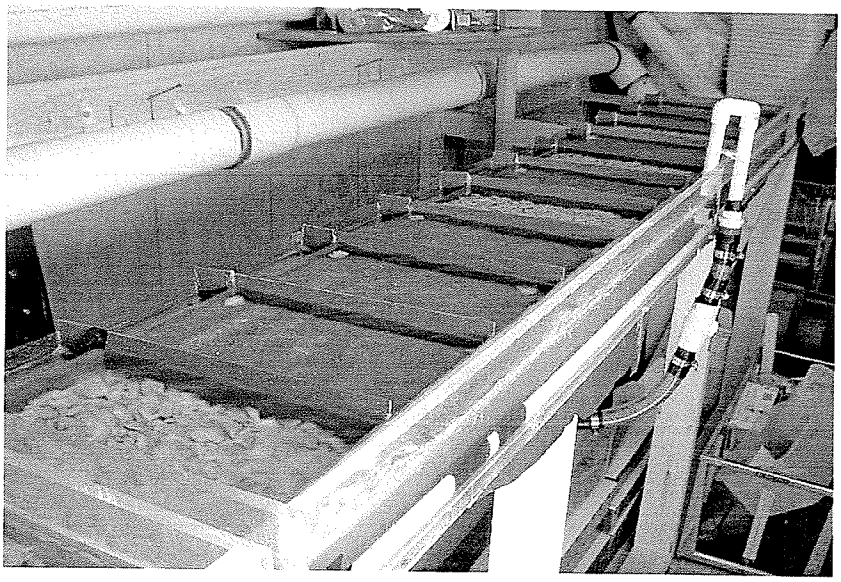


Figure 2.1. Experimental setup in spring 2008 in Pinawa, Manitoba. A recirculating water system was set up with relatively uniform flow across all experimental tanks. The same tanks were employed for temperature and substrate experiments in 2009, conducted in the animal holding facility at the University of Manitoba.

2.2a

2.2b

Figure 2.2a & b: Experimental tanks lined with (a) slate or (b) gravel substrate in spring 2008. Tanks were housed in an aquatic facility in Pinawa, Manitoba.

A third experimental setup was included in the 2008 field season. Six hatchery jars were setup in the same water, and eggs added to each jar (799±164 eggs) for development (fig 2.3). Fertilization of eggs for hatchery jars differed slightly from gravel and slate substrate eggs, with the addition of clay to river water to prevent adherence of eggs to themselves or the hatchery jar walls.

2.2.2: 2009 experimental setup

Hatching, rearing and stress experiments in 2009 were conducted in the animal holding facility in the Duff Roblin building at the University of Manitoba to allow for the establishment and constant monitoring of the various temperature treatments. The

substrate experiment of 2008 was repeated in 2009 with 2 experimental flumes kept at a constant temperature of 12 ± 1°C. These flumes had a mixture of gravel and slate substrate tanks, thereby allowing a replicate of the previous year's experiment. Similar to the 2008 substrate experiment, Perspex sheets were randomly placed in half of these experimental tanks, and removed upon hatching. Eggs and milt were transported separately from the Pointe Du Bois area and fertilized upon arrival at the University of Manitoba.

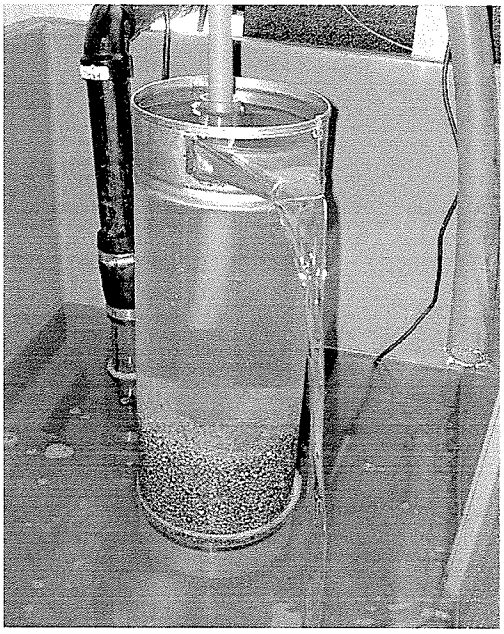


Figure 2.3. Hatchery jars used for rearing lake sturgeon eggs and larvae. The jars were maintained in the aquatic facility in Pinawa, Manitoba.

In addition to this, two treatment groups of 9 and 15°C were also maintained to determine the effects of temperature on the development of lake sturgeon eggs and larvae. Using the same experimental flumes as 2008, each flume was divided into 12 tanks as described previously. Eggs for the 9 and 15°C treatment groups were fertilized

in Pointe Du Bois and transported to the University of Manitoba. Eggs were dispersed for all experimental treatments on the same day. All tanks were initially maintained at 9 ± 1°C. For the 12 and 15 °C treatments water temperature was increased by 1°C daily until the desired treatment temperature was achieved.

Daily maintenance during the egg phase of development involved removal of fungus, while minimizing potential stress to viable eggs. Any eggs accidentally stressed via pressure were removed from the experimental setup. Once eggs had hatched, Perspex sheets were removed as the negatively phototactic prolarvae require crevices to hide from light and water flow. All groups (with the exception of hatchery jars in 2008) were allowed to develop on gravel substrate post hatch. Maintenance of prolarvae simply involved removal of any dead individuals. In preparation for exogenous feeding of the larval phase, brine shrimp were added daily to all experimental tanks and jars at the onset of the larval life stage immediately following absorption of the yolk sac. This was marked with emergence from the gravel substrate and the disappearance of a pigmented 'melanine' or spiral plug, as described by Wang et al. (1985). Brine shrimp eggs (Argentemia certified *Artemia Franciscana* cysts, Redmond, WA, USA) were hatched in an aerated saline aquatic environment maintained at 28°C. Live shrimp were rinsed with freshwater and added 3 times a day to each experimental tank. Excess shrimp were removed from the experimental tanks 60 minutes following feeding.

Following emergence from gravel, and just prior to the onset of exogenous feeding, all prolarvae in both years were transferred to smaller experimental tanks to

ensure each individual had access to the brine shrimp. Fish in 2008 were moved on 21 days post fertilisation (dpf), and fish in 2009 were moved on 20dpf, 12 dpf and 11dpf, which was dependent on the respective temperature treatment of 9, 12, and 15°C respectively. All experimental groups in 2008 and 2009 were fed ad libitum 3 times a day with live brine shrimp to encourage feeding, and to negate any influence of competition for resources in each experimental tank or jar.

2.2.3: Sample collection and processing

For experiments in 2008, 2 individuals from the hatchery jar treatment group were fixed daily in a 10% neutrally-buffered formalin solution (0.17M paraformaldehyde, 0.029M $NaH_2PO_4 \cdot H_2O$, and 0.046M Na_2HPO_4 in 1L deionized water pH 7.4) following an overdose of anaesthetic (240ppm tricaine methanesulfonate). Prolarvae (larvae with the yolk sac present) and larvae (larvae where the yolk sac was absorbed) from gravel and slate bins were fixed weekly. For experiments in 2009, 3 individual eggs or fish were fixed daily from all four flumes. All samples were stored at 4°C prior to measurement.

For measurement, samples were individually transferred to a Petri dish with phosphate buffered solution, and photographed using a light microscope (Nikon, YS100) connected to a digital camera (Sony 3CCD colour camera). Dorsal, ventral and lateral images were obtained depending upon the parameter of interest and measurements of images were obtained using MPIX, Northern Eclipse software. The software was

calibrated when the samples were photographed with images corrected for the magnification used. Care was taken to replicate the orientation of the fish between samples to minimize error associated with measurements between animals. Total area, yolk sac area, eye diameter, head length and width were measured for the substrate comparison experiments conducted in 2008. Total area, yolk sac area, total length, eye diameter and egg diameter were measured for the experiments conducted in 2009. Developmental stages were inferred with reference to Dettlaff et al. (1993). All data were analyzed in Microsoft Excel (Microsoft Office, Microsoft Coorporation). All described experiments in this and subsequent chapters were conducted under approved animal care protocols F05-021 and F04-041 by the University of Manitoba, protocol management review committee as outlined by the Canadian Council for Animal Care.

2.2.4. Statistical Analysis

Data for fish reared on different substrates were analyzed using one-way Analysis of Variance (ANOVA), with Bonferroni's post-test. Due to differences in developmental rates, data for fish reared in different temperatures was not possible to analyze for all time points. Where possible, one-way ANOVA and Bonferroni's post-tests were conducted.

2.3: Results

2.3.1: Developmental rates

Eggs reared in naturally fluctuating temperature (2008) began hatching 7dpf, with all eggs hatched by 11dpf. The first observed hatched egg was in a slate experimental tank. However, there were no significant differences observed in hatching times between the gravel and slate substrates. In comparison, eggs reared in different temperatures (2009) hatched on varying days. Eggs raised at 15 °C hatched from 6 to 8dpf, at 12 °C from 9 to 11dpf, and lastly, at 9 °C from 15 to 17dpf.

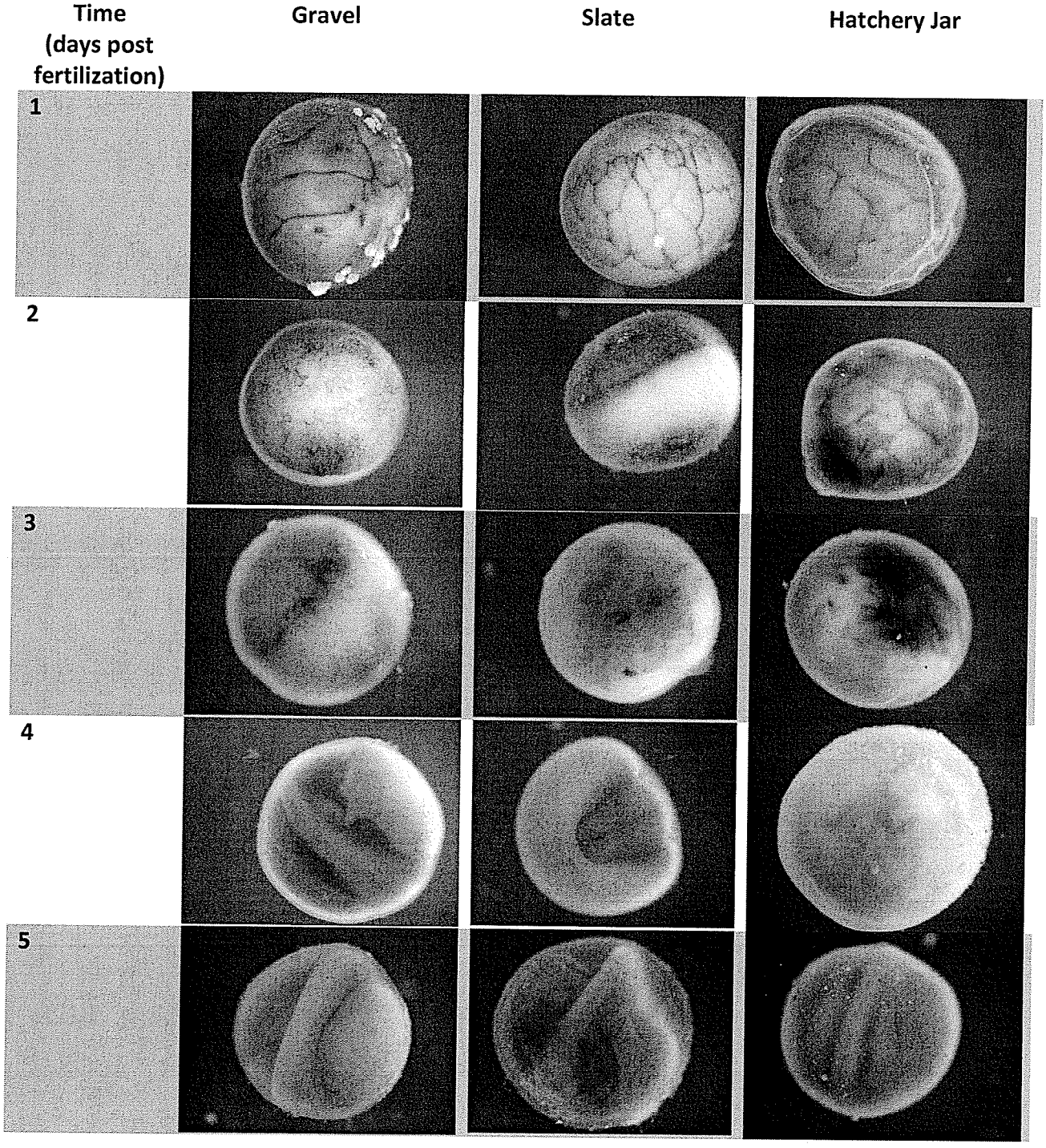
Where it was possible to compare across collection days between treatments figure 2.4 shows no significant differences in the mean diameter of eggs reared on any substrate in both 2008 ($p=0.429$) and 2009 ($p=0.8132$). Further there was no significant difference in egg diameter in any of the temperature treatments during 2009 (Fig. 2.5) ($p=0.9665$). Where it was possible to compare across treatments figure 2.6 shows no significant differences in the mean yolk sac area for prolarvae reared on any substrate in 2008 ($p=0.1693$) and 2009 ($p=0.9292$). In addition no significant differences were observed in mean yolk sac area for prolarvae reared at 9, 12 and 15 °C (Fig 2.7) ($p=0.2546$). Despite error bars indicated in figure 2.7, data was not significantly different, likely due to the small sample size. Furthermore there was no effect of substrate on the total area of prolarvae in 2008 ($p=0.3538$) or 2009 ($p=0.6185$) (Fig 2.8). However, as Figure 2.9 demonstrates there was an effect of temperature on the total

area of prolarvae where prolarvae and larvae were significantly larger in the 12 and 15°C treatment compared to the 9 °C treatment ($p=0.0386$). Figures 2.9 and 2.10 demonstrate the effect of substrate and temperature during development on the percentage of total surface area of the fish that is taken up by the yolk sac. While it is evident that substrate has not affected this ratio it is clear that there is a temperature effect as the slopes for each temperature treatment are very different and follow an anticipated trend in that the higher the temperature the faster the yolk sac is being absorbed.

Development of eggs reared in 9, 12 and 15°C, is depicted in table 2.3. Differences in the rate of development are apparent between temperature treatments. As early as day 3, fish reared in 9°C appear to simply be at stage 2 with the secretion of a hydrophilic colloid, fish in 12°C appear to be at stage 15 of middle gastrula, and fish in 15°C appear to be at stage 17 with a small yolk plug. Differences are further apparent at 6dpf, with 9°C fish at stage 15, 12°C fish at stage 26 with fusion of lateral plates, and 15°C fish at stage 31, immediately prior to hatch (Detlaff et al. 1993). Eggs in the 15°C treatment hatched shortly after (6dpf±2). This accelerated development in higher temperatures is further evident when comparing the rates of yolk sac absorption (fig. 2.10, 2.11).

Table 2.1. Lake sturgeon eggs reared on gravel, slate, and jar substrate in 2008. Images were captured at 2.5X magnification, unless otherwise specified. Daily samples were collected for all three treatments up to 6dpf, following which jar samples alone were collected due to low numbers of viable eggs

(——— = 1.0mm).



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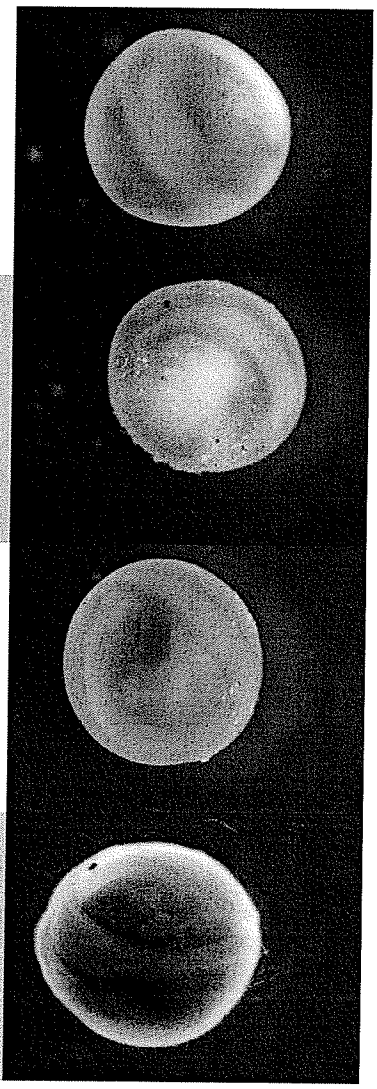

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8

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Table 2.2. General external development on lake sturgeon prolarvae reared in hatchery jars. Numbers associated with each image indicate days post-fertilization. Samples were photographed at a 0.75X magnification unless otherwise specified. See text for image capture details. (—— = 1.0mm)

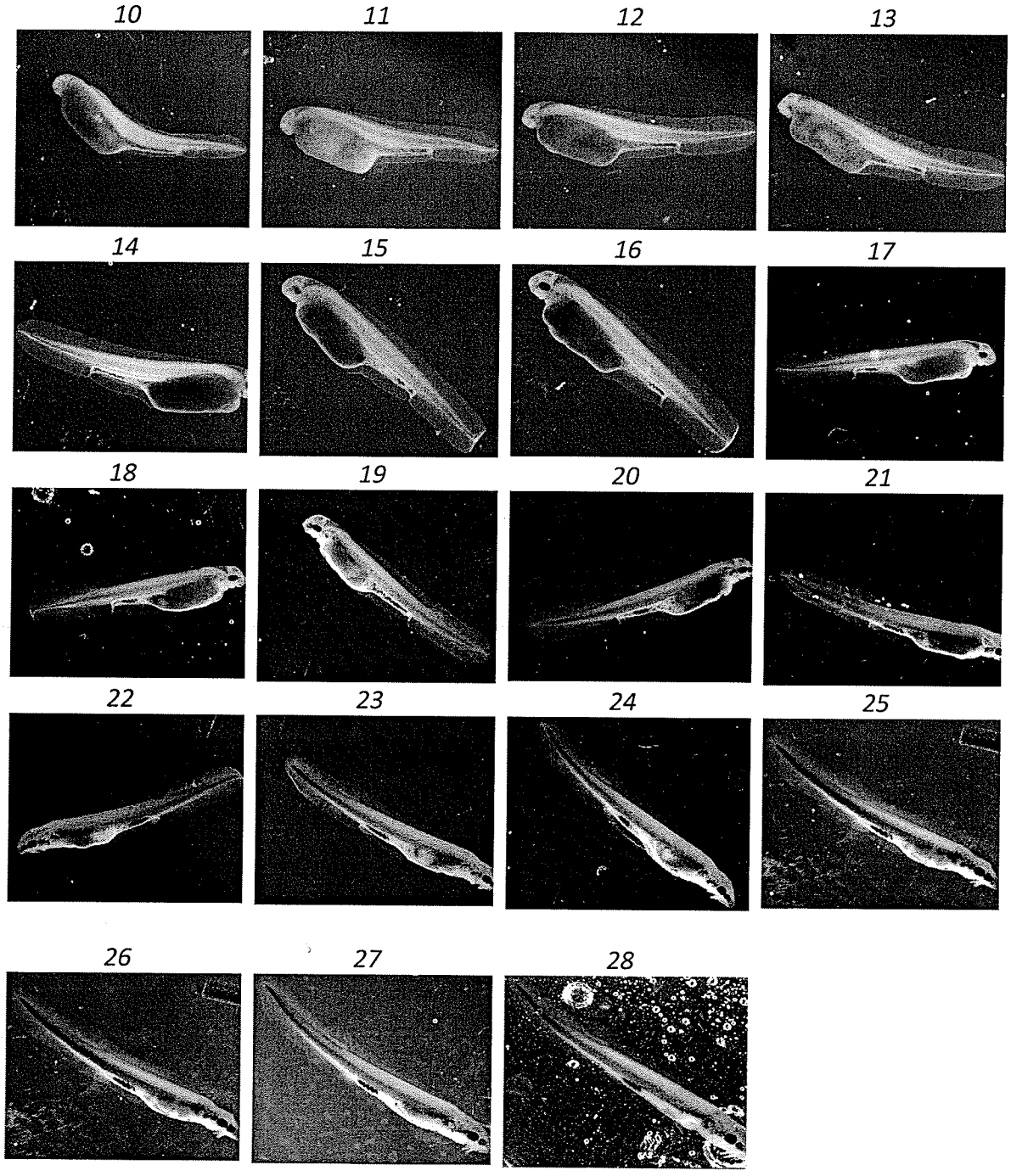
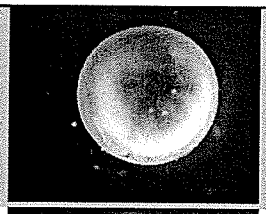
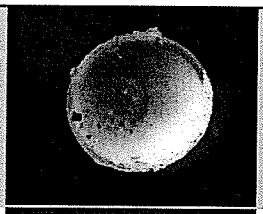
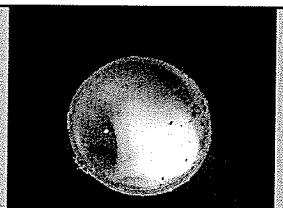
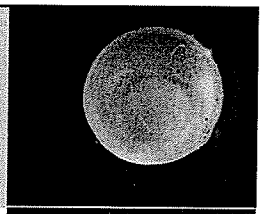
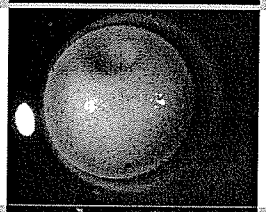
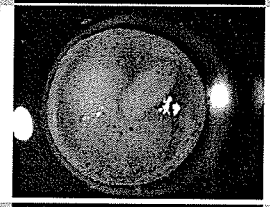
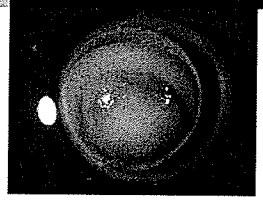
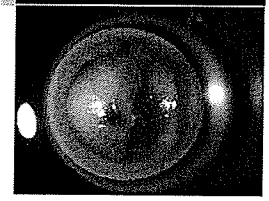
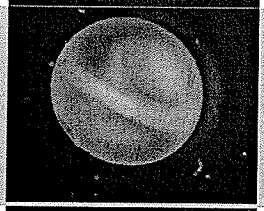
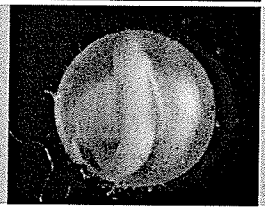
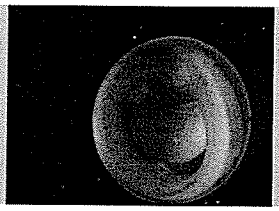
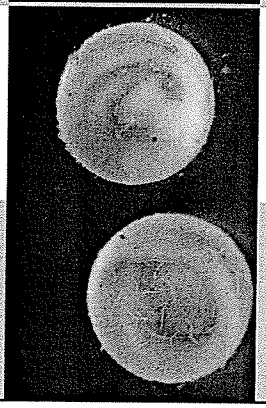


Table 2.3. Lake sturgeon eggs reared in 9, 12 and 15 °C in 2009. Eggs reared on slate substrate at 12 °C are added. Images were captured at 2.5X magnification, following the procedure described in the text. (——— = 1.0mm)

Time (days post fertilization)	9C (gravel)	12C (gravel)	12C (slate)	15C (gravel)
3				
6				
9				
12				
15				

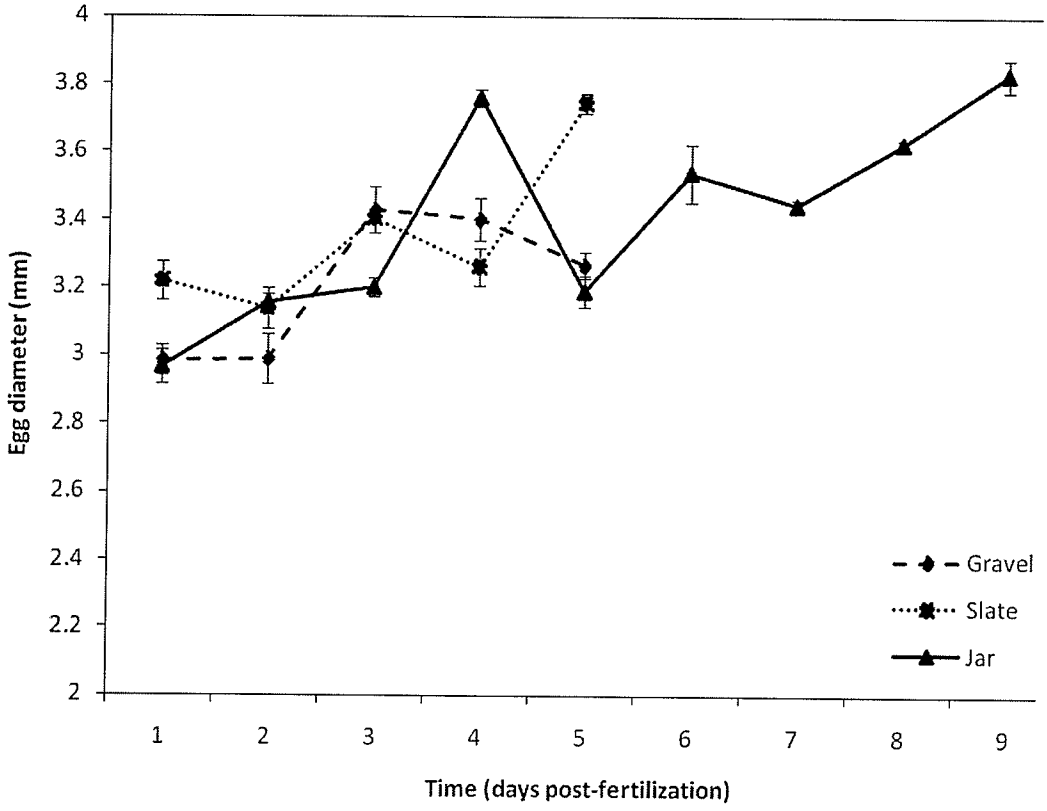


Figure 2.4 a. Mean diameter of fertilized lake sturgeon eggs reared on gravel and slate substrates and in hatchery jars in spring 2008. Values are expressed as mean length (millimeters) ± 1 SEM. ($n \geq 3$).

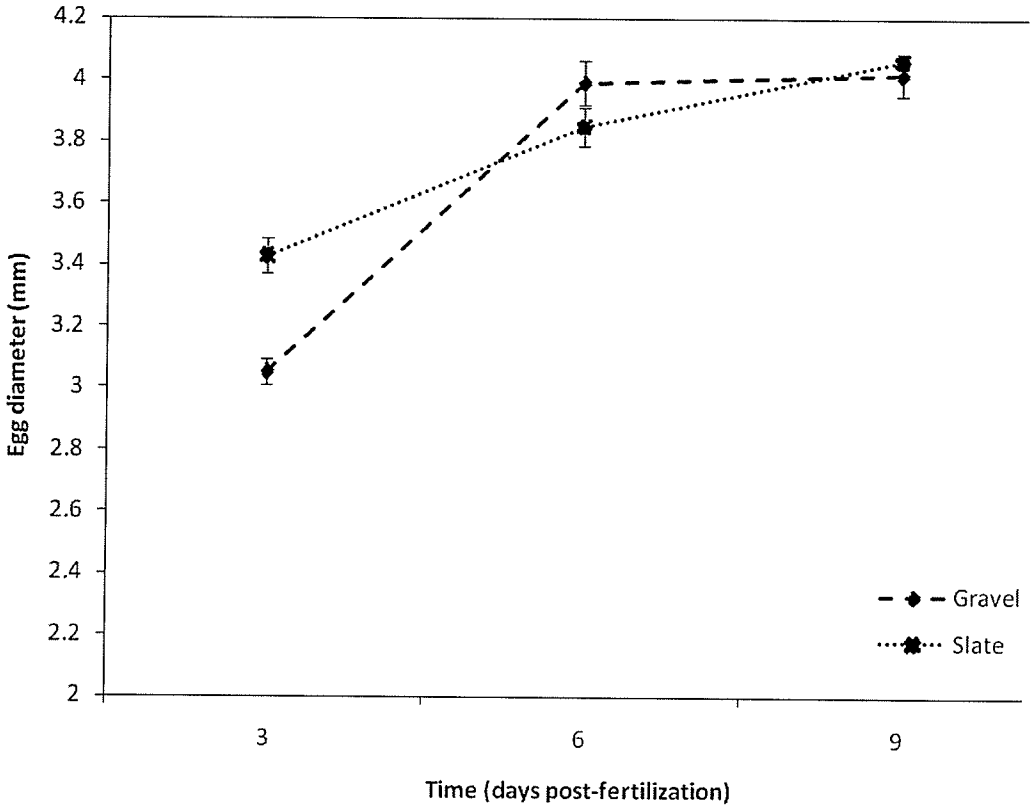


Figure 2.4 b. Mean diameter of fertilized lake sturgeon eggs reared on gravel and slate substrates and in hatchery jars in spring 2009. Values are expressed as mean length (millimeters) ± 1 SEM. ($n \geq 3$).

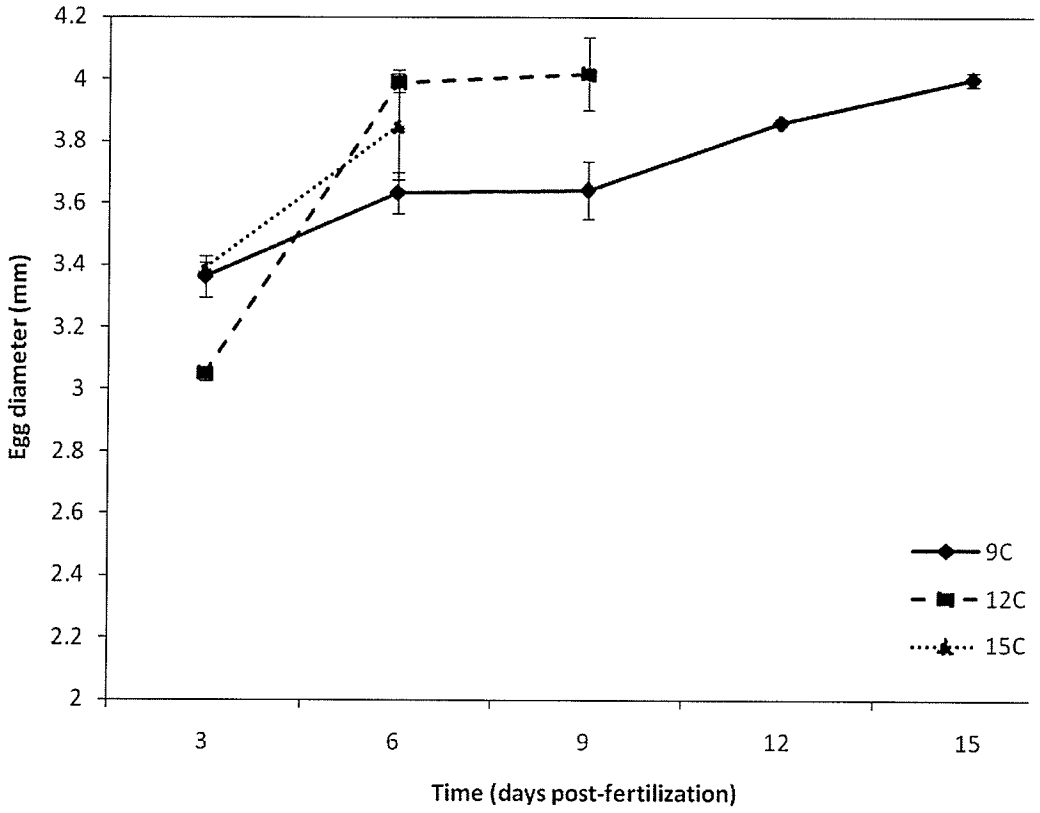


Figure 2.5. Mean diameter of fertilized lake sturgeon eggs reared in 9, 12 and 15°C. Values are expressed as mean length (millimeters) ± 1 SEM. (n = 3).

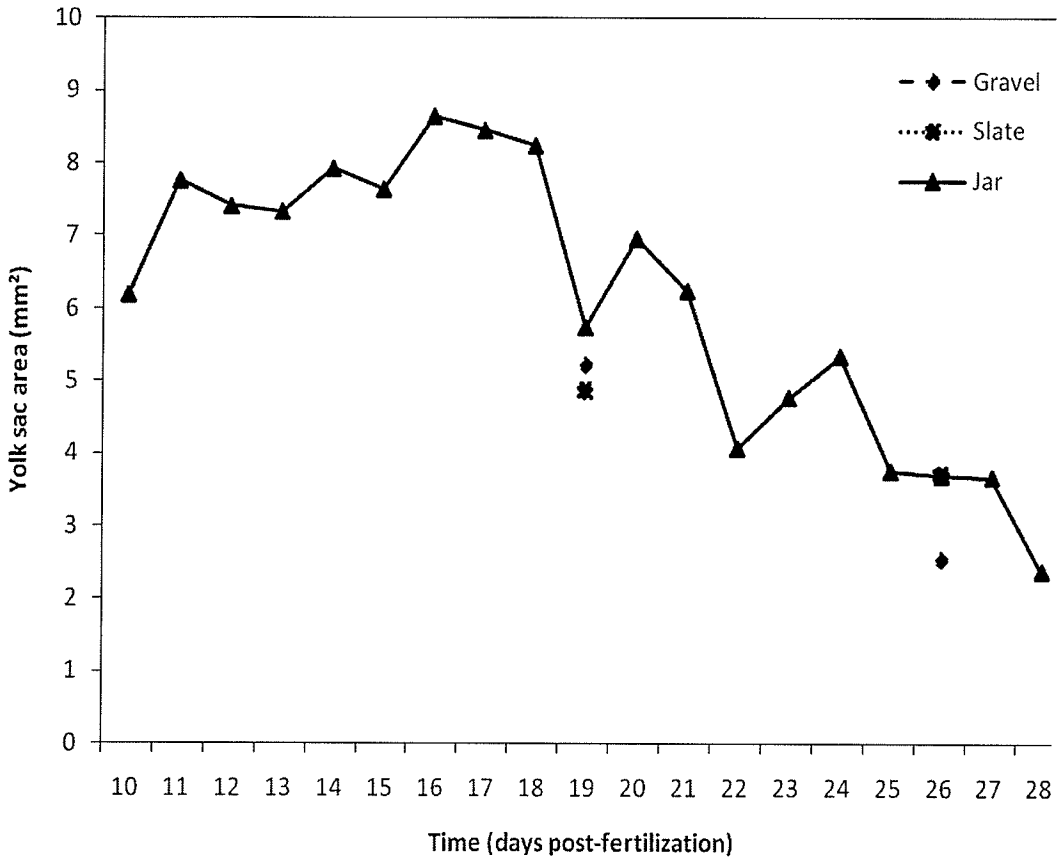


Figure 2.6 a. Yolk sac area of prolarvae and larvae reared on different substrates in spring 2008. Values are expressed as mean surface area (mm^2) ± 1 SEM. (n ≥2).

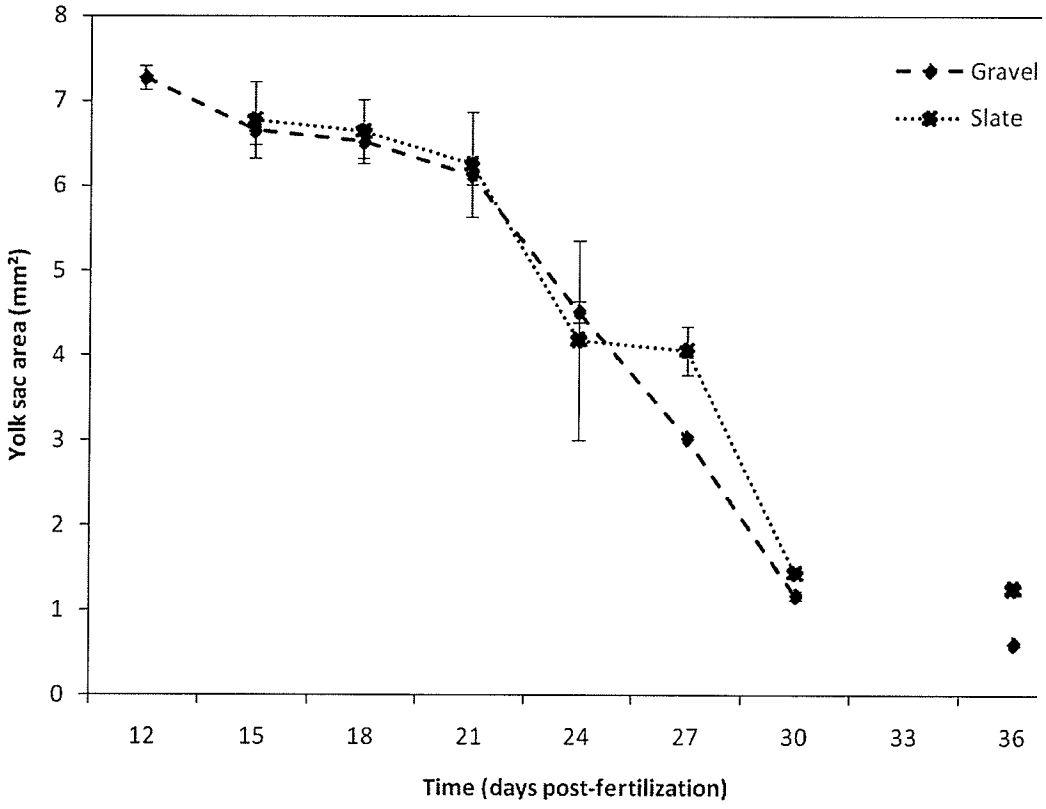


Figure 2.6 b. Yolk sac area of prolarvae and larvae reared on different substrates in spring 2009. Values are expressed as mean surface area (mm^2) ± 1 SEM. (n ≥2).

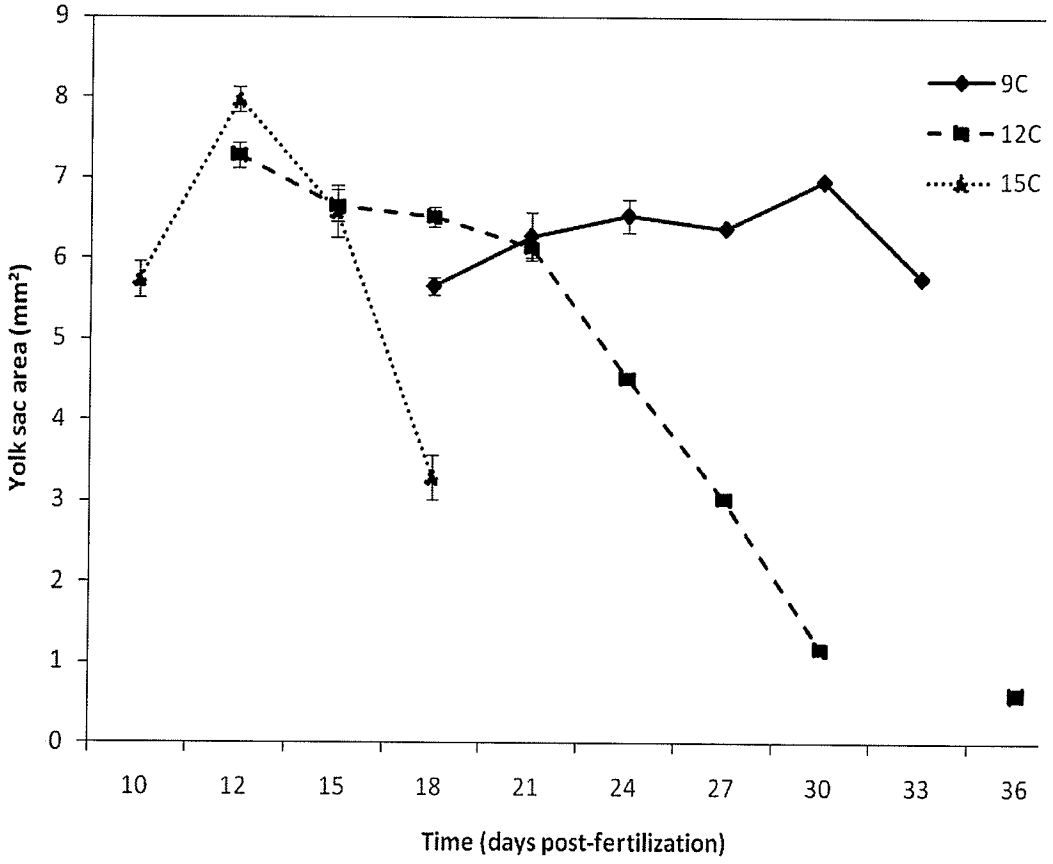


Figure 2.7. Mean yolk sac area of lake sturgeon reared in different temperatures. Values are expressed as mean surface area (mm^2) ± 1 SEM. (n=3).

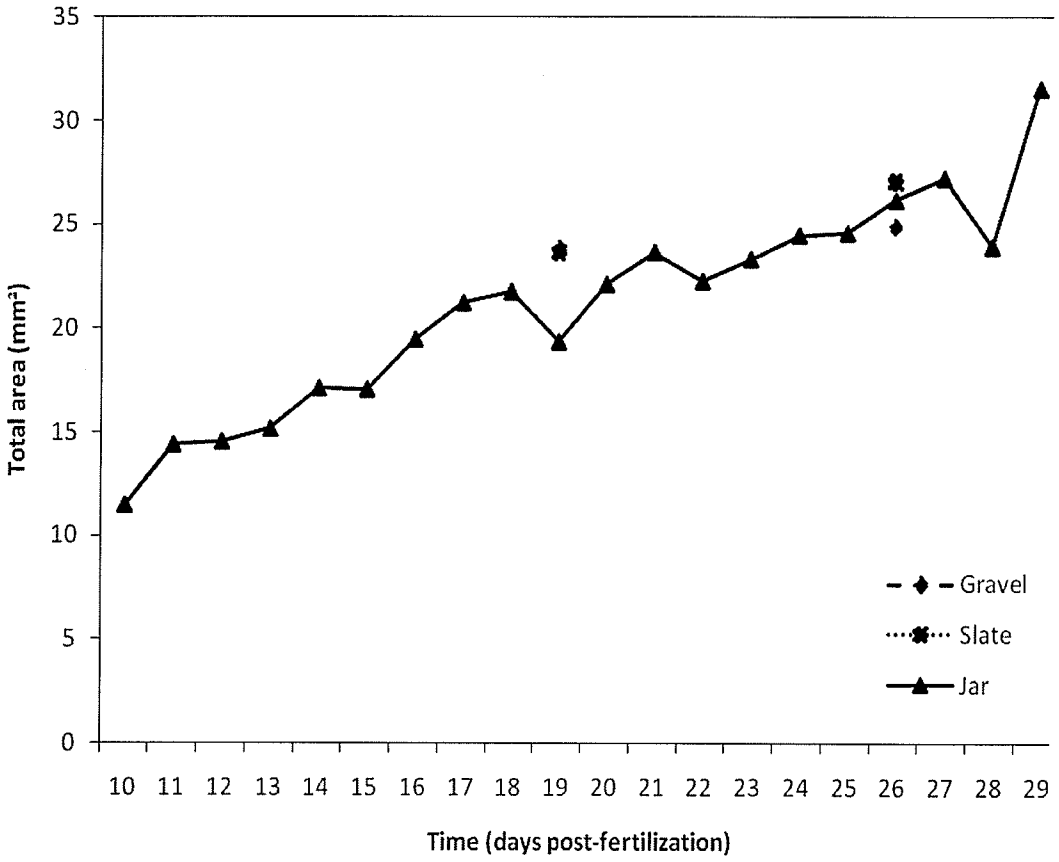


Figure 2.8 a. Total area, including yolk sac, of prolarvae and larvae reared on different substrates in spring 2008. Values are expressed as mean surface area (mm^2) ± 1 SEM. (n ≥ 2).

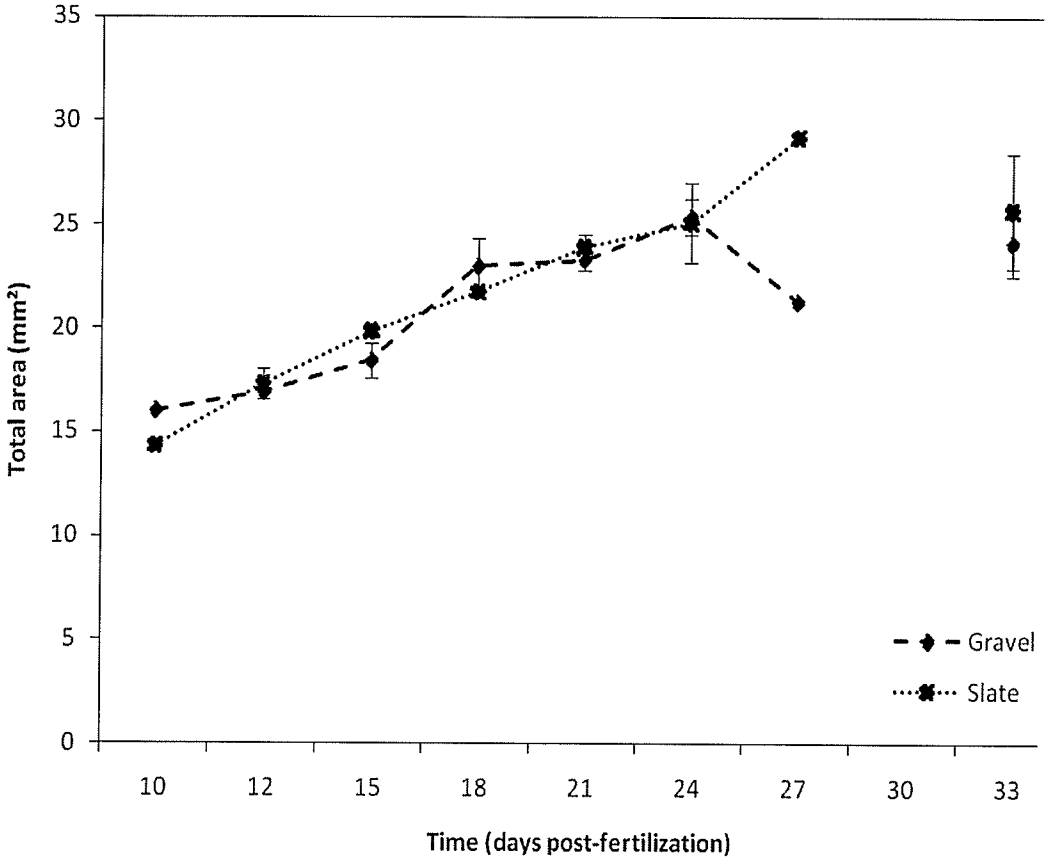


Figure 2.8 b. Total area, including yolk sac, of prolarvae and larvae reared on different substrates in spring 2009. Values are expressed as mean surface area (mm^2) ± 1 SEM. (n ≥ 2).

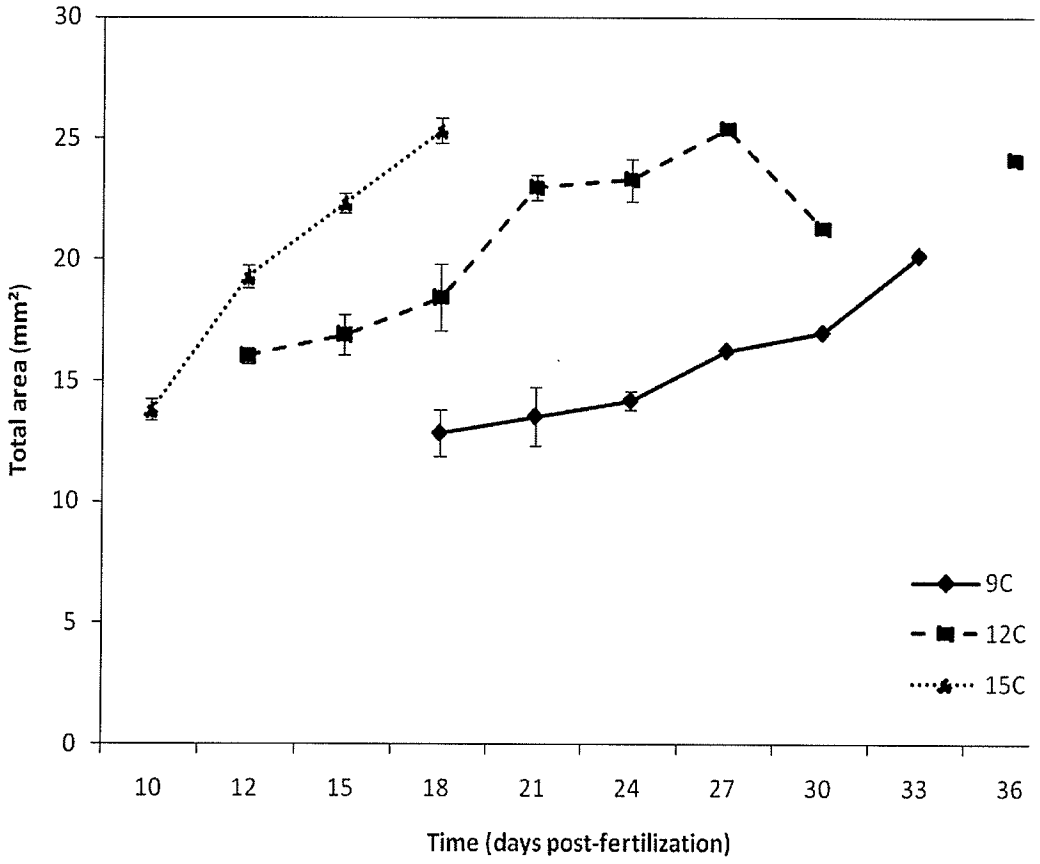


Figure 2.9. Total area, including yolk sac, of prolarvae and larvae reared at 9, 12 and 15°C. Values are expressed as mean surface area (mm^2) ± 1 SEM. (n = 3).

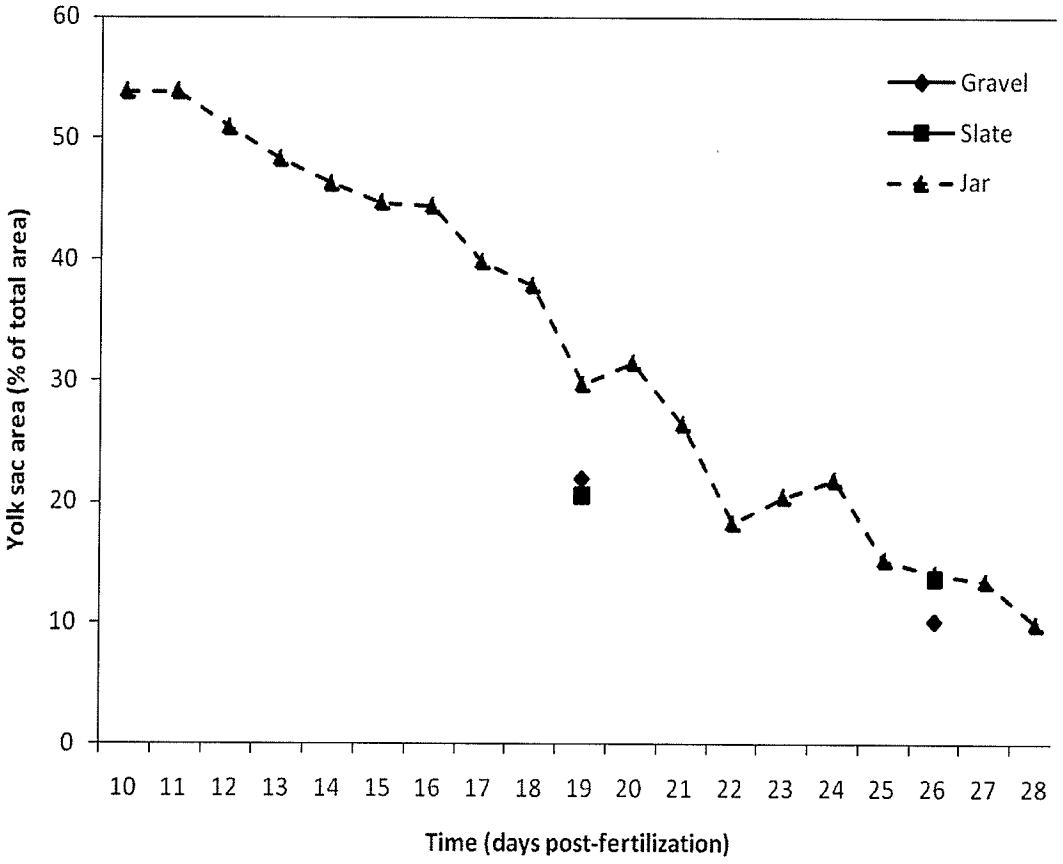


Figure 2.10 a. Yolk sac area expressed as a percent of the total area for prolarvae and larvae reared on different substrates in spring 2008. Values are expressed as mean percentage of total surface area of the fish (n ≥2).

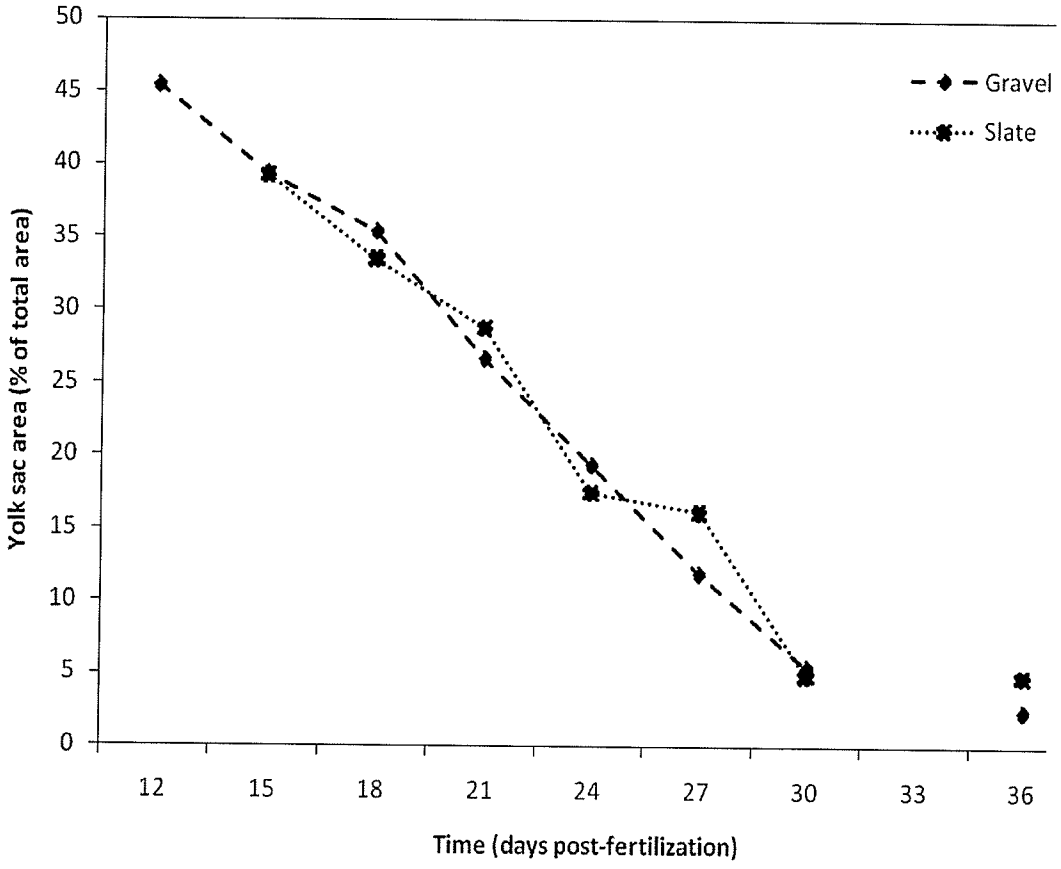


Figure 2.10 b. Yolk sac area expressed as a percent of the total area for prolarvae and larvae reared on different substrates in spring 2009. Values are expressed as mean percentage of total surface area of the fish (n ≥2).

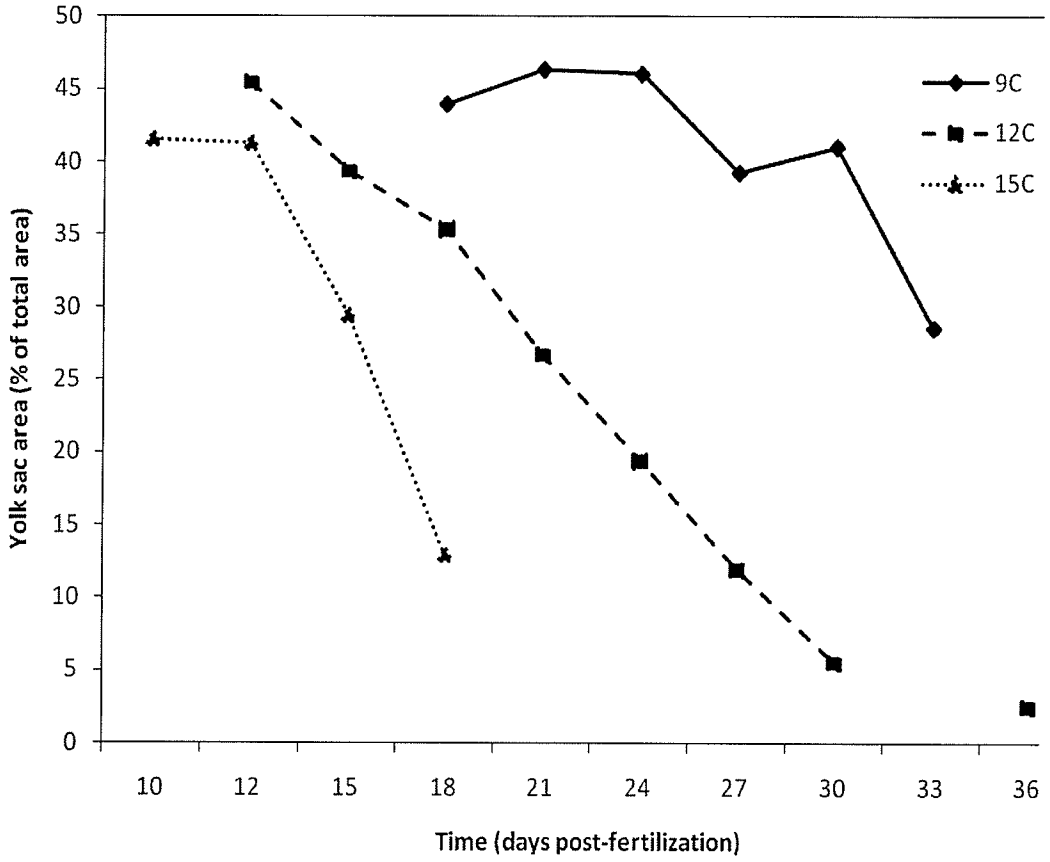


Figure 2.11. Yolk sac area expressed as a percent of total area for prolarvae and larvae reared in 9, 12 and 15 °C. Values are expressed as percentage of total surface area of the fish (mm^2) ($n \geq 2$).

2.4: Discussion

Studying the morphology of an organism during early life stages can provide invaluable information pertaining to external and internal development. A study by Mendoza et al. (2002) on morphophysiological features of alligator gar *Atractosteus spatula* suggests that development impacting external morphology causes three primary physiological changes that should be explored: enzymatic ontogenesis, the digestive tract, corticosteroids and thyroid hormones. As such, it can be inferred that potential morphological changes can be quantified to determine if a trend coincides with the onset of the hypothalamic-pituitary-interrenal stress axis.

Temperature of the immediate environment can impact the yolk absorption rate, with fish utilizing the yolk sac at a greater pace in higher temperatures, as seen in larval shortnose and Atlantic sturgeon (Hardy and Litvak 2003). Hardy and Litvak (2003) propose that such early transition to exogenous feeding may confer a survival advantage by minimizing predation of the vulnerable yolk sac life stage. However, such a trend may be detrimental as it can result in failed coordination between larval food requirements and prey availability in the wild at that particular time.

Several marine teleost species have fully functional organs and organ systems upon exogenous feeding, including functional endocrine system (Falk-Petersen 2005, Tanaka et al. 1995). Interestingly the period of rapid yolk sac absorption immediately prior to exogenous feeding coincides with eye pigmentation in several aquatic species, potentially because exogenous feeding requires visual function (Tanaka et al. 1995).

Based on the fixed samples collected from gravel and slate tanks in 2009, eye pigmentation is first visible at 15dpf, considerably prior to the onset of exogenous feeding. This deviation may simply be due to a difference in species, with lake sturgeon hatching with a fairly large yolk sac (table 2.2). Absorption is not complete at initial appearance of eye pigmentation, thereby eliminating the need for the organism to actively seek food. Furthermore, lake sturgeon are benthic feeders that have poor visual acuity and rely upon tactile stimulation by barbels to find prey, and as such, do not extensively rely on sight (Vecsei and Peterson 2005). Based on these findings, it can be reasoned that the appearance of eye pigmentation does not coincide with the ability to respond to a stressor.

Timing of hatch for eggs reared over gravel and slate substrates in 2008 did not significantly differ, with all eggs hatching 7-11dpf. However, the first observed hatch was noted in a slate experimental tank, and microscopic analysis of fixed samples reveals slightly further development, as compared to eggs on gravel substrate. As seen in table 2.1, eggs reared over gravel appear to be a few stages behind eggs reared on slate. For example, eggs reared on slate substrate were at the 7^{th} cleavage division (stage 9) by 2dpf, while eggs reared on gravel were at 3^{rd} cleavage division (stage 6), as described by Dettlaff et al. (1993). Another example can be seen on 5dpf, where eggs reared on slate appear to be at stage 28, with clear, expanded egg envelope, while eggs reared on gravel appear to be at stage 25, with a rudiment of posterior trunk and tail developing, as seen on 4dpf in slate eggs.

Timing of hatch, as well as development rate during early ontogeny, varied greatly for the different temperature treatments (table 2.1). As for numerous other aquatic species, lake sturgeon prolarvae in this experiment developed at the greatest pace when reared in higher temperatures (Geist et al. 2006, Laurel et al. 2008, Methven and Brown 1990). The incubation period for eggs reared in low temperatures (9°C) was greatly extended as compared to fish from 12 and 15°C treatments (table 2.3). Such delayed development has negative implications in both hatchery practices and in nature. Eggs hatched in hatcheries are particularly vulnerable to fungal infection, leading to high mortality, while eggs in the wild adhere to rocks, and any exposed for extended periods are at a greater risk of predation (Wang et al. 1985).

Size of fertilized eggs, measured as diameter, did not significantly differ for eggs reared on different substrates or temperatures (fig 2.4 a&b, 2.5). Furthermore, no significant difference was noted for yolk sac area of fish reared on gravel as compared to hatched on slate, as well as between the three temperature treatments upon hatch (fig 2.6, 2.7). Lake sturgeon, like other sturgeon species, hatch with a large amount of body fat. Prolarvae survive by absorbing their attached yolk sac, while their internal organ development is occurring (Colombo et al. 2007). Thereby it can be hypothesized that eggs, and hatching larvae, would not differ in the yolk sac area, but rather in the utilization rate.

From a morphological perspective, yolk sac to total area ratio can be used to assess development rate, and thereby whether the relationship between yolk sac

absorption and HPI axis activation exists in lake sturgeon larvae. As seen in figure 2.11, larvae reared in 9, 12 and 15°C treatments exhibit the predicted negative slope, with the ratio of yolk sac to total area decreasing over time. Furthermore, the larvae reared at a high temperature (15°C), have the greatest slope, indicating the most rapid yolk sac absorption, most rapid total area increase, or both, as compared to larvae reared in lower temperatures (9 and 12°C). This finding is supported by numerous studies suggesting development of the prolarval phase is most rapid in higher temperatures (Kappenman et al. 2009, Turner et al. 2007, Wang et al. 1985). Larvae from eggs hatched on gravel and slate substrates similarly exhibited a decreasing yolk sac to total area ratio over time. The rate of yolk sac absorption does not differ between treatments (fig 2.10), suggesting equivalent rates of development. Based on these findings, substrate type during the egg phase does not appear to impact the rate of development.

Total area did not differ between substrate treatments (fig 2.8). However, larvae reared in higher temperatures (12 and 15°C) had a significantly larger total area as compared to fish reared in lower temperatures (9°C) (fig 2.9). This finding coincides with the argument that fish reared in temperatures below their optimal range (spawning in the Winnipeg River typically occurs between 10.5 and 11.5 °C and will continually warm to 14 - 15 °C during the developmental stages examined in this study) may be required to utilize the limited maternally derived resources for immediate survival, thermal tolerance, and gross development as compared to maximizing growth. As such, it can be hypothesized that such larvae are likely to have a higher incidence of physical anomalies, as was observed in the 2009 field season. Fish reared in the coolest

environment exhibited circular swimming behaviour, enlarged clear sacs attached to the abdomen, decreased size at the onset of exogenous feeding, and slowest development. Fish reared in 15°C, on the other hand, exhibited most rapid development, and largest size at the onset of feeding.

While the absorption of the yolk sac corresponds with the onset of exogenous feeding, determining whether this transition coincides with the appearance of cortisol stress responsiveness is explored in chapter 3. The focus of the next chapter of this thesis is to examine the effects of the same environmental parameters on the development of the stress axis in terms of appearance of responsiveness, as well as the magnitude and duration of the cortisol response.

Chapter 3: Development of the cortisol stress response

3.1: Introduction

A plethora of studies on plasma cortisol levels in different organisms is available. This is due to the easy and accurate measurement of circulating cortisol levels, relative ease of obtaining baseline values as a consequence of the response lag time, and a marked increase in plasma cortisol as exposure to stressors increases (Mommsen et al. 1999). As a consequence, circulating cortisol levels are used as primary means of assessing the stress response in fish species, including sturgeon (Allen et al., 2008; Anderson et al. 1991, Barton et al. 2000, Belanger et al. 2001, Cataldi et al. 1998, Cech and Crocker 2002, Maxime et al. 1995).

A study on Atlantic, *A. oxyrinchus*, and shortnose, *A. breviorstrum*, sturgeon tested the effects of hypoxia on the stress axis of juveniles (Baker et al. 2005). Researchers found that juveniles of both species increased their ventilatory rate when exposed to varying reduced oxygen concentrations for a 1 hour period. Data indicated haematological variables, including blood plasma concentrations of cortisol, did not significantly increase for either species when exposed to mildly hypoxic conditions. However a significant increase in plasma cortisol was noted for the more intense hypoxic stressor.

Bayunova et al. (2002) found increased cortisol and glucose levels in response to acute stressors in stellate sturgeon, *A. stellatus*, and Russian sturgeon, *A.*

gueldenstaedtii, and Belanger et al. (2001) similarly found increases in plasma cortisol following water reduction and transportation stress in white sturgeon, *A. transmontanus*.

The cortisol stress response may, however, be species specific as Cataldi and colleagues (1998) tested the effects of temperature and stress on the cortisol response in 4-year-old Adriatic sturgeon, *A. naccarii*, but surprisingly there was no significant increase in plasma cortisol concentrations due to crowding and handling. Here habituation to the rearing conditions may have dampened the stress response in these individuals. However, Lankford et al., (2005) have previously demonstrated that green sturgeon, *A. medirostris*, did not habituate to a repeated stressor.

Aerial exposure has been used as a common stressor in a number of other fish species and the trend of an increase in cortisol levels within 5-10 minutes and a return to baseline within 2-3h following the stressor has frequently been reported. However, what is evident in the acipenserids is that the peak cortisol values reported in the literature are somewhat lower than those values reported for the bony fishes (Baker et al., 2005; Barton et al., 1998; Barton et al., 2000). The implication here is that acipenserids are "less stressed" than teleost fish and that the stimulus for cortisol release and/or the metabolism of cortisol in the circulation is different in this group of fishes (Barton et al. 1998).

The magnitude and duration of cortisol stress response is impacted by genetic, developmental and environmental factors (Falahatkar et al. 2009). Furthermore, the nature of the response is species specific, and can be influenced by the nature and

severity of the stressor itself. Chronically stressed great sturgeon, *Huso huso*, exhibited an increase in plasma cortisol concentration when stressed by aerial exposure, while no significant increase was noted due to handling (Falahatkar et al. 2009).

3.1.1: Substrate

Numerous studies exploring substrate preference by various life stages of different fish species have been conducted. Many actinopterygian species prefer a gravel or rocky substrate during the egg phase, likely due to the availability of surfaces for fertilized egg adhesion, as well as protection from high water currents and predators. For example, experimental studies have noted greater survival of eggs found on rocks and cobble as compared to sand in lake trout, *Salvelinus namaycus* (Marsden et al. 1995). Substrate preference of spawning adults is critical as it determines substrate type for the immobile egg phase. Adult preference of coarse gravel substrates for spawning has been observed in brook charr, *Salvelinus fontinalis*, (Bernier-Bourgault and Magnan 2002), *S. namaycush*, (Kelso et al. 1995), and coho salmon, *Oncorhynchus kisutch*, (Mull 2007).

Several sturgeon species, including Suwannee River Gulf sturgeon, *A. oxyrinchus*, and lake sturgeon, *A. fulvescens*, prefer to spawn over clean gravel substrate (Chiotti et al. 2008, Johnson et al. 2006, Sulak and Clugston 1998). However, a unique population of white sturgeon (*A. transmontanus*) in the Frazer River, British Columbia, Canada,

spawn over a sandy substrate, resulting in decreased recruitment success (Paragamian et al. 2002). This further supports the argument that substrates offering shelter in crevices may allow greatest survival of fertilized eggs. Lake sturgeon, *A. fulvescens*, are defined as 'litho-pelagophil' because their eggs require substrate for attachment (Balon 1975, Harkness and Dymond 1961). Spawning in spring begins with female sturgeon scattering adhesive eggs over boulders and rocky substrate at depths of 0.1-2m (Auer and Baker 2002, Becker 1983, Bruch and Binkowski 2002, LaHaye et al. 1992, Priegel and Wirth 1974). Along with water flow, temperature, and depth, the nature of substrate is critical in determining the viability of fertilized eggs. While differences in preference for habitat do exist between populations of lake sturgeon, many prefer coarse gravel or cobble for spawning (Auer 1996, McKinley et al. 1998). Eggs that are unable to properly adhere to a surface drift away and are often non viable. Upon hatching, the prolarvae are pelagic and negatively phototactic (Harkness and Dymond 1961, Kempinger 1988, Kynard and Horgan 2001, Wang et al. 1985). They actively seek hiding locations within interstitial spaces in their surrounding substrate for most of the prolarvae stage (Auer 1996, Peterson et al. 2007). A study by LaHaye and colleagues (1992) compared spawning, early life history and substrates preferred by lake sturgeon in the L'Assomption and Des Prairie rivers, Quebec, Canada. Spawning and early development sequences were observed to be very similar for both rivers. Once approximately 20mm, the larvae drifted downstream. Researchers noted fewer eggs attached as depth and current velocity increased at both sites. Eggs were observed on a wide range of substrates, ranging from fine and medium sized gravel to boulders, and even on artificial

beds established in Des Prairies River. However, significantly greater numbers of eggs were found on coarse gravel substrates, while no eggs were observed on fine sand, silt and clay beds.

Juveniles may prefer different substrates depending upon their lifestyle, including modes of feeding, diurnal patterns, and predator avoidance. Benthic feeders such as juvenile lake, *A. fulvescens*, pallid, *Scaphirhynchus albus*, and shovelnose, *S. platorynchus*, sturgeon are often found over sand and clay substrates (Peake 1999), where their preferred prey are abundant (Allen et al. 2007, Benson et al. 2005). Hay-Chmielewski and Whelan (1997) suggest that substrate preference in the wild correlates with foods found most frequently in the stomachs of fish. An interesting study by Chiasson and colleagues (1997) divided substrate into classes based on dominant particle size and identified macroinvertebrate abundances at each site. They found the highest concentration of lake sturgeon juveniles over sand and clay substrates where prey species were abundant. This suggests substrate preference, especially at the exogenous feeding stage, is likely a reflection of prey availability. Individual species and populations may, however, utilize multiple substrates dependent on the particular life stage. For example, yearling lake sturgeon, *A. fulvescens*, in Black Lake, Michigan prefer sandy substrates, while juveniles prefer organic substrates (Smith and King 2005).

Juveniles of other sturgeon species are found on varying habitats: sandy mud substrate for juvenile shortnose, *A. breviorstrum,* and Atlantic sturgeon, *A. oxyrinchus*; sand, gravel, boulders and bedrock for juvenile white sturgeon, *A. transmontanus*; and

silt and sand for juvenile shovelnose, *S. platoryhnchus*, and pallid, *S. albus*, sturgeon (Gerrity and Guy 2008, Parsley et al. 1993). An interesting study by Gessner and colleagues (2009) explored physiological responses, including the cortisol stress response, in larval Atlantic sturgeon (*A. oxyrinchus*) reared on gravel and sand substrates, and found preference for gravel immediately following hatch. Larvae reared on sand had the greatest total length, while those on gravel had lowest total length. Interestingly basal cortisol levels did not differ among the treatments prior to feeding, with researchers attributing this to incomplete functioning of the HPI system.

3.1.2: Temperature

Temperature is one of the most influential abiotic variables impacting development of organisms in any environment, and as such, has been extensively studied in numerous fish species, including rainbow trout, *O. mykiss*, haddock, *Melanogrammus aeglefinus*, and green sturgeon, *A. medirostris*, (Martell et al. 2005, Valenzuela et al. 2008, Werner et al. 2007). Due to the link with dissolved oxygen content where warmer waters may have less available oxygen (Geist et al 2006), the temperature of the water can be critical in fish spawning, egg development, and juvenile survival. Water temperature can influence time of hatch, which can in turn affect overall development and growth rate (Methven and Brown 1990). Generally it has been observed that teleosts exhibit a positive correlation between early development and temperature. Fertilized eggs of Pacific cod, *Gadus macrocephalus*, developed slower in

cooler environments, as well as having the poorest hatching success at the lowest tested temperatures (Laurel et al. 2008). Interestingly the researchers noted that early hatching larvae were smaller in size, but had greater post-hatch survival. A study by Geist and colleagues (2006) on chinook salmon, *O. tshawytscha*, had contradictory findings, with no significant difference in sizes across varied temperature treatments. However, similar to the study by Laurel and colleagues (2008), chinook salmon reared in cooler waters had a decreased developmental rate. Green and white sturgeon eggs had greatest hatching success and normal development within an optimal temperature range of 14 to 17°C (Van Eenennaam et al. 2005, Wang et al. 1985). Yellowtail flounder, *Limanda ferruginea*, had no significant difference in egg mortality, but did take longer to hatch in cooler temperatures (Avery et al. 2004). When reared within the confines of their temperature limits, fish generally exhibit increased growth and developmental rates at higher temperatures (Adomski and Caddell 1991), and this has been shown in the present study (see chapter 2). These findings indicate that temperature can act as a prominent variable with potential consequences for later life stages.

Temperature tolerance limits are species specific; thereby the impacts of different temperature treatments may vary depending on the fish being studied. Knowledge of these limits is essential for adequate species management and protection (Van Eenenaam et al. 2005). Heat-shock proteins (hsp) are believed to be involved in stress tolerance by preventing thermal damage and repairing damaged proteins (Werner et al. 2007). Interestingly, larval green sturgeon, *A. medirostris*, reared in warm waters developed deformed notochords, as well as highly expressed hsp proteins, while

fish reared in warm waters and transferred to cooler, optimal temperatures recovered significantly, with a delayed decrease of hsp protein expression (Werner et al. 2006). While most studies suggest a distinct upper tolerance limit for fish species, juvenile shovelnose sturgeon, *S. Platorynchus*, had increased mortality both above and below optimal temperatures. This may be attributed to low temperatures causing depletion of endogenous energy reserves (Kappenman et al. 2009).

The impacts of temperature, substrate and depth on development have been explored for several teleost species (Methratta and Link 2007), including Surubim, *Pseudoplatystoma sp*. The response to temperature in Surubim suggests increased temperatures result in increased development rate and maximum cortisol levels when stressed (Lima et al. 2006). Data on adult green sturgeon acclimated to different temperature treatments suggest that maximum cortisol levels are similar regardless of temperature, but that fish reared at the cooler temperature exhibit an extended stress response (Lankford et al. 2003). Similar work on juvenile Adriatic sturgeon, *A. naccarii*, resulted in higher 'resting' serum cortisol levels in fish reared in high temperatures as compared to fish acclimated to optimal temperatures (Cataldi et al. 1998). Such studies on Acipenseriformes are limited, with little data incorporating the impact of temperature on the ontogeny of a cortisol stress axis.

3.1.3: Objectives and hypothesis

Experiments were designed to examine the role of substrate and temperature on the ontogeny of the cortisol stress response in lake sturgeon, A. fulvescens, over two field seasons. Substrate type during the egg phase was hypothesized to contribute to the ontogeny of the cortisol stress axis, with eggs developing in "natural" conditions ie: gravel substrate exhibiting a decreased response in terms of overall magnitude and duration. Furthermore, it was hypothesized that there would be no difference in the appearance of the stress response, as larvae hatched on any substrate would be expected to exhibit a cortisol response only at the onset of the exogenous feeding stage and substrate would not influence the timing of this. However, it was hypothesised that increased temperature would accelerate the appearance of the cortisol response due to a faster developmental rate in warmer fish. Further, fish raised in warmer waters may have an increased cortisol stress response.

3.2: Materials and Methods

The roles of substrate and temperature on the development of the cortisol stress response were examined over two sampling seasons, spring 2008 and spring 2009. Fertilisation, procedures, maintenance and experimental setup for both 2008 and 2009 have been described in detail in chapter 2 (section 2.2). The methods here will describe the standard protocol used to examine the timing of the appearance of a stress response and the magnitude and duration of the stress response.

3.2.1: Appearance of the cortisol stress response (2008)

Due to high mortality rates and low numbers of available prolarvae, daily sampling for this component of the experiment was conducted using eggs and prolarvae reared in the hatchery jars only. On alternate days, 20 individuals were removed from a random jar, and immediately placed in an overdose of anaesthetic (300ppm tricaine methanesulfonate) before being snap frozen in liquid nitrogen as 'control' samples. 'Experimental' samples were similarly collected for hatchery jar-reared fish on alternate days. Twenty individuals were transferred to a Petri dish and chased for 2 minutes, then left undisturbed for thirty minutes. After 30 minutes, prolarvae or larvae were immediately placed in the overdose of anaesthetic and snap frozen in liquid nitrogen. Time to transfer from the anaesthetic bath to liquid nitrogen was under a minute.

Additional sampling in 2008 was conducted weekly in the gravel and slate substrate treatment groups. Twenty unstressed prolarvae or larvae were collected from randomly selected gravel and slate experimental tanks. Fish were collected, anaesthetised immediately, and frozen as described above.

3.2.2: Appearance of a cortisol response (2009)

Samples were collected daily from all temperature treatments to determine the timing of the appearance of the cortisol stress response. Eight individuals were removed from random 9°C ,12°C and 15°C gravel, and 12°C slate, treatment tanks and placed in an overdose of anaesthetic, then snap frozen in liquid nitrogen as described. The numbers of fish collected during any one sampling event could be reduced based on the whole body cortisol concentrations obtained from the previous years sampling. Control sampling was alternated with experimental sample collection on a daily basis, with the exception of the 9°C treatment group given the significantly lower survival as compared to other treatment groups. The protocol used for stressing individuals was identical to that used in 2008.

3.2.3: Magnitude and Duration of Stress Response

In order to assess the effect of substrate and temperature on the magnitude and duration of the cortisol response, lake sturgeon larvae were exposed to a standard

stressor (chasing for 5 minutes) 3 days sfter exogenous feeding had commenced and the yolk sac and spiral plug had disappeared in all larvae within a given treatment. All sampling was conducted between 9am and 1pm to minimize potential diurnal variation in the stress response. Prior to the first day of the experiment, fish were left undisturbed and unfed for 16 hours.

In 2008 each experimental tank in a treatment group was assigned a time point of 5, 10, 20, 60 or 120 minutes post stress. Each time point had 6 different tanks for each treatment to avoid pseudo–replication, and to account for any tank effect within the experimental design. Time 0, or control, samples were also collected to serve as baseline levels of whole body cortisol, following the 2008 daily 'control' sampling protocol described above. Fish reared in hatchery jars were also tested for the magnitude of their cortisol response at 0, 5, 10, 20, 60 and 120 minutes post-stress following the same protocol as described. However, as the fish were contained within 6 containers from the onset, the procedure required repeated sampling from the same jar. In an effort to counteract any impact from earlier stressing, sampling from each hatchery jar was only conducted once per day, and completed over a 6 day period. All hatchery jars were randomly tested for all six time points over the course of the experiment.

In 2009, based on results obtained in 2008, experimental fish were sampled at 20, 60, 120 and 240 minutes post stress to complement the post-stressed samples collected in 2008. Each flume was subdivided into 24 sections, with 6 sections serving

as replicates for each time point. Larvae were stressed using the standard stressor described within each experimental tank, and up to 6 individuals were collected at the assigned time point. Time points assigned to sections and order of stressing were randomized.

3.2.4: Whole body cortisol extraction

Whole body cortisol concentration for all samples was determined from thawed individuals or groups of individuals depending on the concentration and size of eggs, prolarvae, and larvae. Individual larvae were weighed and placed in separate test tubes with 1.5ml of phosphate buffer solution (PBS) (0.1M Na_2HPO_4 and 0.03M NaH_2PO_4, dissolved in 1L deionized water) per tube. The mixture was sonicated (Sonic dismembrator, XL 200, Fisher Scientific) and vortexed and whole body cortisol was extracted by solid phase extraction techniques. Briefly, using a constant infusion pump, (KD Scientific, Fisher Scientific) samples were passed through Classic C18 Sep-Pak cartridges (360mg 55-105µm, Waters Inc.). Prior to the addition of samples Sep-Pak cartridges were primed with 3ml methanol at a rate of 0.5ml.min^{-1}, and rinsed with de-ionized water at a rate of 2.0ml.min^{-1}. Samples were then loaded onto the cartridges at a flow rate of 1.0 ml.min^{-1} and the Sep-Paks were rinsed again with 5ml de-ionized water at 2.0ml.min^{1}. Samples were then eluted at a rate of 0.5ml.min^{-1} using 3ml methanol. Sep-Paks were then reconstituted using 5ml of 8M urea, followed by a 20ml deionized water wash. No Sep-Pak cartridge was used more than 3 times as this

resulted in an appreciable loss of recovered cortisol, as demonstrated in Table 3.1. Extracted samples in methanol were dried down using a Savant speed vacuum concentrator system (Savant Speed Vac 110S with vapour trap, Fisher Scientific) at a low heat setting. The dried down sample was stored in a -80°C freezer, and processed within 8 weeks of extraction.

3.2.5: Cortisol radioimmunoassay

Individual tubes containing the dried pellet of cortisol were resuspended in 200µl of pH 7.4 radioimmunoassay (RIA) buffer, prepared using 20ml PBS, 180ml water, 1.8g NaCl and 1.0g bovine serum albumen, and briefly vortexed. All extracted samples were analyzed in duplicate. A new standard curve was prepared for each assay using known quantities of cortisol to allow interpolation of the amount of unknown cortisol in samples. A standard 1µg.ml^{-1} stock solution of cortisol (Steraloids,Newport, RI, USA) was separated and frozen in 100µl aliquots. For each assay, a 100µl aliquot was resuspended in 900µl RIA buffer to achieve a concentration of 100ng.ml^{-1}. Serial dilution of the 100ng.ml^{-1} stock yielded a standard curve ranging from 0.0125 to 12.5ng.ml^{-1} (fig 3.1).

Tritiated cortisol was stored in -20°C freezer as a 9:1 toluene:ethanol solution. 7000-disintegrations per minute (dpm) in 100µl was prepared for each assay using RIA buffer. 100µl of tritiated cortisol was added to all assay tubes. 100µl of cortisol-specific antibody (rabbit anti cortisol-3 polyclonal antibody; Fitzgerald Industries, Concord, USA)

was added at a dilution of 1:30,000 to all assay tubes. The employed antibody had cross reactivity of 100% with cortisol, 36% with prednisolone, 5.7% with 11-desoxycortisol, 3.3% with corticosterone, and less than 0.7% for cortisone. Tubes were incubated at 22°C for 60 minutes, and then overnight at 4°C to establish a binding equilibrium between the antibody, labelled cortisol, and cold cortisol. Every assay was terminated with the addition of 100µl of dextran coated charcoal (0.05g Dextran from *Leuconostoc mesenteroides,* and 0.5g activated charcoal dissolved in 10ml RIA buffer) to all tubes, with the exception of total counts that had only the labelled cortisol present. Once added to each tube, samples and standard curves were vortexed and left on ice for 15 minutes. Tubes were then centrifuged at 2,500g for 30 minutes at 4°C before decanting the supernatant into 7ml assay vials. 4ml of Ultima Gold liquid scintillation fluid (Perkin Elmer, Waltham, MA, USA) was added to all vials. Radioactivity for each sample was measured using a liquid scintillation counter (Beckman Coulter, LS6000TA) for 5 (2008) and 3 (2009) minutes per assay vial. No appreciable difference in counting efficiency was noted between 5 or 3 minutes.

Each assay had additional zero and total counts tubes. Zero tubes were treated identically as all standard curve tubes, with the exception that no unlabelled cortisol stock was added to these tubes. However, 100µl of RIA buffer was added to these tubes to maintain equal volume between these and the known and unknown assay tubes. Zero tubes provided a measure of radioactivity bound to the antibody in the absence of competing cold ligand, in order to determine minimum detection limits. Total counts tubes were prepared using 100µl of RIA buffer and 100µl of the tritiated cortisol. After

the overnight incubation, the contents were transferred to assay vials without processing with the dextran-charcoal mixture. Following the addition of 4ml of Ultima Gold liquid scintillation fluid, radioactivity was measured as previously described for samples.

Cortisol concentration in unknown samples was determined with reference to known concentrations in the standard curve using Graph Pad Prism 5 software (La Jolla, Ca, USA) to interpolate unknown values from the standard curve. Values in $ng.ml^{-1}$ were corrected for tissue mass ($ng.g^{-1}$ of tissue) and extraction efficiency. All chemicals used were purchased from Sigma Aldrich (St. Louis MI, USA) unless otherwise stated.

3.2.6: Statistical Analysis

Statistical analysis was completed using Microsoft Excel and Graph Pad Prism 5 software. Due to the nature of daily stressed and unstressed samples, data was analyzed based upon trends and differences based on standard error associated with each sampling point. Magnitude and duration of response data were analyzed using 2-way analysis of variance (ANOVA) with interaction, and a Bonferroni's post-hoc test.

3.2.7: Validation tests

To determine appropriate components and quantities of the radioimmunoassay, several validation tests were conducted.

1. Inter- and Intra- assay variation: Groups of 6 fish were extracted and dried down. Extracted pellets were resuspended in phosphate buffer and combined into one large 'pool' sample. 100µl aliquots of the pool sample were frozen. Approximately 5 pool samples were processed with each assay, providing the amount of variation between assays. Within-assay variation was determined by the addition of 10 pool samples in one assay, and comparing the variability between the individual readings.

2. Extraction Efficiency: Extraction efficiency was determined to correct for sample recovery. Individual larvae were sonicated and homogenized in 1.9ml PBS, with additional control tubes containing 2.0ml PBS alone. All tubes were spiked with 10,000dpm tritiated cortisol. Following vortexing, a 100µl aliquot was removed from all tubes and transferred to individual assay vials. 4ml of scintillation fluid was added and samples read on a liquid scintillation counter to determine pre-extraction counts. Remaining samples were extracted, as described (see section 3.3.4). Pellets were resuspended in equivalent volumes to pre-extraction, and 100µL aliquots transferred to assay vials. Following the addition of 4ml scintillation fluid, samples were read to determine post-extraction counts. The

ratio of pre- and post- extraction counts was used to determine extraction efficiency.

3. Parallelism: A dilution series was used to validate the sample extraction procedure. A large sample pool was extracted and dried pellets resuspended in RIA buffer. 100µl aliquots were frozen. For sample parallelism, 100µl aliquots were used and serially diluted, allowing samples of different concentrations to be used in an assay.

3.3: Results

3.3.1: Validation tests

Extraction efficiency was determined to be 90.18 ± 0.028%, and all cortisol values were adjusted for it (n=7). Diluted series of samples ran parallel to the prepared cortisol standard curve (n=3) (Fig. 3.1). Intra- and inter- assay variation was 3.00% (n=36) and 3.44% (n=10), respectively. The minimum detection limit was 0.072 ± 0.02 $ng.ml^{-1}$.

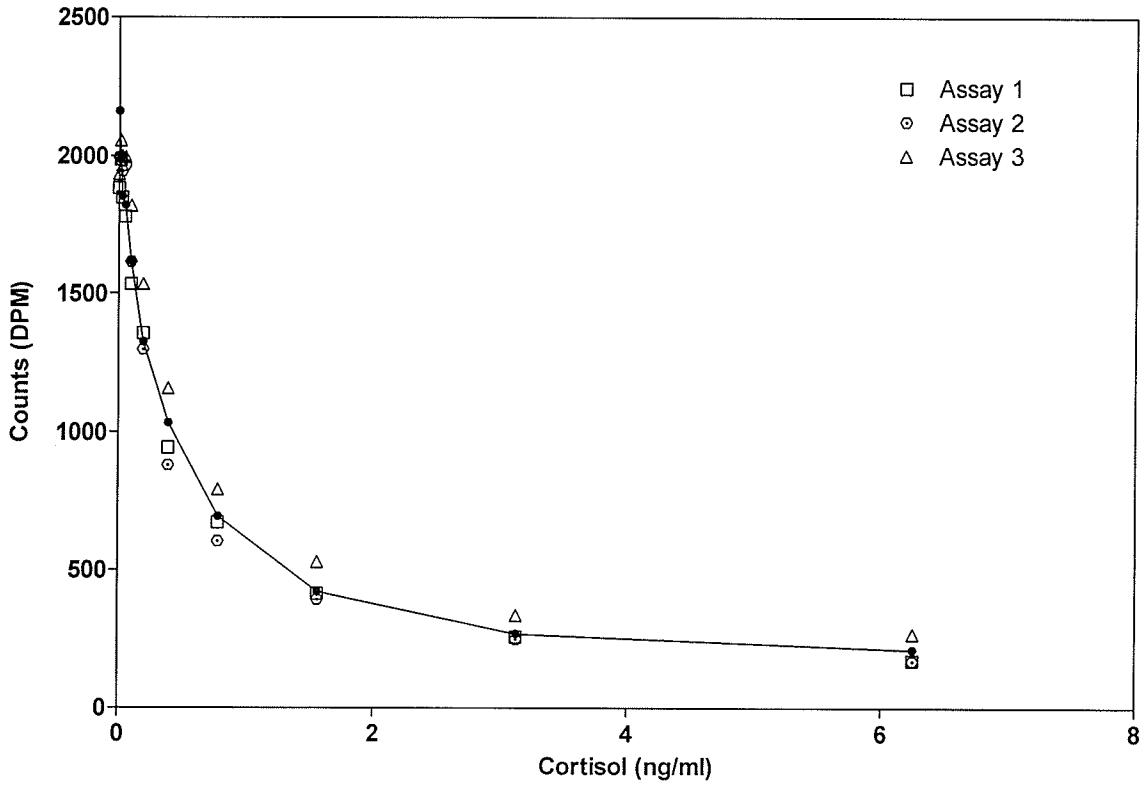


Figure 3.1. Cortisol concentrations of pooled samples paralleling prepared standard curves (n=3). Standard curves were generated based on the addition of 100µl of 0, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.39, 0.78, 1.56, 3.13, and 6.25 $ng.ml^{-1}$ of unlabelled cortisol stock.

Table 3.1. Mean amount of cortisol in identical pool samples extracted. Note the appreciable decline in amount of cortisol recovered from the same sample following the 2nd reconstitution using 8M urea.

Sample Identity	Mean amount of cortisol ($ng.ml^{-1}$)	Std. Error
New Sep-pak	14.73	2.26
1st reconstitution	16.56	1.28
2nd reconstitution	15.54	1.39
3rd reconstitution	7.13	0.71
4th reconstitution	7.97	0.71

3.3.2: Timing of appearance of cortisol response

Whole body cortisol levels for fish at rest are depicted in figure 3.2 for 2008 (a) and 2009 (b). There was no significant difference in cortisol concentration for control fish reared on gravel and slate substrates in either 2008 ($p=0.504$, 2-tailed Student's t-test) (Figure 3.2a) or 2009 ($p= 0.4899$, 2-tailed Student's t-test) (Figure 3.2b). The differences in resting or 'control' levels between 2008 and 2009 may be attributed to the considerably smaller sample size. A more robust data set was utilized in 2009. The 2008 data indicate low levels of control cortisol, with a significant increase after 19dpf.

Experimental individuals collected at 12°C in the substrate treatments demonstrated an appearance of the cortisol response 27dpf when exogenous feeding began (Figure 3.3). Whole body cortisol concentrations in control and experimental fish reared in hatchery jars in 2008 is depicted in figure 3.4. Error associated could not be determined due to low daily sample size due to the fact that numerous fish were required in order to obtain a measurable value using the described assay procedure. Whole body cortisol concentrations for control lake sturgeon reared in 9, 12 and 15°C are depicted in figure 3.5a, and experimental in figure 3.5b. Due to the varying developmental rates of the fish, and thereby the nature of collected data, it was not possible to compare between treatments. In order to analyze the data, trends were evaluated with reference to developmental stages such as hatch and the onset of exogenous feeding.

3.3.3: Magnitude and duration of cortisol stress response

Magnitude and duration of the cortisol response in stressed larval lake sturgeon reared on gravel and slate substrates in 2008 and 2009 are depicted in figures 3.6a and 3.6b. Fish hatched on slate in 2008 had a significant increase in whole-body cortisol concentration within 5 minutes, with levels returning and remaining at rest by 10 minutes (fig 3.6a). Fish hatched on gravel exhibited a significant increase within 5 minutes and return to baseline by 10 minutes. Levels elevated once again to a maximum at 60 minutes post-stress. Final levels remained significantly elevated above rest. Fish reared in hatchery jars exhibited a significant increase by 5 minutes, with levels

returning to baseline by 20 minutes post-stress. Whole-body cortisol concentrations for fish hatched on slate in 2009 increased significantly and reached a maximum at 20 minutes post stress. Levels increased slightly, but not significantly, by 120 minutes, followed by a rapid decrease to resting levels by 240 minutes. Cortisol concentrations in fish hatched on gravel exhibited a significant increase by 20 minutes, with continued ascent at 120 minutes post-stress. Cortisol concentrations are significantly greater at 240 minutes post stress as compared to resting levels (time 0) (fig 3.6b).

For data collected in 2008, both time post stress ($p<0.0001$), and substrate type ($p=0.0005$) significantly influenced whole body cortisol levels. The interaction of time and substrate was also significant ($p<0.0001$) (figure 3.6a). For data collected in 2009, time post stress had a significant impact on the amount of cortisol detected ($p<0.0001$), while substrate did not ($p=0.4111$). The interaction of time and substrate was significant ($p=0.0089$)(Two-way ANOVA with interaction, and Bonferroni's test).

Magnitude and duration of the cortisol response in stressed lake sturgeon reared in 9, 12 and 15°C are depicted in figure 3.7. Time had a significant impact on the amount of cortisol detected post stress ($p=0.0049$), while temperature did not have a significant impact ($p=0.1287$). The interaction of time and temperature was, however, significant ($p=0.01$)(Two-way ANOVA with interaction, and Bonferroni's test) (fig 3.7). No significant change was noted for the 9°C treatment. Cortisol concentration for fish reared in 12°C increased significantly by 20 minutes, and elevated once again at 120 minutes post stress. Final cortisol concentrations at 240 minutes post-stress were

significantly greater than baseline levels (time 0). Cortisol concentration for fish in 15°C treatment increased significantly by 20 minutes, with a decrease to time 0 levels by 60 minutes post-stress.

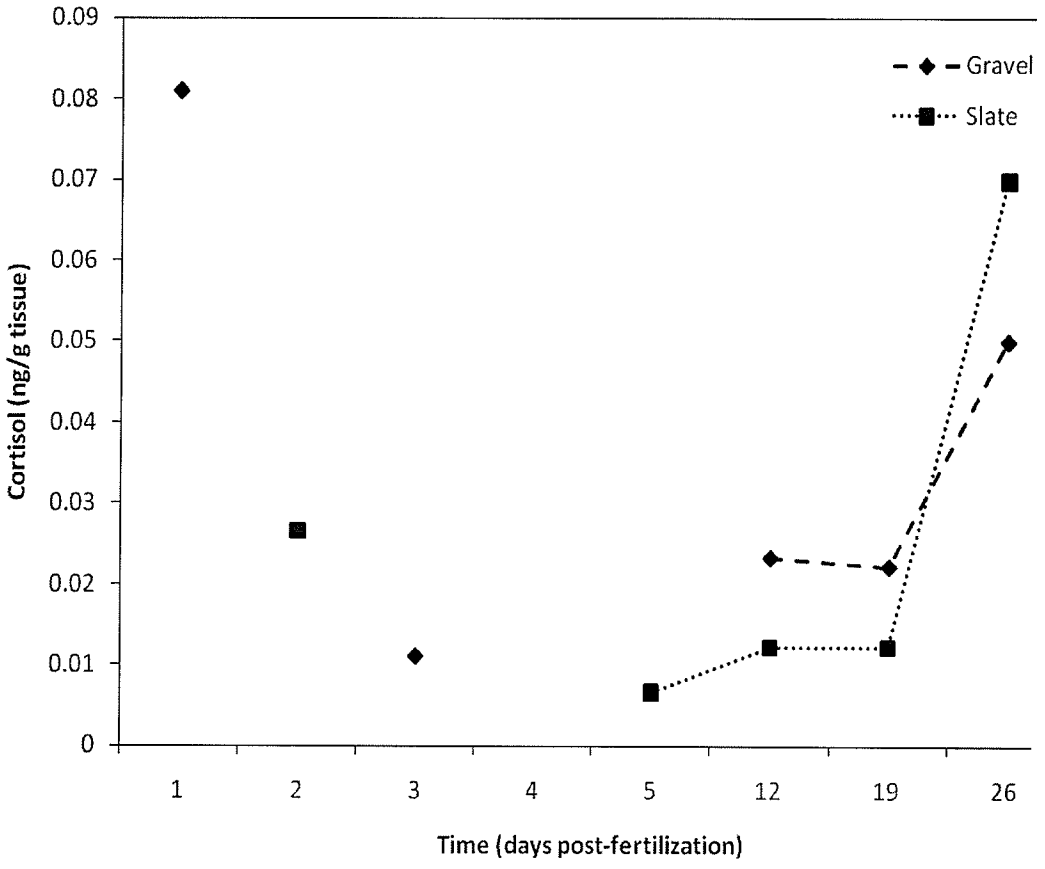


Fig. 3.2 a. Whole body cortisol concentration in control lake sturgeon prolarvae and larvae reared on gravel and slate substrates in 2008. Values are expressed as a mean concentration of cortisol per gram of tissue mass ($ng.g^{-1}$) ± 1 SEM (n=1).

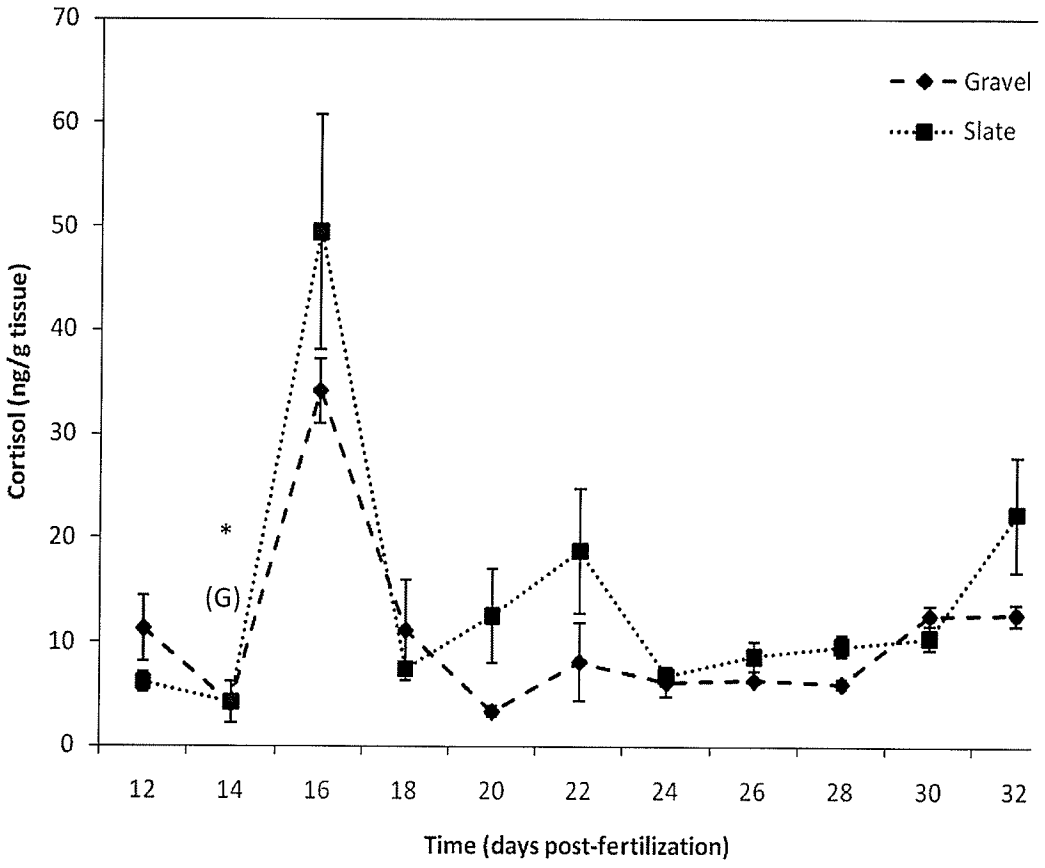


Fig. 3.2 b. Whole body cortisol concentration in control lake sturgeon prolarvae and larvae reared on gravel and slate substrates in 2009. Values are expressed as a mean concentration of cortisol per gram of tissue mass ($ng.g^{-1}$) ± 1 SEM (n=1 for 2008 data, n ≤ 6 for 2009 data, however, some points are as low as 1* due to the availability of samples).

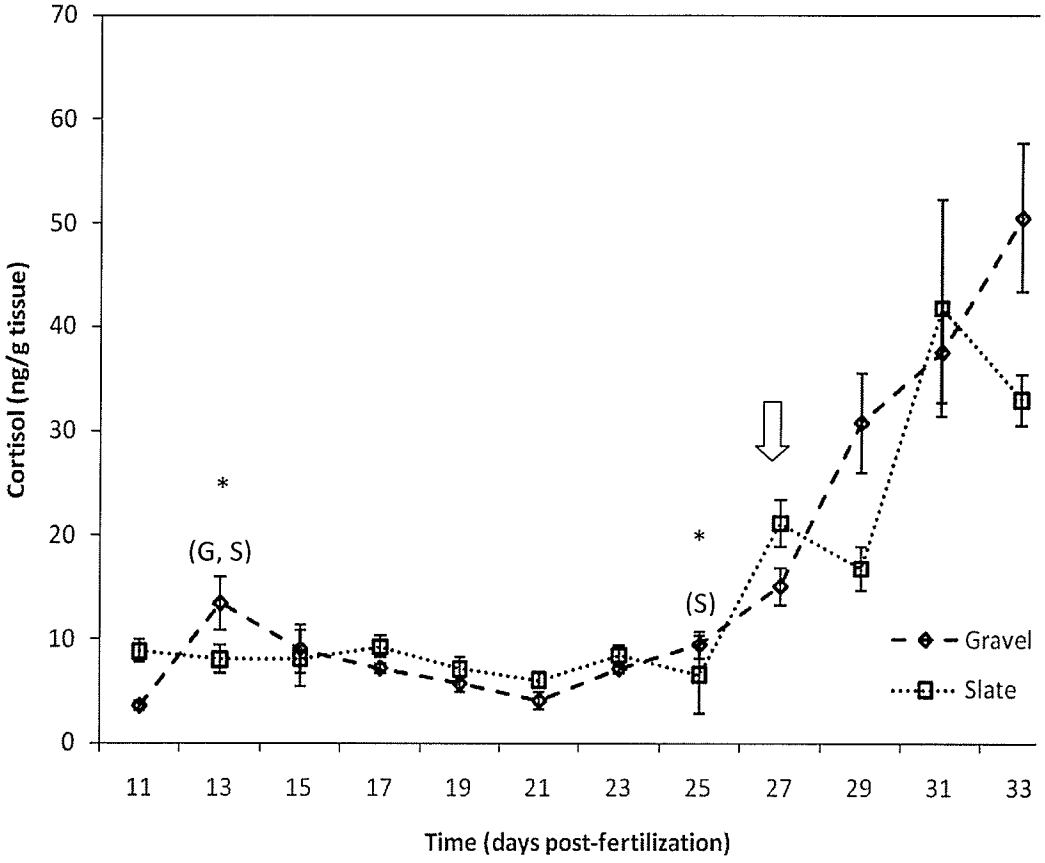


Fig. 3.3. Whole body cortisol concentration in experimental lake sturgeon prolarvae and larvae reared on gravel and slate substrates in 2009. Values are expressed as a mean concentration per gram of tissue mass ($ng.g^{-1}$) ± 1 SEM ($n \leq 6$, however, some points are as low as 2* due to the availability of samples). An arrow marks the onset of exogenous feeding in both treatment groups.

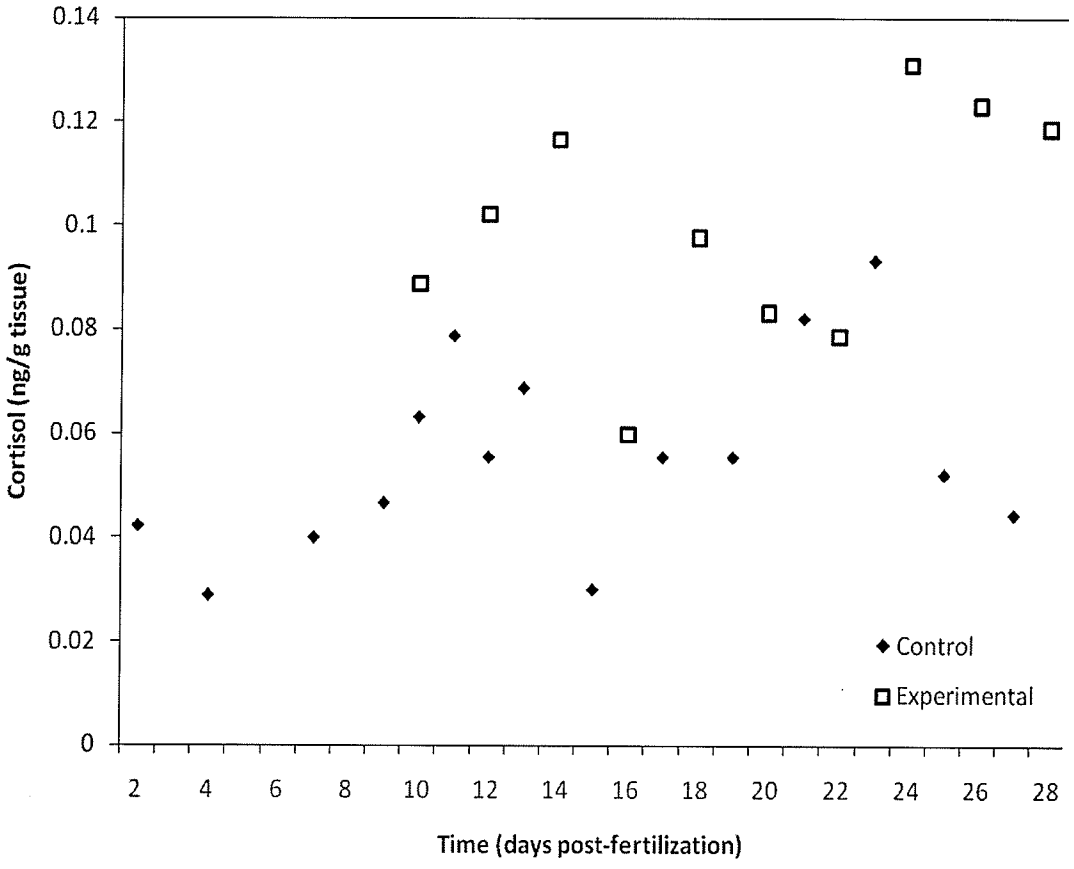


Fig. 3.4. Whole body cortisol concentration in control and experimental lake sturgeon reared in hatchery jars in 2008. Values are expressed as a mean concentration per gram of tissue mass ($ng.g^{-1}$) ± 1 SEM (n =1)).

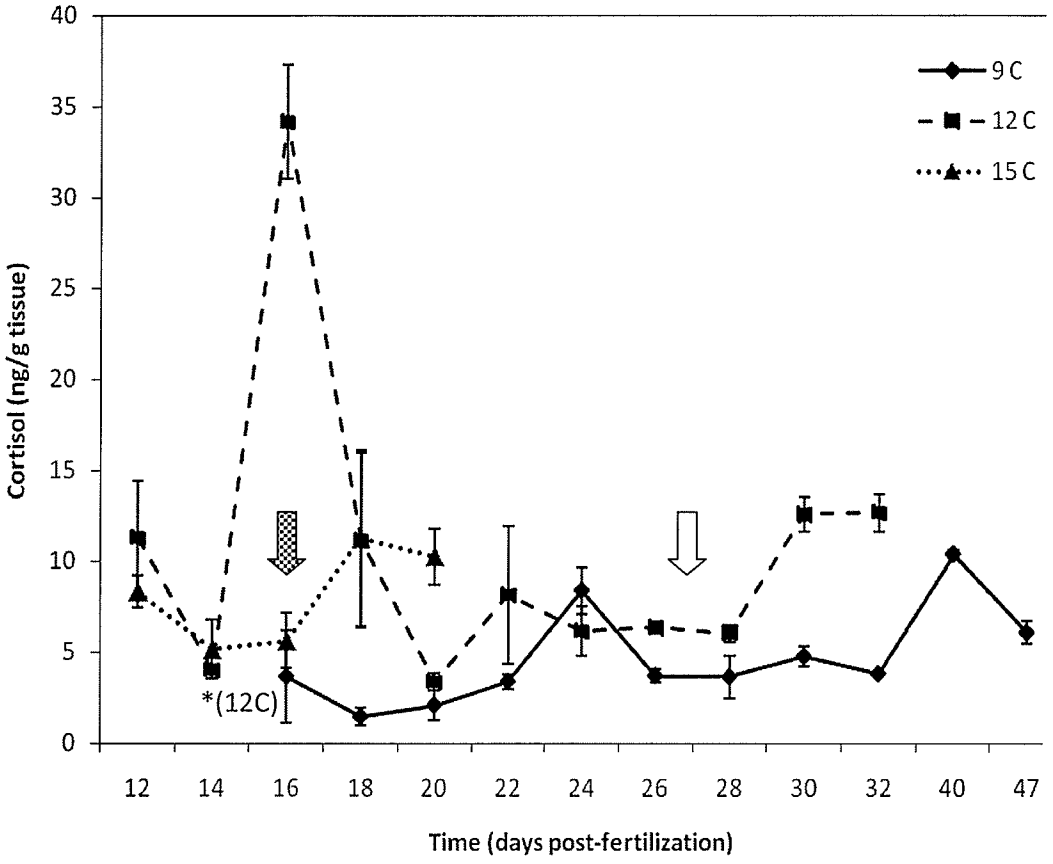


Fig. 3.5 a. Whole body cortisol concentration in control lake sturgeon prolarvae and larvae reared in 9, 12 and 15°C. Values are expressed as a mean concentration per gram of tissue mass ($ng.g^{-1}$) ± 1 SEM ($n \leq 6$, however, some points are as low as 1* due to the availability of samples). Pattern and blank arrows represent the onset of exogenous feeding in 15 and 12°C.

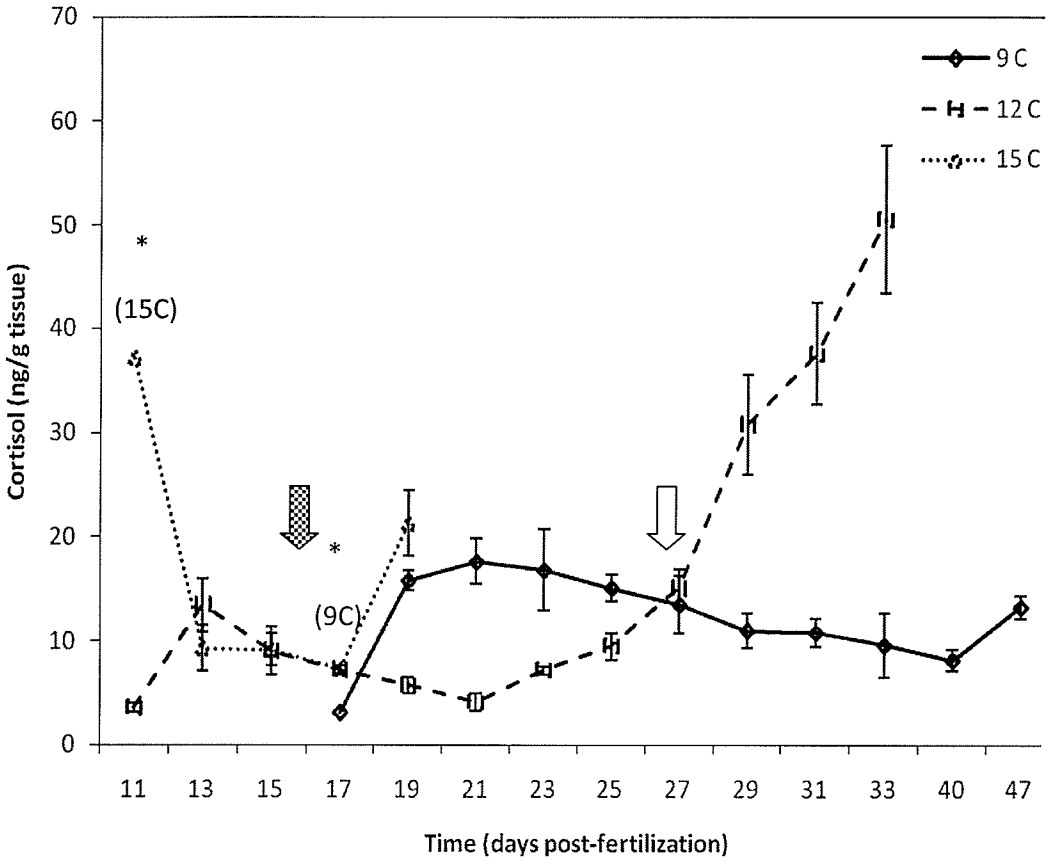


Fig. 3.5 b. Whole body cortisol concentration in experimental lake sturgeon prolarvae and larvae reared in 9, 12 and 15°C. Values are expressed as a mean concentration per gram of tissue mass ($ng.g^{-1}$) ± 1 SEM ($n \leq 6$, however, some points are as low as 1* due to the availability of samples). Pattern and blank arrows represent the onset of exogenous feeding in 15 and 12°C.

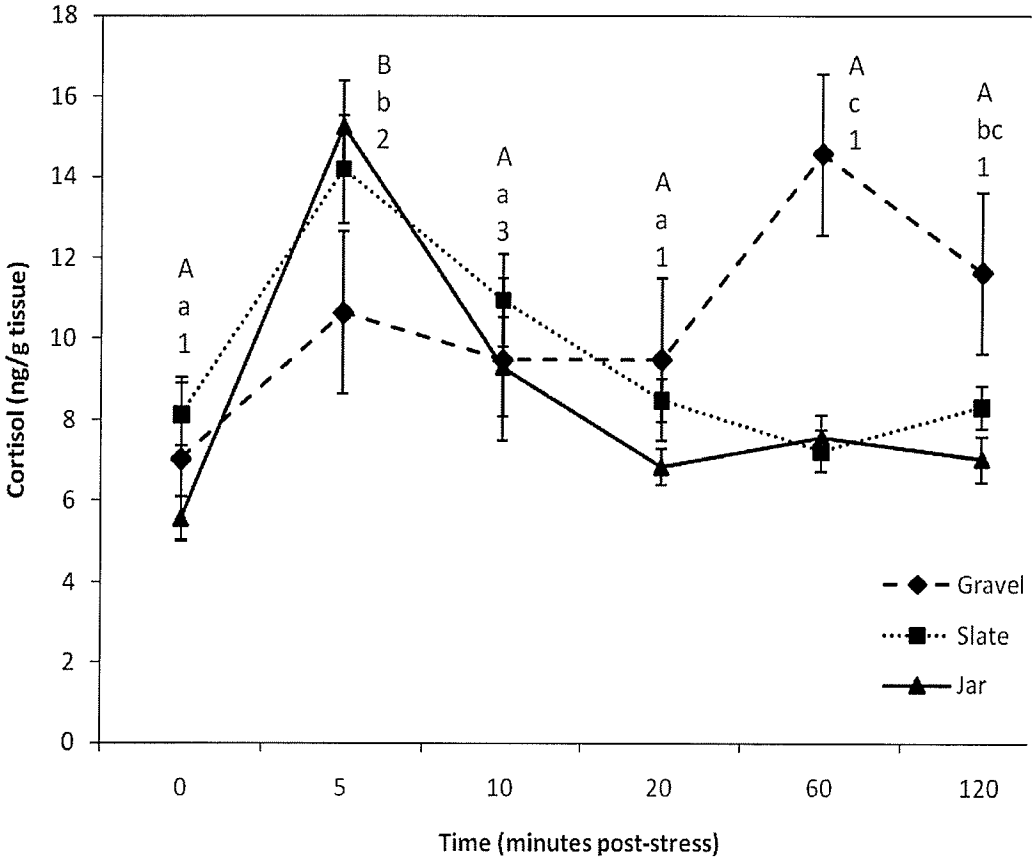


Fig. 3.6 a. Magnitude of whole body cortisol stress response in lake sturgeon larvae reared on gravel and slate substrates in 2008. Values are expressed as a mean concentration of cortisol per gram of tissue mass ($ng.g^{-1}$) ± 1 SEM ($n \geq 5$). Statistical differences within treatments were determined using Bonferroni's post-test. Different letters represent significant differences between time points within the treatment groups, with capital letters for slate, lower case letters for gravel, and numbers for hatchery jars.

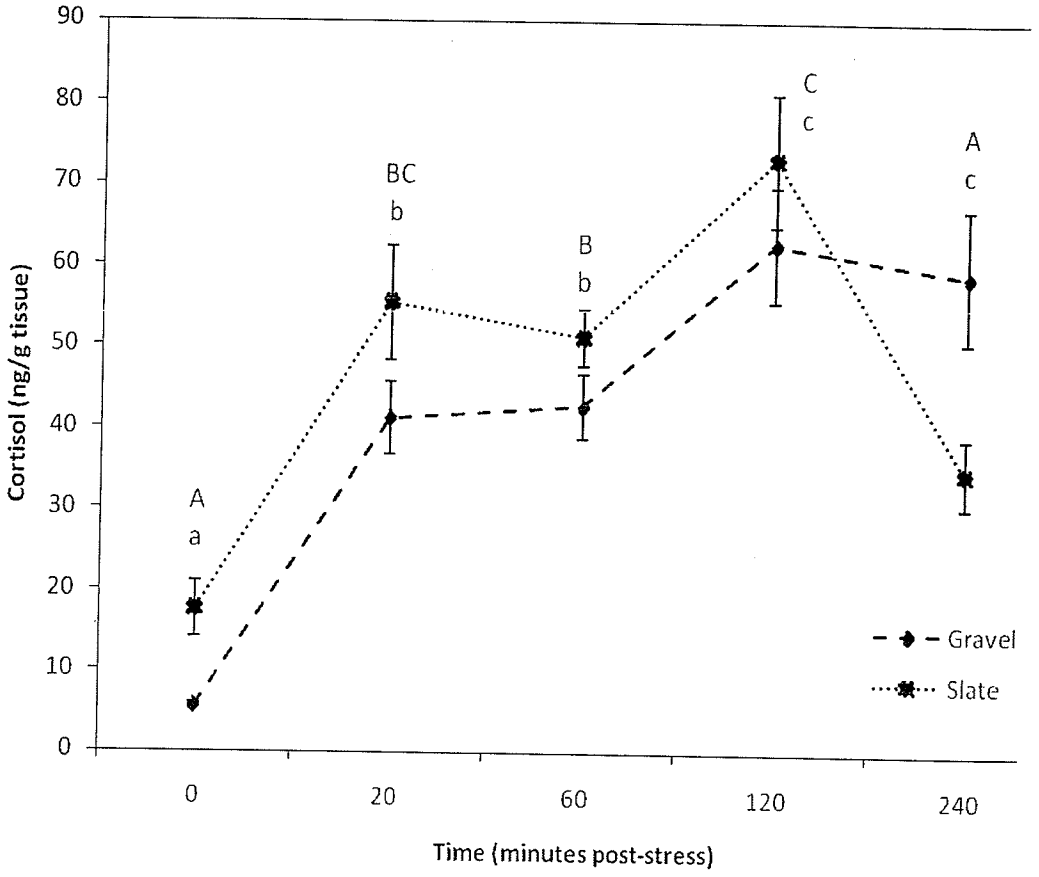


Fig. 3.6 b. Magnitude of whole body cortisol stress response in lake sturgeon larvae reared on gravel and slate substrates in 2009.Values are expressed as a mean concentration of cortisol per gram of tissue mass ($ng.g^{-1}$) ± 1 SEM ($n \geq 5$). Statistical differences within treatments were determined using Bonferroni's post-test. Different letters represent significant differences between time points within the treatment groups, with capital letters for slate, and lower case letters for gravel.

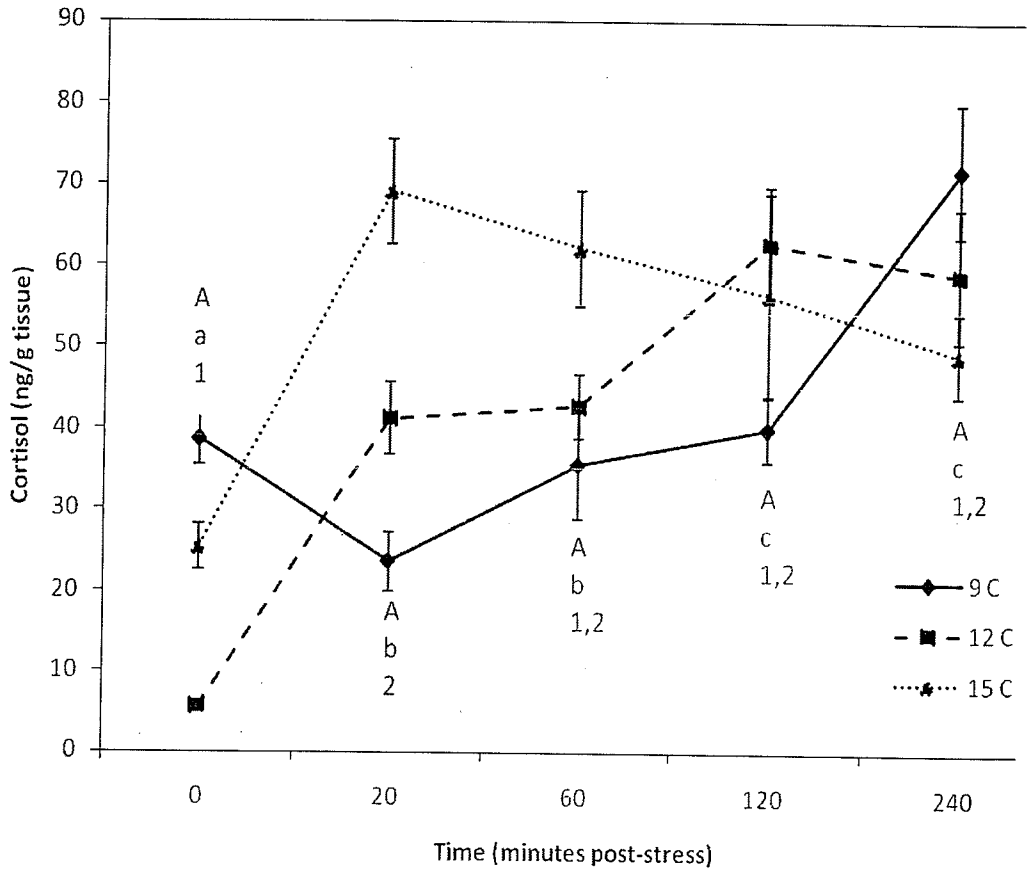


Fig. 3.7. Magnitude of whole body cortisol stress response in lake sturgeon larvae reared in 9, 12 and 15°C in 2009. Values are expressed as a mean concentration of cortisol per gram of tissue mass ($ng.g^{-1}$) ± 1 SEM ($n \geq 5$). Statistical differences within treatments were determined using Bonferroni's post-test. Different letters represent significant differences between time points within the treatment groups, with capital letters for 9°C, lower case letters for 12°C, and numbers for 15°C.

3.3.4: Larvae Survival

In 2008 there was no significant difference in survival of larvae hatched on gravel as compared to larvae hatched on slate (Student's t-test, p=0.43). In 2009 percent survival of larvae reared in different temperature and substrate treatments is depicted in table 3.2. There was no significant difference in survival between larvae reared at 9°C as compared to 15°C (Student's t-test, p=0.67). However, there was significantly greater survival in the 12°C gravel treatment as compared to 9°C (Student's t-test, p=0.024) and 15°C treatment (p=0.0091). When comparing substrates, larvae actually fared better when reared on slate versus gravel as there was greater survival of larvae reared on slate at 12°C, than those reared on gravel at 12°C (Student's t-test, p=0.0011).

Table 3.2. Larvae survival until exogenous feeding for different temperature and substrate treatments in 2009. The surviving larvae are expressed as a percentage of the mean of total fertilized eggs added to each flume ± 1 SEM. Two-tailed t-tests were conducted to determine if significant differences exist between temperature treatments.

Temperature treatments	Surviving larvae (% of total)
9C	1.37 ± 0.21
12C (gravel)	3.45 ± 0.35
12C (slate)	11.73 ± 0.99
15C	1.48 ± 0.18

3.4: Discussion

Current knowledge on the ontogeny of the stress axis in sturgeon species is limited, with none focusing on lake sturgeon. These freshwater species are endangered or threatened, and knowledge of their physiological development is essential for adequate management. Such work could have implications for rearing of juveniles in artificial sites, as well as successful reintroduction into native waters (Kempinger 1988, Peterson et al. 2007, Wang et al. 1985).

Research on cortisol concentrations in several sturgeon species suggests the ancient Acipenseridae family tends to have low baseline levels, as well as decreased response to a perceived stressor (Baker et al., 2005; Barton et al., 1998; Barton et al., 2000). Comparable studies on development of the interrenal stress axis and cortisol stress response have been conducted for teleost species. Cortisol levels can increase 10 to 100 fold in adult teleosts experiencing stress, with levels returning to rest within 3 to 24 hours (Barton and Iwama 1991). Work on larval rainbow trout revealed baseline cortisol concentrations of $6ng.g^{-1}$ in newly fertilized eggs, $0.3ng.g^{-1}$ upon hatching, and finally increasing to $1.4ng.g^{-1}$ in the following week (Barry et al. 1994). Interestingly, Barry and colleagues (1994) noted that resting baseline cortisol concentrations were greatest at the onset of stress responsiveness in larval rainbow trout, a trend also noted in chum salmon (De Jesus and Hirano 1992). Barry and colleagues proposed that this activation prior to feeding may in fact aid in the transition from endogenous to exogenous sources. In the present study lake sturgeon larvae have low resting cortisol

concentration that increased 3 to 14 fold when stressed, depending upon the rearing conditions (fig 3.7). The considerably lower response as compared to teleosts may be a reflection of the lack of stress exposure of these fish in the wild. Historically lake sturgeon are among the largest species found in freshwater environments in North America, with no natural predators. Even juvenile fish possess highly developed protection from potential predators with external body armour in the form of scutes, thereby potentially minimizing selective pressure for a dramatic cortisol stress response (Peterson et al. 2007).

Data from 2008 suggests lake sturgeon eggs contain low levels of cortisol, which gradually decreased over time for both substrate treatments, until 19dpf, followed by rapid increases in resting levels. A similar trend was noted for fish reared on gravel and slate in 2009, with levels decreasing from 16dpf to 30dpf, a time that coincided with the onset of exogenous feeding. Due to greater survival in the 2009 experimental setup, a larger sample set, and thereby more robust data set was possible. Initial corticosteroid levels are maternally derived in numerous aquatic species, and this maternal cortisol is critically involved in early development of fish larvae (Alderman and Bernier 2009, Espmark et al. 2007, Gagliano and McCormick 2009, Tanaka et al. 1995). Measurable levels of CRF system component genes have been detected in unfertilized zebra fish eggs demonstrating maternal contribution in eggs (Alderman and Bernier 2009). Maternally derived cortisol, along with thyroid hormones and growth hormone, are necessary for regulation of embryonic development (Tanaka et al. 1995).

Tanaka and colleagues (1995) describe three major stages during early development: the embryonic phase, the transition phase, and the transformation phase. The embryonic phase involves use of maternal hormones and an inactivated HPI axis, the transition phase involves early functioning of endocrine systems, with low levels of hormones maintained, and the transformation phase involves increased activity of the endocrine systems as well as increased hormone levels. Several marine species have exhibited this trend of low baseline cortisol levels upon hatch that continue to decrease until exogenous feeding, when a rapid increase is observed (Alderman and Bernier 2009, Tanaka et al. 1995). Maternal cortisol may be involved in mediating mineralocorticoid receptor signalling during early development (Alsop and Vijayan 2007). Increase in cortisol levels during early ontogeny, as well as activation of the HPI axis, are immediately preceded by an up regulation of StAR, 11 β-hydroxylase and MCR2 (Alsop and Vijayan 2007). Interestingly, data from this experiment suggests low levels of resting whole-body cortisol from hatch (9dpf ±2days) till 14dpf, with a rapid increase immediately after (fig 3.2b). This initial period of hypo-responsiveness to stress may be to maintain low levels of corticosteroids that may negatively impair other essential development such as neural organization (Barry et al. 1994).

Cortisol concentration in eggs was determined for samples from gravel and slate tanks in 2008 (fig 3.2a). Initial levels of cortisol in eggs coincide with the stress exposure of a female laying the eggs. Several researchers have noted that high stress levels in a spawning female can result in eggs with high cortisol concentrations, and potentially impact the stress responsiveness development (Eriksen et al. 2007, Espmark et al. 2008,

Gagliano and McCormick 2009). To avoid any possible genetic influence of the fertilised eggs, eggs and milt used in both 2008 and 2009 were pooled from several female and male lake sturgeon, and thoroughly mixed prior to fertilization. Work on coral reef damselfish, *Pomacentrus amboinensis* has explored the impacts of maternal cortisol levels, and found that high levels in eggs led to poor hatching success, but possibly longer survival upon hatch (Gagliano 2009). However, the researchers do argue that the increased survival does not strictly confer a long-term survival benefit; rather it reflects immediate response to a stressful environment (Gagliano 2009).

Lake sturgeon prolarvae hatched on slate substrate had a significantly higher control cortisol concentrations at 12, 16, 20, 22 and 28dpf as compared to prolarvae hatched on gravel substrate (fig 3.2b). Levels for the slate treatment plateau at 24dpf, and remain fairly consistent until the onset of exogenous feeding (30dpf), with minor increases noted for both substrate treatments. Final control samples collected upon exogenous feeding were higher for larvae hatched on slate compared to larvae hatched on gravel (32 dpf) (fig 3.2b). These data coincide with higher cortisol levels in fish reared on slate at time 0 samples collected for the magnitude experiment (fig 3.6b). Embryos may adaptively prefer gravel in order to minimize predation and prevent drifting into unsuitable habitats (Gessner et al. 2009, Kempinger 1988). LaHaye et al. (1992) noted greater numbers of lake sturgeon eggs on coarse gravel and none on fine sand, silt or clay. Larval Atlantic sturgeon were tested for their physiological and behavioural preferences for sand and gravel substrates (Gessner et al. 2009). During the initial 5 days post hatch (dph), more embryos were found in the gravel sections. Interestingly the

researchers noted these fish to have the lowest total length, but highest energy content leading to exogenous feeding. Once feeding, gravel fish had the greatest growth rate. Despite the observed morphological differences, Gessner and colleagues (2009) found no significant difference in resting cortisol concentrations between treatments. This trend may, however, arise from the fact that the sampling was conducted for prolarval fish prior to the onset of exogenous feeding. Based on findings in lake sturgeon from this experiment, it appears that sturgeons may not be able to exhibit a cortisol stress response prior to exogenous feeding, so any cortisol concentrations prior to this point may not accurately reflect the impact of stress on the organism. Gessner and colleagues (2009) further conducted sampling at the onset of exogenous feeding, and noted initial increases in both treatments, with fish from gravel treatments decreasing levels following the first feeding while fish from the sand treatment continuing to increase levels up to 14dph. The researchers suggest that this observed difference may be due to sample selectivity and relative ease of sampling that may have skewed the findings.

As seen in control larvae in 2008, lake sturgeon larvae reared in 9 and 15°C temperature treatments in 2009 exhibit low resting whole-body concentration of cortisol. Fish reared in 15°C maintained low cortisol levels up to 16dpf, a period that coincided with the onset of exogenous feeding (15dpf ± 2days), followed by a gradual, but not significant, increase (fig 3.4a). Corresponding samples could not be collected for 9°C fish at the onset of feeding (52dpf ± 2days) due to low survival. However, based on samples collected throughout the course of the experiment, levels remain relatively constant, with any increases returning to baseline levels by the next sampling point.

The minor increases, such as those observed on 24dpf and 40dpf may be attributed to individual variance. Interestingly, time 0 data for exogenous feeding larvae from the 9°C treatment group indicates a relatively high resting cortisol concentration (fig 3.7), suggesting a dramatic increase upon initiation of feeding.

Experimental prolarvae reared in 2009 on gravel and slate were similarly unable to exhibit an increase in cortisol levels early in development, with levels relatively stable at an average of 7.46 ± 1.13 and 7.81 ±0.39 $ng.g^{-1}$ for gravel and slate prolarvae from hatch up to 25dpf (fig. 3.3). The low levels detected prior to 27dpf are likely the maternally derived cortisol also found in unstressed larvae. Data from this experiment suggests that despite the cortisol store, larvae are unable to respond to a stressor likely due to developmental demands. Furthermore, brain or pituitary immaturity may be responsible for the inability to respond to a stressor prior to the exogenous feeding stage (Auperin and Geslin 2008). Barry and colleagues (1994) noted basal cortisol levels present in the 2 weeks immediately following hatch, yet an inability to increase cortisol concentrations when stressed. The researchers suggest that this may occur despite interrenal cells successfully producing cortisol, as well as ACTH found in the pituitary, due to under developed higher brain centres involved in coping with stress. They further suggest the possibility of immature interrenal cells or decreased levels of corticosteroid-binding globulins. Work by Alsop and Vijayan (2009) on larval zebrafish similarly concluded that the delayed activation of the HPI stress response despite basal levels of cortisol present may be due to incomplete development of neural inputs conveying information about the stressor to the hypothalamus.

Cortisol levels in experimental prolarvae upon hatch were found to be 3.09, 3.57 ± 0.50, and 37.21 $ng.g^{-1}$ for eggs hatched at 9, 12 and 15°C respectively (fig 3.5). Fish reared at 9, 12 and 15°C similarly initially responded to a stressor by increasing whole body cortisol concentrations after 17, 25 and 17dpf, respectively. Such findings suggest an activation of the hypothalamic-pituitary-interrenal stress response at the end of the prolarval period. Interestingly experimental fish reared in 9°C water hatched on 15dpf ± 2 days displayed an initial increase in cortisol levels when stressed after 17dpf, before maintaining levels throughout the course of sampling (fig 3.5b).

Environmental variables, particularly during early development, can dramatically impact the ontogeny of the organism. From substrate, temperature, oxygen availability, and invasive species, numerous abiotic and biotic pressures exist. When comparing different temperature treatments, lake sturgeon larvae had significantly decreased percent survival at both temperature extremes (9 and 15°C) as compared to 12°C. Work on lake sturgeon by other researchers has revealed similar findings, with the fish having optimal development in a narrow temperature range (Wang et al. 1985). Lake sturgeon larvae hatched on slate substrate had a greater survival rate as compared to larvae hatched on gravel in 2009 (table 3.2). However, it should be noted that this particular trend was not found in the previous sampling season. The observed greater survival in slate tanks contradicts the notion that a lack of irregular shaped substrate leads to decreased survival. An interesting study by Kihslinger and Nevitt (2005) on rainbow trout, *O. mykiss*, found prolarvae reared over gravel as compared to a bare hatchery tank developed significantly larger cerebella, which enhanced their motor skill, and

presumably had positive effects on overall survival. Researchers suggest this increased brain size may be due to optimal yolk utilization over gravel where prolarvae reared in the gravel substrate may utilize their yolk sacs more efficiently as less energy would be expended for swimming and seeking shelter on a flat surface.

Temperature of the aquatic environment can impact a range of variables, including spawning, hatching success, embryo growth and development, and disease resistance (Armour 1991, Wang et al. 1985). Temperature of the immediate environment is critical to the development and survival of an organism, with many species having lower and upper threshold limits, as well as an optimal temperature range. Water temperature can greatly impact survival. Kappenman and colleagues (2009) noted high mortality at the higher temperature extremes, which they partially attributed to decreased feed efficiency. Low temperatures similarly resulted in mortality, possibly due to a depletion of natural reserves, with fish losing weight despite available food. Lake, *A. fulvescens*, and white sturgeon, *A. transmontanus*, embryo survival was greatest between 14-17°C (Wang et al. 1985). Interestingly, the researcher noted an upper limit of 20°C, but failed to observe a lower temperature limit for these two species, when testing as low as 10°C. Interestingly, fish are capable of surviving at lower or higher temperatures within their tolerance limits, but appear to have costs when doing so, in the form of impaired hatching and development. This could be because limited resources available to the organism, such as a maternally provided yolk sac, may be used to combat the chronic thermal stress and repair damage as compared to essential developmental processes. Exposure to thermal stress in steelhead trout

eggs resulted in increased mortality at higher temperatures. Surviving individuals hatching in higher temperatures were larger in size, and with greater structural asymmetry (Turner et al. 2007). Similarly, green sturgeon, *A. medirostris*, eggs reared in temperatures greater than 22°C have a high incidence of deformities (Mayfield and Cech 2004). Exposure to high temperatures during early ontogeny may result in increased anomalies due to greater cell death, microvascular disturbances, edema, hemorrhage, and infarction (Eriksen et al. 2007).

A study on juvenile shovelnose sturgeon *S. platorynchus* determined optimal growth for the species to occur at 22.4°C, with 10°C being the lowest tolerance limit for growth (Kappenman et al. 2009). Interestingly, maximal growth occurs at temperatures the species experience naturally in the wild at that particular life stage. Juvenile lake sturgeon, green sturgeon, and white sturgeon had maximal growth at 23°C, 24°C, and 20°C respectively (Allen et al. 2006, Wehrly 1995). The identity and specific population temperature preferences can vary. Lake sturgeon in the St. Lawrence River prefer to spawn at 15-16°C, while lake sturgeon in Wolf River Wisconsin had maximum spawn at 10-16°C (Brunch and Binkowski 2002, Johnson et al. 2006, Kempinger 1988). Lake sturgeon in the Winnipeg River, typically spawn when the temperature reaches 10.5 – 11.5°C (C. Barth personal communication).

Eggs and larvae were hatched and reared in hatchery jars during spring 2008 as well. Monitoring hatcheries is essential as practices can greatly affect survival and development of fish. Often these fish are used for restocking natural populations, as

well as for commercial purposes, and their rearing can impact the successes of these projects (Kempinger 1988, Peterson et al. 2007, Wang et al. 1985). Data from 2008 suggest low baseline cortisol concentrations in control fish that exhibited minor increases over time, before returning to levels found in eggs (fig 3.4). Fish in the experimental treatment, on the other hand, appear to exhibit some form of a response fairly early in development (10dpf), with experimental fish having higher levels of whole-body cortisol on all days (except 21-22dpf) than their control counterparts. The greatest difference between experimental and control cortisol levels was observed 24dpf onward. It can be hypothesized a fish would experience minimal stress and develop optimally over gravel, the substrate found in the wild at that particular life stage. An interesting study by Ottesen and colleagues (2007) noted juvenile Atlantic halibut, *Hippoglossus hippoglossus*, housed over a smooth, flat substrate, such as material used for aquaculture tanks, developed skin erosions and epidermal papillomas as compared to fish housed over irregular substrate. Lack of a substrate, as seen in hatchery jars, can lead to increased physical damage such as chafing in African cichlid *Oreochromis mossambicus*. Despite the physical damage, researchers did not note a difference in resting cortisol levels (Correia and Oliveira 2008). Work by Arndt and colleagues (2001) similarly noted rainbow trout *Oncorhynchus mykiss* reared on gravel had greater final weights, growth rate, and better dorsal fin condition as compared to fish reared without substrate.

The observed difference between larvae hatched on gravel, slate or hatchery jars may potentially stem from differences in developmental stage, with one group at a

particular stage better suited to respond to a stressor. Minute differences in development can impact an organism's ability to respond to a stressor, making it challenging to attributing differences solely due to substrate (Glennemeier and Denver, 2002).

At the onset of exogenous feeding, fish in substrate and temperature treatments were stressed via manual chasing to exhaustion, and whole individuals collected when the required time period had elapsed. Once the HPI stress response is activated, the age of an organism can dictate resting levels of cortisol. An example is Adriatic sturgeon, found to have mean concentrations of 11.5±2.9, 79.3 ±46.6, and 73.3±59.5 $ng.ml^{-1}$ in 7-month old, 5-year old, and 7-year old fish (Di Marco et al. 1999). All larvae used to determine magnitude and duration of the cortisol stress response were stressed 3 days following the onset of exogenous feeding. Larvae hatched on slate had significantly higher resting levels of cortisol prior to stress as compared to fish reared in hatchery jars in 2008. Resting concentrations of cortisol were 7.02 ± 0.41 $ng.g^{-1}$ for gravel fish, 8.12 ± 0.78 $ng.g^{-1}$ for slate fish, and 5.55±0.54 $ng.g^{-1}$ for hatchery jar fish. Levels of all three substrate treatments increased following the stressor; however, the magnitude of increase varied, with gravel fish exhibiting a slightly higher peak whole-body cortisol concentration (fig 3.6a). Slate and hatchery-jar fish, by comparison, rose significantly from time 0, and were significantly higher than gravel fish. Levels for both treatments peaked at 5 minutes post-stress, followed by a gradual decline to resting levels by 20 minutes. Final samples had a slightly greater cortisol concentration for fish from the slate treatment. Interestingly, it appears that the magnitude and duration of the whole

body cortisol response was fairly similar in fish from slate tanks and hatchery jars. Such a finding could be due to the strong similarity in slate tanks and hatchery jars. Both have flat surfaces with minimal protection for eggs, currents, light and any other abitoic and biotic disturbance. This study provides further support of the damage occuring when rearing eggs on artificial or flat surfaces, including potentially altered cortisol stress response during early development.

The maximal response was not significantly different for any treatment in 2008, with maximum concentrations of 14.6± 1.58 $ng.g^{-1}$ for gravel, 14.2 ± 1.33 $ng.g^{-1}$for slate, and 15.2± 1.14 $ng.g^{-1}$ for hatchery jar larvae (fig 3.6a). On the other hand, the timing of the maximal response differed for lake sturgeon eggs hatched on gravel. Whole body cortisol concentrations increased significantly by 5 minutes post-stress for fish hatched on slate, followed by a rapid descent to resting levels by 10 minutes. This extremely brief response may be disadvantageous to lake sturgeon larvae in the wild, where stressors are unlikely to be removed entirely within such a short time period. Whole body cortisol concentrations for fish hatched on gravel similarly increased significantly within 5 minutes post-stress, as well as returning to baseline levels by 10 minutes. However, cortisol levels did elevate again to reach a maximum at 60 minutes post-stress, without stimulation by a secondary stressor. Cortisol concentrations remained significantly elevated even at 120 minutes post-stress, suggesting a greatly extended duration of response. Fish reared in hatchery jars also exhibited a significant increase in whole-body cortisol concentrations within 5 minutes post-stress. However, levels failed to return to baseline concentrations until 20 minutes post-stress. Work on larval

rainbow trout had a similar finding, with cortisol levels failing to return to pre-stress levels for 7-week post-fertilization larvae. However, 6-, 8- and 9-week post-fertilization larvae were capable of returning circulating levels of cortisol to pre-stress levels. This may have been in part due to the higher resting cortisol concentrations that would lead to increased corticosteroid binding globulin levels, thereby reducing the potential for a negative feedback on higher neural centres in these experimental groups (Barry et al. 1994). To further explore the duration of the response for fish reared on gravel and slate substrates, greater sampling time points were used in 2009.

Substrate data in 2009 replicated the finding of significantly lower resting cortisol concentration (time 0) in gravel fish (fig 3.6b). Resting cortisol levels were 5.67± 0.41 $ng.g^{-1}$ and 17.59 ± 3.49 $ng.g^{-1}$ for gravel and slate, respectively. Interestingly the trend of the response was initially similar for both treatments, with significant increases at 20 minutes post stress. Larvae hatched on slate substrate maintained heightened levels until 120 minutes post-stress (73.05±8.11 $ng.g^{-1}$), followed by a rapid decrease to resting levels by 240 minutes. Whole body cortisol concentrations in fish hatched on gravel, on the other hand, exhibited a significant secondary increase, with a peak response observed at 120 minutes post stress (62.65± 6.98 $ng.g^{-1}$). Final levels remained elevated as compared to rest at 240 minutes post-stress. This type of stress response, with a delayed and maintained cortisol increase may confer a survival advantage in the wild. If a predator is present in nature, the lake sturgeon larvae with elevated cortisol levels may continue to have a hightened ability to react to a stressor for an extended period. However, such an increase can be costly, with possible desensitization of the

individual fish to stress, as well as negative long term consequences by elevated cortisol concentrations, including altered functions of metabolism, cardiovascular, respiratory, immune and reproductive systems (Barton 2002, Milla et al. 2009, Wendelaar Bonga 1997).

Baseline whole-body cortisol concentrations for fish reared in different temperature treatments differed significantly, with concentrations of 38.6 ±3.1 $ng.g^{-1}$, 5.67±0.41$ng.g^{-1}$, and 25.38 ±2.84$ng.g^{-1}$ for larvae reared in 9, 12 and 15°C, respectively (fig.3.7). Fish reared in 9 and 15°C had elevated resting whole-body cortisol concentrations as compared to fish reared in 12°C. This difference may exist due to fish reared in extreme environments utilizing resources for alternate needs such as immediate survival and coping with the imminent thermal stress, thereby impairing development during a critical phase. Furthermore, larvae in 15°C may be developing too rapidly for normal ontogeny, resulting in developmental errors. Depending upon the nature of habitat these fish normally inhabit, the optimal temperatures can vary. With 10.5 – 11.5°C being the approximate temperature at which lake sturgeon spawn in the Winnipeg river system, and developing eggs and prolarvae will experience a temperature range of approximately 10.5 – 15°C during development, it could be hypothesized that fish are least likely to experience underlying, chronic thermal stress in the 12°C treatment group.

Whole body cortisol concentrations for fish reared in 12°C increased significantly by 20 minutes, and elevated once again to a delayed maximum of 62.65±6.98 $ng.g^{-1}$ at

120 minutes post stress (Fig 3.6). Final cortisol concentration at 240 minutes post-stress remained above concentrations at rest. As compared to the 12°C fish, larvae reared in 15°C had a maximum whole body cortisol concentration of 69.05±6.48ng.ml^{-1} at 20 minutes post stress, with levels returning rapidly to rest within 60 minutes. These resting and stressed cortisol concentrations can be compared to other sturgeon species. An experiment on adult Adriatic sturgeon *Acipenser naccarii* found resting serum cortisol levels to be 9.4 ng.ml^{-1}. Furthermore the researchers noted higher cortisol levels in sturgeon reared in warmer tanks (79. 3ng.ml^{-1}) as compared to cooler tanks (38.5ng.ml^{-1}). This noted trend may be partially influenced by the warmer temperature, as well as potentially more severe stressing at the cooler site by the researchers (Di Marco et al. 1999). Despite minor changes observed within the 9°C group, data was not significantly different from rest throughout the course of the experiment. Such an inability to respond to a stressor that should elicit an increase in circulating levels of cortisol suggests highly impaired ontogeny of the HPI axis, and presumably other physiological parameters, in this thermally extreme treatment group.

Furthermore, there was no significant difference in the maximum cortisol concentration for all temperature treatments, suggesting that temperature, if within the thermal tolerance range, impacts the duration, as opposed to magnitude, of the cortisol stress response (fig 3.6). The lower temperature tolerance limit of lake sturgeon for appropriate development is hypothesized to be 10°C, and is supported by the findings of previous experiments (Wang et al. 1985).

Chapter 4: Catecholamines

4.1: Introduction

Like cortisol, catecholamines are involved in the primary stress response. Study of catecholamines is complementary to the study of cortisol as catecholamines are released instantaneously following stress, while cortisol levels gradually increase to allow an organism to cope. Both catecholamines and cortisol cause a release of glucose by converting glycogen to glucose (Mazeaud et al. 1977). Nerve fibers of the sympathetic nervous system innervate chromaffin cells and relay information from the hypothalamus in the brain. Catecholamines are released from chromaffin tissue upon stimulation of cholinergic receptors (Reid et al. 1998). At a primary level, catecholamine release contributes to an increase in oxygen uptake via higher ventilation rate, higher cardiac output, and hyperglycemia in different fish species (Wendelaar Bonga 1997).

Catecholamines are synthesized by the hydroxylation of tyrosine by tyrosine hydroxylase. The product L-dihydroxyphenylalanine (L-DOPA) is further decarboxylated by the enzyme L-aromatic amino acid decarboxylase (AADC) to dopamine. Dopamine is transferred to secretory vesicles and converted to norepinephrine via cleavage by the enzyme dopamine-B-hydroxylase. A percent of the synthesized norepinephrine is transported to the cell cytoplasm for conversion to epinephrine via methylation by the enzyme phenylethanolamine-N-methyltransferase (Reid et al. 1998). These catecholamines are stored in vesicles ready for immediate release following a stressor.

Release of these catecholamines into the circulation promotes immediate cardio-respiratory and metabolic changes including mobilizing energy stores, increased ventilation rate, and increased lipolysis by epinephrine (Dugan et al. 2007, Magnoni et al. 2008, Reid et al. 1995, Wendelaar Bonga 1997). Work on Antarctic fish has revealed the catecholamine epinephrine causes an increase in arterial pressure by increasing cardiac output and stroke volume (Sandblom et al. 2009). Due to the nature of catecholamine production and storage in vesicles for immediate use, conducting whole-body analysis would not accurately reflect an organism's stress response. Furthermore, due to the nature of catecholamine release within seconds of acute stimulation, it is impractical to obtain baseline catecholamine levels by whole-body analysis. An alternative method is to utilize larger sturgeon that are chronically cannulated to allow plasma collection without stressing the fish. For the purposes of this study, young-of-the-year lake sturgeon juveniles were employed, of sufficient mass to allow repeated plasma collection following a stressful event.

Studies, such as presented in the following chapter, are essential as a plethora of data on the cortisol stress response exists, for teleosts and acipenseriformes, but very limited data are available for the catecholamine stress response in sturgeons. This is particularly relevant given the reported 'low stress' cortisol levels of acipenseriformes. However, this does not necessarily mean that these fish have a low stress response. Experimental data are badly needed for a better understanding of the acute stress response in this and other fish species.

4.1.1: Objective and hypothesis

The objective of this chapter was to determine the magnitude and duration of the nor-epinephrine and epinephrine responses to stressors in young-of-the-year lake sturgeon (*A. fulvescens*). It was hypothesized the responses would be immediate and brief for both catecholamines, with levels returning to rest within 2 hours.

4.2: Materials and Methods

Young-of-the-year lake sturgeon, with an average mass of 359.25±63.25g, were used in catecholamine studies. Fish were reared in the animal holding facility in the Duff Roblin building at the University of Manitoba. Juvenile sturgeon were transferred to individual flow-through, 100L experimental tanks at 12.1 ± 0.3C, and were fed a maintenance diet (2% body mass per day twice daily) of commercial trout pellets. The fish were given one week to acclimate to their new environment.

Three days prior to commencing the experiment fish were removed from the experimental tanks and placed in a 'knock-out' bath of anaesthetic that contained 250ppm tricaine methanesulfonate (MS-222) buffered with an equal volume of sodium bicarbonate ($NaHCO_3$). Following anaesthesia the fish were transferred to a specially designed surgical table and a maintenance dose of buffered anaesthetic (100ppm MS-222 with equal amounts of $NaHCO_3$) was irrigated across the gills during the surgical implantation of a cannula into the caudal sinus. Briefly, a small incision was made posterior to the anal fin and a 1.5m cannula (PE-50, Clay Adams Intramedic tubing) was inserted into the caudal sinus. The cannula was secured with two tie-down points using silk sutures anchored to the posterior end of the fish and filled with heparinised sturgeon physiological saline (in mM) 126 NaCl, 2.2 KCl, 0.45 $CaCl_2$, 3 $MgCl_2$, 4.6 Na_2HPO_4, 0.2 KH_2PO_4, pH 7.6, 100units.ml^{-1} sodium heparin) to prevent clots forming in the cannula. A 2-way luer stopcock was attached to the end of each cannula to seal until blood collection. Fish were then transferred back to their experimental tank and

allowed to recover for 24-48 hours prior to the onset of experimentation. The luer stopcock was placed on the edge of the experimental tank, allowing blood collection with minimal disturbance or detection by the fish.

4.2.1: Baseline sampling

Following the morning feeding, fish were left undisturbed for 3 hours to allow a return of resting catecholamine levels. At this time approximately 600µl of blood was drawn from the luer stopcock attached to each cannula without disturbing fish (time 0). Care was taken to ensure the fish did not sense tugging of the cannula. Fish were reinjected with equal volumes of heparinised sturgeon physiological saline via the cannula. With minimal disturbance, further blood samples were collected at 1, 5, 10, 20, and 120 minutes for baseline values, and fish were reinjected with equal volumes of saline following each time point. Twenty-four hours later, time 0 samples were collected as described above. Water plugs were then removed externally of tanks, to stress all fish via a 30-second aerial exposure when the water had drained. Blood samples were then collected at 1, 5, 10, 20 and 120 minutes post-stress, without further disturbance to the fish. Hematocrit levels were monitored at each sampling point to check on fish health.

Upon collection of blood samples, they were immediately centrifuged at 13000g for 2 minutes at 4°C. The plasma was then transferred to a new tube and snap frozen in liquid nitrogen for storage in a -80°C freezer until analysis.

4.2.2: Standards

Thawed samples were processed immediately prior to measurement on a liquid chromatography unit (Bioanalytical Systems Inc., IN, USA). For each set of samples, new norepinephrine, epinephrine and 3,4-dihydroxybenzylamine (DHBA) were prepared. DHBA was used as an internal standard according to the manufacturer's instructions to determine absolute concentrations of norepinephrine (NE) and epinephrine (E) in samples using the following equation:

$$\text{Conc. of NE}_{unknown} = \frac{(NE/DHBA)_{unknown} \times \text{conc. of NE}_{known}}{(NE/DHBA)_{known}}$$

A stock solution of 100ng.ml^{-1} DHBA was prepared by dissolving 16mg DHBA-HBr in 100ml 0.1M perchloric acid ($HClO_4$). A 100µl aliquot was diluted in 100ml $HClO_4$ (0.1M) and stored at 4 °C. Similar stock solutions of 75ng.ml^{-1} NE and 25ng.ml^{-1} E were prepared by dissolving 14.2mg and 4.6mg NE- and E-bitartrate respectively in 100ml $HClO_4$ (0.1M)and 100µl aliquots further diluted in 100ml of $HClO_4$ (0.1M).

4.2.3: Sample processing

Sample processing was conducted using a modified procedure of Bioanalytical Systems, Inc (BAS). Briefly, samples were prepared by adding 450µl plasma to 12µl DHBA standard and 50mg acid-washed aluminum oxide (AAO) provided by BAS (CF-8010) in a conical reaction vial. Standards for calibration purposes were prepared using

1.0ml phosphate buffer (8.64g Na_2HPO_4, 2.36g KH_2PO_4 and 20.0g Na_2EDTA in 1.0L deionized water), 12µl DHBA standard, 8µl NE or E standard, and 50mg AAO. Additional 1.05ml of Tris buffer, prepared using 45g Tris buffer and 5g disodium ethylenediaminetetraacetic acid (Na_2EDTA) in 250ml water (pH 8.6), was added to all sample vials prior to vortexing. Samples were then collectively shaken for 5 minutes then left undisturbed on ice to allow alumina to settle prior to removal of the supernatant. Alumina was resuspended in deionized water and washed 3 times. After the final wash 0.5ml deionized water was added once again to aid in transferring the alumina slurry to a RC58 membrane microfilter (Bioanalytical Systems Inc.). Samples were centrifuged for 1 minute at 1000g at 4 °C. In the final step, the centrifuged water was discarded, a new receiver tube added to each microfilter, and 100µL of $HClO_4$ (0.1M) was added to the alumina to extract the catecholamines. Samples were vortexed twice with a 5-minute pause between mixings, before a final 1 minute spin at 1000g at 4°C. The alumina was discarded, and the $HClO_4$ containing the extracted catecholamines within the collecting tube was injected onto an analytical column (MF8594, Bioanalytical systems) to separate the NE, E and DHBA in the prepared sample.

An isocratic gradient was used to pump the mobile phase which comprised of 14.175g of monochloroacetic acid, 0.372 g of disodium ethylenediaminetetraacetic acid, 0.465 g of sodium octyl sulphate and 1.5% acetonitrile, methyl cyanide in 2L of milliQ water, pH 3.0, through the column at a flow rate of $1ml.min^{-1}$. 20µL of extracted catecholamine sample was loaded into a Rheodyne injection port and the

chromatography run was started. Retention times and areas under NE, E and DHBA peaks in each chromatogram were analyzed using the Bioanalytical Systems Software to determine unknown catecholamine concentrations. All chemicals used were purchased from Sigma Aldrich (St. Louis MI, USA) unless otherwise stated.

4.3: Results

Resting levels of NE and E in juvenile 'control' lake sturgeon at time 0 were 22.40 ± 6.23 $ng.ml^{-1}$ and 0.89 ± 0.21 $ng.ml^{-1}$ respectively. This experiment resulted in higher levels of NE in stressed juveniles compared to control levels at 0, 1 and 10 minutes (fig 4.1 a). Higher levels of E were also detected in stressed juveniles compared to controls at 0, 5, 10 and 20 minutes (fig 4.1 b). Maximum plasma NE (107.73 ± 28.38 $ng.ml^{-1}$) and E (30.49 ± 10.32 $ng.ml^{-1}$) were detected 10 minutes post stress. Within the experimental stress group, a significant increase in plasma E was observed 1 minute post stress within the stressed group. Percent hematocrit decreased from 0 to 120 minute samples within a treatment, and were significantly lower on the second (experimental) day of sampling (fig 4.2).

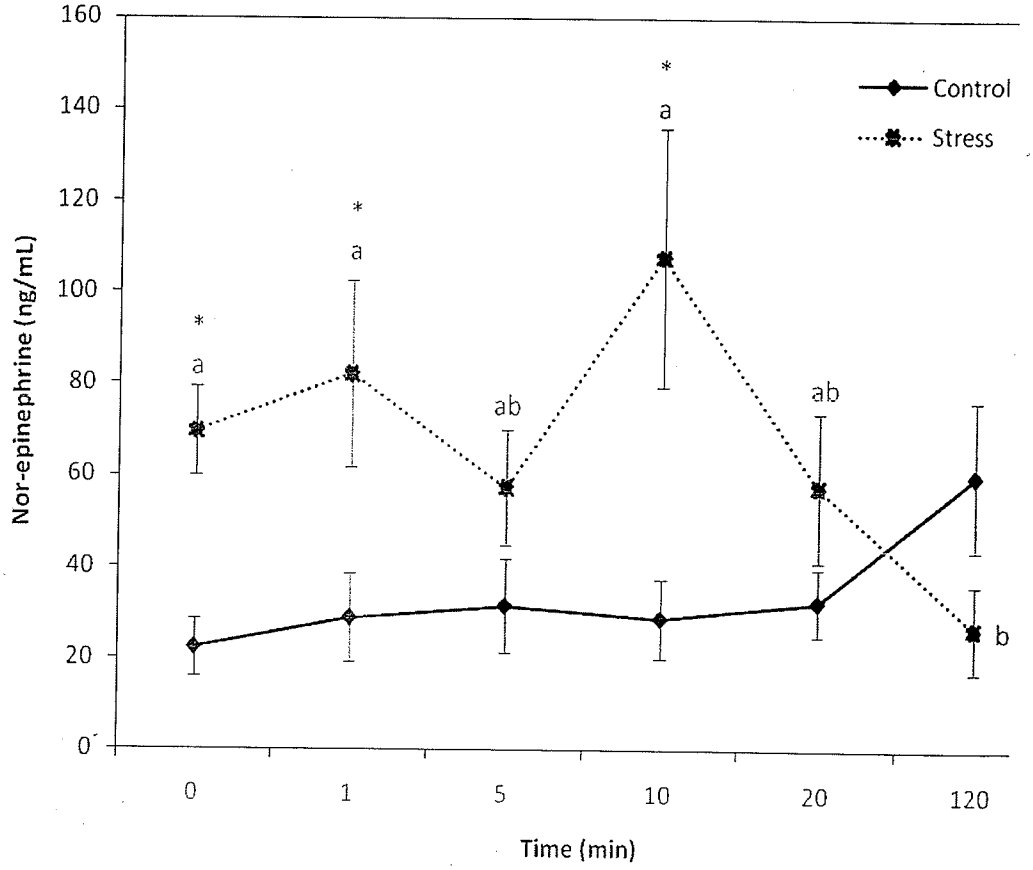


Figure 4.1 a. Plasma norepinephrine concentrations in juvenile lake sturgeon. Values are expressed as a mean concentration in $ng.ml^{-1}$ ± 1SEM. Different letters represent significant differences between time points within a treatment group (Friedman Repeated Measures Analysis of Variance for control data, one-way repeated measures ANOVA and Tukey's multiple comparison for stress data, $P < 0{\cdot}05$). An asterisk represents a significant difference between treatments within a time point (Student's *t*-test, $P < 0{\cdot}05$) (n=7).

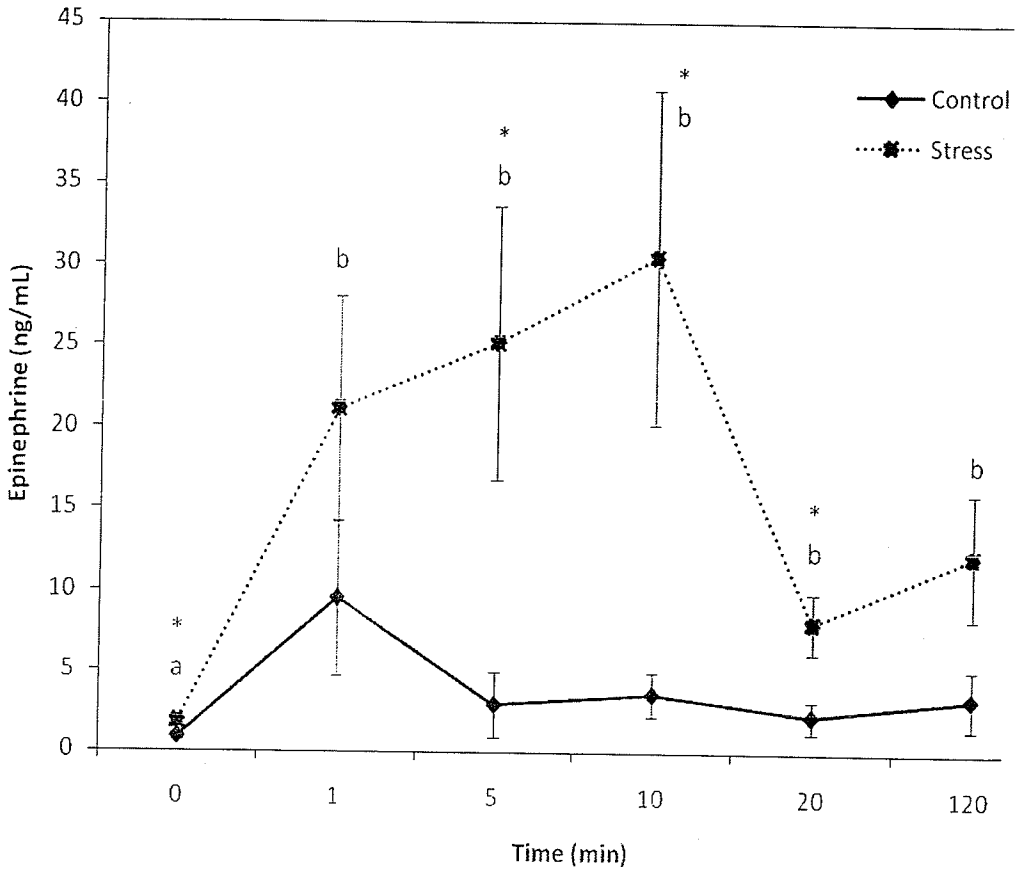


Figure 4.1 b. Plasma epinephrine concentrations in juvenile lake sturgeon. Values are expressed as a mean concentration in $ng.ml^{-1}$ ± 1SEM. Different letters represent significant differences between time points within a treatment group (Friedman Repeated Measures Analysis of Variance for control data, one-way repeated measures ANOVA and Tukey's multiple comparison for stress data, $P < 0{\cdot}05$). An asterisk represents a significant difference between treatments within a time point (Student's *t*-test, $P < 0{\cdot}05$) (n=7).

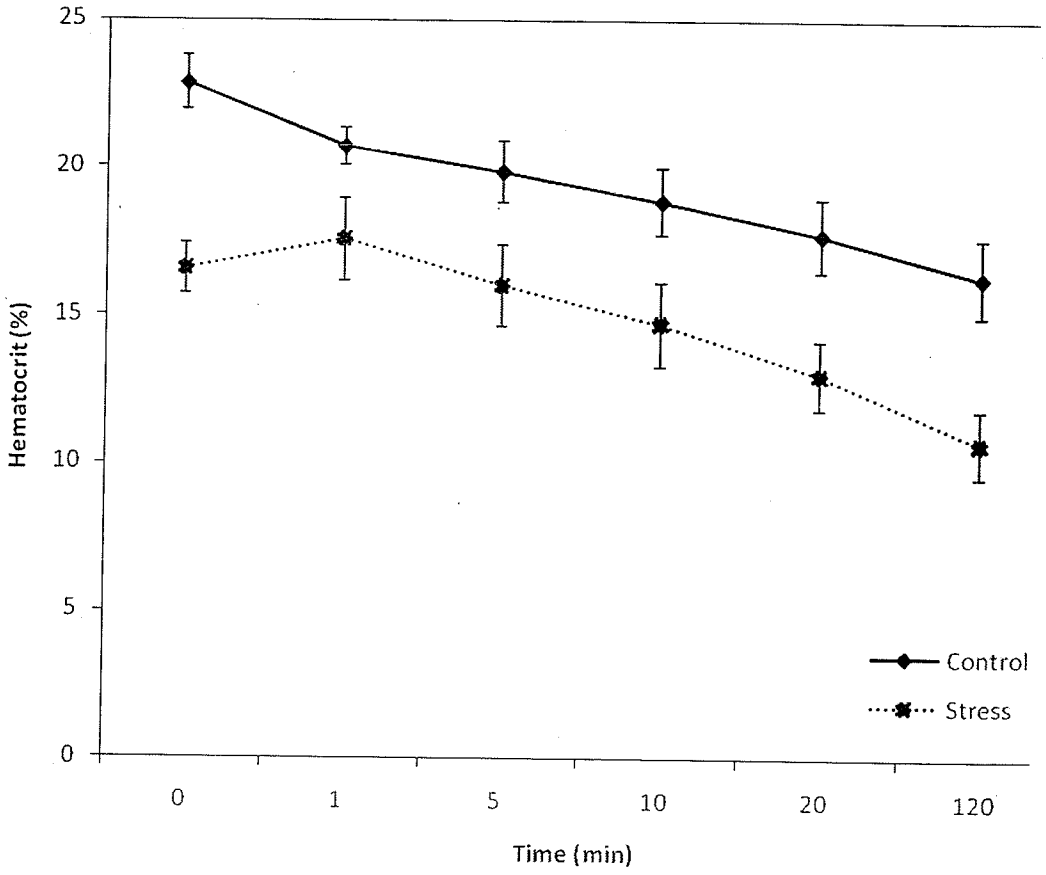


Figure 4.2. Percent hematocrit in juvenile lake sturgeon in control and experimental stress group. Values are expressed as a mean hematocrit ± 1SEM (n=7).

4.4: Discussion

While cortisol concentration is an accepted means of determining the stress response of fish, circulating catecholamines offer insight into the immediate response following a perceived stressor (Reid et al. 1998). Unlike cortisol that is synthesized upon demand, the catecholamines norepinephrine and epinephrine are readily available for release into the bloodstream following stimulation of the sympathetic nervous system (Reid et al. 1998). Data from this study suggest an instantaneous increase in E (fig. 4.1 b). Furthermore, maximal NE and E responses were noted fairly quickly (10 minutes), with increases as early as 1 minute post stress. Interestingly, the catecholamine levels begin declining around 20 minutes post-stress, a time period that coincides with the increase in circulating cortisol levels for the larval lake sturgeon, as seen in chapter 3. This trend may exist due to the delayed production of cortisol now capable of handling the long-term demands of a stressful encounter.

Elevated 'resting' NE and E concentrations (time 0) were detected in experimental group (fig 4.1a&b). Percent hematocrit was significantly lower for fish on the second (experimental) day of sampling (fig 4.2), possibly contributing to a heightened stress state of the fish. However, the trends observed are more likely due to unintentional disturbance causing a relatively instantaneous increase immediately prior to blood collection. The error is likely magnified due to the small sample size.

Catecholamines are present within the bloodstream during periods of rest. Based on this study, lake sturgeon juveniles have higher circulating NE (22.4±6.23 ng.ml

^{1}) as compared to E (0.89±0.21ng.ml^{-1}) levels upon rest (fig 4.1a, b). Data collected suggests elevated NE levels at time 0, prior to aerial exposure for the stress data (fig 4.1a). Several reasons are possible for this observation. It is highly likely that the lake sturgeon detected the presence of the experimentor upon initial sample collection, thereby elevating levels instantaneously and deviating from accurate baseline levels, as seen in the control data set. Despite this variation, the magnitude of the NE response may be meaningful as levels do return to control data by 120 minutes. E levels, similarly, did not significantly differ from control values by 120 minutes post-stress, thereby emphasizing the transient importance of catecholamines in the acute stress response.

Significant release of catecholamines in carp and cod plasma occur immediately in response to an acute stressor, such as aerial exposure (Fuchs and Albers 1988, Nilsson et al. 1976). In the present study circulating levels of both NE and E increased following the 30-second aerial exposure, with the greatest relative increase between pre- and post-stress concentrations of circulating E. The differential storage and release of norephineprine to epinephrine may be a reflection of the particular class of fish, such as great sturgeon *Huso huso* that have a higher concentration of epinephrine (Balashov et al. 1981). Based on a comprehensive review by Reid et al (1998), norepinephrine is the primary catecholamine in elasmobranchs, while epinephrine is generally the primary catecholamine in teleosts, excluding carp. It is suggested that this detected increase in stored epinephrine levels in teleosts may be a result of evolutionary up regulation of the epinephrine synthesis pathway. Furthermore, it is postulated that the enzyme phenylethanolamine-N-methyltransferase that converts NE to E has a higher substrate

affinity, and may be more resistant to degradation in teleosts (Reid et al. 1998). Ratios of circulating NE and E are therefore reliant upon stored levels, ability of chromaffin cells to release catecholamines, accumulation into target tissues, and the rate of metabolic degradation (Reid et al. 1998).

Comparative data for Acipenserids are sparse, however, Maxime et al. (1995), found a significant increase in plasma catecholamines in Siberian sturgeon, *Acipenser baeri*, following stress via 30-minute acute hypoxia. Plasma NE increased to 33.16 ± 5.58 $ng.ml^{-1}$, and E increased to 55.14 ± 9.16 $ng.ml^{-1}$with both catecholamines returning to baseline within 360 min. In white sturgeon, *Acipenser transmontanus*, resting NE and E were 16.33 ± 2.03 $ng.ml^{-1}$, and 7.60 ± 1.01 $ng.ml^{-1}$respectively. Following a 4h exposure to hypercapnic conditions plasma E increased significantly to 10.96 ± 2.13 $ng.ml^{-1}$, while NE remained unaffected (Crocker et al. 2000). In salmonids E values range from <0.55 for resting levels and between 3.66 and 12.82 $ng.ml^{-1}$ in stressed fish (Barton 2000). Like cortisol, the magnitude and duration of the catecholamine stress response is dependent upon the nature and severity of the stressor, as well as the inherent traits of each species (Reid et al. 1998). Researchers argue that the wide range of circulating catecholamine levels in response to a stressor between different species cannot be ascribed entirely due to variance in stored levels, but rather is more likely a reflection of the number of cholinergic receptors found on chromaffin cell surface in teleosts (Reid et al. 1998). However, due to the limited data available on Acipenseriformes and their catecholamine stress response, it is difficult to elucidate the underlying causes for the observed trends. Further studies are essential to determine if catecholamine responses

in sturgeon operate via cholinergic and/or non-cholinergic mechanisms (Reid et al. 1998).

Chapter 5: Conclusion

The objective of the experiments presented in chapters 2 and 3 was to determine the ontogeny of the cortisol response via the hypothalamic-pituitary-interrenal stress axis in larval lake sturgeon. Based on findings in the present study, lake sturgeon larvae are incapable of responding to a stressor prior to the onset of exogenous feeding. Low cortisol concentrations are detectable upon hatch, and this maternally-derived source may influence early development and survival. Fish reared in extreme conditions may utilize this source for immediate survival in the absence of development of the cortisol stress response, as seen in fish reared at 9°C. Maternal cortisol levels gradually decline until the onset of exogenous feeding, a period that coincides with the activation of stress responsiveness in lake sturgeon larvae. Once the endogenous yolk sac food source is depleted, the larvae must commence exogenous feeding and expel the spiral plug (Colombo et al. 2007). As such, it can be inferred that exogenous feeding will coincide with activation of the internal HPI stress axis. Furthermore, larvae do not possess external scutes for protection until the juvenile life stage (Detlaff et al. 1993). From a behavioural perspective, it can be hypothesized that having the ability to respond to a stressor, such as predation, would confer a survival advantage.

Morphological assessment of the ratio of yolk sac area to total area of an organism can provide a guide to detect the onset of stress responsiveness. Other physical parameters, such as the appearance of eye pigmentation, need to be explored

to determine if a correlation between eye pigmentation and the appearance of the cortisol stress response in larval lake sturgeon exists. Studies need to focus on parameters influenced, and contributing to, survival and development at that particular life stage.

Environmental variables, particularly during early development, can dramatically impact the ontogeny of the organism. Lake sturgeon hatched over slate exhibited higher cortisol concentrations at rest, suggesting a chronically stressed fish. Such elevated levels may have negative long-term consequences such as altered metabolism, cardiovascular, respiratory, immune and reproductive systems (Barton 2002, Milla et al. 2009, Wendelaar Bonga 1997). When stressed, larvae hatched on gravel and slate exhibited cortisol responses of similar magnitude. However, the duration of response was greatly extended for larvae hatched on gravel substrate. This extended response is likely more advantageous in nature, where stressors are unlikely to be removed entirely within brief periods.

Rearing in different temperatures greatly impacted the morphology and cortisol stress response of lake sturgeon larvae. Rate of yolk sac absorption, and thereby activation of the HPI stress axis, was most rapid for fish reared in high temperatures. Rapid absorption appears to result in incomplete or improper development of the prematurely activated HPI system, resulting in unusual cortisol responses to a stressor. Fish reared in low temperatures, on the other hand, developed at the slowest pace with high incidence of physical anomalies. Furthermore, these fish were unable to exhibit an

increase in cortisol concentrations when stressed even at the exogenous feeding stage. It can be hypothesized that these larvae were compelled to utilize their maternally derived resources for thermal tolerance and survival as opposed to appropriate development.

Variance between cortisol concentrations observed in this experiment and similar sturgeon work may be due to severity of the stressor. Resting cortisol levels of 4-year old shortnose sturgeon, *Acipenser brevirostrum*, were observed to be 3.56±2.75 $ng.ml^{-1}$ (Belanger et al. 2001, Faulkner and Moberg 1997, Wuertz 2006), and 8.6 to 10 $ng.ml^{-1}$ in white sturgeon, *Acipenser transmontanus*. Increases following stress can range from 30 $ng.ml^{-1}$ in white sturgeon following brief handling, to 100$ng.ml^{-1}$ for *Acipenser stellatus* following intense handling (Bayunova et al. 2002). Molecular work Kusakabe and colleagues (2009) tested expression of StAR, a protein involved in cortisol synthesis. Researchers noted that Actinopterygii fail to exhibit an increase in StAR protein expression (despite an increase in circulating cortisol levels) when administered ACTH or stressed weakly. A higher ACTH dose and higher severity stressor was needed to elicit an increase in protein synthesis. In order to better understand the stress response of lake sturgeon relative to other sturgeon species, further studies incorporating severity and nature of the stressor are necessary.

Like cortisol levels, catecholamines norepinephrine and epinephrine increased in response to a stressor. Catecholamine levels increased nearly instantaneously, allowing the organism to cope immediately in response to a given stressor. Juvenile lake

sturgeon exhibited greater norepinephrine concentrations at rest as compared to epinephrine, while a greater increase in circulating epinephrine concentrations was noted when the fish were stressed. To better understand the trends observed, future studies on enzymes involved in norepinephrine and epinephrine synthesis, such as phenylethanolamine-N-methyltransferase , need to be explored via testing of molecular expression of critical enzymes. Furthermore, to gain a complete understanding of catecholamine concentrations, experiments need to evaluate the numerous parameters that impact circulating levels, such as rates of release by chromaffin cells, accumulation by target tissues, and metabolic degradation. Further studies of catecholamine response are essential to stress research in order to complement data available on the cortisol stress response.

Ontogeny of stress responsiveness of aquatic organisms during early development is undoubtedly critical to determining survival and long-term well being. As such, it significantly impacts restoration programs and aquaculture practices. Future studies on the morphological, physiological, and molecular parameters involved are essential, especially for threatened and endangered species such as lake sturgeon.

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