Thyroid Development in Larval Lake Sturgeon (*Acipenser fulvescens*) and the Potential Thyroid Disruption Associated with Exposure to the Organophosphate Pesticide Chlorpyrifos

By

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Abstract

The thyroid hormone system plays a major role in larval development, growth, and metabolism in fish. Therefore, any anthropogenic alteration in thyroid function could have dramatic effects on individual fitness. In this study Lake Sturgeon, *Acipenser fulvescens*, larvae were exposed to a commercially used organophosphate pesticide, chlorpyrifos (0, 5, 500 and 2000ng.L^{-1}), from hatch until the onset of exogenous feeding (~12 days at 14^{o}C). The presence of thyroid follicles was first observed at 6 days post hatch (dph). Molecular expression of thyroid receptor α (TRα) increased from 3 to 12dph and then decreased from 12 to 21dph. TRα expression was also significantly higher in brain, liver and muscle at 67dph when compared to TRβ. Of the circulating hormones only free-T_3 was consistently measured in larval homogenates from all development time-points sampled. Exposure to chlorpyrifos had no effect on growth or thyroid follicle morphology during the course of the experiment.

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1.0 Introduction

1.1 Lake Sturgeon

Lake Sturgeon, *Acipenser fulvescens*, are large, long-lived freshwater fish species native to the Great Lakes and Hudson-James Bay and Mississippi watersheds (Pikitch et al. 2005). Adult lake sturgeon may live as long as 100 years reaching lengths exceeding 1.5 meters (Auer 1996). They were considered a valuable food source in the 19th and early 20th centuries in Manitoba, thus large commercial fisheries were established to meet demand (Harkness 1936). However, in recent years Lake Sturgeon populations have declined and are classified as a vulnerable species within Manitoba under the Manitoba Endangered Species Act and threatened within Canada and the United States (Williams et al. 1989; Ferguson and Duckworth 1997). Aspects of Lake Sturgeon life history characteristics such as a slow growth rate and delayed sexual maturity have contributed to their decline (Chiotti et al. 2008).

Lake Sturgeon spawn in lakes and streams at the end of spring (Billard and Lecointre 2001) in water temperatures that depend on location but usually fall between 10^{o}C and 15^{o}C (Chiotti et al. 2008). Individuals spawning in rivers tend to choose areas with high flow rates, depths between 1-5 meters, and large substrates such as boulders (Scott and Crossman 1973; Auer 1996). It has been hypothesized the spaces provided by large substrate protects the eggs from potential predators thus lowering egg mortality (Kempinger et al. 1988). Following spawning, fertilized eggs develop for approximately 8-14 days after which the eggs hatch and larvae with a yolk sac emerge (Chiotti et al. 2008).

1.2 Sturgeon Pre-larval Development

In sturgeon and other fish species the yolk-sac larvae, often termed pre-larvae, undergo a series of dramatic and influential changes between hatching and exogenous feeding. These changes have been documented in detail as a 10-stage developmental period by Detlaf et al. (1993) using a number of sturgeon species such as the Russian Sturgeon, *A. gueldenstaedtii*, Starry Sturgeon, *A. stellatus*, Sterlet Sturgeon, *A. ruthenus*, and Beluga Sturgeon, *Huso huso*. Across sturgeon species the difference in morphological characteristics of pre-larvae at hatching are low but become more prominent closer to the onset of exogenous feeding (Alyavdina 1951; Dragomirov 1953). Although morphological differences can sometimes be large the overall development of organ systems such as the digestive system are fairly similar across most sturgeon species (Buddington 1985). Due to the nature of this thesis research and the time-points examined it was important to closely examine key aspects of development at these early life stages. As such, the following description of pre-larval development is an approximate guide to sturgeon pre-larval development from the period of hatch to the onset of exogenous feeding based on sturgeon larval development as described by Detlaf et al. (1993).

Stage 1 represents the period just after hatch where pre-larvae are between 9-10.5mm long with a small head bent towards the large yolk sac and a long tail relative to the rest of the body. Pre-larvae at this stage have a closed intestine (no external opening) and do not have a mouth or gills and therefore rely on cutaneous respiration from blood vessels in the yolk. Distinct muscle bands are present in the trunk region as well as a functional cardiovascular system, which actively pumps blood through the vessels. At this early stage sensory organs are not well developed and there is no pigmentation in the eyes. In Russian sturgeon it has been shown the pre-larvae have no photo-response in stage 1 or any of the pre-larval stages (Detlaf et al. 1993).

In stage 2, pre-larvae are between 10.5-11.5mm long and their mouths are formed and begin to open. The first gill cleft is also present on either side and the beginnings of pectoral fins can be seen. At this stage the head begins to straighten and is no longer curved around the front of the yolk-sac. The formation of sensory barbels occurs as four rounded tubercles or bumps located near the mouth region. Clear differences in the shape of the yolk-sac are observed with the yolk-sac becoming more elongated and less of an obstruction for larval movement. The tail region begins to grow much faster than the rest of the body with the fins beginning to take more shape. At this stage the lateral line system becomes visible in the anterior part of the trunk aiding in the seismosensory function of the pre-larvae (Detlaf et al. 1993).

At stage 3, pre-larvae are between 11.5-12mm long. By now the gill filaments are beginning to develop near the opercular fold and the first branchial arch. The larvae become more pigmented with melanocytes along the dorsal side of the body, the tail, and near the head region. The divide between the developing digestive system and the stomach and intestine begin to narrow, marking the onset of preparation for exogenous feeding. The tip of the tail is bent upwards and muscle segments along the trunk of the body increase in height. The developing opercular fold and branchial arch each contain rudiments of gill leaflets at this stage (Detlaf et al. 1993).

At stage 4, pre-larvae are between 12-13.2mm long. The invagination of the digestive system wall now separates the system into two separate regions: the stomach and the intestine. Pigmentation increases in the eyes and melanocytes are found all over the body of the pre-larvae. Development of the mouth takes place with the lower and upper lips beginning to form as well as rudimentary teeth. Gill leaflets in the first set of gills have elongated slightly and the formation of the second gill cleft and development of the second set of gill leaflets has begun. At this stage the yolk-sac is becoming smaller and less vascularized as the blood supply is shifted elsewhere.

The pectoral fins are becoming larger and more developed as well as the differentiation of fin folds where the anal and dorsal fin will eventually form. The tail fin is now separated from both the anal and dorsal fin by grooves forming a distinct caudal tail region (Detlaf et al. 1993).

Stage 5, pre-larvae are just slightly larger than 13mm in length. Again pigmentation gradually increases with melanocytes aggregating in areas forming different color patterns on the individual pre-larvae. At this stage the sensory barbels are found on the ventral side near the mouth are more elongated and the lateral line system is further developed. The first set of gill filaments is clearly distinguishable, the second set are becoming elongated, and the third gill cleft is not fully complete. The mouth is now developed enough to open and close and is used to aid in mandibular respiration of the gills (Detlaf et al. 1993).

Stage 6, pre-larvae are between 13-14mm in length. The barbels begin to show signs of the first sensory cells and the upper and lower lips develop further. The tailfins keel-like appearance is reduced and the tail bends upwards. Internally the poorly developed liver differentiates into two sections with the rudimentary gall bladder on the right side. The second set of gill filaments are more developed and on the third gill cleft the first row of developing filaments can be seen. At this stage the respiratory movements of the gills and mouth in the pre-larvae have become rhythmic and are much stronger (Detlaf et al. 1993).

Stage 7, pre-larvae are between 14-15mm in length. At this stage the rostrum is elongating and beginning to straighten. Sensory cells are now present on the lower lip and cover more of the barbels. Muscle bands continue to develop and completely cover the ventral and later body surface. The pectoral fins are now lower down and are roughly on the mid-lateral surface of the larvae. As in the 6^{th} stage the keel-like appearance of the tailfin is reduced even further and projections of the ventral fins are now clearly visible and represent the early development of the muscles of the fin. Internally, the liver has divided into two lobes and the

digestive system is more developed with the formation of rudimentary pyloric appendages (Detlaf et al. 1993).

Stage 8, pre-larvae are between 15-16mm in length. The rostrum continues to flatten and now has a horizontal appearance. The keel-like tailfin has been completely reduced in the anterior region and the ventral fins have reached the location of the developing anal fin folds. In the first and second gill sets the formation of secondary filaments can be seen but are less distinguishable in the second set. The third gill set now has its first row of gill filaments and at this stage the fourth gill cleft is visible. Internally, the pyloric appendages begin specializing into different lobes and the opening of the anus is formed. The yolk sac is dramatically smaller by this stage as the yolk reserves have been almost completely used up (Detlaf et al. 1993).

Stage 9, pre-larvae are between 16-18mm in length. Pigmentation of the pre-larvae is continually increasing and melanocytes can now be observed on the pectoral fins as small spots. The head is much larger due to the increase in size of the rostrum and this moves the barbels away from the mouth so they no longer come in contact with the opening of the mouth. Also bony scales can now be seen on the top part of the head region. The pectoral, anal and dorsal fins grow larger and move into their intended positions and become more prominent. Along with fin development a narrow strand appears dorsally representing the early stages of scute development. In addition, secondary gill filaments are present and in the second set of gills and are starting to form in the remaining gills sets. Internally, three distinct lobes of the pyloric appendage can be seen and the digestive tract is almost developed enough for the onset of exogenous feeding. Spiral plugs located within the spiral intestine begin to eject during this stage indicating the pre-larvae are becoming ready to feed exogenously (Detlaf et al. 1993).

Stage 10, pre-larvae are larger than 18mm in length. By this point the vast majority of the yolk sac has been utilized and the spiral plug has been ejected. The larvae are now heavily pigmented and their rostrums continue to grow longer. The sensor systems of the larvae are now more developed with the lateral line system covering most of the length of the body. In addition, larval vision is increased to the point where ocular muscles are formed and help in the detection of food. It is at this stage where the pre-larvae begin exogenous feeding and are now termed larvae (Detlaf et al. 1993).

The timing of the 10 stages of pre-larval development described by Detlaf (1993) is heavily dependent on water temperature. High temperatures increase metabolic rate thus increasing the yolk absorption rate and decreasing the incubation time (Zubair et al. 2012). Pre-larvae reared at colder temperatures will take longer to reach exogenous feeding compared with those held at higher temperatures (Zubair et al. 2012). Russian sturgeon held at 18.5^{o}C took 8 days to reach the stage of exogenous feeding whereas pre-larvae of the same species held at 16^{o}C took 10 days (Detlaf 1993). Comparatively, Lake Sturgeon in this study held at 16^{o}C took ~12 days to begin exogenous feeding a full 2 days longer than Russian sturgeon held at the same temperature.

1.3 Sturgeon larvae

Timing for the onset of exogenous feeding varies depending on species and temperature (Detlaf et al. 1993). Lake Sturgeon larvae reared at 15^{o}C were found to start feeding exogenously at 17 days post fertilization (dpf) but larvae held at 9^{o}C took until 52dpf to begin feeding exogenously (Zubair et al. 2012). The above results indicate the significance of water temperature on the timing of larval development and the onset of exogenous feeding. Once

exogenous feeding starts Lake Sturgeon larvae and juveniles feed on small freshwater invertebrates such as Baetidae nymphs and Dipteran larvae (Kempinger 1996). In laboratory/hatchery studies using cultured Lake Sturgeon the larvae are frequently transitioned to live brine shrimp (*Artemia* spp.) for a period of 2-4 weeks before being switched to a more nutritious food source such as bloodworm or commercial trout pellets (Klassen and Peake 2008). In nature, Lake Sturgeon larvae spend the first few weeks on the sediment floor in sandy areas with low water flow and may then be carried downstream or remain in these locations until the juvenile stage (Billard and Lecointre 2001). Larval stages including that of Lake Sturgeon exhibit high rates of mortality due to their small size and inadequate defense mechanisms (Caroffino and Sutton 2010). Little research has been conducted on larval Lake Sturgeon from hatching to about 80dph partly due to the small size of larvae and ineffective capture methods. Therefore further exploration into both the effects of pesticides on this important and vulnerable life stage and the proper development of the thyroid system are warrented.

1.4 Pesticides

Contamination of aquatic ecosystems by pesticides is occurring at a quick rate (Gadgil and Heda 2009). Consequently the study of their effect on freshwater organisms is imperative. Pimentel (1995) estimated only 0.1% of all applied pesticides reach the target organism with the remaining 99.9% contaminating other areas of the environment. Pesticides have been found to induce a number of serious effects on non-target aquatic organisms and studies have shown exposure of fish to pesticides can lead to lower oxygen consumption (Heath et al. 1993; Barbierri and Ferriera 2011), decreased acetylcholinesterase synthesis (Klaverkamp et al. 1977), increased

oxidative stress (Oruç 2010; Kavitha and Rao 2008), and endocrine disruption (Dogan and Can 2011; Makynen et al. 2000; Tian et al. 2010).

Organophosphate pesticides (OP's) are common agricultural pesticides and their more rapid degredation in the environment has led to their use in replacing the far more persistent organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT), aldrin, and endosulfan (Botaro et al. 2011; Piazza et al. 2011). Consequently OP's account for approximately 50% of all pesticide applications worldwide (Casida and Quinstad 2004). A common mode of action of OP's is the inhibition of acetylcholinesterase (AChE) synthesis, an essential enzyme involved in the breakdown of the neurotransmitter acetylcholine (ACh) within the cleft of the cholinergic synapse (Kwong 2002; Carr et al. 1997). When ACh is released into the synaptic cleft it causes an excitatory post-synaptic potential by binding to nicotinic or muscarinic receptors on the post-synaptic cell (Hulme et al. 2006). The decrease in AChE level as a result of OP application decreases the removal of ACh from the synaptic cleft leading to prolonged nerve excitation (Samaranayaka 1977). Neurons in a prolonged excitatory state are incapable of transmitting messages across a synapse leading to a total breakdown in neuronal signaling (Samaranayaka 1977). Toxicity symptoms observed in estuarine fish and invertebrates are characteristic of neural transmission failure due to AChE inhibition and include hyperactivity, paralysis, and eventual death (Fulton and Key 2001).

OP's such as chlorpyrifos (Oruç 2010; De Angelis et al. 2009) and piperophos (Viswanath et al. 2010) are also known to influence endocrine systems leading to alteration of steroid hormone levels and thyroid function, thus affecting reproduction, sex, development, growth, and metabolism (Mantovani 2006; Mantovani and Maranghi 2005; Bernal et al. 2003; De Angelis et al. 2009).

1.5 Chlorpyrifos

Chlorpyrifos, (O,O- diethyl *O*-[3,5,6-trichloro-2-pyridinyl]-phosphorothioate) a popular broad-spectrum insecticide (Fig. 1.1), is used to manage insects such as coleopterans (beetles), dipterans (flies), lepidopterans (moths and butterflies), and homopterans (sucking insects) (Benedetto et al. 2010) and is one of the most highly used organophosphate pesticides in the world (Carr et al. 1997; Kavitha and Rao 2008; Asperlin 1994) and measurable quantities have been described in the Great Lakes watershed (Braun and Frank 1980) Like most pesticides chlorpyrifos enters aquatic ecosystems as spray drift or in water run-off where it can cause unwanted side effects in fish and other aquatic organisms. Measured toxicity values for adult Lake Trout exposed to chlorpyrifos show 50% mortality at concentrations between 0.007-0.051mg.L^{-1} for a period of 96 hours (Benedetto et al. 2010). Like most OP's, chlorpyrifos acts by inhibiting the activity of AChE but other means of toxicity have been identified. Chlorpyrifos has been found to interfere with endocrine function in fish (Tripathi and Shasmal 2010; Thangavel et al. 2005; Saxena and Mani 1988), mice (De Angelis et al. 2009) and ewes (adult female sheep) (Rawlings et al. 1998). Specifically it is the documented effect chlorpyrifos exposure has on the thyroid hormone system, which is of particular concern in this thesis. In ewes administered 12.5mg.kg^{-1} chlorpyrifos for 36 days plasma T_4 levels were noticeably lower than control values (Rawlings et al. 1998) and in CD1 mice exposure to 6mg.kg^{-1} chlorpyrifos resulted in a significant decrease in plasma T_4 levels (De Angelis et al. 2009). These findings suggest a potential for chlorpyrifos to interfere with the thyroid system at least in mammals.

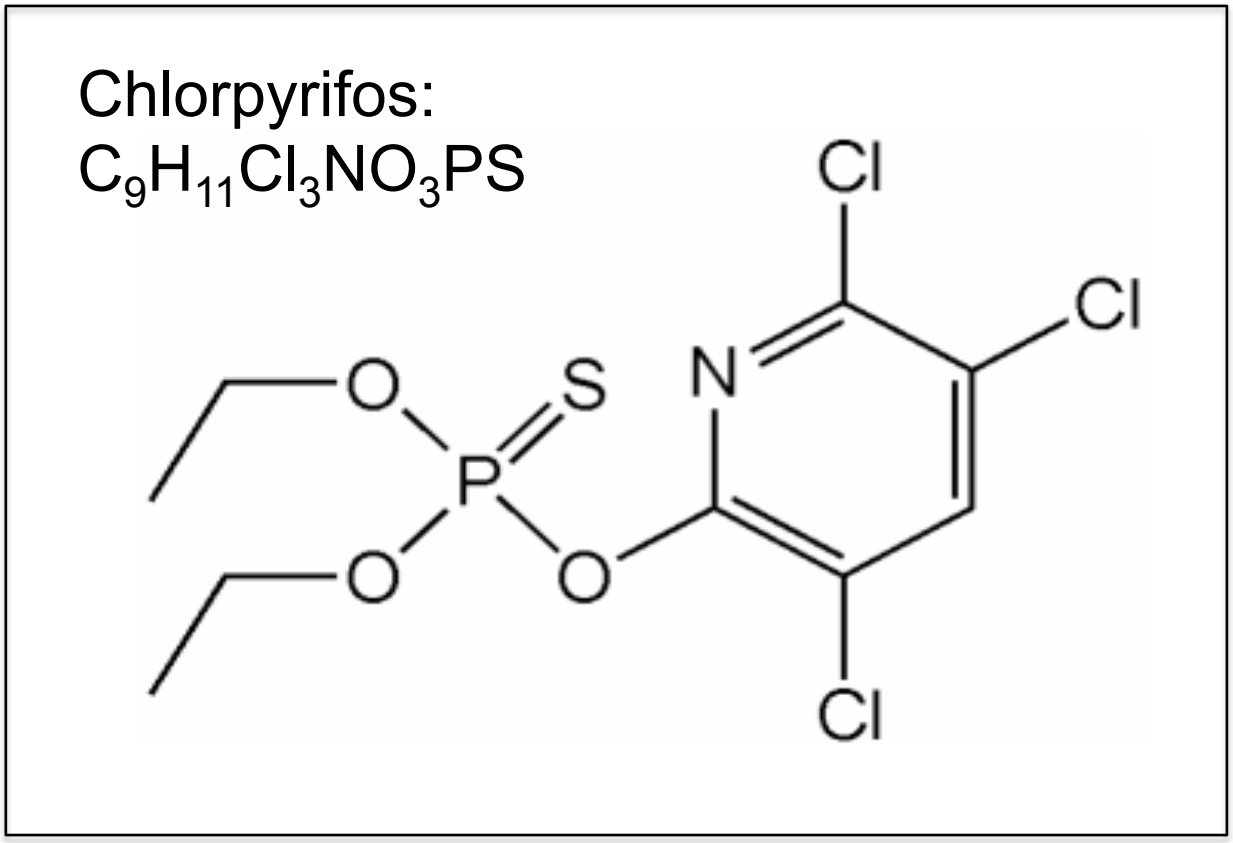

Figure 1.1: Chemical Structure of the organophosphate pesticide chlorpyrifos showing the phosphate group which is characteristic of phosphorous based pesticides. Open Source: http://en.wikipedia.org/wiki/Chlorpyrifos

Toxicity investigations of chlorpyrifos in exposed fish have focused mainly on AChE inhibition and oxidative stress (Oruç 2010; Kavitha and Rao 2008; Carr et al. 1997). In Nile Tilapia, *Oreochromis niloticus*, exposure to 5µg.L^{-1}, 10µg.L^{-1}, and 15µg.L^{-1} of chlorpyrifos in the water for 15 or 30 days caused significantly lower AChE activity in all treatment groups and exposure lengths when compared to control levels (Oruç 2010). Similarly, Mosquito Fish, *Gambusia holbrooki*, exposed to 297µg.L^{-1} chlorpyrifos for 4 days had significantly lower AChE

activity and chlorpyrifos also decreased the activity of key antioxidant enzymes such as catalase, superoxide dismutase, and glutathione reductase (Kavitha and Rao 2008). While a direct link between thyroid hormone disruption and chlorpyrifos exposure in fish remains to be established, other OP's such as dimecron and fenitrothion significantly alter the thyroid system in fish. Freshwater adult Mozambique Tilapia, *Sarotherodon mossambicus*, exposed to 0.001ml.L^{-1} dimecron had significantly lower levels of the biologically active hormone T_3 when compared to control samples (Thangavel et al. 2005). In the Freshwater Murrel, *Channa punctatus*, exposure to fenitrothion at a concentration of 1-5mg.L^{-1} (~50% EC50) caused significant thyroid follicle hypertrophy in adult fish. After 30 days of exposure both the follicle and colloid heights were smaller than the controls indicating a negative response in exposed fish. Following 120 days of exposure the follicular epithelium showed signs of degeneration and red blood cells were observed in the follicular colloid (Saxena and Mani 1988). Analysis of the chemical structures of chlorpyrifos (Fig. 1.1), dimecron (E-[3-Chloro-4-(diethylamino)-4-oxobut-2-en-2-yl] dimethyl phosphate), and fenitrothion (O,O-Dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate) (Fig. 1.2) and their grouping may provide more information into the potential of chlorpyrifos to interfere with the thyroid system. Based on chemical structure alone chlorpyrifos and fenitrothion have a more similar structure when compared to dimecron. Chlorpyrifos and fenitrothion are both in the same class of organophosphates termed Thiophosphoric acid thiophosphoryl-type whereas dimecron is not designated within a particular group (Pope 1999). The ability of OP's like dimecron and fenitrothion to interfere with the thyroid axis in fish outlines the potential of chlorpyrifos to play a similar role in inhibiting thyroid development and function in Lake Sturgeon. However, the differences in structures of OP's may impart varying activities in the environment and in biological systems indicating the importance of further testing of organophosphate pesticides such as chlorpyrifos (Pope 1999).

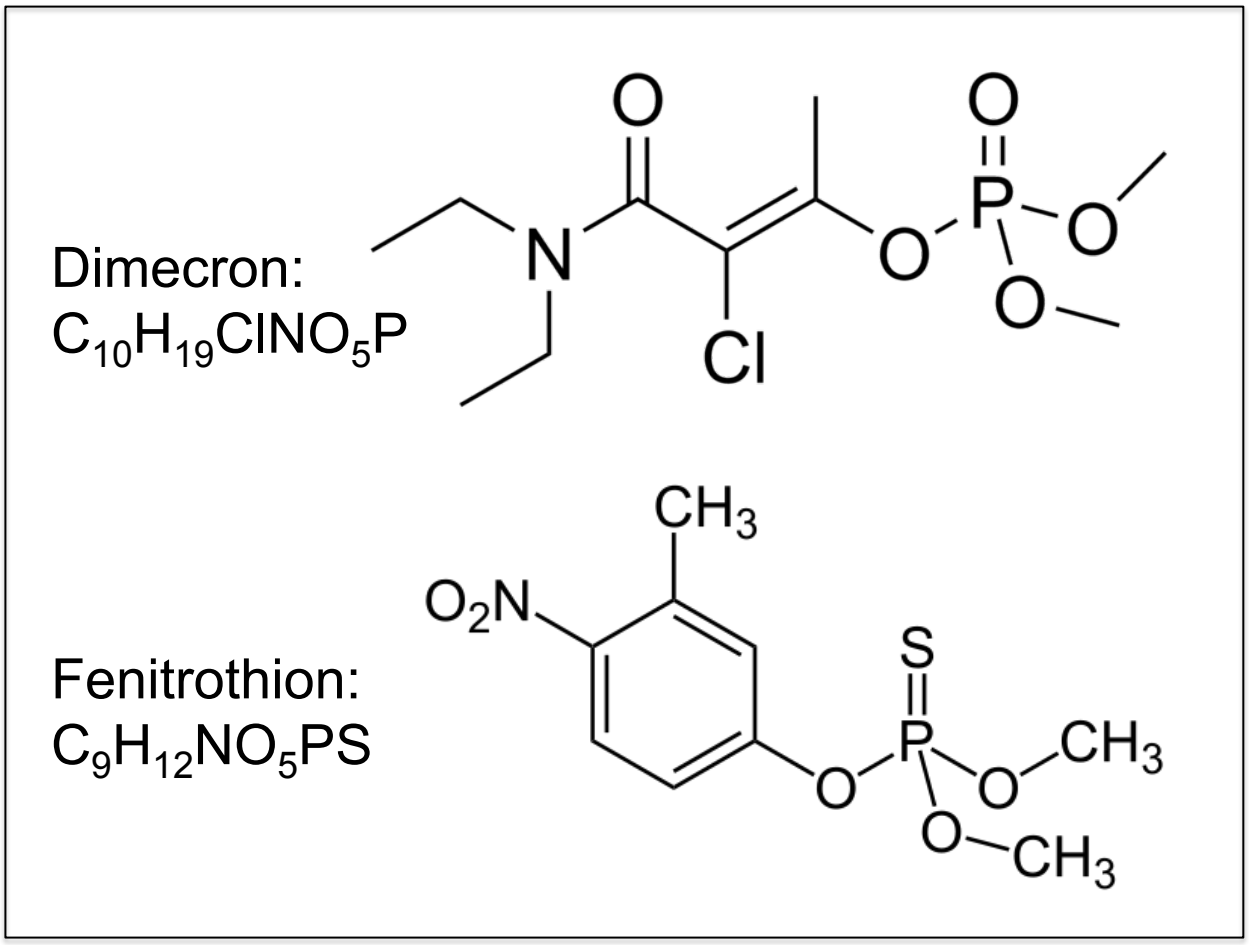

Figure 1.2: The chemical structure of two organophosphate pesticides linked to thyroid disruption in fish. Open Source: Dimecron: http://en.wikipedia.org/wiki/Phosphamidon Fenitrothion: http://en.wikipedia.org/wiki/Fenitrothion

1.6 Thyroid System

The main thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) are essential in a number of biological processes such as metabolism, osmoregulation, growth, and development (Eales et al. 1999; Szkudlinski et al. 2002). The thyroid system in vertebrates is regulated through the hypothalamus-pituitary-thyroid axis (HPT axis; Figure 1.3). Stimulation of

neurosecretory fibers within the hypothalamus causes the release of thyrotropin releasing hormone (TRH), which acts directly on receptors in the adenohypophysis of the pituitary to release thyroid-stimulating hormone (TSH) (Bently 1998). In fish there are two known TRH receptors, both of which are structurally similar to the mammalian TRH receptor (Harder et al. 2001). However, the relationship between TRH and the release of TSH in fish is not well understood.

TSH in vertebrates is a multi-subunit molecule containing a hormone-specific subunit termed TSHβ and a glycoprotein subunit termed GSUα, which is shared with other gonadotropins such as luteinizing hormone and follicle stimulating hormone (MacKenzie et al. 2009). The TSHβ subunit is responsible for binding to the TSHR and has been sequenced in a number of different teleost species such as Atlantic Salmon, *Salmo salar*, Rainbow Trout, *Oncorhynchus mykiss*, and the European Eel, *Anguilla anguilla*. Studies have shown TRH has the potential to up-regulate the production of TSHβ mRNA in the Bighead Carp, *Aristichthys nobilis*, indicating a possible role for TRH in the release of TSH (Chatterjee et al. 2001). In that study, pituitary cell cultures harvested from adult Bighead Carp were incubated in the presence of synthetic TRH for 36 hours. Following the incubation period, cells were harvested and the total RNA was extracted to analyze the expression level of TSHβ where it was found TSHβ was up-regulated.

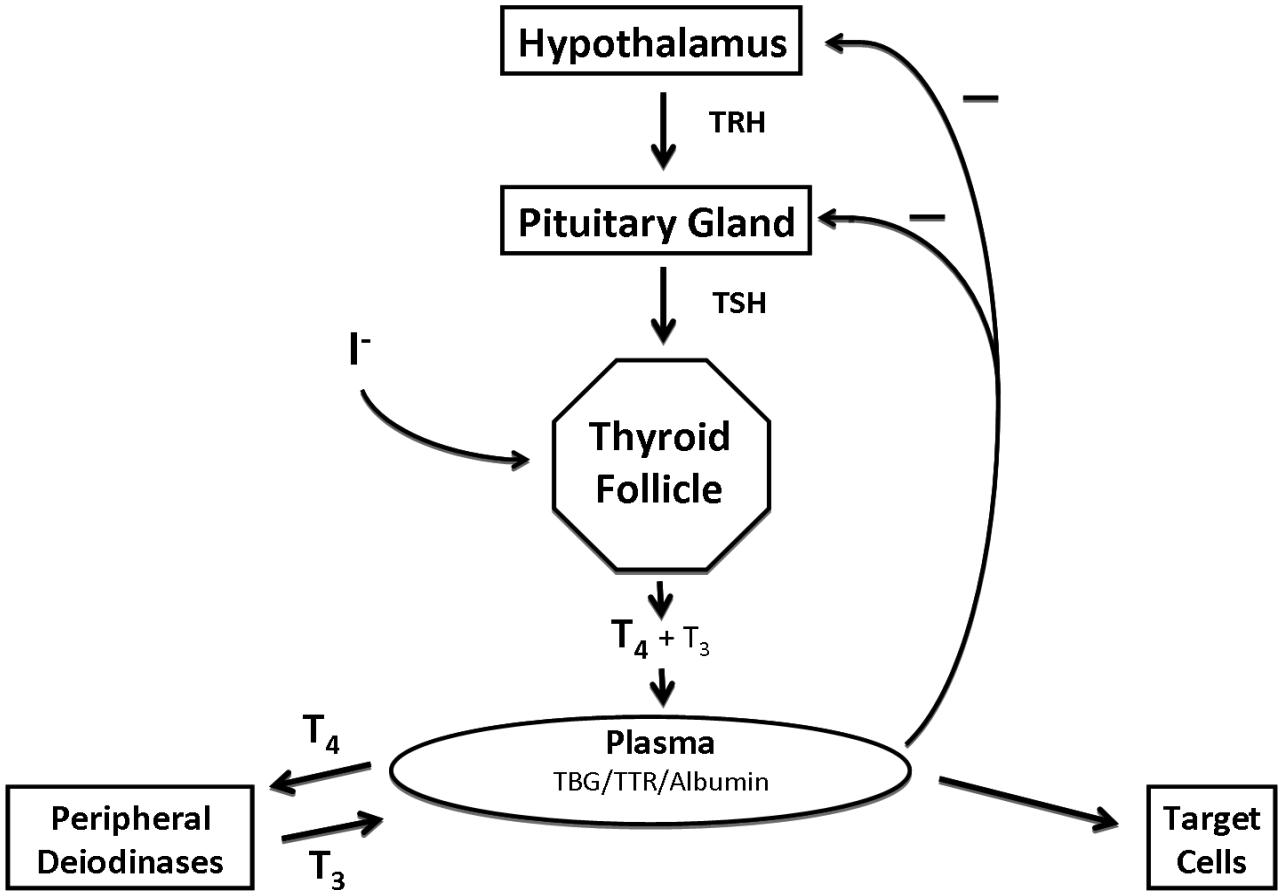

Figure 1.3: The Hypothalamus-Pituitary-Thyroid axis in vertebrates showing the pathway of thyroxine (T_4) and triiodothyronine (T_3) production through the release of thyrotropin releasing hormone (TRH) in the hypothalamus and thyroid stimulating hormone (TSH) in the pituitary. Negative feedback loops are indicated by "—" in the figure. TBG = Thyroxine Binding Globulin, TTR = Transthyretin and I^- = iodide. Figure adapted from Blanton and Specker 2007; Bentley 1998; Hadley and Levine 2006; Norris and Carr 2013.

After TRH binds, TSH is released from the thyrotrope cells in the pituitary and is transported in the plasma to the thyroid follicle where it binds to the TSH receptor (TSHR). There has been only one TSHR isolated in fish and it is a G-protein coupled receptor (Blanton and Specker 2007; Farid and Szkudlinski 2004). The TSHR is part of a family of glycoprotein hormone receptors (GPHRs) containing a large extracellular domain with leucine-rich repeats to aid in ligand binding (Szkudlinski et al. 2002). It is believed a TSHR distinct from other gonadotropin receptors was present prior to the divergence of the teleost lineage from tetrapod's, which provides evidence to support the proposed functional and structural similarity between mammalian and teleost TSHRs (Oba et al. 2001). However, the expression and function of the TSHR in fish is not well known partly due to the dispersed nature of the thyroid follicular tissue in fish (MacKenzie et al. 2009). In Striped Bass, *Morone saxatilis*, TSHR mRNA transcripts were detectable in skeletal muscle, heart, and brain tissues as well as being highly expressed in thyroid and gonadal tissue (Kumar et al. 2000). When bound to TSH the TSHR elicits a response through a number of pathways including the activation of cyclic AMP-dependent transduction pathways, protein kinase A pathways and calcium pathways (Kumar at al. 2000). Stimulation of this pathway initiates the uptake of iodide and production of T_3 and T_4 within the thyroid follicle (Eales et al. 1999; Szkudlinski et al. 2002). In addition to physiological responses TSH also elicits structural changes in the thyroid tissue itself. There are clear dose-dependent changes in thyroid follicular histology when the system is stimulated. Increases in follicular cell height, vascularization, colloid vacuolization, as well as decreased colloid density, and changes in nucleus size and shape are often observed (Eales and Brown 1993). Increased colloid vacuolization demonstrates the active transport of TH's into the cell lumen and across the basal membrane into the blood stream. Both T_3 and T_4 help to regulate the release of TSH from the pituitary through negative feedback processes but T_4 is believed to be the main hormone involved

in negative feedback regulation of TSH in fish (see Fig 1.3) (Yoshiura et al. 1999; MacKenzie et al. 2009).

TH production in all vertebrates including fish requires the presence of iodine in its ionic form, iodide (I^-), which is the form found in the plasma. Fish are capable of getting iodide from their diet or directly from the water they live in, but the concentrations vary depending on food type and water salinity. Iodide concentrations in seawater are normally around 5μg.dl^{-1} but in freshwater iodide concentrations are much lower, between 0.01-1μg.dl^{-1} (Eales 1979). Based on the difference in iodide concentration between seawater and freshwater it was previously believed freshwater fish species would have lower concentrations of iodide in their plasma. However this is not the case as studies on freshwater salmonids have documented plasma iodide concentrations of 100-200μg.dl^{-1}, much greater than recorded in humans, 1μg.dl^{-1} (Leloup 1970). In addition, it has been shown that iodide concentrations are higher in tissues of freshwater fish compared to seawater species. Evidence of this is found in freshwater Burbot, *Lota lota*, where it was shown the skin is an important area for iodide storage (Wiggs 1971). Certain species from the order clupeiforme have a specific plasma pre-albumin protein unique to fish, which reversibly binds iodide (Leloup 1970). It is hypothesized the protein reduces the loss of iodide across the kidneys, gills, and gut surfaces thereby maintaining high plasma iodide levels. The level of iodide in plasma samples of teleost fish is not consistent and there is a high degree of variability among species and habitat type (Leloup 1970).

One of the mechanisms proposed for the uptake of iodide into both the plasma and thyroid follicles is the sodium iodine symporter (NIS) (Moren et al. 2008). The NIS in terrestrial animals has been shown to be heavily involved in the transport of iodide into both the thyroid follicle and the gastric mucosa of the intestine. The presence of the NIS in the intestinal mucosa allows for the uptake of iodide present in their diet directly into their blood stream (Vayre et al. 1999). The use of the NIS to transport iodide into the plasma and thyroid follicle has also been suggested in fish with research performed on species such as the Zebrafish, *Danio rerio*, Fathead Minnow, *Pimephales promelas*, and Mosquito Fish. In each of these species exposure to environmental concentrations of the known NIS inhibiting substance, perchlorate, significantly decreased thyroid hormone levels and reduced reproductive success (Muhki and Patino 2007; Crane et al. 2005; Park et al. 2006). Although a decrease in TH was observed in all the species the TH pathway was not completely shut down indicating there are other methods fish may use to accumulate iodide for use in TH biosynthesis. Fish are also capable of actively pumping iodide into the blood stream through their gills (Hunn and From 1966). An energy dependent carrier mediated branchial iodide pump has been identified in fish and is believed to be responsible for the high levels of plasma iodide in freshwater fish (Leloup 1970). In Brook Trout, *Salvelinus fontinalis*, starved for several weeks plasma iodide increased instead of decreased indicating the importance of the branchial iodide pump to the TH system (Higgs and Eales 1971). Based on these results it is clear iodide is crucial for the normal functioning of the thyroid system in all vertebrates. Fish have acquired useful mechanisms to avoid shortages in iodide even when living in iodine deplete environments. Because of these mechanisms cases where fish have been found to be suffering from low iodide levels in the wild or in artificial environments are very rare.

After uptake of iodide into the plasma the circulating ions must enter the thyroid follicular cell in order to be incorporated into TH molecules (Fig 1.4). The thyroid follicle structure in fish is similar to that of mammals with a single layer of thyroid follicular cells surrounding a colloidal space (Salvatore 1969; Power et al. 2001). The main difference between mammalian and fish thyroid tissue is that mammals have a distinct thyroid gland comprised of an aggregation of thyroid follicles usually in the pharyngeal region. In fish, however, thyroid follicles are often loosely interspersed within connective or fatty tissue and are often located near or on vascular tissue for direct access of TH to the blood (Blanton and Specker 2007). In Fathead Minnows the thyroid tissue is located in the pharyngeal region (Wendelaar-Bonga 1993), in Medaka, *Oryzias latipes*, it is located near the ventral aorta (Raine et al. 2001), and in some freshwater cyprinids and poecilids the thyroid tissue can even associate with the kidney (Blanton and Specker 2007). The appearance of follicular tissue is different across fish species. In some teleost species such as Coho Salmon, *Oncorhynchus kisutch*, and Chinook Salmon, *Oncorhynchus tschawytscha*, thyroid follicles become active in developing embryos 3 to 4 weeks after fertilization (Greenblatt et al. 1989). Early formation of thyroid follicles has also been demonstrated in fathead minnows and Atlantic Salmon (Lam 1994). The majority of teleost species do not have active follicles until the absorption of their yolk sac, which is the primary source of TH in the developing larvae and is maternally derived (Lam 1994; Power et al. 2001).

It is believed the general pathway for the synthesis of TH is the same in teleost fishes as it is in other vertebrates (Brown and Eales 1993). As previously discussed the NIS plays a role in the transport of iodide across the cell surface and into the lumen of the thyroid follicular cell (Moren et al. 2008). Iodide is also pumped across the cell membrane via the thyroid iodide pump (Leloup and Fontaine 1960). These two processes combined aid in the process of iodide

accumulation in the thyroid and the production of TH. Once inside the thyroid follicular cell iodide is not directly incorporated into TH instead it is bound to a special glycoprotein within the cell called thyroglobulin (TG). TG mRNA is released from the nucleus of the follicular cell and is then transported to the rough endoplasmic reticulum where the mRNA is converted into protein. Once properly translated the TG dimer is transported to the golgi apparatus for the addition of carbohydrates and sulfate moieties (Ring et al. 1987; Zoeller et al. 2007; Eales and Brown 1993).

The TG molecule has been identified in all vertebrates studied to date and amino acid analysis shows the protein is similar across vertebrate groups (Marchelidon et al. 1971). With the exception of hagfish, the main iodine-binding protein, TG, is a molecule with four tyrosyl-binding sites and acts as a storage site for TH components (Dunn and Dunn 2000; Suzuki et al. 1975). In order for TG to bind iodide it must be oxidized by either H_2O_2 or O_2 at the apical membrane of the follicular cell (Taurog 2000). Once oxidized, iodide is incorporated into TG with the help of thyroperoxidase (TPO), a highly conserved enzyme among vertebrates, near the apical cell membrane and is either exocytosed from the follicular cell into the colloid or remains in the lumen of the cell (Zoeller et al. 2007; Eales and Brown 1993). Iodination within the TG protein creates two different iodothyronines: monoiodotyrosine (MIT) and diiodotyrosine (DIT) both of which are important for the synthesis of TH's (Brown and Eales 1993). MIT and DIT are the iodine molecules coupled together to form the main thyroid hormones T_4 and to a lesser extent T_3. When the thyroid follicle is stimulated by the binding of TSH to its receptor various cellular mechanisms and pathways are activated causing the follicular cell to reabsorb TG from the colloid back into the lumen via pinocytosis. Once absorbed back into the cell it is joined with a lysosome containing phagocytic enzymes and peroxidases (Eales and Brown 1993). Within the

lysosomal complex TPO and H_2O_2 help to couple MIT and DIT, or more commonly, DIT and DIT couple together by way of an ether bond between the two molecules. The ether bond created between MIT and DIT forms T_3 and the more common configuration between DIT and DIT creates T_4 (Eales and Brown 1993; Zoeller et al. 2007). From there T_4 and to a lesser extend T_3 is released across the basal membrane into the blood stream where they are bound to carrier proteins and transported throughout the body. Uncoupled MIT and DIT as well as unbound TG produced by the lysosomal complex are metabolized and the resulting amino acids and iodide are recycled within the follicular cell for further use (Eales and Brown 1993).

There are a number of important thyroid hormone transport proteins found in vertebrates such as transthyretin (TTR), thyroxine binding globulin (TBG), albumin, and lipoproteins (Power et al. 2000). The use of TH transport proteins is a result of the highly lipophilic nature of the TH's (Richardson et al. 2005). The partition coefficient of THs has been established at roughly 20,000:1 between lipid and aqueous conditions (Distefano et al. 1993). Due to the high preference for lipid rich environments unbound TH in the plasma do not distribute evenly among cells and will cross lipid membranes at the first opportunity. Cells of rat livers perfused in an aqueous buffer solution containing ^{125}I-T_4 without thyroid-binding proteins showed uneven ^{125}I-T_4 uptake with the labeled T_4 entering the first cells they came in contact with. Follow up experiments where thyroid-binding proteins were added to the buffer solution ensured an even distribution of the labeled T_4 among the liver cells further demonstrating their importance (Mendel et al. 1987). The presence of thyroid-binding proteins and the lipophilic nature of T_4 and T_3 both contribute to the very low concentrations of unbound TH in the plasma of vertebrates. It has been recorded in vertebrates that unbound concentrations in the plasma can be as low as 0.03% of total T_4 and 0.4% for total T_3 (Kawakami et al. 2006a). In humans and most

mammals the main thyroid-binding protein is TBG, which shows the highest affinity for T_4 followed by TTR and lastly albumin (Richardson et al. 2005). However, TBG or a TBG-like molecule has yet to be found in fish leading researchers to believe other thyroid-binding proteins such as albumin and certain lipoproteins may be responsible (Santos et al. 2002). Richardson et al. (1994) found the only T_4 binding protein in amphibians, reptiles and fish was albumin based on the uptake of labeled T_4 from plasma samples. This led researchers to believe albumin was the main T_4 transporting protein in fish, reptiles, and amphibians.

These findings were brought into question when a thyroid binding protein was discovered in the Sea Bream, *Sparus auratus*, later identified as TTR (Santos and Power 1999). In Masu Salmon, *Oncorhynchus masou*, TTR has also been identified but its presence in other fish species has not been reported. TTR is a tetramer composed of 4 separate subunits each 130 amino acids long with a large leader peptide of 20 amino acids (Morgado et al. 2008; Santos et al. 2002). In fish, each subunit has a molecular weight of ~15 kDa, which is similar to the TTR subunits seen in mammals. Tissue related expression and production of TTR has not been studied extensively in fish but its production in mammals occurs mainly in the liver and choroid plexus where it helps to transport T_4 across the blood brain barrier (Morgado et al. 2008). One such study dealing with TTR tissue expression in a teleost fish, the Sea Bream, found TTR production was abundant in the liver but was also identified in much smaller levels in cardiac and skeletal muscle, intestine, skin, kidney, brain, gills, and the pituitary (Santos and Power 1999). Although the amino acid sequence in fish TTR has only a 40-55% similarity with mammalian TTR the amino acids responsible for binding TH are highly conserved between the two groups (Santos et al. 2002). However, slight amino acid differences between mammalian and fish TTR do have an important affect on the binding of T_4 and T_3 in fish TTR. In the amino acid sequence of TTR in

sea bream the addition for three amino acids, aspartic acid, lysine, and histidine, increases the length of the N-terminus and leads to a decrease in hydrophobicity near the active binding site of TH. In addition, the surface potential at the TH binding site is more negative in fish species when compared to mammalian TTR. The more negative binding site in fish makes T_3 a more preferable ligand as it is in a neutral form at physiological pH compared to T_4, which is ionic (Peterson 1971). The change in hydrophobicity and electronegativity has been implicated in the preference of fish TTR for T_3 compared to T_4 in mammals. In Masu Salmon and Sea Bream, binding studies indicate TTR has a three times higher affinity for T_3 than T_4 indicating the potential for TTR in fish to be the main T_3 binding protein (Santos et al. 2002). The preferential binding of TTR to T_3 is not unique to fish as the same trend is seen in other non-mammalian vertebrates such as birds and amphibians. In the American Bullfrog, *Rana catesbeiana*, the affinity of TTR for T_3 is higher than for T_4 and in birds such as pigeons, *Columba livia*, the affinity of TTR for T_3 is four times higher than observed in mammals (Chang et al. 1999). These findings suggest ancestral TTR was originally a T_3 binding protein but has evolved in mammals to become a T_4 binding protein (Santos et al. 2002). Once TH's are bound to their transport proteins whether it be T_4 bound to lipoproteins and albumin or T_3 bound to TTR they are transported in the blood stream to their target site where they bind to thyroid hormone receptors.

Thyroid receptors (TR) play a large role in the thyroid system and their functions have been identified in a number of mammalian species (Nelson and Habibi 2009). However, the role of TR in fish is still being determined. TR belong to a large group of nuclear receptors, which includes the steroid hormone receptors and act to regulate the expression of TH related DNA domains (Evans 1988). Mammal and fish TR share a similar DNA domain structure containing an N-terminal domain, a DNA binding domain, a hinge-region which is highly variable, a ligand

binding domain (LBD) which is the site of TH binding, and a C-terminal domain (Nelson and Habibi 2009). In mammalian studies TR have been shown to be closely associated with DNA sites known as thyroid response elements (TRE) but little is known about the presence of TRE's in fish or their function (Nelson and Habibi 2009). Unbound TR in mammals act by repressing gene transcription in association with co-repressor proteins bound together in a co-repressor complex (Collingwood et al. 1999). Once bound by TH, mainly T_3, the TR complex becomes activated and subsequent transcription of the specific genes is induced (Lee and Yen 1999). It should also be noted in fish species such as Rainbow Trout that T_3 has been found to bind to TR with a 10-fold higher affinity than T_4 thus demonstrating its importance in the process (Bres and Eales 1986). Two different receptor genes have been found in vertebrates, TRα and TRβ, each with various sub-types depending on the species being examined (Harvey and Williams 2002; Lazar 1993). TR sub-types have been described in mammals and are believed to be splice variants of the original TRα and TRβ genes with N-terminus, C-terminus, or LBD alterations. There are five functional receptor sub-types identified in mammals TRα1, TRα2, TRβ1, TRβ2, and TRβ3 (Kawakami et al. 2006b). The presence of TR sub-types similar to those observed in mammals have also been identified in a number of fish species indicating the relatively conserved nature of this group of nuclear receptors (Nelson and Habibi 2009). Despite confirmation of their presence in fish little is known about their functionality outside of mammalian species.

Although little is known regarding the mechanism of differential TR function in fish the structure, formation, and expression level of receptor sub-types has been documented in a number of species such as Goldfish, *Carassius auratus*, TRα1, TRα2, TRαt, and TRβ (Nelson and Habibi 2006), Black Rockfish, *Sebastes melanops*, TRα and TRβ (Shafi et al. 2012), Japanese Flounder, *Paralichthys olivaceu*, TRαA, TRαB, TRβ1, and TRβ2 (Yamano and Miwa 1998), Nile Tilapia,

and Atlantic Salmon, TRαA and TRαB (Marchand et al. 2001). In goldfish both TRα sub-types, TRα1 and TRα2, were expressed in all tested tissue (gonad, liver, pituitary, brain, heart, gut and muscle) with the highest expression levels observed in the pituitary and lower brain, however, no difference in expression levels was documented between males and females (Nelson and Habibi 2006). TR expression of Black Rockfish during development was examined by Shafi et al. (2012) where they found TRα expression was higher in all tissues at all time-points when compared to TRβ. The expression levels for TRα were higher in gonad, liver, brain, and kidney compared to other tissues with gonad tissue having the highest expression. Based on their results they hypothesized a clear link between TR expression and larval growth and gonad development particularly TRα. A TRα subtype named TRα1 has been identified in the early stages of embryo development in Zebrafish when other TR sub-types are absent indicating TRα may play a more important role in early development in Zebrafish and possibly other fish species (Essner et al. 1997). In addition to TR expression differing among tissues it also differs in response to increases or decreases in plasma T_3 concentration. Work performed on Fathead Minnows by Lema (2009) and the Striped Parrotfish, *Scarus iseri*, by Johnson and Lema (2011) showed a clear increase in TRα and TRβ expression within the liver and brains of fish kept in tanks dosed with T_3. These results indicate a clear relationship between the TR's and their ligand and the subsequent control of gene expression.

TR's can play an even bigger role in species which undergo a TH dependent larval/juvenile metamorphosis and their expression level is often tissue specific. In larval flounder Yamano and Miwa (1998) demonstrated a clear pattern between the up-regulation of TRα and the stages of larval development and metamorphosis. They found the level of TRα expression peaked at the most active stage of metamorphosis and then decreased post-metamorphosis whereas TRβ expression peaked after metamorphosis and remained relatively high compared to TRα in the juvenile flounder. The highest expression levels were found in tissues undergoing the largest morphological and physiological changes such as skeletal muscle and the inner mucosa of the stomach. The peak in TRα expression correlated with the measured peak in T_4 indicating the importance of ligand mediated activation and expression in the TR genes.

In vertebrates the TH deiodinase enzymes act to regulate both circulating and bound levels of TH and their activity is often tissue specific (Figure 1.4). The deiodinase enzymes make up a group of at least three different selenoenzymes each with a characteristic function in the removal of outer or inner ring iodine molecules from specific TH's. In fish and other vertebrates deiodinase enzymes are tissue specific and vary between different species. The D1 enzyme, which can deiodinate both the inner and outer phenol rings but has a preference for reverse T_3 (rT_3), is mainly found in the kidney but can also be found in lower concentrations in the liver and thyroid of certain fish species. The D3 enzyme, which only has inner ring deiodination activity prefers T_3 over T_4 converting T_3 to diiodothyronine (T_2) and is found mainly in brain tissue (Mol et al. 1997, 1998). The most biologically significant TH enzyme in vertebrates, T_4 outer ring deiodinase (T4ORD) often referred to as D2, is responsible for the conversion of T_4 to T_3 (Klaren et al. 2005). D2 acts to remove an iodine molecule from the outer phenol ring of the T_4 molecule

thus converting T_4 to the more biologically active T_3 (Darras and Van Herck 2012). In teleost fish the D2 enzyme is found mainly in the liver of most species tested where it has the highest activity. Because of D2's high activity in liver tissue and its response to T_3 concentration it is believed D2 is the main regulatory enzyme of circulating T_3 (Van der Guyten et al. 1998). Comparatively, studies on two teleost fish species, the Fathead Minnow and Striped Parrotfish, demonstrated that levels of D2 activity were low in other tissues such as brain, kidney, and heart and no activity was observed in muscle tissue (Johnson and Lema 2011).

In more ancient vertebrates such as the Sea Lamprey, *Petromyzon marinus*, the same trends regarding deiodinase tissue expression are not evident. In un-metamorphosed sea lamprey, D2 activity occurs in all tissue types but the highest activity is primarily in the intestine. Similarly, in adult (spawning-phase) sea lamprey D2 activity was highest in intestinal tissue but was low in kidney and muscle and was not observed in liver tissue. There was no recorded T3ORD (D1) activity in any tissues of both larval and adult lamprey. T4IRD and T3IRD (D3) activity was negligible in tissue of un-metamorphosed lamprey but was detectable in kidney and to a greater extent in the intestines of upstream spawning lamprey (Eales et al. 1997). In the other vertebrates mentioned thus far D2 activity is high in liver tissue but the lack of D2 activity in the liver of adult sea lampreys may be a function of the release of TH directly from the endostyle, the primitive thyroid gland, into the lumen of the digestive tract.

Like TR gene expression deiodinase gene expression and subsequent activity is linked to levels of T_4 and T_3 via feedback regulation. In all teleost species studied D2 mRNA expression increases during periods of hypothyroidism and decreases during periods of hyperthyroidism (Johnson and Lema 2011). These changes in mRNA expression are then translated into increases in enzyme activity during hypothyroidism and decreases in activity during hyperthyroidism (Van der Guyten et al. 2001). During this process in mammals and potentially in other vertebrates T_4 levels also help to regulate D2 activity at the post-translational level through ubiquitination leading to the degradation of D2 enzymes by specific proteasomes (Arrojo and Bianco 2011).

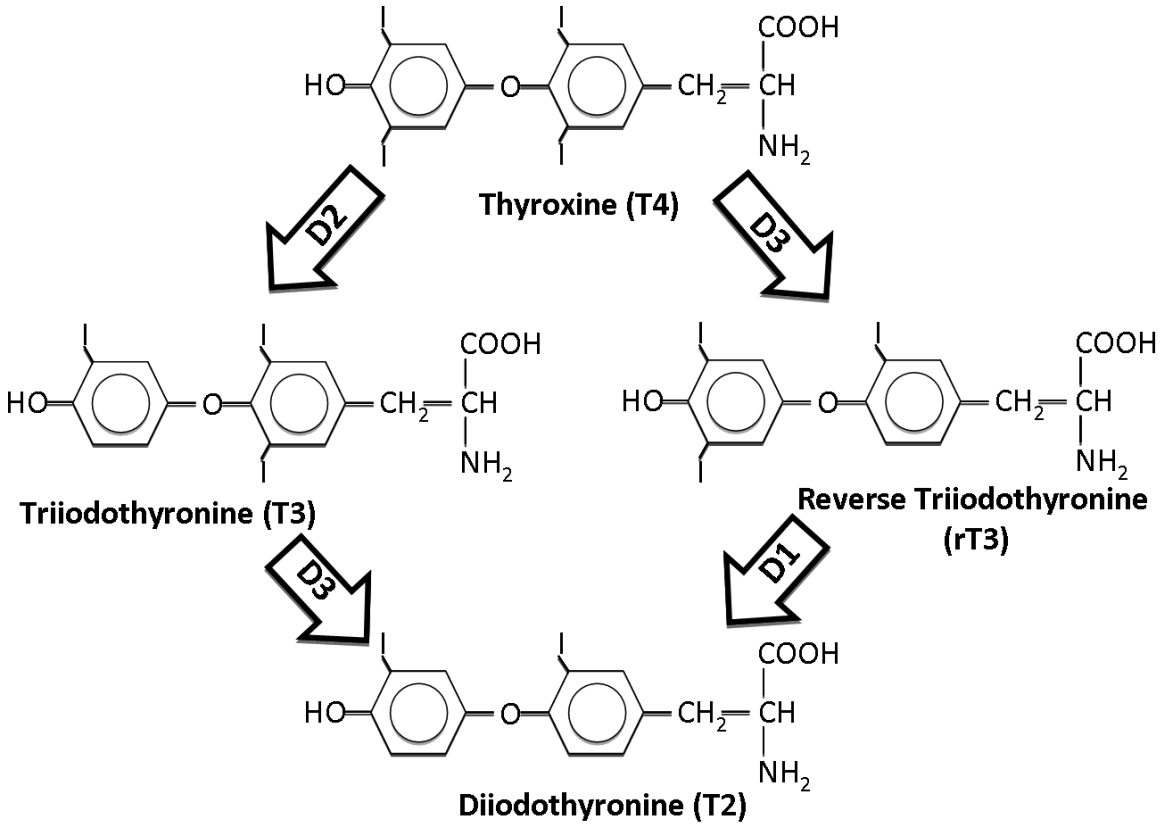

Figure 1.4: The thyroid hormone metabolic pathway showing the conversion of thyroid hormones into different forms by deiodinase enzymes. The enzyme D3 converts thyroxine (T4) to reverse triiodothyronine (rT3) and triiodothyronine (T3) to diiodothyronine (T2). The enzyme D1 converts rT3 to T2. The most important enzyme D2 converts T4 to T3. Figure adapted from Darras and Van Herck 2012; Bentley 1998; Hadley and Levine 2006; Norris and Carr 2013.

1.7 TH in Fish Development

The role of T_3 and T_4 in egg and larval development as well as metamorphosis has been studied extensively in a wide variety of fish and amphibian species. TH's have been found in the developing eggs of all teleost species examined to date such as Rainbow Trout, Mozambique Tilapia, Coho Salmon, Red Sea Bream, *Pagrus major*, Striped Bass, and the goldfish but

concentrations are highly variable (Yamano 2005; Tagawa et al. 1990). The eggs of freshwater species tend to have greater T_4 concentrations and the eggs of seawater species tend to have higher T_3 concentrations. Fish that live in both fresh and seawater have intermediate concentrations of both hormones (Tagawa et al. 1990). Studies on the changes in TH concentration during egg development in a number of teleost species show TH concentrations decline throughout embryogenesis and into the early larval life-stages until endogenous TH production starts (Lam 1994). The steady decline in TH levels indicates the consumption of TH's during the stages of early development. Since most species of fish do not produce TH endogenously at the stage of the developing embryo it is believed the TH present in the eggs of teleost fish is maternal in origin (Yamano 2005). The transfer of TH's from the female to her eggs has been demonstrated in a number of species of fish by maternal injection of TH and analysis of TH content in the eggs. In Striped Bass, maternal injection of TH increased the concentration of TH within the eggs and larvae further demonstrating the important role of maternal TH (Brown et al. 1988). In the same study it was shown eggs from T_3-injected ($20mg.kg^{-1}$) mothers had greater survival and growth during the early life-stages and were more likely to have successfully inflated swim bladders. Similarly, maternal injection of T_4 ($20mg.kg^{-1}$) into Goldstriped Amberjack, *Seriola lalandi*, and Japanese Parrotfish, *Oplegnathus fasciatus*, increased larval survival in both species but had no affect on growth (Tachihara et al. 1997). In Mozambique Tilapia T_3 and T_4 levels dropped steadily post-fertilization until 7 days post-hatch coinciding with the progression from embryonic to larval development (Reddy et al. 1992). They hypothesized the tilapia embryos and larvae were using the T_3 and T_4 in early development. After 7 days post-hatch T_3 and T_4 levels began to rise with T_4 increasing from $0.02ng.larvae^{-1}$ to $1.58ng.larvae^{-1}$ at 25dph. T_3 levels showed a similar trend but concentrations were much lower than those of T_4. They proposed the increase in T_3 and T_4 might be responsible in assisting the

larvae to adapt to their new environment as the timing corresponds with larval emergence from the safety of their mothers mouth.

TH appears to be vital in a number of physiological and morphological changes at the larval stage in many species of fish. For several teleost species, such as flounder, stomach development does not fully occur until the larval-juvenile transition (Tanaka 1971). In Tanaka et al. (1995) larval Japanese flounder held in tanks containing 30ppm water-borne T_4 for 15 days demonstrated large differences in digestive system development when compared to control samples. The appearance of granules in the upper portion of the rectal epithelium consistent with activation of pinocytosis of proteins from the gut lumen occurred much earlier in T_4-treated larvae. In addition, there was also an increase in the presence of vacuolated intestinal epithelial and hepatic cells, which were confirmed to contain high quantities of lipids and glycogen respectively. When larval Japanese Flounder and Summer Flounder, *Paralichthys dentatus*, were exposed to TH the appearance of the gastric gland was also accelerated and therefore the production of pepsin and pepsinogen occurred earlier. The reverse effects were observed when flounder larvae were exposed to thiourea a known thyroid inhibiting compound which acts by blocking the synthesis and secretion of TH (Miwa et al. 1992; Huang et al. 1998a; Baumann et al. 1944). These results indicate the potential of T_4 to stimulate protein, lipid and glycogen uptake in developing flounder larvae. However, in larval Striped Bass water-borne exposure to 1, 10, or 100ppb T_3 had mixed effects on the development of the stomach and gastric gland. Larvae treated with the highest dose had a significantly thicker layer of stomach muscle but development of the gastric blind-sac where the gastric glands are located was inhibited in fish treated with the medium and high T_3 doses (Huang et al. 1998b).

TH's have also been implicated in the development and maturation of erythrocytes (Power et al. 2001; Inui 1995), visual systems (Browman and Hawryshyn 1992; Beatty 1972; Reddy and Lam 1992) and pigmentation (Reddy and Lam 1992). The involvement of TH in the development of erythrocytes has been documented in mammals, birds, reptiles, and amphibians and to a lesser extent in fish. Pre-metamorphic larval flounder erythrocytes are large round cells with round nuclei but during the period of transition to the juvenile stage a shift in erythrocyte morphology takes place. Treatment with T_4 induced a premature shift towards the juvenile/adult erythrocyte morphology where cells are small and elliptical with a large round nucleus (Inui et al. 1995). In Rainbow Trout and Black Goldfish exposure to TH's aided in the development of the visual system. Rainbow Trout exposed to $300\mu g.L^{-1}$ water-borne T_4 prematurely lost UV sensitivity and had recorded differences in the retinal photoreceptor distribution. The changes observed in rainbow trout correspond to events that occur during natural development when it is believed the fish enter deeper waters (Browman and Hawryshyn 1992). In goldfish larvae T_4 and T_3 exposure led to increased telescoping of the eyes, which does not occur until later in the developmental process. Pigmentation was also increased in TH treated goldfish with melanophore concentration on the scales significantly increased compared to controls (Reddy and Lam 1992).

The potential for TH's to influence brain development and function has been established in teleost species such as Atlantic Salmon and Singi fish, *Heteropneustes fossilis*, (Morin et al. 1997; De et al. 1992; De et al. 1993). In salmonid species at the period of parr-smolt transformation (PST) plasma levels of T_4 increase and it is believed T_4 may play a role in altering brain levels of dopamine (DA), serotonin (5-HT), and their metabolites. Atlantic salmon exposed to $500\mu g.L^{-1}$ T_4 prior to PST had elevated plasma levels of T_4 similar to values measured during

natural PST. Salmon were analyzed for differences in dopamine (DA) and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) as well as serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA). T_4-treated salmon had decreased levels of DOPAC and a lower DOPAC/DA and 5-HIAA/5-HT ratio in the olfactory system when compared to control fish. This supports that T_4 had an affect on specific brain regions in Atlantic salmon at a key point in development and may play a role in regulating normal brain function (Morin et al. 1997). De et al. (1992; 1993) also demonstrated a T_3 effect on the Na^+/K^+ ATPase, Mg^{2+} ATPase, and AChE activities in the brain of Singi fish. One injection per day for three days of $0.10\mu g.g^{-1}$ T_3 significantly increased Na^+/K^+-ATPase and Mg^{2+}-ATPase activity in Singi fish brain. Additional support of the link between T_3 and the activation of these enzymes was carried out using the Singi fish exposed to the thyroid inhibitor thiourea where a significant decrease in ATPase activity was observed (De et al. 1992). T_3 has also been implicated in the maintenance of AChE activity where exposure to T_3 in Singi fish increased AChE activity and exposure to the thyroid and protein synthesis inhibitor cycloheximide decreased AChE activity (De et al. 1993).

Interestingly, thyroxine has been implicated in the sex determination and gonadogenesis of zebrafish larvae. Mukhi et al. (2007) exposed larval zebrafish to two concentrations (100 and 250ppm) of water-borne perchlorate, a known thyroid hormone synthesis inhibiting substance, for 30 days and the sex ratio, thyroid histology and gonadal structure were examined. They found perchlorate exposure shifted the sex ratio in the female direction in a dose-dependent fashion. Co-treatment with T_4 was successful in reversing the feminizing effects of perchlorate and even advanced the onset of spermatogenesis (Mukhi et al. 2007). Further, support for their results was published by Sharma and Patino (2013) where 72-hour post-fertilized Zebrafish

larvae were exposed to methimazole, an anti-thyroid drug which acts by inhibiting thyroid peroxidase and therefore TH synthesis within the thyroid follicle (Rutland et al. 2009), and T_4 for a period of 30 days. Zebrafish exposed to perchlorate (820,000nM) and methimazole (150,000 and 300,000nM) exhibited signs of hypothyroidism and their sex ratios were shifted towards females compared to control fish. Comparatively, Zebrafish exposed to T_4 (1 and 10nM) had sex ratios shifted in the opposite direction towards males. In addition, spermatogenesis was inhibited in Zebrafish larvae exposed to perchlorate and methimazole but was not affected in T_4 treated larvae. The previous two studies indicate the potential for TH to play a large role in gonadogenesis and establishing sex in developing Zebrafish larvae.

In larval Japanese Flounder, T_3 and T_4 are the primary hormones responsible for metamorphosis to the juvenile life stage. Inui and Miwa (1985) demonstrated the importance of T_4 by immersing flounder larvae in water containing thyroxine ($0.1mg.L^{-1}$) or thiourea ($30mg.L^{-1}$). After a period of 6 days, the eyes of thyroxine treated larvae had completely moved to the left side and the large dorsal fin had been fully reabsorbed. In comparison, larvae treated with thiourea, a compound capable of interfering with iodide uptake were prevented from metamorphosing resulting in oversized larvae. In addition, Yamano and Miwa (1998) recorded an increase in both T_3 and T_4 during Japanese Flounder metamorphosis in whole-body larval homogenates. The concentrations of T_3 and T_4 in flounder were significantly lower post-metamorphosis indicating the importance of both hormones during metamorphosis. Also important during flounder metamorphosis is the conversion of larval muscle fibers into the juvenile/adult versions. In the larval form, flounder muscle is composed of thin muscle fibers with relatively few myofibrils. However, during metamorphosis when TH levels are high muscle fibers become thick with many myofibrils present. The role of TH in the process was established

by treatment with thiourea, which inhibited the development of adult muscle fibers in the metamorphosing larvae (Inui et al. 1995). Other fish species such as Zebrafish and Orange-spotted Grouper, *Epinephelus coioides*, have a documented reliance on T_3 and T_4 for larval metamorphosis. Zebrafish larvae exposed to substances, which interfere with the thyroid system such as thiourea, 6-n-propyl-2-thiouracil (PTU), potassium perchlorate ($KClO_4$), and methimazole have inhibited shifts from the larval to juvenile form. Zebrafish exposed to waterborne thyroid inhibitors at varying concentrations exhibited underdeveloped pectoral and pelvic fins, scales, and reduced pigment pattern (Brown 1997). Additionally, when TH was added to the tanks already dosed with thyroid inhibitors the affects of the inhibitors were reduced. Similar results were observed in the Orange-spotted Grouper where larvae reared in 0.01, 0.1, and 1ppm T_4 or T_3 all metamorphosed earlier than controls in a dose-dependent relationship irrespective of size (de Jesus et al. 1998).

Interestingly, the opposite trend is observed in agnathan species studied prior to metamorphosis (Youson et al. 1997). Agnathan species are some of the most ancient vertebrate species on the planet and their evolutionary transition is said to have been conservative over the last ~350 million years with very little evolutionary change (Forey and Janvier 1994). In the Sea Lamprey, metamorphosis is characterized by a decrease in circulating levels of both T_4 and T_3 even though levels of the two hormones rise steadily during larval development (Youson et al. 1994). In a study conducted in 1994 and 1995 on premetamorphic sea lampreys exposed to water-borne T_3 metamorphosis was severely altered. In 1994, 90% of the premetamorphic lampreys exposed to T_3 at a concentration of $1mg.L^{-1}$ for a period of 4 months did not metamorphose. The same trend was observed in the 1995 study where only 36% of T_3 treated lampreys metamorphosed. Comparatively, in both year classes, T_4 exposure ($10mg.L^{-1}$) had no

affect on metamorphosis when compared to control tanks (Youson et al. 1997). The role of TH in lamprey metamorphosis was tested further by exposure to the thyroid inhibiting substance thiourea where exposure to thiourea was successful in inducing premature larval metamorphosis (Holmes and Youson 1993).

1.8 Thyroid System in Sturgeon

Due to the divergence of the Actinopterygii (ray-finned fish) over 200 million years ago the thyroid systems of the two sub-classes, Chondrosteii (includes the sturgeon order Acipenseriformes) and the Neopterygii (includes the Teleostean lineage), may differ. In addition, the Acipenseriformes order is one of the most primitive fish orders and is near the base of the Actinopterygean evolutionary tree further indicating the potential for differences in the thyroid systems between sturgeon and teleost species (Chourdhury and Dick 1998). Although the thyroid system has been studied quite extensively in a number of teleost species such as Atlantic Salmon, Rainbow Trout, Fathead Minnow, and Zebrafish very little work has been conducted on the thyroid system in sturgeon. In the Russian Sturgeon, levels of T_3 and T_4 have been measured in eggs, embryos, and developing larvae and levels of T_4 were reported to be higher than those of T_3 in all life-stages examined (Boiko et al. 2004). During embryogenesis the level of T_3 and T_4 decreased indicating the potential involvement of both hormones in embryo development as the maternal supplies of the two hormones were exhausted. The importance of both TH's during the early life-stages was also shown in Stellate Sturgeon where TH was essential in the development and maturation of the eggs (Detlaf and Davydova 1974).

T_4 concentrations began to rise in hatched larvae prior to the onset of exogenous feeding reaching a maximum value of 0.36ng.larvae^{-1} in Russian Sturgeon (Boiko et al. 2004). The fluctuations in T_4 and to a lesser extent T_3 in Russian sturgeon larvae are more in line with teleost species undergoing some form of metamorphosis in which the thyroid hormones play an important role. Boiko et al. (2004) were able to demonstrate the significance of T_4 in larval survival by showing that larval mortality decreased with higher thyroid status. It was also suggested the rise in T_4 aids in larval imprinting of the environment further boosting survival. Studies performed on juvenile White Sturgeon showed low plasma TH levels as well as seasonal variability in both T_4 and T_3 (McEnroe and Cech 1994).

1.9 Thyroid System in Lake Sturgeon

Several studies have looked at the thyroid system in cultured and wild juvenile Lake Sturgeon but little is known about the role of the thyroid system in earlier life stages and throughout development. Plohman et al. (2002a) examined TH concentrations in serum and tissue of both cultured and wild juvenile sturgeon. Serum taken from cultured sturgeon held at temperatures between 12-15^{o}C had T_4 and T_3 concentrations of 0.29 ± 0.25ng.ml^{-1} and 0.19 ± 0.01ng.ml^{-1} respectively. In wild Lake Sturgeon serum concentration were mostly lower but highly variable with a few adult individuals having very high T_4 and T_3 levels reaching 2.9ng.ml^{-1} and 9.65ng.ml^{-1} respectively. It was hypothesized the high levels of TH in a few wild individuals may be due to a surge in TH related to spawning (Eales and Brown 1993). Additionally, unbound levels of T_4 (FT4 = 1.10%) and T_3 (FT3 = 0.44%) were higher in sturgeon than Rainbow Trout (FT4= 0.18% and FT3 = 0.08%), however, calculated plasma concentrations were similar to those observed in salmonids indicating sturgeon tissues are likely exposed to

similar levels of unbound TH but with reduced access to bound TH reserves (Eales et al. 1983; Eales and Shostak 1985; Weirich et al. 1987).

Tissue concentrations of T_4 and T_3 in juvenile cultured Lake Sturgeon were also examined by Plohman et al. (2002a). Levels of T_4 were low in most tissues (liver = 0.06 ± 0.03ng.g^{-1}; kidney = 0.10 ± 0.03ng.g^{-1}; brain = 0.62 ± 0.06ng.g^{-1}) except for the gall bladder (6.02 ± 2.88ng.g^{-1} and thyroid (2.42 ± 0.97ng.g^{-1}). T_3 tissue concentrations were also low (liver 0.07 ± 0.04ng.g^{-1}; kidney 0.07 ± 0.05 ng.g^{-1}; intestine 0.77 ± 0.26ng.g^{-1}) with the exception of the brain (5.88 ± 3.12ng.g^{-1}) and thyroid (21.31 ± 2.02ng.g^{-1}). Comparatively, the T_4 values in Coho Salmon liver, brain, and kidney are 10-100 times higher than recorded in Lake Sturgeon (Specker et al. 1992). The T_3 concentrations in liver, kidney and intestine were also lower in Lake Sturgeon than recorded for Rainbow Trout (2.5-3.5ng.g^{-1}) (Fok et al. 1990). In most tissues studied the T_3:T_4 ratio was greater than 1 indicating a higher tissue concentration of T_3 when compared to other fish species. Although for the most part tissue TH concentration was comparatively low in Lake Sturgeon, brain and liver samples showed high levels of TH, particularly T_3. In brain tissue the high T_3 concentration was believed to be a result of the high affinity shown for T_3 by the thyroid-binding protein TTR, which can be produced in the brain of vertebrates (Power et al. 2000). Both brain and thyroid tissue had high T_3:T_4 ratios, 11.32 and 10.55, respectively but it was the large difference between T_4 (2.42 ± 0.97ng.g^{-1}) and T_3 (21.31 ± 2.02ng.g^{-1}) in the thyroid, which was of particular interest. In most teleost models T_4 is believed to be the primary hormone released from the thyroid but it appears T_3 may also be released in high quantities in Lake Sturgeon (Plohman et al. 2002a; Eales and Brown 1993). The low level of T_4 and high level of T_3 in the sturgeon thyroid may indicate a less significant role of peripheral deiodination pathways at the tissue level (Plohman et al. 2002a).

Research examining the deiodination pathways and the enzymes involved was carried out on cultured 2-year-old Lake Sturgeon by Plohman et al. (2002b). D2 activity was found in the liver, intestine, and thyroid but not in the brain, skeletal muscle, kidney, or gonad. D2 activity was highest in the liver of Lake Sturgeon with rates of 0.120 ± 0.153pmoles T_4 deiodinated.hr^{-1}.mg $protein^{-1}$. Although D2 was the most active in the liver other tissues were shown to have D1 and D3 activity as well. Intestinal deiodination was detectable in Lake Sturgeon samples but is usually low or undetectable in other species of fish such as Atlantic Cod, *Gadus morhua*, (Cyr et al. 1998) and American Plaice, *Hippoglossoides platessoides*, (Adams et al. 2000). However, it is the principal tissue for deiodination in Sea Lampreys (Eales et al. 1999) and Pacific Hagfish, *Myxine glutinosa*, (McLeese et al. 2000) indicating Lake Sturgeon may still possess, to a lesser extent, the primitive deiodination trait. Despite low D2 activity in the brain D3 activity via inner-ring deiodination of T_4 and T_3 was higher which is similar to studies performed on teleost species (Mol et al. 1998; Adams et al. 2000). Low D2 activity and T_4 concentrations in the brain suggests very little production of T_3 occurs in the brain itself, instead the high levels of T_3 recorded in brain tissue by Plohman et al. (2002a) are a direct result of uptake from the plasma pool. Another important tissue where D2 activity was measured above detectable limits is the thyroid gland where activity was 0.044 ± 0.02pmole.hr^{-1}.mg $protein^{-1}$. The high T_3 concentrations in thyroid tissue along with measurable D2 activity indicates the potential for internal conversion of T_4 to T_3 within the follicle itself (Plohman et a. 2002a; 2002b). Combined, the previous results suggest the Lake Sturgeon thyroid is capable of releasing large amounts of T_3 directly into the plasma, which is in direct contrast to other fish species where peripheral control is favored.

A possible reason for the low levels of TH measured in Lake Sturgeon is their reliance on entirely freshwater habitats, which are characteristically low in iodine thus limiting the amount of available iodine for the synthesis of TH's (Plohman et al. 2002a). Evidence of this can be seen with Lake Sturgeon having some of the lowest plasma iodine levels (0.008-0.015µl.ml^{-1}) of any fish species (Leloup 1970). As a cartilaginous fish and the only sturgeon species in North America to live exclusively in freshwater habitats Lake Sturgeon physiology is different from that of freshwater teleosts and this may affect the thyroid system in larval Lake Sturgeon. In most teleost fish the conversion of T_4 to T_3 occurs in the peripheral tissues such as the brain, liver, kidney and gills (Blanton and Specker 2007).

As previously discussed T_3 and T_4 have been shown to directly influence early embryonic and larval development of a number of fish species. Exposure to thyroid inhibiting compounds prevented larval development and metamorphosis in flounder (Inui and Miwa 1985) and Zebrafish (Brown 1997). Environmental contaminants known to interfere with thyroid function include the organophosphate dimecron (Thangavel et al. 2005), the polybrominated diphenyl ether DE-17 (Yu et al. 2011), and the organochlorine endosulfan (Sinha et al. 1991). The results of these studies highlight the potential damaging effects thyroid disrupting chemicals could have on developing fish larvae. Consequently, any negative effect on the thyroid system during the larval life-stage could severely influence growth and survivability of a particular fish species.

1.10 Objectives

The main objective of this thesis was to examine and document the development of the thyroid hormone system in larval Lake Sturgeon. This was achieved through a combination of histological, biochemical and molecular techniques. In addition I aimed to assess any potential endocrine disruptive effects by the organophosphate pesticide, chlorpyrifos on TH development through exposure at environmentally relevant concentrations during larval development. Specific objectives included:

1) Identify Lake Sturgeon growth characteristics and condition factor in developing larvae in control and chlorpyrifos treatments.
2) Examine thyroid gland follicular development throughout development in larval Lake Sturgeon in control and chlorpyrifos treatments using histological techniques
3) Analyze the expression of TH receptor subtypes in different tissue types of larval Lake Sturgeon in control and chlorpyrifos treatments throughout development
4) Determine the whole-body levels of the main thyroid hormones T_3 and T_4 in larval Lake Sturgeon in control and chlorpyrifos treatments throughout development
5) Quantify the activity of deiodinase enzymes, specifically the outer-ring deiodinatition of T_4, among different tissue types in young of the year, 1 year old, and 2 year old sturgeon juveniles

1.11 Hypotheses

$H1_0$: The presence of thyroid follicles or thyroid tissue will be evident in larvae with their yolk-sac still present and will become more developed following the onset of exogenous feeding.

$H2_0$: TR expression will change throughout development and TRα expression will be higher than TRβ expression at all developmental time-points and in all tissue types.

$H3_0$: T4ORD activity will be highest in liver and brain tissue of YOY, one year old, and two year old Lake Sturgeon when compared to muscle and heart tissue.

$H4_0$: Whole-body TH levels will correlate with key developmental stages in larval development such as the onset of exogenous feeding and the development of active thyroid follicles.

$H5_0$: Chlorpyrifos will negatively alter thyroid development in Lake Sturgeon larvae by decreasing thyroid follicle development, whole-body TH levels, TR expression, and growth.

2.0 Methods

2.1 Experimental Protocol

2.1.1 Lake Sturgeon Larvae

Eighteen adult Lake Sturgeon (9 female and 9 male) were captured by gill net during the spawning season for Lake Sturgeon in May of 2012 just downstream of the Pointe du Bois generating station on the Winnipeg River, Manitoba (est. 1909, 50º17'52N, 95º32'51W). Fish were then transported to the animal holding facility at the University of Manitoba where they were transferred to 2275 L flow through aquaria maintained at 12°C under a 12h:12h light:dark photoperiod. Following a 24h acclimation period 5 female and 4 male fish were induced to spawn through the intraperitoneal injection of 10μg kg^{-1} gonadotropin releasing hormone (GnRH, (Des-Gly^{10},D-Ala^{6},Pro-$NHEt^{9}$)-LHRH, Bachem, Torrance, CA, USA) followed by a 20μg.kg^{-1} dose 20h later. Eggs and milt were harvested within the following 24h from these fish. The remaining adults not induced to spawn with GnRH administration failed to produce any viable eggs or milt. Eggs were then incubated in flow through MacDonald hatching jars (round bottomed, 13L capacity) at 14°C at the Department of Fisheries and Oceans Freshwater Institute, Winnipeg, MB, Canada. During incubation the hatching jars were checked twice daily and dead eggs and any fungus build up was carefully removed by pipette. Approximately 7 days post fertilization larvae began to hatch and were immediately separated from un-hatched larvae and placed in 15L flow through aquaria maintained at 14°C with a 12h:12h light:dark cycle. All adult sturgeon used for collection of eggs and milt were released back into the Winnipeg River at or near the location of capture. All methods employed were approved by the University of Manitoba Protocol Management Review Committee in accordance with the Canadian Council for Animal Care.

2.1.2 Chlorpyrifos Exposure and Larval Feeding

Once all pre-larvae had hatched out they were placed into sixteen 15L flow through aquaria maintained at 14^{o}C and a flow rate of 100ml.min^{-1}. The tanks were divided into four treatments as follows with four tanks per treatment; control (0ng.L^{-1}), low (5ng.L^{-1}), medium (500ng.L^{-1}), and high (2000ng.L^{-1}) final in tank chlorpyrifos (LORSBAN 500 EG, Dow AgroSciences, 500g/L emulsified concentrate) concentrations. Exposure began at 3dph for all pre-larvae. Chlorpyrifos concentrations prepared from addition of concentrated chlorpyrifos preparation to water were maintained using a peristaltic pump at a flow rate of 1ml.min^{-1} for a period of 10 days during which time all larvae still had the yolk sac attached and had not begun exogenous feeding. Exposure concentrations and duration was designed to mimic the exposure typically seen in the environment when commercial crops are treated with pesticides such as chlorpyrifos (Rawn and Muir 1999). After completion of the exposure period fish were transitioned to exogenous feeding by the introduction of live brine shrimp to the flow through tanks. Larval Lake Sturgeon were fed live brine shrimp using a dropper for one hour 2-3 times per day (9am, 3pm, and 9pm) for a period of 5 weeks. After 5 weeks the larvae were transitioned to commercial trout feed (starter crumble #0, Skretting USA, Tooele, Utah) for the remainder of the experimental period. Tanks were cleaned daily to remove excess food and fecal matter and were kept in darkness when possible.

2.1.3 Larval Sampling

Larvae were randomly sampled on 3, 6, 9 and 12 days post hatch (dph). At 3dph larvae were sampled from each treatment tank just prior to the start of chlorpyrifos exposure. Samples

were taken at 6, 9 and 12dph during the exposure to chlorpyrifos. Three sample points were performed post exposure at 21, 36, and 67dph. Samples from 12dph represent larvae in stage 8 of development just prior to exogenous feeding Detlaf (1993). A total of thirty six larvae were sacrificed per tank every sample period and randomly distributed such that thirty larvae were used for whole-body T_3 and T_4, three for molecular analysis, and three for histological assessment of thyroid follicles. All larvae harvested from each tank during the sample period were anesthetized with a fatal dose (200ppm) of buffered Tricaine Methanesulfonate (MS222) before: a) being placed directly a sample vial then snap frozen in liquid nitrogen and stored at -80°C prior to whole body T_3 and T_4 analysis; b) being placed in a sample vial containing RNALater (Ambion, Life Technologies, Burlington, ON, Canada) then snap frozen in liquid nitrogen and stored at -80°C prior to thyroid receptor expression analysis; c) being placed in a sample vial containing Bouins fixative for 24 hours then transferred to 70% ethanol for storage prior to microscopy analysis. Body mass and total body length was assessed at each sampling time-point for those larvae destined for microscopy analysis. Body mass was taken to the nearest tenth of a mg and body length was taken using a set of electronic calipers to the nearest μm (L.S. Starret Company, Athol, MA) before the larvae were placed in fixative. All chemicals used were obtained from Sigma Aldrich (Mississauga, ON, Canada) unless otherwise stated. All growth measurements and results were analyzed for statistical difference using a two-way ANOVA with a Tukeys HSD test to compare against controls (Graphpad Prism 5, La Jolla, CA, USA). Calculations of condition factor were performed using Fulton's condition factor formula where K is condition factor, W is weight in grams, and L is length in cm's (Froese 2006):

$$K = 100(W/L^3)$$

2.2 Histology

2.2.1 Tissue Fixation and Slide Preparation

Samples fixed in Bouins solution were put in labeled histocasettes and placed in a Tissue Tek VIP 5 tissue processor (Sakura Finetek USA, Torrance, CA). In the processor, samples were dehydrated from 70% ethanol to 100% ethanol, cleared in toluene, and infiltrated with molten paraffin. The histocasettes were then taken from the tissue processer and individual larval samples were removed and solidified in paraffin wax molds. The molds were left for 2-3 days to solidify at room temperature before sectioning. Sectioning was performed on two samples per tank per time-point (providing a total of 8 fish per treatment per timepoint) using a microtome at a thickness of 5um per section. Sections taken through the glossopharyngeal region (in large ribbons) were laid out in rows and every 4-5 sections were fixed to microscope slides using albumin fixative for use in both Harris' Hematoxylin and Eosin (H&E) staining as well as Immunohistochemistry (IHC). Once sections were added to the slides, slides were placed in an oven at 40^{o}C overnight to allow the wax sections to fully adhere to the slides. Slides for H&E staining were then stored in a dry environment at room temperature until H&E staining whereas slides for IHC were stored at 4^{o}C.

2.2.2 Harris Hematoxylin and Eosin Staining

Slides marked for H&E staining were stained 20 slides at a time according to the protocol described by Edwards (1967). Briefly, slides were first submerged in a series of toluene and toluene/ethanol solutions starting with 5 minutes in 100% toluene followed by 2.5 minutes in a 1:1 toluene:ethanol mix in order to remove the wax from the sections. Second, slides were hydrated using a series of ethanol solutions starting with one rinse in 100% ethanol for 2 minutes followed by one rinse in 90% ethanol for 2 minutes, one rinse in 75% ethanol for 2 minutes and

two rinses in water for 2 minutes. Once the sections were hydrated they were placed in Harris' Hematoxylin solution for 5 minutes and then rinsed 4 times in running tap water. Slides were then submerged in acid alcohol (0.2% HCl in 70% ethanol) for 15 seconds followed by 2 rinses in running tap water. Slides were then submerged in 0.33% phosphotungstic acid (by volume) for 30 seconds followed by 30 seconds in 0.33% citric acid (by volume) and then 5 minutes in running tap water. Slides were then counterstained using eosin for 2.5 minutes and were then rinsed 2 times in running water until excess stain was removed. Once rinsed slides were dehydrated in a series of ethanol steps starting with 1 minute at 95% ethanol, followed by two rinses in 100% ethanol for 2 minutes each. Slides were then immersed in a 1:1 toluene:ethanol mix for 2 minutes followed by two 100% toluene baths for 2 minutes each. The slides were then removed from the toluene and were mounted with a cover slip using mounting media and allowed to set for 1-2 weeks.

2.2.3 Immunohistochemistry

Slides were de-waxed using toluene and hydrated using an alcohol series from 100% ethanol to pure water. Briefly, slides were first submerged in a series of toluene and toluene/ethanol solutions starting with 5 minutes in 100% toluene followed by 2.5 minutes in a 1:1 toluene:ethanol mix in order to remove the wax from the sections. Slides were then hydrated using a series of ethanol solutions starting with one rinse in 100% ethanol for 2 minutes followed by one rinse in 90% and 75% ethanol for 2 minutes each and two rinses in water for 2 minutes. IHC staining of thyroid follicular tissue using T_4 antibodies was carried out using the VectaStain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA) according to previously published literature (Raine and Letherhead 2000; Grandi and Chicca 2004). Slides were then incubated in BLOXAL (Vector Laboratories Inc.) for 10 minutes and rinsed in 0.1M phosphate buffered

saline (PBS) pH 7.4 for 5 minutes in order to block endogenous peroxidase and alkaline phosphatase activity. 10X PBS solution was made by combining 10.9g Na_2HPO_4, 3.2g NaH_2PO_4, and 90g NaCl in a 1L volumetric flask and then adding distilled water to a final volume of 1L. 10X PBS stock solution was then diluted 1:10 with MilliQ water for use in IHC. Blocking serum was prepared according to the protocol provided by the VectaStain Elite ABC kit and was applied to the slides and left for 20 minutes. Slides were then blotted dry and rabbit anti-thyroxine at a dilution of 1:4000 was applied to slides and left at room temperature for 30 minutes. Excess primary antibody was then removed and the slides were rinsed in PBS for 5 minutes. Slides were then incubated for 30 minutes in biotynilated secondary antibody prepared according to the protocol provided by the VectaStain Elite ABC kit. After incubation with secondary antibody, slides were rinsed in PBS for 5 minutes. The slides were then incubated with the VectaStain ABC reagent for 30 minutes prepared according to the manufacturers protocol. Excess ABC reagent was removed and the slides were rinsed in PBS for 5 minutes. All slides were stained using the 3,3'-diaminobenzidine (DAB) peroxidase (Vector Laboratories Inc.). Slides were stained for 7-8 minutes and then rinsed in distilled water to stop the staining process. After peroxidase staining slides were counterstained in Harris Hematoxylin for 20 seconds, rinsed in running water for 1 minute, dehydrated through an alcohol series from 60% ethanol to 100% ethanol, and mounted for observation using light microscopy.

2.2.4 Histological Measurements

Sections from 3, 6, 9 and 12dph were assessed for the presence of thyroid tissue using previously discussed IHC techniques. Tissue samples from 21, 36, and 67dph were assessed for the presence of thyroid follicles using light microscopy as follicles were clearly visible in all samples. All samples with confirmed thyroid follicular tissue using the described IHC and light

microscopy techniques were analyzed for follicular cell height and nucleus height using a Nikon Eclipse 50i light microscope (Nikon Canada, Mississauga, ON) and the computer program ImagePro Plus version 7.0 (Media Cybernetics Inc., Rockville, MD). Three to six follicles per sample from each tank and time-point were measured for cell height and nucleus height depending on the clarity of the sections and number of follicles present according to a modified protocol published by Park et al. (2011). For each selected follicle, four cell height and four nucleus height measurements were taken at approximately the north, south, west, and east sides of the follicle as well as colloid area where possible. Values from each of the four measurements of follicle height and nucleus height were averaged and the results were grouped together into treatments. All measurements and pictures were taken at 400X magnification using a QImaging Q3 digital camera (QImaging, Surrey, BC) and pictures were stored for future reference. All histological measurements and results were analyzed for statistical difference using both a one-way and two-way ANOVA with a Tukeys HSD test to compare against controls (Graphpad Prism 5, La Jolla, CA, USA).

2.3 Molecular Biology

2.3.1 RNA Extraction

RNA samples from control tanks at 3, 12, 21, and 67dph were used for molecular analysis of thyroid hormone receptor α (TRα) and β (TRβ) expression. Only control samples were chosen for the PCR analysis because evidence provided from histological and growth analysis demonstrated no effect of chlorpyrifos on either growth or follicular development. Therefore running control samples only allowed for comparison between time-points as all samples could be fitted to a single RT-PCR 96-well plate

Larvae samples frozen in RNAlater at -80°C were thawed on ice and the RNA was extracted from six randomly selected control larvae at each of the three time-points 3, 12, and 21dph. Samples from 67dph were large enough for dissection therefore individual tissue samples (liver, brain, and muscle) were removed and extracted separately from 6 larvae. Extractions were carried out using the Trizol Reagent method (Ambion) according to the manufacturers protocol. Briefly, individual larvae from 3, 12, and 21dph were homogenized in 250, 500, and 500µl Trizol respectfully using a Qiagen TissueLyser II (Qiagen, Venlo, Netherlands) and allowed to incubate for 5 minutes at room temperature. After incubation molecular grade chloroform was added to each sample (3dph = 50µl, 12dph = 100µl, and 21dph = 100µl) and they were shaken vigorously for 15 seconds, incubated for 3 minutes at room temperature, and centrifuged at 12,000g for 15 minutes and 4°C. The separated solution contains a lower red phenol-chloroform phase and an upper colorless aqueous layer containing the RNA. The top aqueous layer was pipetted and transferred to a clean PCR tube. 100% molecular grade isopropanol was added to each sample in order to precipitate the RNA (3dph = 125µl, 12dph = 250µl, and 21dph = 250µl). Each sample was allowed to incubate at room temperature for 10 minutes and was then centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was then pipetted and discarded leaving the RNA pellet at the bottom of the tube. RNA pellets were washed with 500µl 75% molecular grade ethanol by vortexing each sample briefly and centrifuging at 7,500g for 5 minutes at 4°C. The supernatant from the washed RNA samples was carefully pipetted and the pellet was allowed to air dry for 2-3 minutes. RNA samples were then re-suspended in 30µl RNA storage solution (Ambion). RNA quantity and quality was measured using A260/280 and A260/230 absorbance ratios on a NanoDrop 2000c Spectrophotometer (Thermo Scientific) and results were recorded.

2.3.2 DNase Treatment

RNA samples were then DNase treated in order to remove any potential genomic contamination. A deoxyribonuclease I amplification grade kit (Invitrogen, Carlsbad, CA, USA Cat. No. 18068-015) was used according to the provided protocol. For each sample 0.5µg of RNA was combined with 1µl 10X DNase I reaction buffer, 1µl DNase I amplification grade ($1U.\mu l^{-1}$), and DEPC-treated water to a total volume of 10µl. Tubes were incubated at room temperature for 15 minutes and the reactions were then deactivated with 1µl of 25mM EDTA. After deactivation samples were incubated at $65^{o}C$ for 10 minutes. After DNase treatment the absence of genomic DNA contamination was confirmed in each sample by PCR analysis and gel electrophoresis. If no contamination was observed the DNase treated RNA was reverse transcribed into cDNA to be used for real time analysis. If the presence of genomic contamination was detected after the first DNase treatment, samples were treated a second time with DNase according to the described protocol.

2.3.3 PCR Primers

Specific TRα and TRβ primers for sturgeon were designed by Dr. Hamid Habibi and Shaelen Konschuh at the University of Calgary, AB. The internal standard chosen for the molecular work was elongation factor 1α (EF-1α) based on its stable tissue specific expression level demonstrated in a previously published molecular study performed on Lake Sturgeon by Allen et al. (2011). Forward and reverse primer sequences for TRα and TRβ receptors were ordered according to the specifications provided by Dr. Habibi's lab and the EF-1α primer pairs were ordered according to Allen et al. (2011).

All primer sets (Table 1) were ordered through Sigma Aldrich and once received, primer sets were tested on Lake Sturgeon cDNA using PCR and gel electrophoresis. Gel products from TRα, TRβ, and EF-1α primer sets were then cut out of the gel for purification. Excised gel fragments were weighed and placed in 1.5ml snap-cap vials and were purified using a commercially available E.Z.N.A gel extraction kit (Omega Bio-tek, Norcross, GA). Briefly, 1 volume of Binding Buffer (XP2) was added to each tube containing a known gel weight and assuming a gel density of $1 g.ml^{-1}$. Samples were then incubated at $60^{o}C$ for 7 minutes or until the gel completely melted making sure to vortex every 2-3 minutes. Once completely dissolved, solutions were pipetted (700µl at a time) into separate HiBind DNA Mini Columns enclosed within a 2 ml collection tube (provided with the kit). Columns were then centrifuged at 10,000g for 1 minute at room temperature and the filtrates were discarded and the tube reused. 300µl of Binding Buffer was then added to each column and the samples were centrifuged at 13,000g for 1 minute at room temperature. The filtrates were discarded and the tubes reused. 700µl SPW Wash Buffer (provided in the kit) was then added to each column and centrifuged at 13,000g for 1 minute at room temperature. The filtrates were discarded and the tubes reused. The empty columns were then spun down at 15,000g for 2 minutes at room temperature in order to dry the column matrix and remove any residual ethanol from the SPW Wash Buffer. The columns were then transferred to newly labeled 1.5ml tubes and 40µl of Elution Buffer (provided in the kit) was added directly onto the column membrane and allowed to sit for 2 minutes. Columns were then centrifuged at 15,000g for 1 minute at room temperature and the eluted products were stored for sequencing. Isolated gel products were sent to the DNA Sequencing Facility at the Robarts Research Institute (London, ON, Canada) for DNA sequencing. Once the sequences were determined they were entered into BLAST and compared to known DNA sequences for TRα,

TRβ, and EF-1α receptors where it was confirmed the primers were amplifying the correct products.

Table 2.1: Forward (F) and reverse (R) primer sequences used for real-time analysis of thyroid hormone receptor and elongation factor expression.

Real-time PCR Primer	Nucleotide Sequence (5' to 3')	Annealing Temp. (^{o}C)	Product Size (bp)
TRalpha F	CCACTGGAAACAGAAGCGCAAGTT	66.4	~110
TRalpha R	TATCTTGGTGAACTCGCTGAAGGC	66.4	
TRbeta F	AGATCGTCCTGGTCTTGCAAGTGT	66.4	~110
TRbeta R	TTTGGGCCAGAAGTGAGAGACGTT	66.4	
EF-1α-RT-F1	TGGCATCACCATTGACATCT	64.0	237
EF-1α-RT-R1	AGCTGCTTCACACCCAGAGT	64.0	

2.3.4 cDNA Synthesis

Reverse transcription of the DNase treated RNA samples was performed using an iScript cDNA Synthesis Kit (Bio-Rad, Cat. No. 170-8891) according to the manufacturers protocol. In clean PCR tubes 4µl 5X iScript reaction mix, 1µl iScript reverse transcriptase, 5µl DNase treated RNA, and 10µl Nuclease free water was added to each tube. Samples were then placed in a thermocycler (Vapo-protect, Eppendorf, Mississauga, ON) and run for 5 minutes at 25^{o}C, 30 minutes at 42^{o}C, and 5 minutes at 85^{o}C. The cDNA samples were then tested using PCR analysis and gel electrophoresis to verify production of a cDNA product (Fig 3.11).

2.3.5 RT-PCR Procedure

cDNA samples generated from the previous step were then used to quantify the expression level of both thyroid receptor sub-types TRα and TRβ. Real-time analyses were performed using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, Cat. # 172-5201) at a

reaction volume of 10µl. The 10µl reaction volume contained 5µl EvaGreen Supermix, 3.6µl molecular clean water, 0.2µl of the forward primer, 0.2µl reverse primer and 1µl cDNA. A master mix solution was prepared for a total of 50 reactions and 9µl of master mix was carefully pipetted into each well. After pipetting the master mix, cDNA samples were pipetted into the corresponding wells and the caps were placed on the wells. Samples were shaken to mix solutions and centrifuged briefly (2-5 seconds) to ensure the complete 10µl solution was at the bottom of each tube. PCR strips containing the reaction mixtures were then placed in a MJ Mini Personal Thermocycler connected to a MiniOpticon Real-Time PCR System (Bio-Rad, Hercules, CA). Specific PCR parameters were programmed using CFX Manager Software (Bio-Rad) and were different for each of the two target genes and the internal standard. TRα and TRβ expression samples were held at 95^{o}C for 3 minutes followed by 40 cycles of: 20 seconds at 95^{o}C and 15 seconds at 66.4^{o}C. For EF-1α expression samples were held at 95^{o}C for 3 minutes followed by 40 cycles of: 15 seconds at 95^{o}C and 20 seconds at 64^{o}C. Melt curves were performed at the end of each PCR run at 1^{o}C intervals from 60^{o}C to 95^{o}C in order to ensure no contamination was amplified. EF-1α expression was assessed to confirm expression level did not differ significantly among treatment groups, tissue samples, and time-points. Expression results from the extracted tissue samples for TRα and TRβ were normalized using corresponding values from the internal standard EF-1α. Results from whole-body homogenates at early developmental time-points were presented as absolute values in femtograms cDNA in a manner similar to that previously described by Cruz et al. (2013) due to the lack of a suitable internal standard. All molecular measurements and results were analyzed for statistical difference using a one-way ANOVA with a Tukeys HSD test to compare expression level throughout development and between tissues (Graphpad Prism 5, La Jolla, CA, USA).

2.4 Thyroid Hormone Levels

2.4.1 Whole-body Thyroid Hormone Extraction

Whole-body larval samples stored at -80^{o}C were used for TH extraction according to a modified protocol published by Shi et al. (2009). Larvae from each tank were pooled together up to a maximum mass of 1.4g (~20-30 larvae depending on time-point) and final weights were recorded. Samples older than 12dph were minced on ice with a scalpel to aid homogenization After weighing, samples were placed in 2ml eppendorf snap cap vials and immersed in 0.5ml ice-cold methanol containing 1mM 6-N-propylthiouracil (PTU) and 1% by volume 1M ammonium hydroxide. Samples were then homogenized using a TissueLyser II (Qiagen) for 2 minutes or until tissue was completely broken down. Once homogenized, samples were sonicated on ice for 20 seconds using a 150T Ultrasonic Dismenbrator (Fischer Scientific) at 70% output and vortexed vigorously. Samples were then centrifuged using a Heraeus Multifuge X3R centrifuge (Thermo Scientific) at 3500g for 20 minutes at 4^{o}C. The supernatants were then transferred to clean 70mm plastic assay tubes and kept on ice. The pellets were then resuspended in 0.5ml methanol containing 1mM PTU and 1% by volume 1M ammonium hydroxide. The samples were then re-sonicated, vortexed, and centrifuged. The supernatants were then pooled and dried down completely at room temperature using a Savant ISS110 SpeedVac Concentrator (Thermo Scientific). Once dried, samples were re-suspended in 0.2ml methanol, 0.8ml chloroform, and 0.2 ml barbital buffer and vortexed vigorously for 1-2 minutes. The samples were then centrifuged at 3500g for 15 minutes at 4^{o}C and the aqueous supernatant was removed and placed in clean 1.5ml snap cap vials. The supernatants were dried down again as described above and the samples were then re-suspended in 0.4ml phosphate buffer (pH 7.4) and stored at -80^{o}C until analysis using a

commercially available kit for total and free T_3 and T_4 (MP Biomedicals, Diagnostic Division, Orangeburg, NY, USA).

2.4.2 Thyroid Hormone Radioimmunoassay

Free T_3 (Cat. No. 06B-258710) and total T_3 (Cat. No. 06B-254216) as well as free T_4 (Cat. No. 06B-257214) and total T_4 (Cat. No. 06B-254029) were measured using commercially available kits (MP Biomedicals). Assays were carried out according to the protocols provided with each kit. Briefly, samples were removed from -80^{o}C and thawed on ice. Once thawed a specified amount of sample specific to each kits protocol (free T_3: 100µl, total T_3: 100µl, free T_4: 50µl, and total T_4: 25µl) was added to the anti-body coated tubes provided in the kits. Standard curve solutions were also added using the same volumes used for the samples. Once standards and samples were pipetted into individual tubes 1ml [^{125}I] Tracer solution was added to each tube and the contents were shaken to ensure complete mixing. All tubes were then incubated at 37 ± 1^{o}C in a water bath. Incubation times were dependent on the type of kit being used: 60 minutes for total T_3 and T_4, 90 minutes for free T_4, and 150 minutes for free T_3. After incubation the tracer/sample solutions were poured off and blotted dry and 1ml of water was added to each tube. Tubes were gently shaken again and the water was poured off and the tubes blotted dry for a second time. Once blotted dry the tubes were measured for radioactivity using a Wizard^2 2480 Automatic gamma counter (PerkinElmer, Woodbridge, ON). Thyroid hormone concentrations were determined by comparing the counts per minute (CPM) measured from the samples (single assay) with the known standard concentrations provided in the assay kits. All TH measurements and results were analyzed for statistical difference using a a non-parametric Kruskal-Wallis test with a Dunn's multiple comparison post hoc test to compare TH levels throughout development (Graphpad Prism 5, La Jolla, CA, USA). Due to measurement limitations in the assay kits used

for free T3 only undetectable samples were assigned a value randomly selected between zero and the minimal detectable limit of the assay.

2.5 Deiodinase Activity

2.5.1 Tissue Collection

Liver and plasma samples were taken from ten young-of-the-year (YOY), ten one-year-old, and ten two-year-old Lake Sturgeon held at the animal holding facility at the University of Manitoba. Sturgeon were sacrificed by immersion in a lethal dose of buffered MS222 (250ppm) and blood samples were immediately collected from the caudal sinus according to Palace et al. (2010). Blood samples were centrifuged at 8000g for 3 minutes and the plasma was removed, placed in clean 1.5ml screw cap tubes, and flash frozen in liquid nitrogen. Upon blood collection individual liver samples were taken and their masses recorded. Liver samples were then flash frozen in liquid nitrogen and stored at -80^{o}C.

2.5.2 Enzyme Extraction and Isolation

Extraction of deiodinase and production of the fractions was performed using a modified version of the extraction procedure described by Tomy et al. (2007). Samples were removed from -80^{o}C and allowed to thaw on ice. Individual tissues were then removed from their original vials and placed in 15ml Falcon tubes on ice. Samples were suspended in 0.1M sodium phosphate buffer (combine 80ml 1M K_2HPO_4 and 20ml 1M NaH_2PO_4 then dilute with water to 1L) spiked with 1mM Ethylenediaminetetraacetic acid (EDTA) and 20mM Dithiothreitol (DTT) at pH 7.4. Once samples were suspended in buffer they were homogenized on ice using a Polytron PT2100 for two minutes. 1ml of the tissue homogenates were taken and transferred to

clean 1.5ml eppendorf tubes and centrifuged at 28,000g for 10 minutes at 4°C. The supernatants (S9) were carefully pipetted off and placed in new 1.5ml tubes and frozen at -80°C.

2.5.3 Original Deiodinase Assay

Four glass tubes were prepared per S9 fraction according to table 2.2. 1ml of ethanol was added to each of the two "denatured" tubes to deactivate the enzyme prior to the addition of the T_4 substrate in order to prevent any conversion of T_4 to T_3. The two denatured tubes acted as negative controls and their values were subtracted from those containing the active enzyme. All tubes were incubated at 37°C for 90 minutes in a water bath. After 90 minutes the tubes were placed on ice and the reaction was stopped by the addition of 1ml 100% ice-cold methanol. The production of T_3 in all the samples was measured by radioimmunassay using a commercially available T_3 kit (see section 2.4.2 for the detailed assay procedure).

Table 2.2: Original deiodinase assay setup including volumes of buffer used per tube and concentrations of T_4 substrate.

	Active	**Denatured**
# of Incubations	2	2
Deiodinase Buffer	15 µl	15 µl
S9 Fraction	10 µl	10 µl
Ethanol	-	1000 µl
Thyroxine (600ng/µl)	5 µl	5 µl

2.5.4 Modified Deiodinase Assay

Due to the large cross-reactivity observed at the specific substrate concentrations (T_4) used in the protocol described above, the substrate concentration was reduced and combined with a slightly modified experimental design, measureable deiodinase activities were obtained. The

assay used here involves incubating the enzyme samples with excess substrate (T_4) and measuring the amount of T_3 created by outer-ring deiodination in each sample. Briefly, each S9 sample was run in triplicate. In all tubes minus controls 15µl deiodinase buffer, 10µl S9 fraction, and 5µl T_4 spike at a concentration of 8ng/µl for a total T_4 substrate concentration of 40ng was added. Control tubes, also assessed in triplicate, contained 15µl deiodinase buffer, 10µl spiked deiodinase buffer (1mM EDTA and 20mM DTT) and 5µl T_4 spike (8ng/µl). All tubes were then incubated at 37^{o}C for 90 minutes in a water bath. After 90 minutes the reaction in all tubes was stopped with the addition of 1ml ice-cold methanol and samples were gently mixed. The production of T_3 in all the samples was measured by radioimmunassay using a commercially available free T_3 kit (see section 2.4.2 for the detailed assay procedure). All deiodinase measurements and results were analyzed for statistical difference using a one-way ANOVA with a Tukeys HSD test to compare against controls (Graphpad Prism 5, La Jolla, CA, USA).

Protein content of samples for deiodinase measurement was analyzed using a Quick-Start Bradford protein assay kit (Bio-Rad, Cat. #500-0201) according to the protocol provided in the assay kit. Standard curve concentrations (25, 20, 15, 10, 5, 2.5, 1.25µg.ml^{-1}) were made using the same phosphate buffer used in the deiodinase assay starting from a stock bovine serum albumin (BSA) concentration of 2mg.ml^{-1}. The 1X dye reagent provided with the assay kit was removed from the fridge and allowed to come to room temperature prior to running the assays. 150µl of the protein standards and deiodinase samples were added to micro-plates in duplicate and combined with 150µl of 1X Dye reagent for a total assay volume of 300µl. Samples were then allowed to sit at room temperature for 10 minutes before analysis. After incubation samples were analyzed using a PowerWave XS2 micro-plate reader set at a wavelength of 595nm and the

computer program Gen5 (BioTek, Winooski, VT, USA). The protein content of each sample was used to calculate deiodinase activity.

3.0 Results

3.1 Larval Growth

No difference in larval mortality was observed between any of the chlorpyrifos treatments when compared to controls (Fig 3.1). At the beginning of the exposure larvae had an average total length of 12.81 ± 0.06mm and at the onset of exogenous feeding larvae had an average total length of 21.13 ± 0.09mm across all treatments (Fig 3.2). There was no statistically significant difference in larval length between control and treatment tanks at 3, 6, 9, 12 and 21dph. However, at 36 and 67dph mean larval total length in the low chlorpyrifos treatment (41.35 ± 1.77mm and 58.40 ± 1.54mm respectively) was significantly greater when compared to all other treatments.

Larval body mass at the beginning of the exposure was 18.5 ± 0.1mg and at the onset of exogenous feeding larval body mass was 33.8 ± 0.2mg (Fig 3.3). There was no statistically significant difference in larval body mass in control and treatment tanks at 3, 6, 9, 12, and 21dph, however, at 36 and 67dph average body mass from larvae in the low treatment tanks (219 ± 25mg and 638 ± 45mg respectively) was significantly greater when compared to average body mass from larvae in the control treatment (153 ± 11mg and 446 ± 29mg respectively).

Larval growth was also analyzed using Fulton's condition factor (CF) (Fig 3.4). The only significant difference in condition factor was at the first time-point, 3dph, where control and high tank values (0.86 ± 0.02 and 0.86 ± 0.03 respectively) were significantly lower than the medium tanks (0.92 ± 0.03).

3.2 Thyroid Follicle Development

Primordial thyroid tissue was first observed in Lake Sturgeon histological sections at 6dph from each of the control and chlorpyrifos treatments and was tracked throughout development at all subsequent time-points (Fig. 3.5; all images of control samples). Distinct thyroid follicles with a clear colloid were first observed at 12dph. Structural development of thyroid follicles continued from 12dph culminating in fully developed and active follicles observed in all larvae sampled at 67dph. The identity of thyroid tissue at the early larval life stages particularly larvae with the yolk-sac still present (6, 9, and 12dph) was confirmed using T_4-antibody labeled immunohistochemistry (IHC) (Fig. 3.6). Tissues containing T_4 are represented by dark brown stained areas. Fully developed and active thyroid follicles can be seen in the IHC micrograph from 67dph which shows the dark brown stained colloid indicating the presence of T_4 within the thyroid follicles. The presence of colloid vacuoles at 67dph also indicates the presence of active thyroid follicles (Fig 3.7).

Thyroid follicular cell height, although not statistically so, was highest for all treatments at 6dph but was quite variable between treatments for the remainder of the developmental time-points (Fig 3.8). Measurements from each treatment and time-point represent means calculated with an n=8 individuals. At 6dph follicular cell height was greatest in larvae from the low treatment tanks (12.33 ± 0.42μm) when compared to larvae from all other treatments (control: 10.62 ± 0.45μm, medium: 10.78 ± 0.44μm, high: 10.56 ± 0.32μm). At 9dph the follicular cell height was lowest in larvae from the control treatment (8.67 ± 0.34μm) when compared to all other treatments (low: 9.77 ± 0.22μm, medium: 9.73 ± 0.34μm, high: 9.65 ± 0.27μm) (Fig 3.8). At 12dph follicular cell height was lowest in the control treatment group (7.49 ± 0.30μm) and highest in the medium treatment group (8.99 ± 0.40μm) but no difference was observed between the remaining treatments (Fig 3.8). At 21dph there were no statistically significant differences

among treatment groups when compared to the control tanks, however, the follicular cell height in the medium chlorpyrifos treatment group (7.67 ± 0.24μm) was significantly lower than the low (8.80 ± 0.40μm) and high (8.79 ± 0.19μm) exposure treatments (Fig 3.8). There was no significant difference in follicular cell height between any treatment groups at 39dph but at 67dph follicular cell height in the high treatment group (8.29 ± 0.15μm) was the lowest (Fig 3.8).

Thyroid follicle nucleus cell height was not as variable between treatments or across timepoints, the only significant differences were found at the 6 and 21dph timepoints (Fig 3.9). At 6dph the follicle cell nucleus height was significantly different between the low and medium treatment groups (6.03 ± 0.13μm and 5.57 ± 0.15μm respectively) and at 21dph follicle cell nucleus height was significantly lower in the medium treatment (4.90 ± 0.10μm) when compared to the control (5.46 ± 0.11μm) and high (5.44 ± 0.14μm) treatment groups (Fig. 3.9).

Thyroid follicle cell height and nucleus height measurements from treatments where there was no significant difference between treatments were pooled together at each time-point ($n \geq 18$; Fig 3.10). Follicle cell height was significantly higher in 6dph and 9dph larvae (11.06 ± 0.22μm and 9.52 ± 0.15μm respectively) when compared to 12, 21, 36, and 67dph larvae (8.23 ± 0.17μm, 8.39 ± 0.15μm, 8.76 ± 0.12μm, and 8.67 ± 0.12μm respectively) (Fig 3.10A). Thyroid follicle nucleus height was significantly lower in 12 and 21dph larvae (5.31 ± 0.07μm and 5.27 ± 0.06μm respectively) when compared to 6, 9, 36, and 67dph samples (5.79 ± 0.07μm, 5.88 ± 0.07μm, 5.70 ± 0.05μm, and 6.05 ± 0.07μm respectively). Follicular nucleus height at 67dph was significantly higher than all other time points.

3.3 Thyroid Receptor Expression

The absolute expression level of TRα and TRβ, was measured in whole larval homogenates at 3, 12 and 21dph (Fig. 3.12A). The three time-points chosen for analysis were identified as important developmental markers. Samples from 3dph represent the first sampled time-point and the baseline for receptor expression, samples from 12dph represent a time-point just prior to the onset of exogenous feeding, and samples from 21dph represent a time-point where larvae were actively feeding exogenously. The expression level of TRα increased significantly from 3 to 12dph but was significantly lower in the 21dph larvae when compared to 12dph larvae. The expression level of TRβ was not significantly different between 3 and 12dph larvae but there was a significant decrease in expression observed in the 21dph larvae when compared to both 3dph and 12dph samples (Fig. 3.12B).

Relative expression of both thyroid receptor α and β was also established in three tissue types (brain, liver, and muscle) from 67dph larvae (Fig. 3.13). No significant difference in TRα expression was observed in either of the three tissue types. However, there was a difference in tissue expression with respect to TRβ where both brain and liver had significantly higher relative expression when compared to muscle. There were also significant differences between TRα and TRβ expression within tissues. In all three tissues relative expression of TRα was significantly higher than TRβ expression. Fig 3.11 is a gel electrophoresis image showing the gel products (TRα, TRβ, and EF-1α) of cDNA amplified via PCR from larval Lake Sturgeon.

3.4 Thyroid Hormone Levels

Whole-body levels of free T_3 (FT3), total T_3 (TT3), free T_4 (FT4), and total T_4 (TT4) were measured in Lake Sturgeon larvae throughout development. FT4 was not detectable in any of the

samples analyzed (36 and 67dph samples). TT3 and TT4 levels were only detected in a few samples throughout development whereas FT3 was detectable in samples at all time-points throughout development (Table 3.1, 3.2). There were no significant difference in whole-body FT3 plotted using pg FT3.g tissue^{-1} between 3, 6, 9, 12, and 21dph, however, FT3 levels at 36 and 67dph (0.041 ± 0.006 and 0.08 ± 0.02pg.g tissue^{-1} respectively) were significantly lower than 3, 6, 9, and 21dph (Table 3.1). When FT3 samples were plotted using pg FT3.larvae^{-1} levels were significantly higher at 21dph when compared to all other time-points. Percent FT3 (%FT3) values were determined by dividing FT3 by TT3 and multiplying by 100. The %FT3 decreases prior to 12dph and then rises from 12 to 21dph but results were only measurements of one sample and could not be analyzed statistically (Table 3.1).

Plasma TT3 and TT4 was also measured in ten young of the year (YOY), ten one year old and ten two year old Lake Sturgeon (Fig 3.14). TT4 was not detectable at any of the time-points. The mean TT3 levels in plasma samples of YOY Lake Sturgeon (n=8,) and one year old Lake Sturgeon (n=2) was 26.36 ± 2.99ng/dl and 29.88 ± 4.87ng/dl respectively, interestingly TT3 was not detectable in any of the ten plasma samples analyzed from two year old Lake Sturgeon.

Table 3.1: Whole-body levels of TT3, FT3, and TT4 as well as %FT3 in Lake Sturgeon larvae throughout development. Values represent means ± SE. The n values are represented as the number of detectable samples measured at the particular time-point over the total number of samples analyzed. Significant differences between time-points were established using a Kruskall-Wallace non-parametric test.

Time-point	TT3 (pg.g tissue^{-1})	FT3 (pg.g tissue^{-1})	%FT3	TT4 (ng.g tissue^{-1})
3dph	128.8 (n=1/4)	0.13 ± 0.06 (n=2/4)	0.18	Not Detectable
6dph	Not Detectable (n=0/16)	0.07 ± 0.03 (n=4/16)	N/A	Not Detectable
9dph	151.1 (n=1/16)	0.07 ± 0.03 (n=4/16)	0.16	Not Detectable
12dph	154.1 (n=1/16)	0.08 ± 0.03 (n=7/16)	0.11	Not Detectable
21dph	107.2 ± 20 (n=3/16)	0.12 ± 0.04 (n=7/16)	0.23	Not Detectable
36dph	Not Detectable (n=0/16)	0.02 ± 0.01 (n=7/16)	N/A	5.4 ± 0.2 (n=2/16)
67dph	Not Detectable (n=0/16)	0.08 ± 0.02 (n=16/16)	N/A	5.66 (n=1/16)

Table 3.2: Whole-body levels of TT3 and FT3 in Lake Sturgeon larvae throughout development. Values represent means ± SE. The n values are represented as the number of detectable samples measured at the particular time-point over the total number of samples analyzed. Significant differences between time-points were established using a Kruskall-Wallace non-parametric test.

Time-point	TT3 (pg.larvae^{-1})	FT3 (pg.larvae^{-1})
3dph	2.7 (n=1/16)	0.003 ± 0.001 (n= 60/120)
6dph	Not Detectable	0.002 ± 0.001 (n= 80/320)
9dph	5.4 (n=1/16)	0.002 ± 0.001 (n=120/480)
12dph	5.9 (n=1/16)	0.003 ± 0.001 (n=210/480)
21dph	7.4 ± 1.4 (n=3/16)	0.008 ± 0.002 (n=210/320)
36dph	Not Detectable	0.002 ± 0.001 (n=210/480)

3.5 Deiodinase Activity

The activity of the T_4 outer ring deiodinase (T4ORD) was measured in liver tissue of young of the year (YOY), one year old, and two year old Lake Sturgeon juveniles. T4ORD activity was detectable in liver tissue of all three life-stages. T4ORD activity was significantly higher in the liver of YOY sturgeon (0.24 ± 0.03pmol.hr^{-1}.mg protein^{-1}) compared to one and two year old sturgeon (0.11 ± 0.01 and 0.121 ± 0.008pmol.hr^{-1}.mg protein^{-1}) (Fig 3.15).

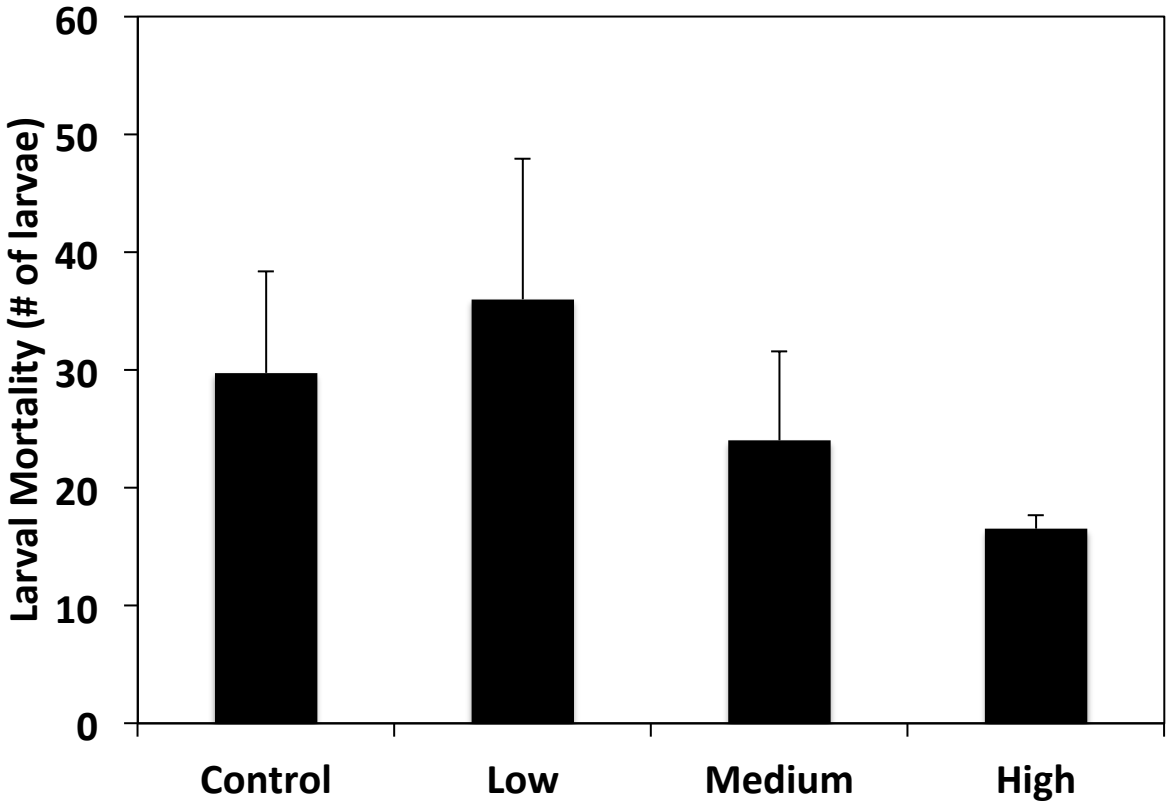

Figure 3.1: Mortality of larval Lake Sturgeon held in flow-through aquaria and exposed to low (5ng.L^{-1}), medium (500ng.L^{-1}), and high (2000ng.L^{-1}) environmental chlorpyrifos concentrations for ten days during development. Values represent means ± SEM, n=4 tanks for each treatment. Significant differences between treatments were established using a one-way ANOVA with no difference between treatments observed ($P<0.05$).

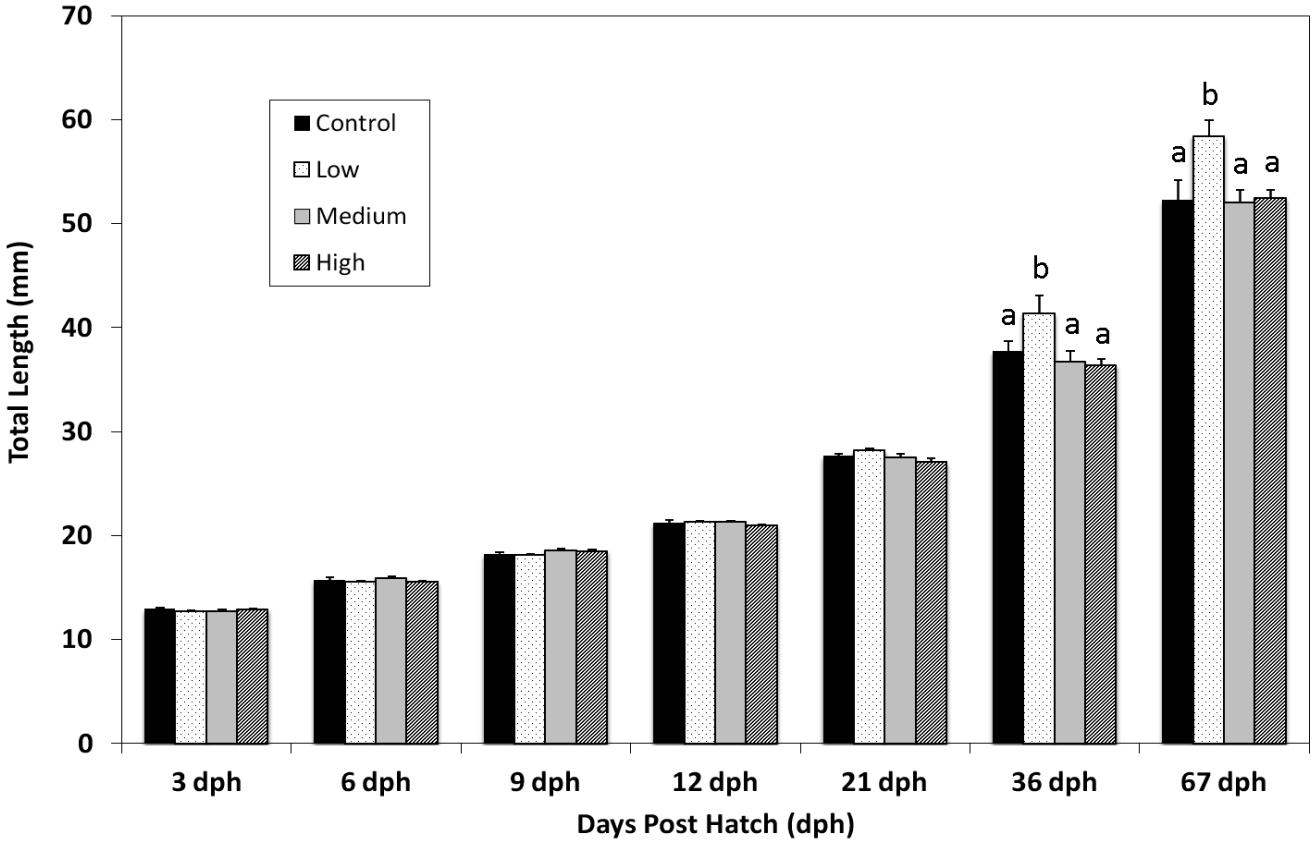

Figure 3.2: Larval Lake Sturgeon total body length when held in flow-through aquaria and exposed to low (5ng.L^{-1}), medium (500ng.L^{-1}), and high (2000ng.L^{-1}) environmental chlorpyrifos concentrations for ten days during development. Values represent means ± SEM, n=12. Significant differences between treatments were established using a two-way ANOVA. Means labelled with different letters are significantly different from each other ($P<0.05$).

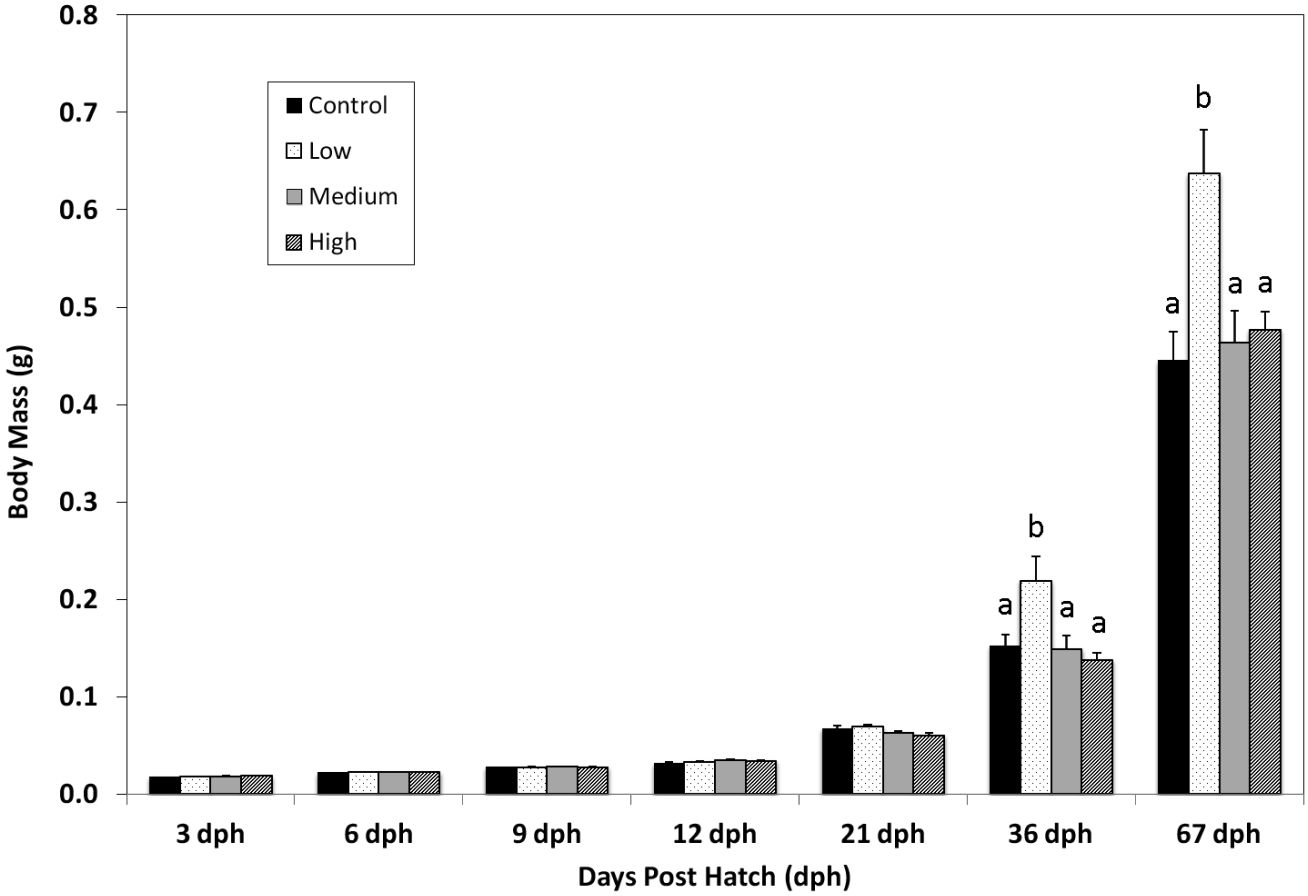

Figure 3.3: Larval Lake Sturgeon body mass when held in flow-through aquaria and exposed to low (5ng.L^{-1}), medium (500ng.L^{-1}), and high (2000ng.L^{-1}) environmental chlorpyrifos concentrations for ten days during development. Values represent means ± SEM with an n=12. Significant differences between treatments were established using a two-way ANOVA. Means labelled with different letters are significantly different from each other ($P<0.05$).

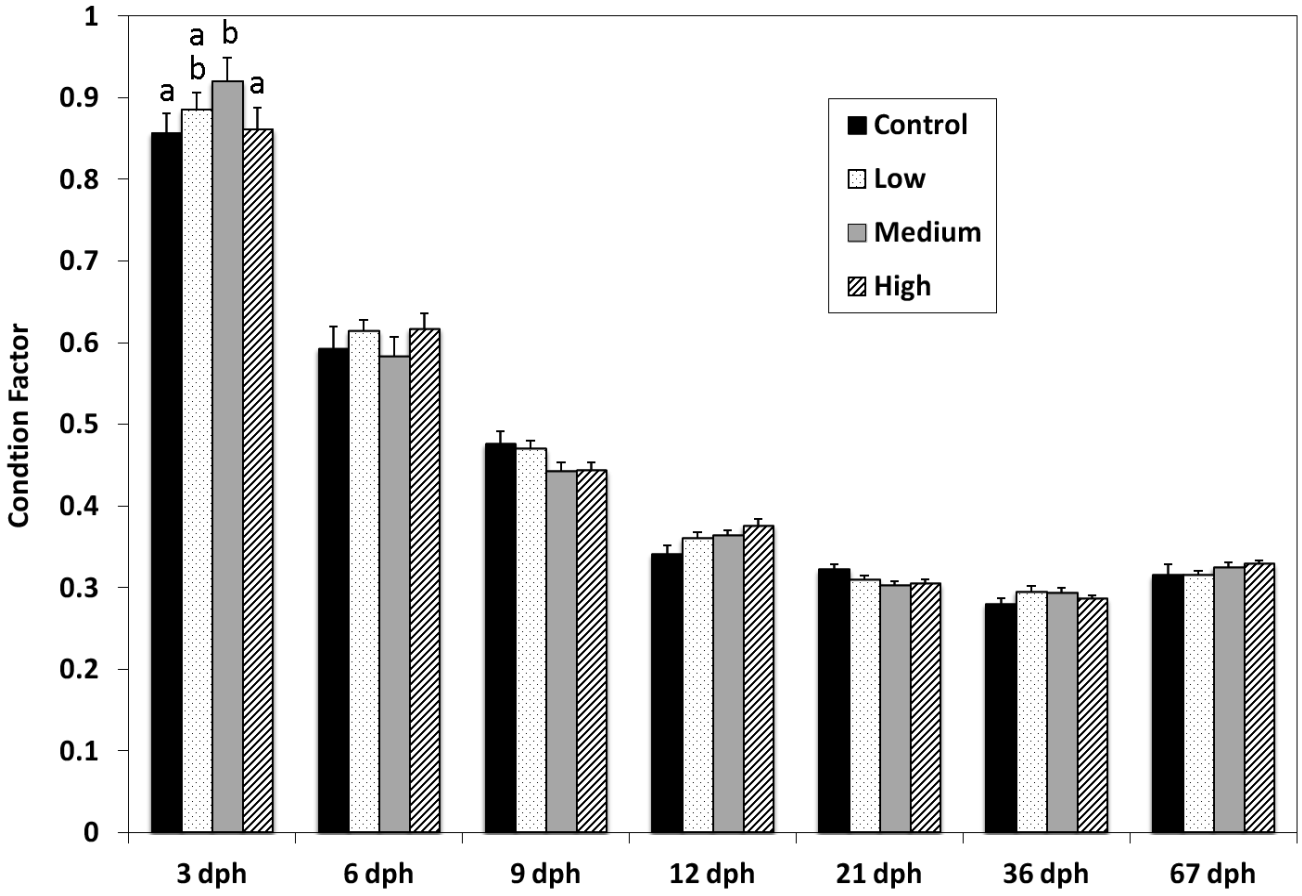

Figure 3.4: Larval Lake Sturgeon condition factor (CF) when held in flow-through aquaria and exposed to low (5ng.L^{-1}), medium (500ng.L^{-1}), and high (2000ng.L^{-1}) environmental chlorpyrifos concentrations for ten days during development. Values represent means ± SEM with an n=12. Significant differences between treatments were established using a two-way ANOVA. Means labelled with different letters are significantly different from each other ($P<0.05$).

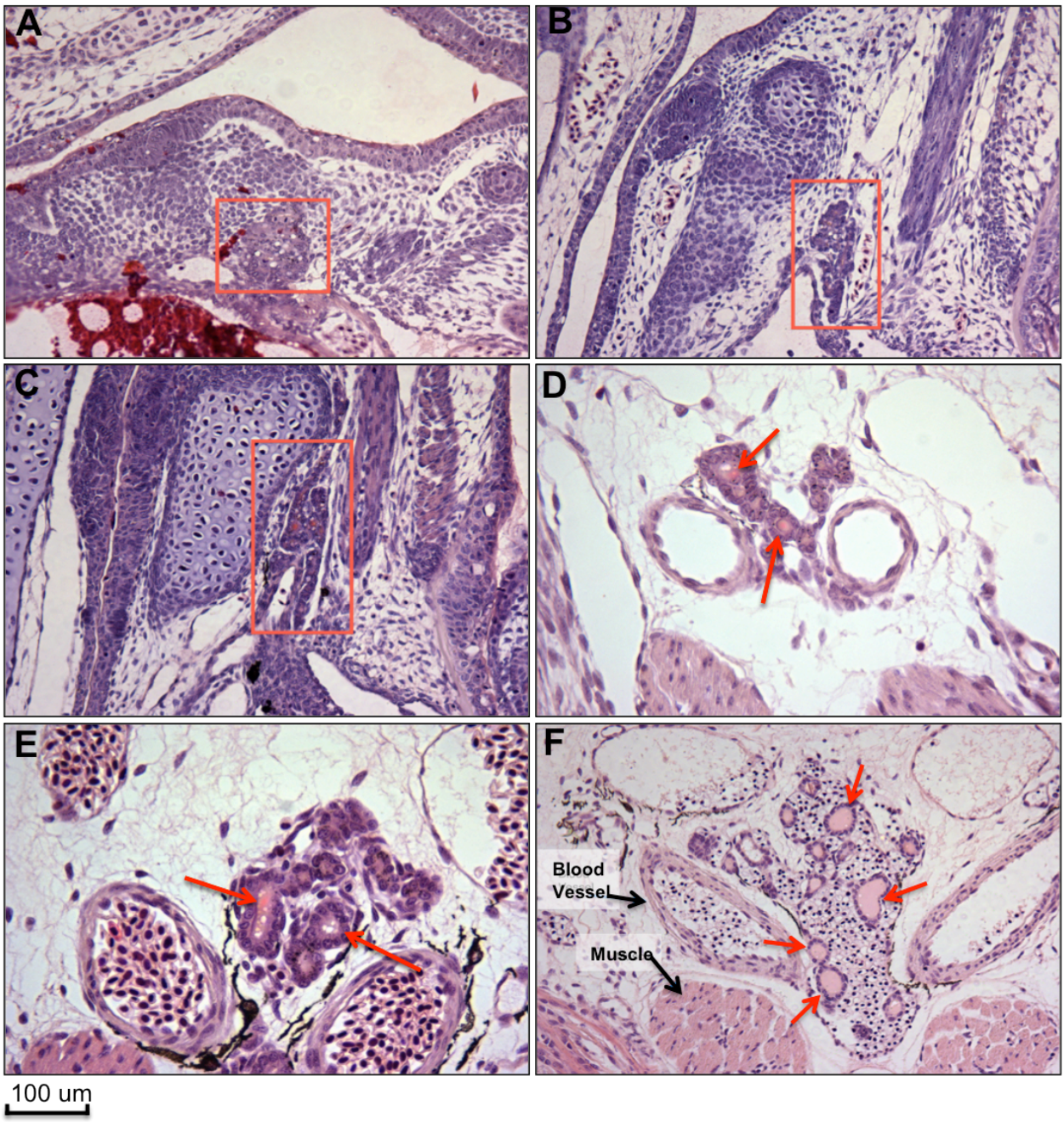

Figure 3.5: Histological sections of Lake Sturgeon larvae taken through the glossopharyngeal region in control fish (magnification: 400X). Sections were stained with Harris's Haematoxylin and Eosin and were used to track thyroid development. Location of developing thyroid tissue is indicated by a box in the early time-points and in later time-points arrows indicate thyroid follicles. A: 6 days post hatch (dph) B: 9dph C: 12dph D: 21dph E: 36dph F: 67dph.

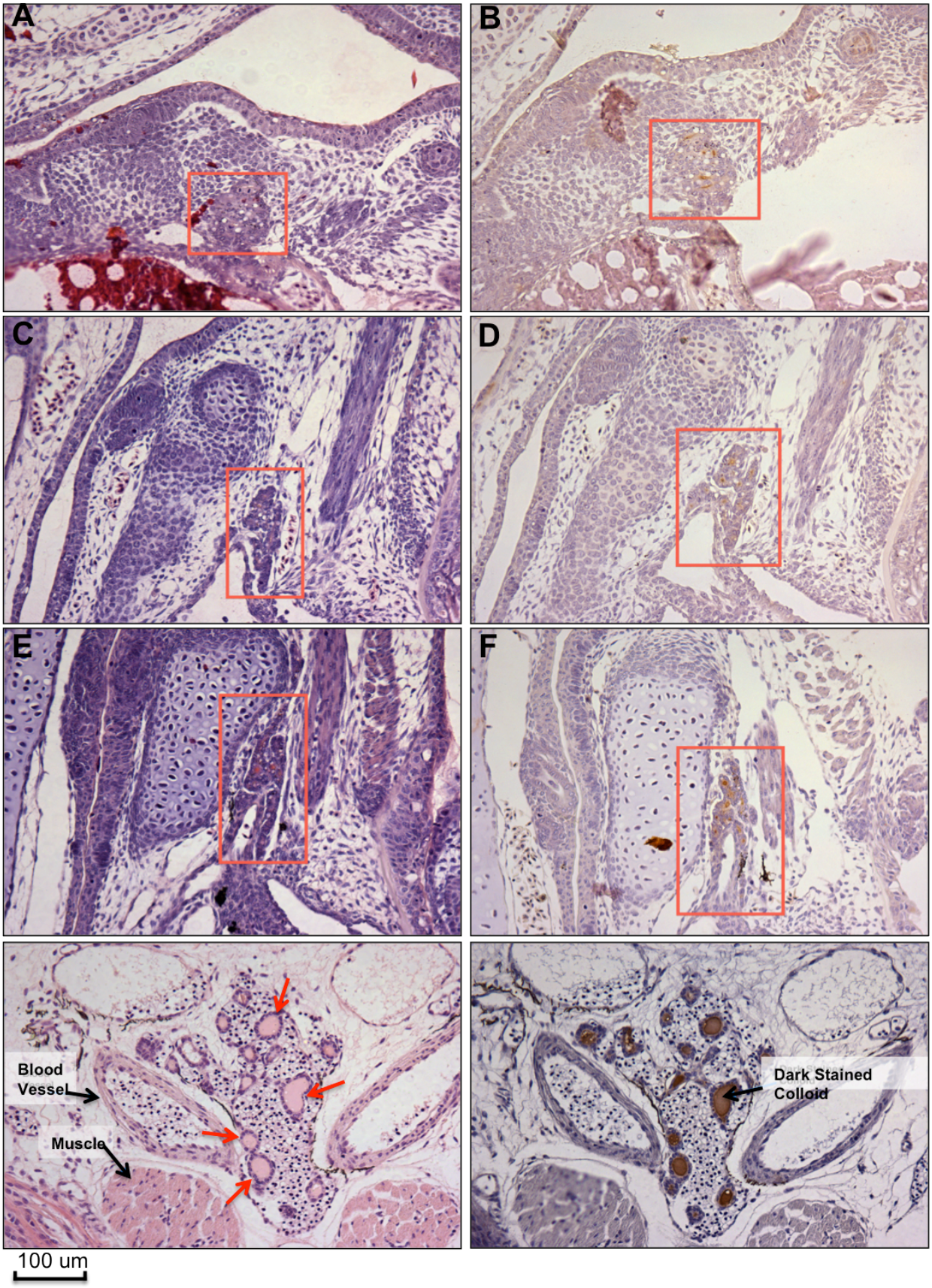
A
B
C
D
E
F
Blood Vessel
Muscle
Dark Stained Colloid
100 um

Figure 3.6: Histological sections of Lake Sturgeon larvae taken through the glossopharyngeal region in control fish (magnification: 400X). Sections were stained with Harris' Haematoxylin and Eosin (H&E) and by T_4-antibody labeled Immunohistochemistry (IHC) to confirm the presence of thyroid tissue. A: H&E stained 6 days post hatch (dph) larvae B: IHC stained 6dph larvae C: H&E stained 9dph larvae D: IHC stained 9dph larvae E: H&E stained 12dph larvae F: IHC stained 12dph larvae G: H&E stained 67dph larvae H: IHC stained 67dph larvae.

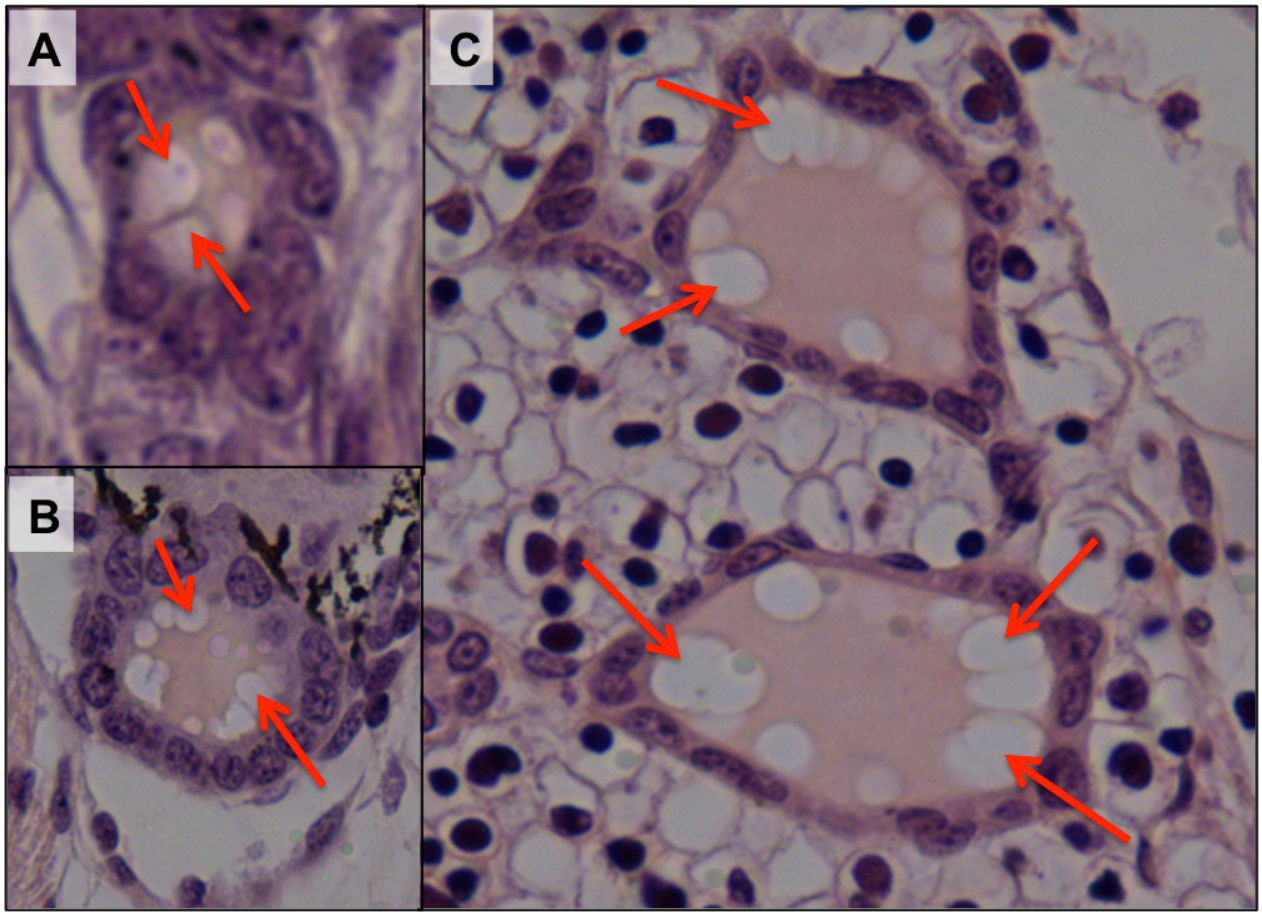

Figure 3.7: Enlarged histological sections of the glossopharyngeal region of 21dph (A), 36dph (B), and 67dph (C) Lake Sturgeon larvae containing thyroid follicles (magnification: 400X). The section was stained with Harris's Haematoxylin and Eosin and the red arrows indicate colloid vacuoles within the thyroid follicle, outlined with purple stained epidermal cells, which is indicative of follicular activity.

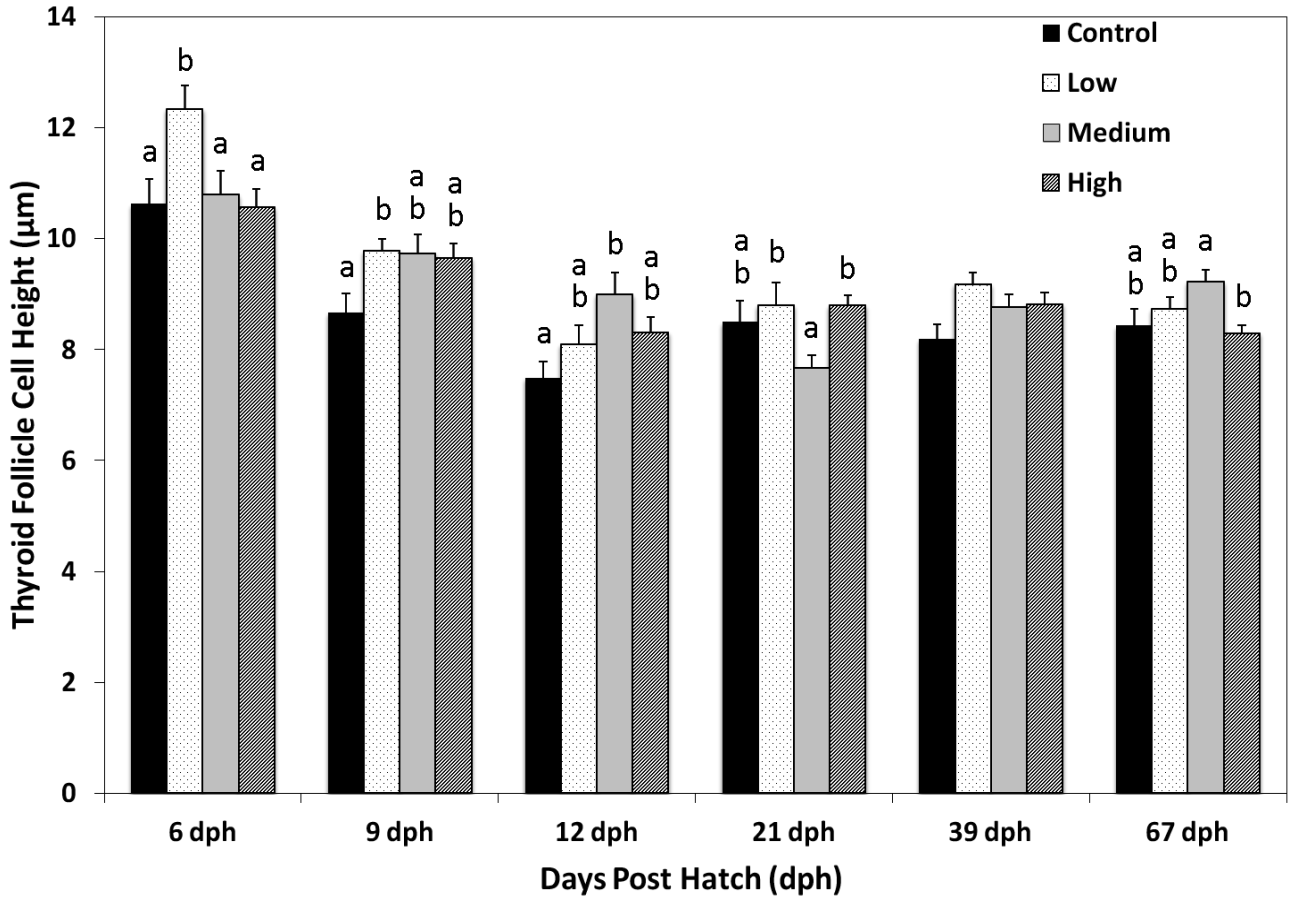

Figure 3.8: Larval Lake Sturgeon thyroid follicle cell height when held in flow-through aquaria and exposed to low (5ng.L^{-1}), medium (500ng.L^{-1}), and high (2000ng.L^{-1}) environmental chlorpyrifos concentrations for ten days during development. Values represent means ± SEM with an n=8. Significant differences between treatments were established using a two-way ANOVA. Means labelled with different letters are significantly different from each other ($P<0.05$).

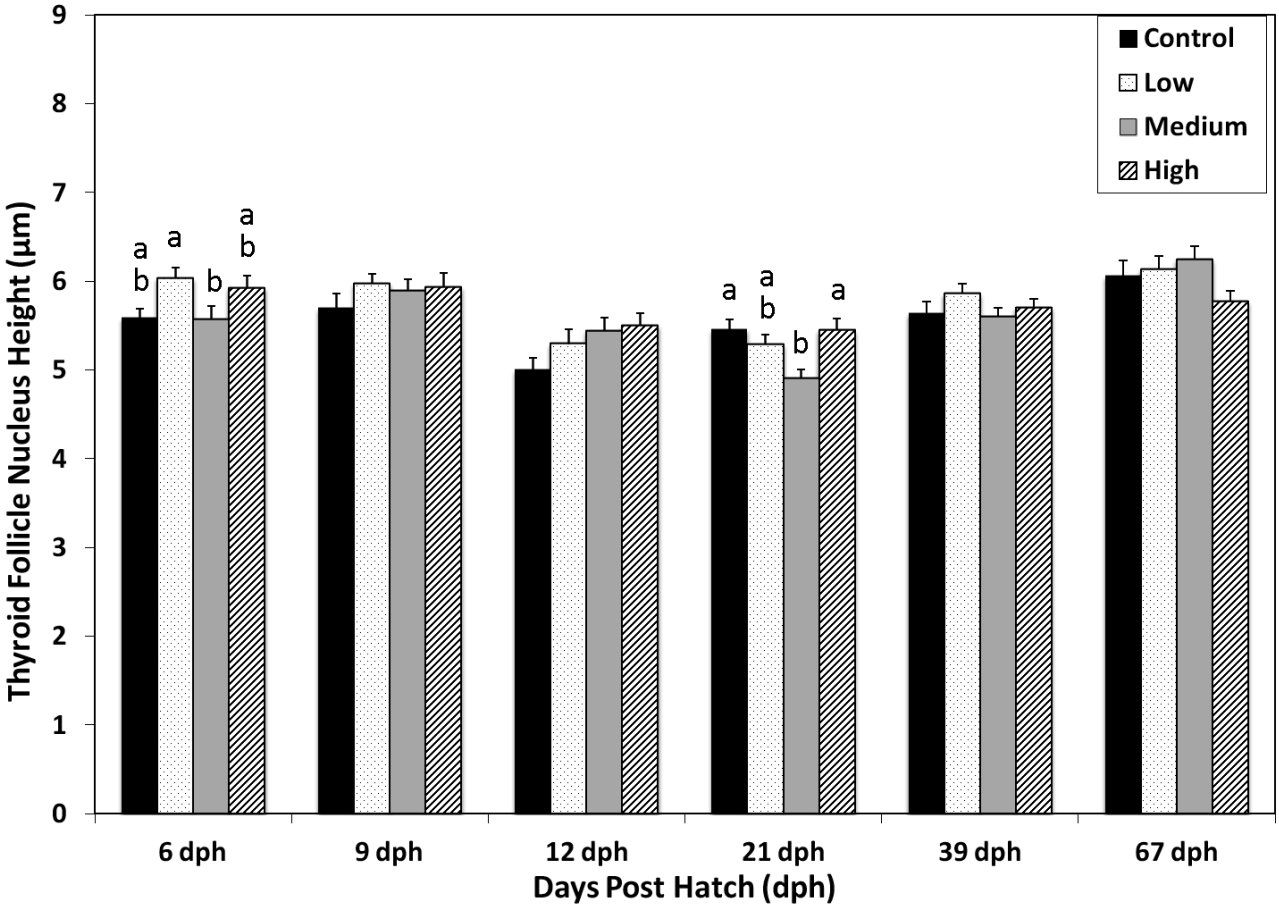

Figure 3.9: Larval Lake Sturgeon thyroid follicle nucleus height when held in flow-through aquaria and exposed to low (5ng.L^{-1}), medium (500ng.L^{-1}), and high (2000ng.L^{-1}) environmental chlorpyrifos concentrations for ten days during development. Values represent means ± SEM with an n=8. Significant differences between treatments were established using a two-way ANOVA. Means labelled with different letters are significantly different from each other ($P<0.05$).

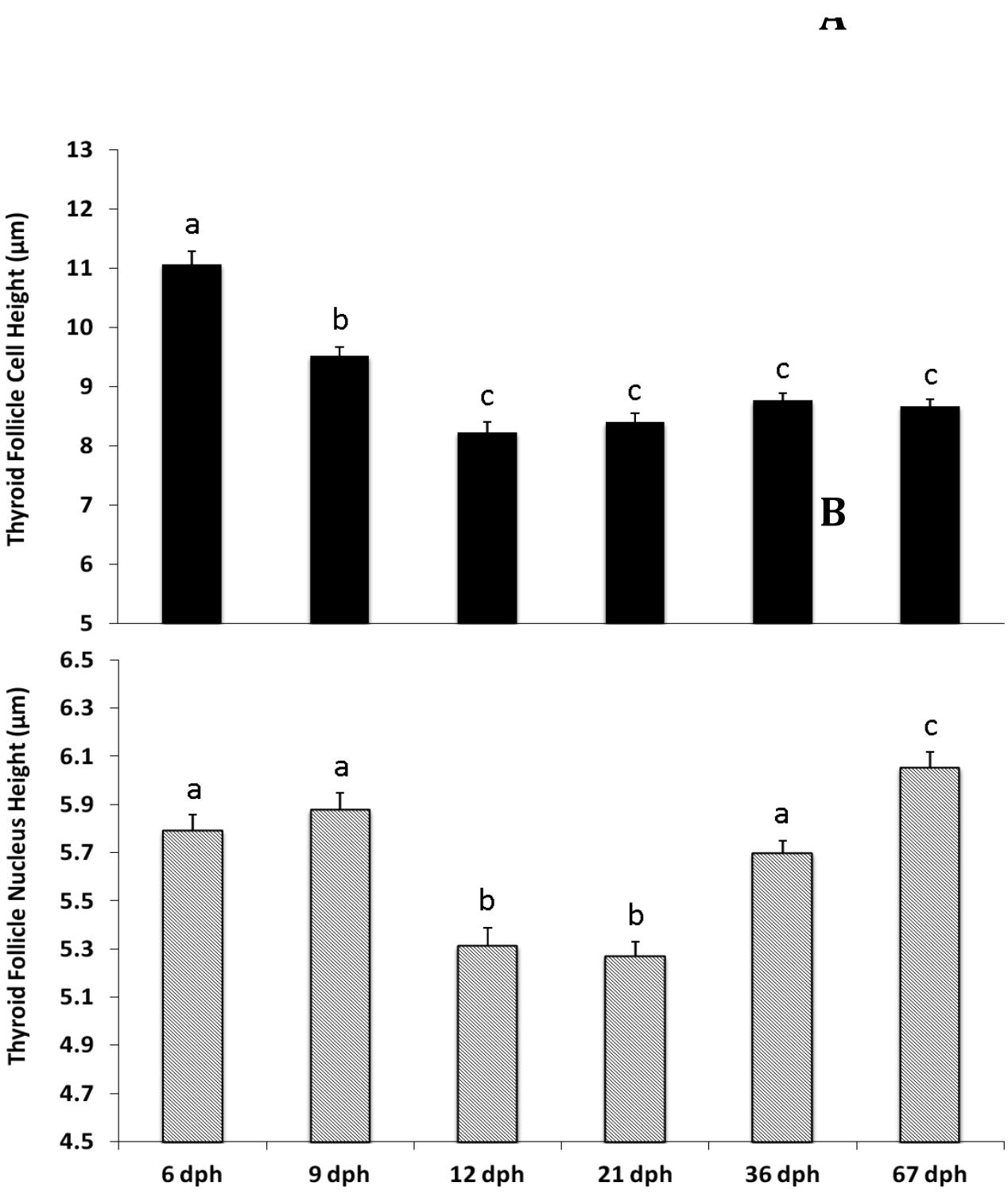

Figure 3.10: Pooled larval Lake Sturgeon thyroid follicle cell height (A) and nucleus height (B) measurements (values are expressed as means ± SEM, with an n ≥ 18 larvae). Significant difference in follicular cell height and nucleus height between time-points was determined using a one-way ANOVA. Means labelled with different letters are significantly different from each other ($P<0.05$).

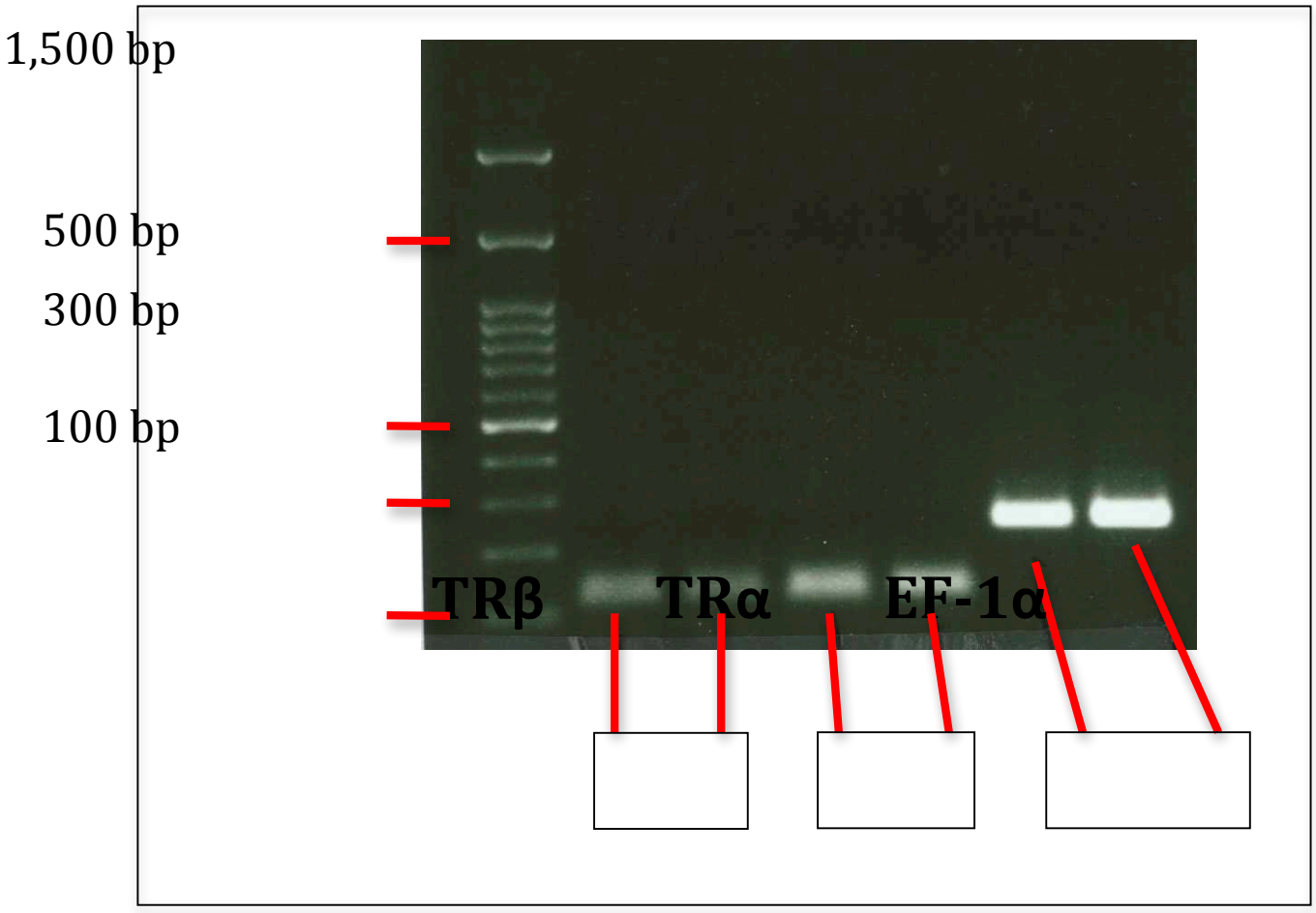

Figure 3.11: A gel electrophoresis image of amplified cDNA created from mRNA isolated from Lake Sturgeon larval samples. The figure shows clear bands indicating the two main genes in question, TRα and TRβ, just above 100 base pairs (bp) and the internal standard, EF-1α, just under 300bp. The lane on the far left of the figure represents the TECDNA-06R ladder with a range from 100-3000bp.

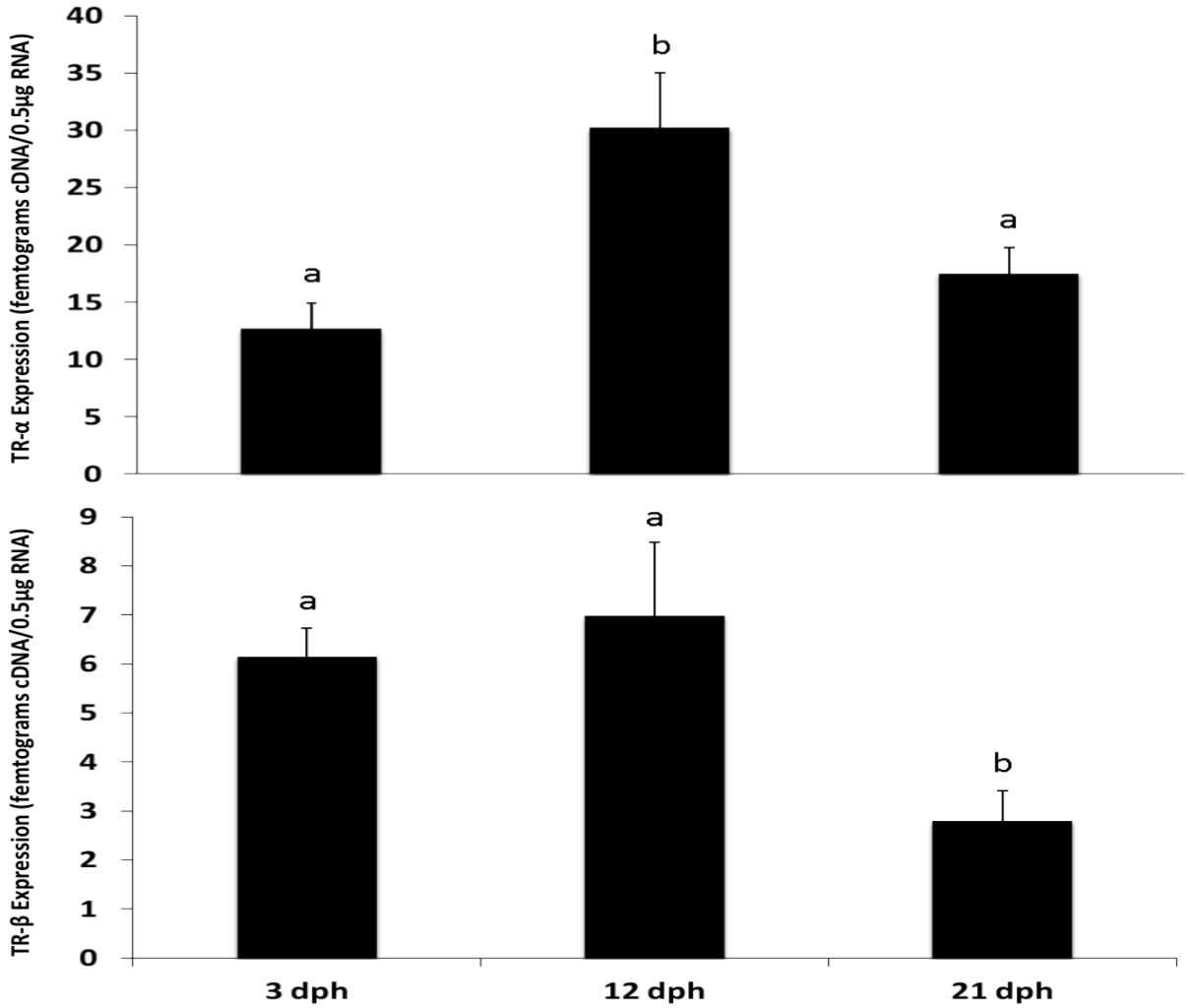

Figure 3.12: Whole-body absolute TR-α (A) and TR-β (B) expression in Lake Sturgeon larvae from control tanks over three developmental time-points measured using RT-PCR. The cDNA used for RT-PCR was reverse transcribed from DNase treated RNA isolated from whole-body Lake Sturgeon homogenates. Values represent means ± SEM, with an n=12 for 3dph and n=6 for 12 and 21dph. Any significant difference in expression between time-points was established using a one-way ANOVA. Means labelled with different letters are significantly different from each other ($P<0.05$).

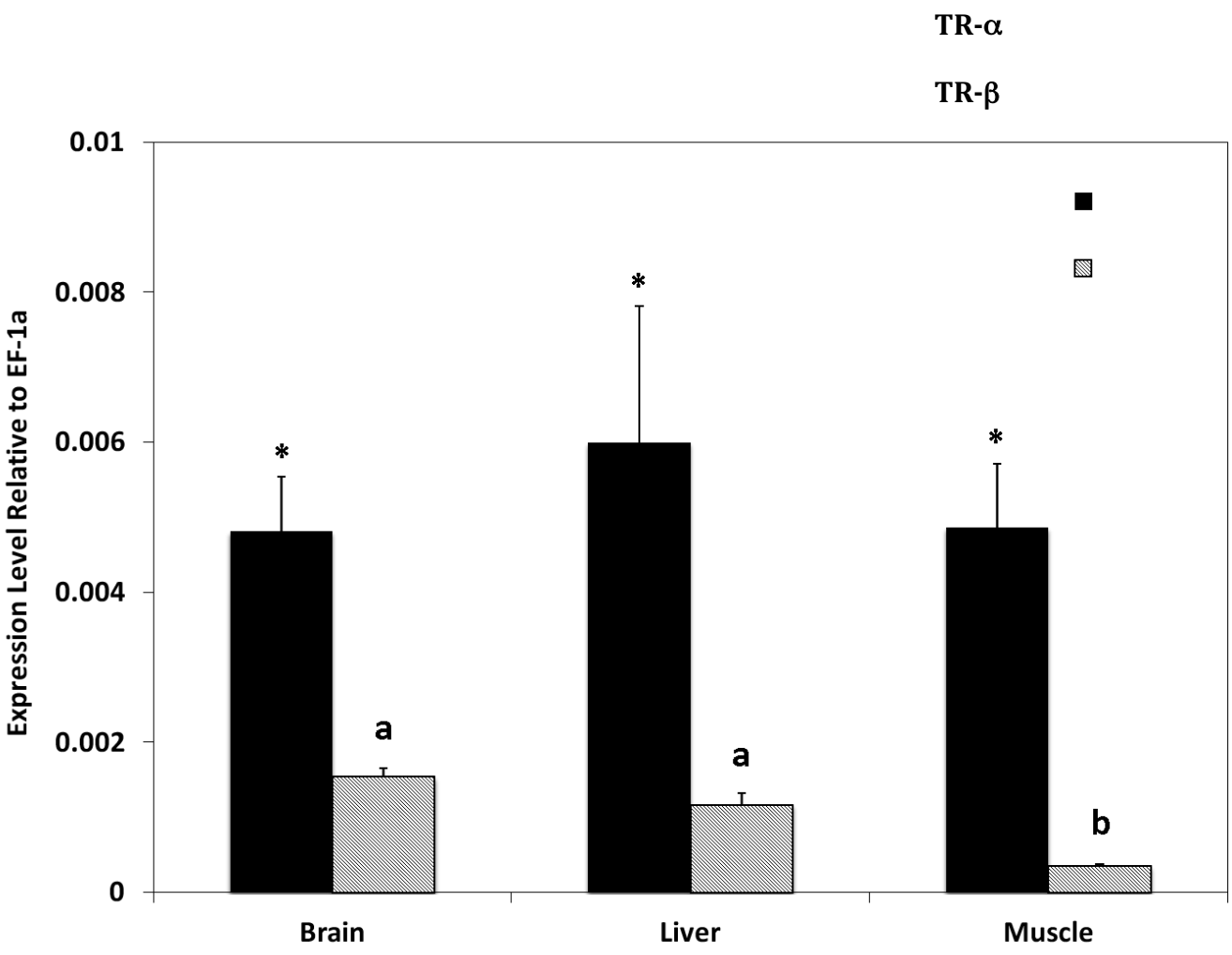

Figure 3.13: Relative expression level of TR-α and TR-β normalized using EF-1α in three different tissues (Brain, Liver, Muscle) of 67 days post hatch (dph) Lake Sturgeon larvae. The cDNA used in RT-PCR was reverse transcribed from RNA isolated from individual tissue samples collected from larval Lake Sturgeon (values are expressed as means ± SEM, with an n=6). Significant difference in expression between tissues was determined using a two-way ANOVA with a $P<0.05$. Different letters denote significant differences in expression within receptor sub-type and the * symbol denotes significant differences in expression between receptor sub-types.

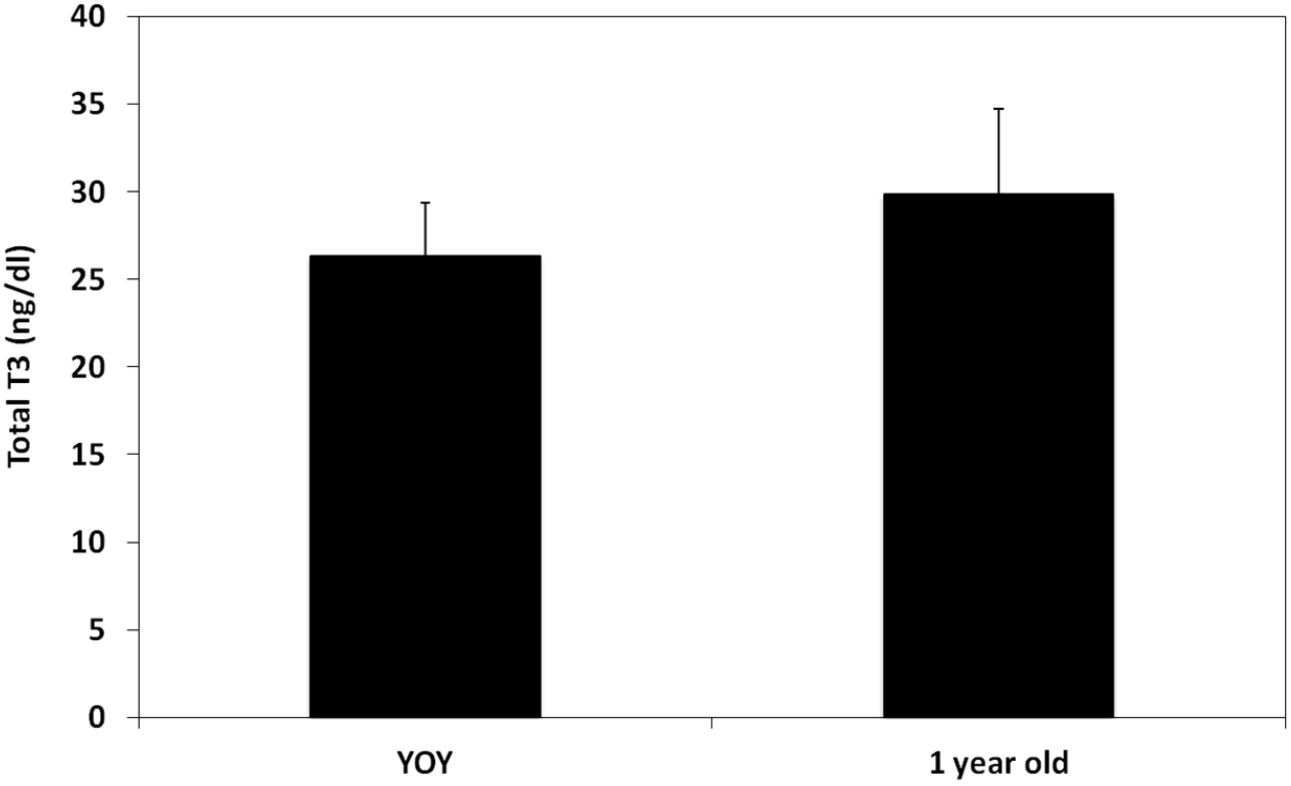

Figure 3.14: Total T_3 in plasma samples of young of the year (YOY) and one year old cultured juvenile Lake Sturgeon. Values are expressed as a means ± SEM, with an n=8 for YOY and n=2 for one year old samples.

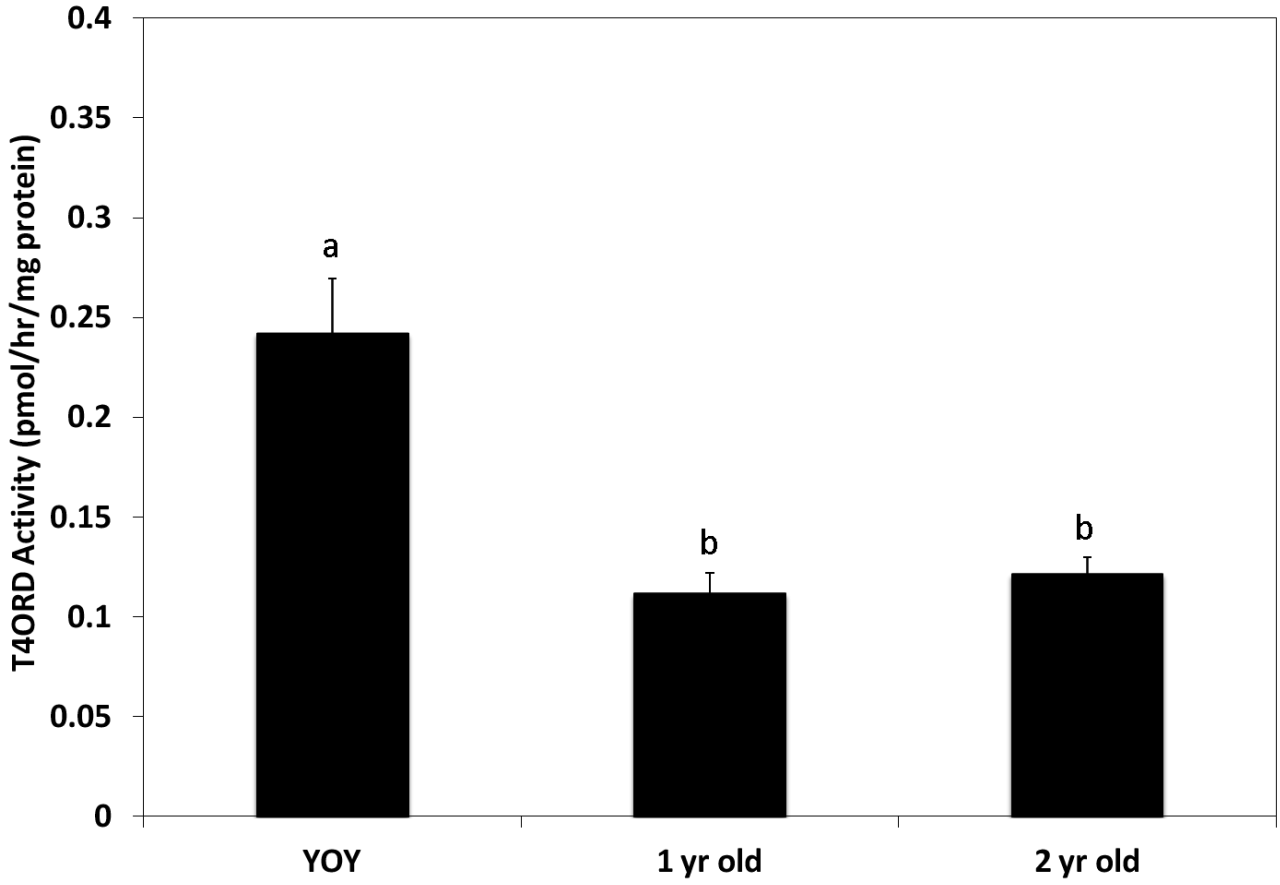

Figure 3.15: T4 outer-ring deiodinase (T4ORD) activity in liver tissue taken from young of the year (YOY), 1 year old, and 2 year old cultured Lake Sturgeon. Values represent means ± SEM, with an n=10. Significant differences between tanks were established using a one-way ANOVA. Means labelled with different letters are significantly different from each other ($P<0.05$).

4.0 Discussion

4.1 Larval Growth

The first objective of this thesis was to examine growth rate of larval Lake Sturgeon throughout early development and to determine any potential effects of chlorpyrifos on larval growth and survival. The chosen exposure concentrations were designed to be well below the lethal concentration of chlorpyrifos and therefore no larval mortality was expected however mortality was tracked throughout the experiment to confirm this. In addition, the low treatment concentration of 5ng.L^{-1} was chosen based on its environmental relevance within Manitoba water systems. The medium (500ng.L^{-1}) and high (2000ng.L^{-1}) concentrations represent values ten to fifteen orders of magnitude higher than recorded environmental levels (Rawn and Muir 1999). Comparatively, experiments carried out on Lake Trout demonstrated an LC50 value, the concentration at which mortality totals 50% of the exposed population, for chlorpyrifos between 7000ng-51000 ng.L^{-1} (Benedetto et al. 2010). Overall, exposure to chlorpyrifos at the concentrations used in this thesis did not affect larval mortality throughout the experimental period. Mortalities did not differ significantly between treatments and therefore it can be concluded chlorpyrifos did not influence the natural mortality rate under test conditions.

Total length and body mass was recorded in each treatment at each sample time-point in order to accurately track larval Lake Sturgeon growth rate over the course of the experiment. Mean total lengths for larvae from each treatment at 3, 6, 9, 12, and 21dph were not statistically different from each other but at 36 and 67dph larvae in the low chlorpyrifos treatment were significantly longer than all other treatments. It was evident therefore that exposure to chlorpyrifos from 3 to 12dph at the concentrations used in this study did not affect larval Lake Sturgeon growth rate at least until 21dph. However, significant differences in total length and

body mass between treatments were observed at 36 and 67dph although these are more likely the result of other factors, which will be discussed below.

When compared to length measurements reported for Sterlet Sturgeon (Rybnikar et al. 2011), over a similar developmental timeframe, Lake Sturgeon larvae in the present study had similar but slightly greater total lengths than Sterlet Sturgeon up until 36dph, however, total length at 67dph in Lake Sturgeon from the present study was much shorter than that reported for Sterlet Sturgeon at 64dph (Rybnikar et al. 2011). Similarly, total body mass of Lake Sturgeon larvae from the present study at 3, 6, 9, and 12dph had higher mean body masses than Sterlet Sturgeon at comparable time-points (5, 10, and 13dph) (Rybnikar et al. 2011), however, body mass in Sterlet Sturgeon beyond 13dph were always higher than those for larval Lake Sturgeon.

Based on research examining the effects of stocking density on growth in a number of different fish species such as Atlantic Sturgeon, Climbing Perch, *Anabas testudineus*, and European Perch, *Perca fluviatilis*, one factor that may explain the observed differences in total length and body mass between treatments in the present study is stocking density (Szczezepkowski et al. 2011; Aminur-Rahman and Marimuthu 2010; Romare 2000). Juvenile Atlantic Sturgeon held at three different stocking densities for 64 days were shown to have significantly different growth rates with the lowest stocking density not surprisingly having the highest growth rate when compared to the higher stocking densities (Szczezepkowski et al. 2011). Lake Sturgeon larvae in this experiment were divided as evenly as possible into 16 flow through tanks but at the conclusion of the study when initial stocking density was determined it was discovered the low treatment tanks, particularly tanks 5, 6, and 8, had lower initial stocking densities when compared to other treatment tanks. The average larval density in the low treatment tanks were 21%, 24%, and 27% less than the control, medium and high tanks

respectively. Consequently, larvae in the low chlorpyrifos treatment likely had high growth rates due to the reduced stocking density when compared to the other treatments.

Interestingly the observed differences in growth between treatments did not appear until later on in development, after the absorption of the yolk sac and when larvae were switching to an exogenous food source. It has been demonstrated in Siberian Sturgeon, *Acipenser baeri*, that larval growth significantly increases with the transition to exogenous feeding (Gisbert and Williot 1997). Competition for food is a widely researched topic in the aquaculture industry and it has been documented in a number of fish species that increased competition for food leads to decreased growth rate (Davey et al 2006; Amundsen et al. 2007; Aminur-Rahman and Marimuthu 2010). Larvae in the treatment tanks with lower stocking density would presumably have less competition for food and would therefore have a faster growth rate as a) more food may be available for each individual and/or b) less energy is spent on the consumption of that food.

Larval growth was also analyzed using Fulton's condition factor (CF), which presents growth as a factor of both total length and body mass. Because CF accounts for differences in length and body mass it could be considered a more appropriate indicator of whether or not there are differences in growth between treatments. The only significant differences between CF in the present study were prior to chlorpyrifos exposure, at 3dph. Therefore any observed differences could not have been the result of chlorpyrifos treatment and may instead be due to slight differences in the size of the larvae within treatments prior to the beginning of the experiment. Larval CF was ~0.9 across treatments at the beginning of the experiment and decreased gradually until 21dph where CF leveled at ~0.3. Although differences in total length and body mass were observed between treatments at 36 and 67dph the CF was not statistically different between treatments at those time-points. The decrease in CF from 3 to 21dph is likely the result of the total length increasing at a greater rate than body mass due to resorption of the yolk-sac and

utilization of the nutrients for metabolic purposes. Similar results were observed in Sterlet Sturgeon where CF decreased during the period when yolk-sac was present but eventually leveled off after the onset of exogenous feeding (Rybnikar et al. 2011).

Although it appears density is the main reason for the differences in growth observed in the low treatment tanks other factors such as sample bias, water temperature, and genetic differences may have played a role. When sampling larval fish it is possible to inadvertently remove the larger and more accessible fish and subsequently skew the results towards a particular direction. If this were the case one would expect to see differences in growth between treatment tanks throughout the experiment and the trend would not be the same from time-point to time-point. However, differences in total length and body mass were not different until 36 and 67dph and there was a clear trend with the larvae in the low tanks having higher measurements than the other treatments. The link between increased water temperature and accelerated larval growth has been studied extensively in a number of different fish species including a wide variety of sturgeon such as Shovelnose Sturgeon, *Scaphirhynchus platorynchus* (Kappenman et al. 2009), Shortnose Sturgeon, *Acipenser brevirostrum*, Atlantic Sturgeon (Hardy and Litvak 2004), and Siberian Sturgeon (Ronyai 1999). It is unlikely that temperature played a role in the differential growth rates observed in the present as water temperature in each treatment tank was the same. Finally, all larvae used in this experiment were from the same brood stock and were treated in the same way therefore genetic variability does not explain the observed growth rate differences.

4.2 Thyroid Follicle Development

The second objective in this thesis was to examine thyroid follicular development throughout the larval life-stage of Lake Sturgeon. This was accomplished using two histological

techniques: H&E staining to illustrate the structure of thyroid follicles and surrounding tissues and IHC staining to isolate the location, if present at all, of thyroid follicles in the developing larvae.

The genesis of thyroid follicles in developing fish larvae is known to differ among fish species. In Coho, Chinook, and Atlantic salmon, active thyroid follicles have been shown to develop prior to hatch, as early as 3-4 weeks post-fertilization (Greenblatt et al. 1989; Lam 1994). However, for most teleost fish species thus far examined active follicles do not appear until the absorption of the yolk-sac, which is believed to be the primary source of TH in developing larvae (Lam 1994; Power et al. 2001). Lake Sturgeon larval samples from this study demonstrate the presence of thyroid follicles at the yolk sac stage, prior to the onset of exogenous feeding. Based on the T_4-antibody labeled IHC staining performed on larval sections from 6, 9, and 12dph (Fig 3.6) the thyroid tissue and developing follicles appear to be synthesizing T_4 endogenously or alternatively are sites of T_4 storage within the larvae. It is likely that the yolk-sac is an additional source of TH's, however, it is unknown if this is the predominant source of T_4 as it is clear that de-novo synthesis of T_4 is occurring in these larvae during the yolk sac stage. Based on the success of the T_4-antibody IHC staining you might also expect the yolk-sac to immunostain positively if it was in fact a source of T_4. Unfortunately, due to the relatively poor fixation and staining of the yolk tissue there was very little yolk on the sections and they did not stain well enough to analyze using IHC.

It was hypothesized that thyroid follicles would become more developed after the onset of exogenous feeding when maternal TH in the yolk-sac would no longer be present. Larval sections from 21, 36, and 67dph stained with H&E were clearly more developed than those observed in the earlier time-points (Fig 3.5). Within the thyroid follicular cells, TG is iodinated and transported into the colloid where upon stimulation of the follicle the iodinated TG is

processed into vacuoles and either macro- or micro-pinocytosed back into the lumen of the follicular cell. Research performed on the Spotted Ratfish, *Hydrolagus colliei*, showed that activation of the thyroid gland via TSH administration increased the abundance and size of the colloid vacuoles (Nakai and Gorbman, 1969). Their results demonstrate the importance of the presence of vacuoles in determining the relative activity of the thyroid follicle. Therefore, the presence of vacuoles (Fig 3.7) within the follicular colloid at 21, 36 and 67dph in larval Lake Sturgeon indicates the possibility of follicular responsiveness to TSH and active synthesis and secretion of TH's into the circulation (Eales and Brown 1993; Kmiec et al. 1998; Nakai and Gorbman 1969).

Morphological metrics of the thyroid gland have been considered an important tool in determining the activity of the thyroid system in many organisms due to the structural response shown by thyroid follicles to physiological changes. Measurements of thyroid follicle cell height and nucleus height were taken from histological sections at all time-points in order to assess any changes as a result of development and chlorpyrifos exposure. Statistical differences in thyroid follicle cell height were observed between treatments at 6, 9, 12, 21, and 67dph but the differences had no dose-response relationship to chlorpyrifos and trends between treatments were often not repeated in the following time-points. Thyroid follicle nucleus height was also measured and the only significantly different values were seen at 6 and 21dph. Similar to cell height, the differences observed in the nucleus height measurements were not dose-dependent and no trend was observed between the two time-points. Based on these results it was concluded that at the concentrations tested, chlorpyrifos exposure had no pathological effect on Lake Sturgeon thyroid follicular development.

Despite earlier results there were clear observable trends in the thyroid follicle cell height and nucleus height measurements across the developmental time-points. To improve statistical

strength in the data, measurements within time-points but between treatments that were not significantly different from each other were pooled. Follicle cell height measurements in Lake Sturgeon from this study were higher than those recorded for other fish species such as Zebrafish (Ortiz-Delgado et al. 2006), Rainbow Trout (Raine and Letherland 2000), Fathead Minnow (Crane et al. 2005), and European Sea Bass (Schnitzler et al. 2008) at comparable developmental time-points. Thyroid follicle cell height was highest at 6dph and decreased until 12dph where cell height leveled off and remained constant for the remainder of the study. A similar result was observed in Zebrafish larvae (Raine and Leatherland 2000) where the large thyroid cell height measured at early developmental time-points may be due to a reduction in the number of thyroid follicle cells and therefore larger cells are required to synthesize sufficient TH. As the fish develops there is hyperplasia and therefore individual cell size is smaller.

Thyroglobulin (TG) mRNA transcription in thyroid follicular cells is stimulated through second messenger pathways initiated by TSH binding to receptors on the follicular cell membrane. Follicular activation has been shown to cause morphological changes in the nucleus such as a more visible nucleolus, increased size, and a more spherical shape (Eales and Brown 1993). As such it would be expected to see nucleus height increase just prior to exogenous feeding when the majority of maternal TH from the yolk-sac has been absorbed. However, nucleus height did not increase but instead remained the same from 12 to 21dph having decreased from 9 to 12dph. Interestingly, nucleus height increased significantly from 21 to 36dph and again from 36 to 67dph where it was greatest within the timeframe of the present study. This may indicate that at later life-stages (36 and 67dph) the nuclei of the follicular cells are more active and are playing a more important role in the production of TG and subsequently T_4 and T_3, a result that is supported by the presence of vacuoles in the colloid space at the same time-points, as described above.

4.3 Thyroid Receptor Expression

The third objective of this thesis was to examine thyroid hormone receptor, TRα and TRβ, expression in Lake Sturgeon larvae by molecular means, specifically, ontological and tissue specific differences in TRα and TRβ receptor expression was determined. Based on the histological results and life stages whole body receptor expression was examined in 3dph larvae as this was the earliest post-hatch sampling point for all other aspects of the study, at 12dph as this was just prior to exogenous feeding and 21dph as all larvae that had survived until this time-point had successfully transitioned to exogenous feeding. At 67dph larvae were of sufficient size to allow for dissection of individual tissues for the examination of receptor expression in the liver, brain, and muscle.

The rapidly developing larvae used for TR expression in whole-body homogenates meant that selection of an internal standard was problematic as all cells were undergoing significant morphological and potentially functional changes during the experimental timeframe. As a consequence internal standards normally suitable for expression data were highly variable and therefore results are presented as absolute expression levels. TRα expression was highest just prior to the onset of exogenous feeding, an important developmental milestone in larval Lake Sturgeon. Similarly, in larval flounder TRα expression peaked during the most active phase of metamorphosis and then decreased afterwards (Yamano and Miwa 1998). This could mean the observed increase in larval Lake Sturgeon TRα expression was due to the increased need for TR's at the crucial developmental period of exogenous feeding. A second possible explanation for the increase in TRα expression at 12dph is increased TH levels, which may stimulate transcription of TRα mRNA. Indeed, a positive relationship between T_3 and TR expression was

demonstrated in Striped Parrotfish where environmental T_3 increased both TRα and TRβ expression in the liver and brain of exposed fish (Johnson and Lema 2011). However, in the Lake Sturgeon larvae there was no increase in TH observed at 12dph as described in Striped Parrotfish. Furthermore, TRβ expression levels in larval Lake Sturgeon were not significantly different between developmental time-points. In light of the results other unknown mechanisms may be responsible for the differential regulation of TRα and TRβ transcriptional activity observed in this study. Interestingly, larval flounder TRβ expression did not peak until after metamorphosis and remained higher than TRα thereafter (Yamano and Miwa 1998). Results from expression of TR's during early development in larval Lake Sturgeon indicate a dominance of TRα expression. Similar findings were observed in Black Rockfish where TRα expression was higher than TRβ at all developmental time-points (Shafi et al. 2012). Also a TRα sub-type (TRα-1) in Zebrafish has been identified in early development particularly when other subtypes were not expressed pointing to a possible role of TRα in early larval development (Essner et al. 1997).

Although expression levels of TRα and TRβ have been studied in a number of fish species their functional capabilities are not fully understood but it is believed they act through transcriptional repression and activation (Nelson and Habibi 2008, 2009). In mammals, the function of both TR's and their sub-types is known in greater detail and in transgenic mice it has been shown TRβ sub-types are more important in the inner ear (Forrest et al. 1996a), liver, and in TH homeostasis (Forrest et al. 1996b) whereas TRα sub-type are important in the heart, GI tract, and for temperature regulation (Mai et al. 2004). The functional roles of both TR's has been established in mammals using transgenic mice where the deletion of TR sub-types enables researchers to monitor the effect of a particular gene on set physiological endpoints. For

example, when the TRβ gene was knocked out in mice auditory function was decreased but there was no decrease in metabolic, neurological, or developmental function (Forrest et al. 1996a). Whereas when TRα1 and TRα2 were both knocked out animals suffered from decreased growth, neurological development, a lower metabolism, and eventually premature death (Fraichard et al. 1997). In mice lacking just TRα1 growth and body temperature both decreased compared to wild type mice (Wikstrom et al. 1998). As in mammals, fish TR's have specific functional roles and it is believed their modes of action are similar to those of mammalian TR's. In goldfish, one of the roles of TRα and TRβ was determined using RNA interference (RNAi) to block the expression of TR genes in order to determine their role in the deiodinase pathways (Nelson and Habibi 2008). By blocking the majority of TRα−1 and TRβ translation they observed a 50 and 85% reduction in T_3 induced expression of D3 respectively. However, blocking the genes alone without T_3 induction had no effect on D3 gene expression or the expression level of the other TR genes. Their findings demonstrate the potential role of TR's in the control of gene transcription particularly deiodination pathways. In addition, they demonstrated that a truncated form of TRα (TRα-t) present in goldfish may act as a repressor of the process and when its expression was blocked D3 expression increased both with and without induction using T_3. Based on the larval Lake Sturgeon expression results and previously published literature in both mammals and fish it appears TRα may play a much larger role in the thyroid system of developing larval Lake Sturgeon compared to TRβ. However, the measureable levels of TRβ mRNA at all developmental time-points suggest a functional role of this receptor that cannot be discounted. Clearly further work is required to understand the importance of each receptor during development.

Tissue specific expression of TRα and TRβ from 67dph Lake Sturgeon larvae again suggested a dominance of TRα as mRNA expression was significantly higher than TRβ in the brain, liver and muscle. Similar trends in tissue expression of TR's were reported in Black Rockfish (Shafi et al. 2012) where TRα expression was higher than TRβ in all tissues tested. Surprisingly TRα expression in Lake Sturgeon larvae showed no tissue specific differences, which was unexpected. As previously mentioned tissues such as the liver and brain are key sites where TH binds and generates a developmental response. It is possible that important developmental processes that require integration by the thyroid system remain active even in 67dph larval Lake Sturgeon and therefore there remains a need for all tissues to have high expression levels of TRα. However, TRβ expression was significantly different between tissue types with brain and liver tissue having higher expression than muscle tissue. These results are similar to those found in developing embryonic chickens where TRα was found to be expressed uniformly in all tissues indicating a more general role in development whereas TRβ appeared to be more tissue specific with expression levels higher in the eyes, lungs, kidney, yolk-sac, and particularly the brain (Forrest et al. 1990). The high level of TRβ in the brain of developing chicks was attributed to a period where brain development is rapid and TH dependent indicating its role in T_3 mediated brain maturation (Forrest et al. 1990). Similarly, rat hepatic TR expression is largely the result of TRβ1 which makes up ~80% of the T_3-induced hepatic activity (Montesinos et al. 2006). In Lake Sturgeon it is possible TRβ has more of a tissue specific role as seen in developing chicks whereas TRα has more of a dominant but general function. These results underline the potential for disparate functional roles of TRα and TRβ in other vertebrates and developing Lake Sturgeon and could provide an exciting research opportunity going forward.

4.4 Thyroid Hormone Levels

The fourth objective of this thesis was to examine whole-body levels of TH in developing Lake Sturgeon larvae. Unfortunately, limitations on the kits used meant that the detection of measurable values of T_4 and T_3 from larval Lake Sturgeon was unreliable. With the exception of FT3 no more than one or two samples in any time-point had detectable levels of TH's making interpretation of results problematic. FT3 was present at detectable values in a few samples from all time-points whereas TT3 was detectable intermittently throughout development. The more reliable detection of FT3 and TT3 over TT4 and FT4 may have been the result of the parameters of the commercial assays used in the present study. The detection limits for the TT3, FT3, TT4, and FT4 assays were 6.7, 0.006, 760, and 0.045ng.dl^{-1} respectively. TT4 and TT3 plasma samples from two-year-old Lake Sturgeon reported by Plohman et al. 2002a would fall below the detection limits for both the FT3 and FT4 commercial assay kits used in this experiment. The kits were designed for human analysis therefore the TT4 detection limit is set at a much higher level than TT3.

In most organisms T_4 is released at high concentrations and it is the peripheral conversion of T_4 to T_3 that provides the majority of the active hormone. When TT3 was detectable it was at concentrations roughly 500 fold greater than FT3. The high lipophilic nature of unbound T_3 hinders its transport within the bloodstream and therefore the concentrations of unbound T_3 are normally far less than bound T_3 in fish and other vertebrates (Eales and Brown 1993). Due to low sample size and detection limit the discussion will primarily focus on FT3 rather than TT3, FT4, and TT4. However, given the unreliable measurement capacity of the assays used the following discussion is based on observed trends in the data as opposed to significant differences between time-points.

Percent Free-T_3 (%FT3) in two-year-old Lake Sturgeon was previously reported to be 0.44% compared to 0.08% in Rainbow Trout (Plohman et al. 2002a). In the Lake Sturgeon larvae from this experiment the %FT3 values are less than those previously recorded for two-year-old Lake Sturgeon but are higher than those for rainbow trout. However, this value is based on a low sample size for measured TT3 in larval Lake Sturgeon and therefore an increased sample size is required to provide a more definitive estimation of %FT3 in the larval Lake Sturgeon. Nonetheless, it is likely that the difference in % FT3 observed between the Lake Sturgeon larvae and the two year old juveniles is life-stage related and is the result of different demands on the thyroid hormone system.

Analysis of FT3 was assessed as pg/g of tissue and pg/individual larvae . When expressed as pg/larvae (table 3.2) there was an observed increase in measured FT3 at 21dph compared to all other time-points but the same trend was not observed when the results were plotted as pg/g tissue (table 3.1). There was however an observed decrease in FT3 from 21 to 36dph in both data-sets, which may be the result of a reduced need for FT3 at that particular larval life-stage. By 36dph Lake Sturgeon larvae were actively feeding and had been for over two weeks therefore the potential need for FT3 may have been lower than at 21dph, a vital stage just after the onset of exogenous feeding. In Russian Sturgeon, a peak in T_4 and to a lesser extent T_3 just prior to exogenous feeding was reported by Boiko et al. (2004) but this did not appear to be similar in larval Lake Sturgeon from this study. The maximal value for TT3 in Russian Sturgeon (0.025 ± ng/larvae) were recorded just after the onset of exogenous feeding and were much higher than recorded in Lake Sturgeon samples at a similar time-point in this study.

Provided FT3 accounts for 0.44% of the total T_3 in circulation, according to measurements by Plohman et al. (2002a), then FT3 levels would be approximately ten times higher in Russian Sturgeon when compared to the measured Lake Sturgeon concentrations.

Similarly, Nile Tilapia T_3 concentrations recorded by Reddy et al. (1992) were much higher than recorded in Lake Sturgeon. Interestingly, the values recorded for Nile Tilapia were also higher than T_3 levels in Russian Sturgeon recorded by Boiko et al. (2004) reaching a maximal concentration of $0.19 \pm 0.02 ng.larvae^{-1}$ at 25dph. It was suggested by Plohman et al. (2002b) the low concentrations of TH observed in the plasma of two-year-old Lake Sturgeon samples were partly the result of a completely fresh water existence, which is typically low in available iodine. Furthermore, Lake Sturgeon have been shown to have some of the lowest plasma iodide concentrations of any fish species and therefore the production and utilization of TH appears to be different in Lake Sturgeon (Leloup 1970). If iodide concentrations are low in two-year-old Lake Sturgeon it is possible they are even lower in the larval stage and this might account for the differences observed in TH levels between Lake Sturgeon and other fish species.

Plasma levels of TT3 in YOY Lake Sturgeon in the present study were similar to those previously reported for juvenile Lake Sturgeon (Plohman et al. 2002a) which are much lower with respect to similar aged teleost fish (Plohman et al. 2002a). In addition, TT4 was not detectable in samples from one- and two-year-old Lake Sturgeon plasma further demonstrating the low levels of TH present in Lake Sturgeon juveniles. Despite the low levels of TT3 in the YOY it appears levels drop even lower in two-year-old sturgeon as evidenced by the lack of detectable TH in any of the two-year-old plasma samples using the available commercial kits. The low levels of TT3 in YOY and one year old sturgeon when compared to other teleost species appears to indicate T_3 may play less of a role in the juvenile life-stage of Lake Sturgeon and a more prominent role in the YOY life-stage. TH levels have been shown to be higher in developing fish and have been implicated in the development of adult muscle fibers in Japanese Flounder (Inui et al. 1995), brain development in Atlantic Salmon (Morin et al. 1997; De et al. 1992; De et al. 1993), and metamorphosis in Zebrafish (Brown 1997), Japanese Flounder

(Yamano and Miwa 1998), and Orange Spotted Grouper (deJesus et al. 1998). YOY sturgeon are predictably smaller and less developed than one and two year old sturgeon and it is possible the increased TT3 recorded in their plasma is needed to aid their growth and survival during an important and vulnerable life-stage. Indeed Boiko et al. (2004) demonstrated a positive relationship between thyroid status and survival in larval Russian Sturgeon.

4.5 Deiodinases

The final objective of this thesis was to determine the activity of the important deiodinase enzyme, T4ORD, in the liver tissue of YOY, one year old, and two year old Lake Sturgeon. T4ORD was present at detectable levels in YOY, one-year-old, and two-year-old fish and interestingly was highest in the liver of the YOY fish. Deiodinase activity is linked to proportional levels of T_4 and T_3 in fish where lower levels of circulating TH lead to increased T4ORD activity in the tissues and increased TH leads to decreased enzyme activity (Johnson and Lema 2011; Eales and Brown 1993; Van der Guyten et al. 2011). Thus YOY Lake Sturgeon may require more T_3 and therefore T4ORD is more active in converting T_4 to the biologically active T_3 directly at the target site, in this case the liver.

Similar to the deiodinase results presented in this project previously published Lake Sturgeon data demonstrates T4ORD activities for two-year-old Lake Sturgeon are close to the values observed in this study but YOY and one-year-old sturgeon were not analyzed (Plohman et al. 2002b). Comparatively, hepatic T4ORD activity has been recorded at higher levels in Rainbow Trout when compared to the Lake Sturgeon measured in this experiment. It was found that T4ORD activity in trout liver was similar in activity to that found in mammalian pituitary, cerebral cortex, and brown adipose tissue, however, hepatic T4ORD activity is generally not

observed in mammals (Orozco et al. 1997). In Rainbow Trout the high hepatic T4ORD is linked with high circulating levels of T_3 but the level of association between the two processes is unknown (Eales 1985; Orozco et al. 1992, 1997).

4.6 The Thyroid System

The overall objective of this thesis was to track the development of the thyroid system in developing Lake Sturgeon larvae and to assess any potential effect of chlorpyrifos on that system. Based on growth and histological analysis it was concluded there was no effect of chlorpyrifos on growth and thyroid follicle development in Lake Sturgeon larvae. Although there were differences among treatments in both growth and histological measurements the differences were not dose dependent and therefore were most likely the result of other variables such as stocking density.

When tracking the development of any system within an organism it is important to evaluate the results not just individually but as a whole in order to gain a more holistic understanding of how the system operates. In this project Lake Sturgeon thyroid development was studied at the histological, molecular, and physiological level by assessing thyroid follicle development, thyroid receptor gene expression, whole-body levels of TH's, and deiodinase activity. Based on the histological results Lake Sturgeon appear to be comparable to salmonids where thyroid gland development occurs prior to the complete absorption of the yolk-sac. But even with the observed presence of thyroid follicles and confirmed presence of T_4 within the colloid at 12dph the level of TH's recorded in the larvae were very low and in most cases were undetectable using the TH assay kits. Lake Sturgeon TH levels remained low and for the most part undetectable in the assays used throughout larval development even with the presence of

active follicles at 21, 36, and 67dph. However, FT3 was detectable in a larger proportion of samples at 21 and 36dph and was detectable in all samples at 67dph at which point follicular histology demonstrated the larvae have fully developed and active thyroid follicles complete with large colloid vacuoles. It is possible the larval thyroid follicles examined prior to the 67dph time-point were producing TH's but at levels undetectable to the assay used in this study.

The morphological characteristics of thyroid follicles are also responsive to changes in hormone concentrations such as TH and TSH. Increased TSH levels have been shown to increase follicular cell height and nucleus activity in fish suggesting an increase in activity (Eales and Brown 1993). TSH is regulated via negative feedback loops controlled by TH (Fig 1.3) and therefore changes in TH concentration can indirectly influence follicular morphology. In the Lake Sturgeon larvae examined in this experiment a decrease in both thyroid follicle cell height and nucleus height at 12 and 21dph was observed. The decrease corresponds to the time-points where the measured FT3 concentrations were observed to be slightly higher than other time-points. It is possible the potential increase in FT3 acts via negative feedback pathways to lower the level of TSH and therefore lower follicular cell height and nucleus height. Unfortunately the previous conclusion is weakened by a lack of TT3 and TT4 data and the successful analysis of both forms would significantly increase the understanding of the feedback loops involved in Lake Sturgeon and their impact on follicular morphology.

TRα expression increased at 12dph and given what is known from the literature we would expect an associated peak in T_3 or T_4. However, there was no observed peak in FT3 levels at 12dph and while it is possible a peak in TT3 occurred at 12dph, the concentrations in the larval samples were below the detectable limit of the assay. Although there was no significant increase in FT3 ($pg.larvae^{-1}$ or $pg.g\ tissue^{-1}$) at 12dph when the observed TRα increase occurred there was

an observed increase in FT3 (pg.larvae^{-1}) at 21dph when TRα expression was lower. In Fathead Minnows (Lema et al. 2009) and Striped Parrotfish (Johnson and Lema 2011) exposure to T_3 caused an increase in both TRα and TRβ expression whereas the results previously mentioned for Lake Sturgeon do not. Comparatively, in the liver tissue of hypothyroid rats (low TH) TR expression increased and in hyperthyroid rats (high TH) TR expression decreased (Zandieh-Doulabi et al. 2004). Therefore, based on the current study it may be that TH regulation of TR expression in Lake Sturgeon is more comparable to the mammalian system.

Lake Sturgeon larvae appear to have an active thyroid system as seen in the histological sections tracking follicular development and the TR expression data, yet whole-body levels of T_4 and T_3 are lower than most fish species. These data could be interpreted as TH's being less critical in Lake Sturgeon during early development, or perhaps more likely, an increased sensitivity to changes in circulating levels of the active hormone T_3, during early development.

Analysis was carried out on YOY, one-year-old, and two-year-old sturgeon to determine deiodinase activity and plasma TH concentrations. YOY Lake Sturgeon had the highest proportion of plasma samples with detectable values for TT3 and it was also YOY sturgeon that had the highest hepatic T4ORD activities. There is a likely relationship therefore between the high proportion of samples with detectable TT3 in YOY sturgeon and the high T4ORD activity in the liver of samples from the same time-point. TT3 concentrations in two year old cultured Lake Sturgeon suggest concentrations that are lower than the measured values for TT3 in the YOY sturgeon from this study (0.26 ± 0.03ng.ml^{-1}) (Plohman et al. 2002a). By comparing the two studies it can be seen that a decrease in T4ORD enzyme activity recorded in this study may be reflected in a decrease in circulating levels of T_3. The decrease in T4ORD activity could still lead to an increase in T_3 but only if T_4 concentrations were high, however, no such relationship

was observed in the TH results. One possible explanation is that deiodination of T_4 primarily occurs in the thyroid tissue of one and two year old sturgeon and therefore peripheral conversion at the liver is no longer as important. Preferential follicular release of T_3 instead of T_4 was hypothesized by Plohman et al. (2002a) based on results indicating thyroid tissue concentrations of T_3 being 10.6 times higher than T_4. If T_3 were released at higher concentrations from the sturgeon thyroid compared to other fish species then peripheral conversion of T_4 would not be as important and would reflect the relatively low levels observed in this experiment.

In conclusion, chlorpyrifos was found to have no effect on the thyroid system in developing Lake Sturgeon larvae. Thyroid development was monitored throughout early development by assessing thyroid follicular morphology, thyroid receptor expression, and whole-body TH levels. Thyroid follicular cells were first observed in larval samples at 6dph and there was continued morphological development throughout the experiment. The expression levels of both TR genes, TRα and TRβ, changed over developmental time with TRα increasing from 3 to 12dph and then decreasing from 12 to 21dph. TRβ expression did not change from 3 to 12dph but decreased from 12 to 21dph. In all tissues tested at 67dph TRα expression was significantly higher than TRβ. Surprisingly, only TRβ demonstrated any tissue specific expression with both liver and brain expression significantly higher than muscle. Only FT3 was consistently measured in all time-points and it appears Lake Sturgeon larvae have low concentrations of TH when compared to other fish species. T4ORD activity was only detectable in liver tissue from YOY, one-year-old, and two-year-old Lake Sturgeon. YOY Lake Sturgeon had significantly higher hepatic T4ORD activity when compared to one and two-year-old sturgeon.

4.7 Future Directions

The present study has described development of a key hormonal system in larval Lake Sturgeon, from a physiological and indeed developmental point the most obvious next step would be to examine functional aspects of the TH system during larval development. Based on the results demonstrated it was clear the thyroid system was present and functioning early on in development but appeared, at least in regard to circulating levels of the hormone, to be operating at levels well below those of other fish species. In order to experimentally test the link between the thyroid system and larval Lake Sturgeon development it would be interesting to expose developing Lake Sturgeon to varying concentrations of a known thyroid inhibiting substance such as thiourea. Within the literature the use of thyroid inhibiting substances is extensive and has demonstrated the importance of the thyroid system in a number of different fish species such as Japanese Flounder (Inui et al. 1995; Inui and Miwa 1985; Yamano and Miwa 1998), Zebrafish (Brown 1997), and Singi fish (De et al. 1992). Exposing Lake Sturgeon larvae to a thyroid inhibiting compound such as thiourea and comparing development to unexposed fish would provide important information regarding the functional role of TH in Lake Sturgeon development. For comparison larvae could be exposed to water-borne concentrations of T_3 or T_4 to stimulate the thyroid system above the normal level. Each of the proposed study alternatives would provide invaluable information regarding the role of the thyroid system in larval Lake Sturgeon and would fill crucial gaps in the literature.

As previously mentioned in section 4.4 the detection limits of the RIA kits used to measure TH were not sensitive enough to measure the low concentrations present in Lake Sturgeon larvae. Therefore the lack of consistent measurable values for TT3, TT4, and FT4 provides an opportunity for future work dealing with larval TH. Other RIA protocols and detections methods such as the use of Sephadex column chromatography and HPLC analysis as

described by Brown and Eales (1977) and performed on juvenile Lake Sturgeon tissue and plasma by Plohman et al. (2002a; 2002b) would provide an alternative and more sensitive method for the detection of TH within Lake Sturgeon larvae.

Expression of TRα and TRβ was examined in the developing Lake Sturgeon larvae, however, primers were not developed for possible TR subtypes as described in other fish species (Nelson and Habibi 2006; Yamano and Miwa 1998; Marchand et al. 2001). If alternative sub-types were found and their sequences determined it would be interesting to go one step further and compare sequences and expression levels between other species in order to establish the degree of genetic conservation within the receptor sub-types. In addition it would be extremely valuable to determine the functionality of the described receptor sub-types in order to establish their relevance and importance within the thyroid system of Lake Sturgeon. Little is known about the functionality of TR's and their sub-types in fish and the possibility of examining them in an ancient fish species such as Lake Sturgeon would be a great opportunity.

5.0 References Cited

Adams, B.A, Cyr, D.G. and Eales, J.G. 2000. Thyroid hormone deiodination in tissues of American Plaice, *Hippoglossoides platessoides:* Characterization and short-term responses to polychlorinated biphenyls (PCBs) 77 and 126. *Comparative Biochemistry and Physiology.* **127C:** 367–378.

Allen, P.J., Weihrauch, D., Grandmaison, V., Dasiewicz, P., Peake, S.J. and Anderson, W.G. 2011. The influence of environmental calcium concentrations on calcium flux, compensatory drinking, and epithelial calcium channel expression in a freshwater cartilaginous fish. *The Journal of Experimental Biology.* **214**: 996-1006.

Alyavdina, L.A. 1951. On the biology and systematics of Acipenseridae in the early stages of their development. *Trudy Saratov Fil VNIRO*. **1**: 33-73.

Aminur-Ramen, M. and Marimuthu, K. 2010. Effect of different stocking density on growth, survival and production of endangered native fish climbing perch (*Anabas testudineus,* Bloch) fingerlings in nursery ponds. *Advances in Environmental Biology*. **4**(2): 178-186.

Amundsen, P.A., Knudsen, R., and Klemetsen, A. 2007. Intraspecific competition and density dependence of food consumption and growth in Arctic Char. *Journal of Animal Ecology*. **76**: 149-158.

Arrojo, E.D.R. & Bianco, A.C. 2011. Type 2 deiodinase at the crossroads of thyroid hormone action. *International Journal of Biochemistry & Cell Biology*. **43**: 1432–1441.

Asperlin, A. 1994. Pesticide industry sales and usage 1992 and 1993 market estimates. US Environmental Agency Report No. 733-K-94-001.

Auer, N.A. 1996. Importance of habitat and migration to sturgeons with emphasis on Lake Sturgeon. *The Canadian Journal of Fisheries and Aquatic Sciences*. **53**: 152-160.

Barbieri, E. and Alves Ferreira, L.A. 2011. Effects of the organophosphate pesticide folidol 600 (R) on the freshwater fish, Nile Tilapia (*Oreochromis niloticus*). *Pesticides, Biochemistry and Physiology*. **99**(3): 209-214.

Baumann, E.J., Metzger, N., and Marine, D. 1944. Mode of action of thiourea on the thyroid gland of rabbits. *Endocrinology*. **34**(1): 44-49.

Beatty, D.D. 1972. Visual pigment changes in salmonid fishes in response to exogenous L-thyroxine, bovine TSH and 3-dehydroretinol. *Vision Research*. **12**: 1947-1960.

Benedetto, A., Abete, M.C., Prearo, M., Fioravanti, F., and Squadrone, S. 2010. Chlorpyrifos effects on Rainbow Trout (*Oncorhynchus mykiss*): analysis of gene expression profiles. *Journal of Biotechnology.* **150**: S125-S126.

Bentley, P.J. 1998. Comparative Vertebrate Endocrinology, 3rd ed. Cambridge University Press, Cambridge, UK.

Bernal, J., Guadano-Ferraz, A., and Morte, B. 2003. Perspectives in the study of thyroid hormone action on brain development and function. *Thyroid.* **13**(11): 1005-1012.

Billard, R. and G. Lecointre. 2001. Biology and conservation of sturgeon and paddlefish. *Reviews in Fish Biology and Fisheries*. **10**(4): 355-392.

Blanton, M.L. and Specker, J.L. 2007. The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. *Critical Reviews in Toxicology*. **37**(1-2): 97-115.

Boiko, N.E., Vorob'eva, O.A., Gigor'yan, R.A., and Kornienko, G.G. 2004. Dynamics of thyroid hormones at early stages of development of the sturgeon *Acipenser guldenstadti*. *Journal of Evolutionary Biochemistry and Physiology*. **40**(2): 176-181.

Botaro, D., Machado Torres, J.P., Malm, O., Rebelo, M.F., Henkelmann, B., and Schramm, K. 2011. Organochlorine pesticides residues in feed and muscle of farmed Nile Tilapia from Brazilian fish farms. *Food and Chemical Toxicology*. **49**(9): 2125-2130.

Braun, H.E. and Frank, R. 1980. Organochlorine and organophosphorous insecticides – their use in 11 agricultural watersheds and their loss to stream waters in southern Ontario, Canada 1975-1977. *Science of the Total Environment*. **15**(2): 169-192.

Bres, O. and Eales, J. G. 1986. Thyroid hormone binding to isolated trout (*Salmo gairdneri*) liver nuclei in vitro: binding affinity, capacity, and chemical specificity. *General and Comparative Endocrinology*. **61**: 29-39.

Browman, H.I. and Hawryshyn, C.W. 1992. Thyroxine induces precocial loss of ultraviolet photosensitivity in Rainbow Trout (*Onchorhynchus mykiss*, Teleostei). *Vision Research*. **32**(12): 2303-2312.

Brown, S. and Eales, J.G. 1977. Measurement of L-thyroxine and 3.5.3'-triiodo-L-thyronine levels in fish plasma by radioimmunoassay. *The Canadian Journal of Zoology*. **55**: 293-299.

Brown, C.L., Doroshov, S.I., Nunez, J.M., Hadley, C., Vaneenennaam, J., Nishioka, R.*S.* and Bern, H.A. 1988. Maternal triiodothyronine injections cause increases in swimbladder inflation and survival rates in larval Striped Bass *Morone saxatilis. The Journal of Experimental Zoology*. **248**: 168-176.

Brown, D.D. 1997. The role of thyroid hormone in zebrafish and axolotl development. *Proceedings of the National Academy of Science USA*. **94**: 13011-13016.

Buddington, R.K. 1985. Digestive secretions of Lake Sturgeon, *Acipenser fulvescens*, during early development. *Journal of Fish Biology*. **26**: 715-723.

Carrofino, D.C. and Sutton, T.M. 2010. Predation on early life stages of Lake Sturgeon in the Peshtigo River, Wisconsin. *Transactions of the American Fisheries Society*. **139**:1846–1856.

Carr, R.L., Ho, L.L., and Chambers, J.E. 1997. Selective toxicity of chlorpyrifos to several species of fish during an environmental exposure: Biochemical mechanisms. *Environmental Toxicology and Chemistry.* **16**(11): 2369-2374.

Casida, J.E. and Quinstad, G.B. 2004. Organophosphate toxicology: Safety aspects of nonacetylcholinesterase secondary targets. *Chemical Research Toxicology*. **17**(8): 983-998.

Chang, L., Munro, S.L., Richardson, S.J. and Schreiber, G. 1999. Evolution of thyroid hormone binding by transthyretins in birds and mammals. *European Journal of Biocemistry*. **259**: 534–42.

Chatterjee, A., Hsieh, Y.L., and Yu, J.Y.L. 2001. Molecular cloning of cDNA encoding thyroid stimulating hormone beta subunit of Bighead Carp *Aristichthys nobilis* and regulation of its gene expression. *Molecular and Cellular Endocrinology*. **174**(1-2): 1-9.

Chiotti, J.A., Holtgren, J.M., Auer, N.A., and Ogren, S.A. 2008. Lake Sturgeon spawning habitat in the Big Manistee River, Michigan. *North American Journal of Fisheries Management*. **28**(4): 1009-1019.

Chourdhury, G. and Dick, T.A. 1998. The historical biogeography of sturgeons (Osteichthyes; Acipenseridae): A synthesis of phylogenetics, palaeontology, and palaeogeography. *Journal of Biogeography.* **25:** 623– 640.

Collingwood, T.N., Urnov, F.D., Wolffe, A.P. 1999. Nuclear receptors: co-activators, co-repressors and chromatin remodeling in the control of transcription. *Journal of Molecular Endocrinology*. **23**: 255–275.

Crane, H.M., Pickford, D.B., Hutchinson, T.H. and Brown, J.A. 2005. Effects of ammonium perchlorate on thyroid function in developing Fathead Minnows, *Pimephales promelas*. *Environmental Health Perspective*. **113**: 396–401.

Cruz, M.J., Sourial, M.M., Treberg, J.R., Fehsenfeld, S., Adlimoghaddam, A., and Weihrauch, D. 2013. Cutaneous nitrogen excretion in the African clawed frog *Xenopus laevis*: Effects of high environmental ammonia (HEA). *Aquatic Toxicology*. **136-137**: 1-12.

Cyr, D. G., Idler D. R., Audet, C., McLeese, J. M. and Eales, J. G. 1998. Effects of long-term temperature acclimation on thyroid hormone deiodinase function, plasma thyroid hormone levels, growth, and reproductive status of male Atlantic Cod, *Gadus morhua. General and Comparative Endocrinology.* **109:** 24–36.

Darras, V.M. and Van Herck, S.L.J. 2012. Iodothyronine deiodinase structure and function: from ascidians to humans. *Journal of Endocrinology*. **215**: 189-206.

Davey, A.J.H., Turner, G.F., Hawkins, S.J., and Doncaster, C.P. 2006. Mechanisms of density dependence in stream fish: exploitation competition for food reduces growth of adult European Bullheads (*Cottus gobio*). *Canadian Journal of Fisheries and Aquatic Sciences*. **63**: 597-606.

De, S. Dasmahapatra, A.K. and Medda, A.K. 1992. Alterations of ion-dependent ATPase activities in the brain of Singi Fish, *Heteropneustes fossilis* (Bloch), by triiodothyronine. *General and Comparative Endocrinology*. **86**: 162-169.

De, S., Dasmahapatra, A.K. and Medda, A.K. 1993. Stimulation of acetylcholinesterase activity by triiodthyronine in the brain of Singi Fish, *Heteropneustes fossilis. Fish Physiology and Biochemistry.* **10**(5): 419-424.

De Angelis, S., Tassinari, R., Maranghi, F., Eusepi, A., Di Virgilio, A., Chiarotti, F., Ricceri, L., Venerosi Pesciolini, A., Gilardi, E., Moracci, G., Calamandrei, G., Olivieri, A., and Mantovani, A. 2009. Developmental exposure to chlorpyrifos induces alterations in thyroid and thyroid hormone levels without other toxicity signs in CD-1 mice. *Toxicological Sciences: an official journal of the Society of Toxicology*. **108**(2): 311-319.

de Jesus, E.G.T., Toledo, J.D. and Simpas, M.S. 1998. Thyroid hormones promote early metamorphosis in grouper (*Epinephalus coioides*) larvae. *General and Comparative Endocrinology.* **112**: 10-16.

Detlaf, T.A. and Davydova, S.I. 1974. Effect of triiodothyronine on ripening of oocytes in Stellate Sturgeon after the action of low temperatures and reservation of females. *Ontogenez.* **5:** 454–462.

Detlaf, T.A., Shmalgauzen, O.I., and Ginzburg, A.S. 1993. Sturgeon Fishes: developmental biology and aquaculture. Springer-Verlag, Berlin.

Distefano J.J., Nguyen T.T. and Yen, Y.M. 1993. Transfer kinetics of 3,5,3'-triiodothyronine and thyroxine from rat blood to large and small intestines, liver, and kidneys in vivo. *Endocrinology.* **132**: 1735–1744.

Dogan, D. and Can, C. 2011. Endocrine disruption and altered biochemical indices in male *Oncorhynchus mykiss* in response to dimethoate. *Pesticide Biochemistry and Physiology*. **99**(2): 157-161.

Dragomirov, N.I. 1953. Specific characters of newly-hatched larvae of Acipenseridae. *Proceedings of the USSR Academy of Science*. **93**: 551-554.

Dunn, J.T., and Dunn, A.D. 2000. Thyroglobulin: Chemistry, biosynthesis, and proteolysis. In: The Thyroid: A Fundamental and Clinical Text, Eighth Edition, L.E. Braverman and R.D. Utiger, eds., pp. 91– 104. Lippincott Williams and Wilkins, Philadelphia.

Eales, J.G. 1979. Thyroid functions in cyclostomes and fishes. In Barrington, E.J.W., ed. *Hormones and Evolution,* Vol. I. London: Academic Press, pp. 341-436.

Eales, J. G., Omeljaniuk, R. J. and Shostak, S. 1983. Reverse T3 in Rainbow Trout, *Salmo gairdneri. General and Comparative Endocrinology.* **50:** 395– 406.

Eales, J.G. 1985. The peripheral metabolism of thyroid hormones and regulation of thyroidal status in poikilotherms. *Canadian Journal of Zoology*. **63**: 1217-1231.

Eales, J. G. and Shostak, S. 1985. Free T4 and T3 in relation to total hormone, free hormone indices, and protein in plasma of Rainbow Trout and Arctic Char. *General and Comparative Endocrinology.* **58:** 291–302.

Eales, J.G. and Brown, S.B. 1993. Measurement and regulation of thyroidal status in teleost fish. *Reviews in Fish Biology and Fisheries*. **3**: 299-347.

Eales, J.G., Holmes, J.A., McLeese, J.M. and Youson, J.H. 1997. Thyroid hormone deiodination in various tissues of larval and upstream-migrant Sea Lampreys, *Petromyzon marinus*. *General and Comparative Endocrinology*. **106**: 202-210.

Eales, J.G., Brown, S.B., Cyr, D.G., Adams, B.A., and Finnson, K.R. 1999. Deiodination as an index of chemical disruption of thyroid hormone homeostasis and thyroidal status in fish. Environmental Toxicology and Risk Assessment: Standardization of Biomarkers for Endocrine Disruption and Environmental Assessment: Eighth Volume. **1364**: 136-164.

Edwards, J.E. 1967. Methods for the demonstration of intercellular substances of the connective tissues. In McClung Jones R, ed, *McClung's Handbook of Microscopical Technique.* Hafner, New York, NY, USA.

Essner, J.J., Breuer, J.J., Essner, R.D., Fahrenkrug, S.C. and Hackett Jr., P.B. 1997. The Zebrafish thyroid hormone receptor α1 is expressed during early embryogenesis and can function in transcriptional repression. *Differentiation.* **62**: 107–117.

Evans, R.M. 1988. The steroid and thyroid hormone receptor super-family. *Science.* **240**: 889–895.

Farid, N.R. and Szkudlinski, M.W. 2004. Minireview: Structural and functional evolution of the thyrotropin receptor. *Endocrinology*. **145**(9): 4048-4057.

Ferguson, M.M. and Duckworth, G.A. 1997. The status and distribution of Lake Sturgeon, *Acipenser fulvescens*, in the Canadian provinces of Manitoba, Ontario and Quebec: A genetic perspective. *Environmental Biology of Fishes*. **48**(1-4): 299-310.

Fok, P., Eales, J. G. and Brown, S. B. 1990. Determination of 3,5,3'-triiodo-L-thyronine (T3) levels in tissues of Rainbow Trout (*Salmo gairdneri)* and the effect of low ambient pH and aluminum. *Fish Physiology and Biochemistry.* **8:** 281–290.

Forey, P. and Janvier, P. 1994. Evolution of the early vertebrates. *American Scientific*. **82**: 554-565.

Forrest, D., Sjoberg, M., and Vennstrom, B. 1990. Contrasting developmental and tissue specific expression of α and β thyroid hormone receptor genes. *The EMBO Journal*. **9**(5): 1519-1528.

Forrest, D., Erway, L.C., Ng, L., Altschuler, R., and Curran, T. 1996a. Thyroid hormone receptor β is essential for development of auditory function. *National Genetics*. **13**: 354–357.

Forrest, D., Hanebuth, E., Smeyne, R.J., Everds, N., Stewart, C.L., Wehner, J.M., and Curran, T. 1996b. Recessive resistance to thyroid hormone in mice lacking thyroid hormone receptor β: evidence for tissue-specific modulation of receptor function. *European Molecular Biology Organization Journal*. **15**: 3006–3015.

Fraichard, A., Chassande, O., Plateroti, M., Roux, J.P., Trouillas, J., Dehay, C., Legrand, C., Gauthier, K., Kedinger, M., Malaval, L., Rousset, B., and Samarut, J. 1997. The T3Rα gene encoding a thyroid hormone receptor is essential for post-natal development and thyroid hormone production. *The EMBO Journal*. **16**(14): 4412-4420.

Froese, R. 2006. Cube law, condition factor and weight-length relationships: history, meta-analysis and recommendations. *The Journal of Applied Ichthyology*. **22**: 241-253.

Fulton, M.H. and Key, P.B. 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environmental Toxicology and Chemistry*. **20**(1): 37-45.

Gadgil, M. and Heda, N. 2009. Of rivers, fish and poison. *International Journal of Ecology and Environmental Sciences.* **35**(1): 1-11.

Grandi, G. and Chicca, M. 2004. Early development of the pituitary gland in *Acipenser nacarii* (Chondrostei, Acipenseriformes) an immunocytochemical study. *Anatomy and Embryology*. **208**: 311–321.

Gisbert, E. and Williot, P. 1997. Larval Behavior and effect of the timing of initial feeding on growth and survival of Siberian Sturgeon (*Acipenser baeri*) larvae under small scale hatchery production. *Aquaculture*. **156**: 63-76.

Greenblatt, M., Brown, C.L., Lee, M., Dauder, S., and Bern, H.A. 1989. Changes in Thyroid-Hormone Levels in Eggs and Larvae and in Iodide Uptake by Eggs of Coho and Chinook Salmon, *Oncorhynchus kisutsch* and *Oncorhynchus tschawytscha*. *Fish Physiology and Biochemistry*. **6**(5): 261-278.

Hadley, M.E. and Levine, J.E. 2007. Endocrinology 6th edition. Prentice Hall, New Jersey, USA.

Harder, S., Dammann, O., Buck, F., Zwiers, H., Lederis, K., Richter, D., and Bruhn, T.O. 2001. Cloning of two thyrotropin-releasing hormone receptor subtypes from a lower vertebrate (*Catostomus commersoni):* Functional expression, gene structure, and evolution. *General and Comparative Endocrinology.* **124(2)**: 236–245.

Hardy, R.S. and Litvak, M.K. 2004. Effects of temperature on the early development, growth, and survival of Shortnose Sturgeon, *Acipenser brevirostrum*, and Atlantic Sturgeon, *Acipenser oxyrhynchus*, yolk-sac larvae. *Environmental Biology of Fishes*. **70**: 145-154.

Harkness, W.J.K. 1936. Report on the sturgeon situation in Manitoba. *Manitoba Department of Natural Resources Manuscript Repor*t. 80-31980:18.

Harvey, C.B. and Williams, G.R. 2002. Mechanism of thyroid hormone action. *Thyroid.* 12: 441–446.

Heath, A.G., Cech, J.J., Zinkl, J.G., Finlayson, B., and Fujimura, R. 1993. Sublethal effects of methyl parathion, carbofuran, and molinate on larval Striped Bass. *Water Quality and the Early Life Stages of Fishes*. **14**: 17-28.

Higgs, D.A. and Eales, J.G. 1971. Iodide and thyroxine metabolism in the Brook Trout, *Salvelinus fontinalis* (Mitchill) during sustained exercise. *Canadian Journal of Zoology.* **49**: 1255-69.

Holmes, J.A. and Youson, J.H. 1993. Induction of metamorphosis in landlocked Sea Lampreys, *Petromyzon marinus*. *Journal of Experimental Zoology.* **267**: 598-604.

Huang, L., J.L. Specker, and D.A. Bengtson. 1998a. Thyroxine (T4) accelerates gastric gland formation and function in metamorphosing Summer Flounder (*Paralichthys dentatus*). *Journal of Experimental Zoology*. **280**: 231-237.

Huang, L., Miwa, S., Bengtson, D.A. and Specker, J.L. 1998b. Effoct of triiodothyronine on stomach formation and pigmentation in larval Striped Bass (*Morone saxatilis*). *The Journal of Experimental Biology.* **280**: 231-237.

Hulme, E.C., Soper, A.K., McLain, S.E., and Finney, J.L. 2006. The hydration of the neurotransmitter acetylcholine in aqueous solution. *Biophysical Journal.* **91**(6): 2371-2380.

Hunn, J,B. and From, P.O. 1966. *In vivo* uptake of radioiodide by Rainbow Trout. *Journal of Water Pollution Control Federation*. **38**: 1981-1985.

Inui, Y. and S. Miwa. 1985. Thyroid hormone induces metamorphosis of flounder larvae. *General and Comparative Endocrinology*. **60**: 450-454.

Inui, Y., Yamano, K. and Miwa, S. 1995. The role of thyroid hormone in tissue development in metamorphosing flounder. *Aquaculture*. **135**: 87-98.

Johnson, K.M. and Lema, S.C. 2011. Tissue specific thyroid hormone regulation of gene transcripts encoding iodothyronine deiodinases and thyroid hormone receptors in Striped Parrotfish. *General and Comparative Endocrinology*. **172**: 505-517.

Kappenman, K.M., Fraser, W.C., and Toner, M. 2009. Effect of temperature on growth, condition, and survival of juvenile Shovelnose Sturgeon. *American Fisheries Society*. **138**: 927-937.

Kavitha, P. and Rao, J.V. 2008. Toxic effects of chlorpyrifos on antioxidant enzymes and target enzyme acetylcholinesterase interaction in Mosquito Fish, *Gambusia affinis*. *Environmental Toxicology and Pharmacology*. **26**(2): 192-198.

Kawakami, Y., Seoka, M., Miyashita, S., Kumai, H. and Ohta, H. 2006a. Characterization of transthyretin in the Pacific Blue Tuna, *Thunnus orientalis*. *Zoological Science*. 23: 443-448.

Kawakami, Y., Shin, D., Kitano, T., Adachi, S., Yamauchi, K., and Ohta, H. 2006b. Transactivation activity of thyroid hormone receptors in fish (*Conger myriaster*) in response to thyroid hormones. *Comparative Biochemistry and Physiology*. **144B**(4): 503-509.

Kempinger, J.J. 1988. Spawning and early life history of Lake Sturgeon in the Lake Winnebago system, Wisconsin. *American Fisheries Society Symposium*. pp. 110-122.

Kempinger, J.J. 1996. Habitat, growth, and food of young Lake Sturgeon in the Lake Winnebago system, Wisconsin. *North American Journal of Fisheries Management*. **16**: 102-114.

Klaren, P.H.M., Haasdijk, R., Metz, J.R., Nitsch, L.M.C., Darras, V.M., Van der Guyten, S. and Flik, G. 2005. Characterization of an iodothyronine 5'-deiodinse in Gilthead Seabream (*Sparus auratus*) that is inhibited by dithiothreitol. *Endocrinology*. **146(12)**: 5621-5630.

Klassen, C.N. and Peake, S.J. 2008. Effect of diet switch timing and food source on survival and growth of Lake Sturgeon. *Journal of Applied Ichthyology*. **24**: 527-533.

Klaverkamp, J.F., Duangsawasdi, M., Macdonald, W.A., and Majewski, H.S. 1977. An evaluation of fenitrothion toxicity in 4 life stages of Rainbow Trout *Salmo Gairdneri*. *American Society for Testing and Materials*. **634**: 231-240.

Kmiec, Z., Kotlarz, K., Smiechowska, B., and Mysliwski, A. 1998. The effect of fasting and refeeding on thyroid follicle structure and thyroid hormone levels in young and old rats. *Archives of Gerontology and Geriatrics*. **26**: 161–175.

Kumar, R.S., Ijiri, S., Kight, K., Swanson, P., Dittman, A., Alok, D., Zohar, Y., and Trant, J.M. 2000. Cloning and functional expression of a thyrotropin receptor from the gonads of a vertebrate (bony fish): potential thyroid-independent role for thyrotropin in reproduction. *Molecular and Cellular Endocrinology.* **167**(1-2): 1-9.

Kwong, T.C. 2002. Organophosphate pesticides: Biochemistry and clinical toxicology. *Therapeutic Drug Monitoring*. **24**(1): 144-149.

Lam, T.J. 1994. Hormones and egg/larval quality in fish. *Journal of the World Aquaculture Society*. **25**(1): 2-12.

Lazar, M.A. 1993. Thyroid hormone receptors: multiple forms, multiple possibilities. *Endocrine Reviews*. **14**: 184–193.

Lee, H. and Yen, P.M. 1999. Recent advances in understanding thyroid hormone receptor co-regulators. *Journal of Biomedical Sciences*. **6**: 71–78.

Leloup, J. and Fontaine, M. 1960. Iodine metabolism in lower vertebrates. *Annals New York Academy of Science.* **86**: 316-53.

Leloup, J. 1970. The mechanisms of blood iodine regulation and their endocrine control in teleosts in freshwater. *Mémoires du Muséum national d'histoire naturelle. Série A, Zoologie.* **62:** 1–108.

Lema, S.C., Dickey, J.T., Schultz, I.R., and Swanson, P. 2009. Thyroid regulation of mRNAs encoding thyrotropin b-subunit, glycoprotein a-subunit, and thyroid hormone receptors a and b in brain, pituitary gland, liver, and gonads of an adult teleost, *Pimephales promelas*. *Journal of Endocrinology*. **202**: 43–54.

MacKenzie, D.S., Jones, R.A., and Miller, T.C. 2009. Thyrotropin in teleost fish. *General and Comparative Endocrinology*. **161**: 83-89.

Mai, W., Janier, M.F., Allioli, N., Quignodon, L., Chuzel, T., Flamant, F., and Samarut, J. 2004. Thyroid hormone receptor alpha is a molecular switch of cardiac function between fetal and postnatal life. *Proceedings of the National Academy of Science U.S.A*. **101**: 10332–10337.

Makynen, E.A., Kahl, M.D., Jensen, K.M., Tietge, J.E., Wells, K.L., Van der Kraak, G., and Ankley, G.T. 2000. Effects of the mammalian antiandrogen vinclozolin on development and reproduction of the Fathead Minnow (*Pimephales promelas*). *Aquatic Toxicology*. **48**(4): 461-475.

Mantovani, A. and Maranghi, F. 2005. Risk assessment of chemicals potentially affecting male fertility. *Contraception*. **72**(4): 308-313.

Mantovani, A. 2006. Risk assessment of endocrine disrupters - The role of toxicological studies. *Annals of the New York Academy of Sciences*. **1076**: 239-252.

Marchand, O., Safi, R., Escriva, H., Van Rompaey, E., Prunet, P. and Laudet, V. 2001. Molecular cloning and characterization of thyroid hormone receptors in teleost fish. *Journal of Molecular Endocrinology.* **26**: 51–65.

Marchelidon, J., Brisson-Martin, A. and Lachiver, E. 1971. Composition en acides aminos de la thyroglobuline de divers poissons. *General and Comparative Endocrinology.* **18**: 601-602.

McEnroe, M. and Cech, J.J. 1994. Measurements of thyroid hormones, T3 and T4, in juvenile white sturgeon over the annual cycle. *In:* High Performance Fish: Proceedings of an International Fish Physiology Symposium (D. D. Machinlay, Ed.), pp. 182–185. University of British Columbia, Vancouver.

McLeese, J.M., Wright, G.M., Youson, J.H. and Eales, J.G. 2000. Deiodination activity in extrathyroidal tissues of the Atlantic Hagfish, *Myxine glutinosa. Journal of Experimental Zoology.* **287:** 445–452.

Mendel, C.M., Weisiger, R.A., Jones, A.L. and Cavalieri, R.R. 1987. Thyroid hormone binding proteins in plasma facilitate uniform distribution of thyroxine within tissues - a perfused rat liver study. *Endocrinology.* **120**: 1742–1749.

Miwa, S., Yamano, K. and Inui, Y. 1992. Thyroid hormone stimulates gastric development in flounder larvae during metamorphosis. *Journal of Experimental Biology.* **261**: 424–430.

Mol, K.A., Van der Guyten, S., Darras, V.M., Visser, T.J. and Kuhn, E.R. 1997. Characterization of the iodothyronine outer ring and inner ring deiodinase activities in the Blue Tilapia, *Oreochromis aureus. Endocrinology*. **138(5)**: 1787-1793.

Mol, K.A., Van der Guyten, S., Burel, C., Kuhn, E.R., Boujard, T. and Darras, V.M. 1998. Comparative study of iodothyronine outer ring and inner ring deiodinase activities in five teleostean species. *Fish Physiology and Biochemistry*. **18**: 253-266.

Montesinos, M.M., Pellizas, C.G., Velez, M.L., Susperreguy, S., Masini-Repiso, A.M., and Coleoni, A.H. 2006. Thyroid hormone receptor β1 gene expression is increased by dexamethasone at transcriptional level in rat liver. *Life Science*. **78**: 2584-2594.

Moren, M., Sloth, J.J., and Hamre, K. 2008. Uptake of iodide from water in Atlantic Halibut larvae (*hippoglossus hippoglossus L.*). *Aquaculture*. **285**: 174-178.

Morgado, I., Melo, E.P., Lundberg, E., Estrela, N.L., Sauer-Eriksson, A.E., and Power, D.M. 2008. Hormone affinity and fibril formation of piscine transthyretin: the role of the N-terminal. *Molecular and Cellular Endocrinology*. **295**: 48-58.

Morin, P.P., Winberg, S., Nilsson, G.E., Hara, T.J. and Eales, J.G. 1997. Effects of l-thyroxine on brain monoamines during parr-smolt transformation of Atlantic Salmon (*Salmo salar* L.). *Neuroscience Letters.* **224**: 216-218.

Muhki, S. and Patinõ, R. 2007. Effects of prolonged exposure to perchlorate on thyroid and reproductive function in zebrafish. *Toxicological Science*. **96**: 246–254.

Muhki, S., Torres, L. and Patinõ, R. 2007. Effects of larval-juvenile treatment with perchlorate and co-treatment with thyroxine on Zebrafish sex ratios. *General and Comparative Endocrinology*. **150**: 486-494.

Nakai, Y. and Gorbman, A. 1969. Cytoogical and functional properties of the thyroid of Chimaeroid fish *Hydrolagus colliei*. *General and Comparative Endocrinology*. **13**: 285-302.

Nelson, E.R. and Habibi, H.R. 2006. Molecular characterization and sex-related seasonal expression of thyroid receptor subtypes in goldfish. *Molecular and Cellular Endocrinology*. **253**: 83–95.

Nelson, E.R. and Habibi, H.R. 2008. Functional significance of a truncated thyroid receptor subtype lacking a hormone-binding domain in goldfish. *Endocrinology*. **149**(9): 4702-4709.

Nelson, E.R. and Habibi, H.R. 2009. Thyroid receptor sub-types: structure and function in fish. *General and Comparative Endocrinology*. **161**: 90-96.

Norris, D.O. and Carr, J.A. 2013. Vertebrate Endocrinology 5th Edition. Academic Press. Waltham, USA.

Oba, Y., Hirai, T., Yoshiura, Y., Kobayashi, T., and Nagahama, Y. 2001. Fish gonadotropin and thyrotropin receptors: the evolution of glycoprotein hormone receptors in vertebrates. *Comparative Biochemistry and Physiology*. **129B**: 441–448.

Orozco, A., Ruiz, J.A., and Valverde, R.C. 1992 The importance of employing homologous serum free of thyronines in radioimmunoassays to assess circulating thyroid hormones in rainbow trout. *Boletín de Estudios Médicos y Biológicos*. **40**: 41-47.

Orozco, A., Silva, J.A., and Valverde-R, C. 1997. Rainbow trout liver expresses two iodothyronine phenolic ring deiodinase pathways with the characteristics of mammalian types I and II 5'-deiodinases. *Endocrinology*. **138**(1): 254-258.

Ortiz-Delgado, J.B., Ruane, N.M., Pousão-Ferreira, M., Dinis, M.T., and Sarasquete, C. 2006. Thyroid gland development in Senegalese Sole (*Solea senegalensis*) during early life stages: A histochemical and immunohistochemical approach. *Aquaculture*. **60**: 346–356.

Oruç, E.O. 2010. Oxidative stress, steroid hormone concentrations and acetylcholinesterase activity in *Oreochromis niloticus* exposed to chlorpyrifos. *Pesticide Biochemistry and Physiology*. **96**(3): 160-166.

Palace, V., Park, B., Pleskach, K., Gemmill, B., and Tomy, G. 2010. Altered thyroxine metabolism in Rainbow Trout (*Oncorhynchus mykiss*) exposed to hexabromocyclododecane (HBCD). *Chemosphere*. **80**: 165–169.

Park, J.W., Rinchard, J., Liu, F., Anderson, T.A., Kendall, R.J. and Theodorakis, C.W. 2006. The thyroid endocrine disruptor perchlorate affects reproduction, growth, and survival of Mosquito Fish. *Ecotoxicology and Environmental Safety*. **63**: 343–352.

Park, B.J., Palace, V., Wautier, K., Gemmil, B., and Tomy, G. 2011. Thyroid axis disruption in juvenile Brown Trout (*Salmo trutta*) exposed to the flame retardant β-tetrabromoethylcyclohexane (β-TBECH) via the diet. *Environmental Science and Technology*. **45**(18): 7923-7927.

Peterson, P.A. 1971. Studies on the interaction between pre-albumin, retinol-binding protein, and vitamin A. *The Journal of Biological Chemistry*. **246**: 44–49.

Piazza, Y.G., Pandolfi, M., and Lo Nostro, F.L. 2011. Effect of the Organochlorine Pesticide Endosulfan on GnRH and Gonadotrope Cell Populations in Fish Larvae. *Archives of Environmental Contamination and Toxicology*. **61**(2): 300-310.

Pikitch, E.K., Doukakis, P., Lauck, L., Chakrabarty, P. and Erickson, D. 2005. Status, trends and management of sturgeon and paddlefish fisheries. *Fish and Fisheries.* **6**: 233-265.

Pimentel, D. 1995. Amounts of Pesticides Reaching Target Pests - Environmental Impacts and Ethics. *Journal of Agriculture and Environmental Ethics*. **8**(1): 17-29.

Plohman, J.C., Dick, T.A., and Eales, J.G. 2002a. Thyroid of Lake Sturgeon, *Acipenser fulvescens* I. hormone levels in blood and tissues. *General and Comparative Endocrinology*. **125**(1): 47-55.

Plohman, J.C., Dick, T.A., and Eales, J.G. 2002b. Thyroid of Lake Sturgeon, *Acipenser fulvescens* II. deiodination properties, distribution, and effects of diet, growth and a T3 challenge. *General and Comparative Endocrinology*. **125**(1): 56-66.

Pope, C.N. 1999. Organophosphorous pesticides: do they all have the same mechanism of toxicity? *Journal of Toxicology and Environmental Health, Part B: Critical Reviews.* **2**(2): 161-181.

Power, D.M., Elias, N.P., Richardson, S.J., Mendes, J., Soares, C.M., and Santos, C.R.A. 2000. Evolution of the thyroid hormone-binding protein, transthyretin. *General and Comparative Endocrinology*. **119**: 241-255.

Power, D.M., Llewellyn, L., Faustino, M., Nowell, M.A., Bjornsson, B.T., Einarsdottir, I.E., Canario, A.V.M., and Sweeney, G.E. 2001. Thyroid hormones in growth and development of fish. *Comparative Biochemistry and Physiology*. **130C**: 447-459.

Raine, J.C. and Leatherland, J.F. 2000. Morphological and functional development of the thyroid tissue in Rainbow Trout (*Oncorhynchus mykiss*). *Cell and Tissue Research*. **301**: 235–244.

Raine, J.C., Takemura, A., and Leatherland, J.F. 2001. Assessment of thyroid function in adult medaka (*Oryzias latipes)* and juvenile Rainbow Trout (*Oncorhynchus mykiss)* using immunostaining methods. *Journal of Experimental Biology.* **290**(4): 366–378.

Rawlings, N.C., Cook, S.J., and D. Waldbillig. 1998. Effects of the pesticides carbofuran, chlorpyrifos, dimethoate, lindane, triallate, trifluralin, 2,4-D, and pentachlorophenol on the metabolic endocrine and reproductive endocrine system in ewes. *Journal of Toxicology and Environmental Health*. Part A: **54**: 21–36.

Rawn, D.F.K, and Muir, D.C.G. 1999. Sources of chlorpyrifos and decthal to a small Canadian prairie watershed. *Environmental Sciences and Technology*. **33**: 3317-3323.

Reddy, P.K. and Lam, T.J. 1992. Effect of thyroid hormones on morphogenesis and growth of larvae and fry of telescopic-eye Black Goldfish, *Carassius auratus*. *Aquaculture.* **107**: 383-394.

Reddy, P.K., Brown, C.L., Leatherland, J.F., and T.J. Lam. 1992. Role of thyroid hormones in tilapia larvae (*Oreochromis massambicus*): II. changes in the hormones and 5' monodeiodinase activity during development. *Fish Physiology and Biochemistry*. **9(5/6)**: 487-496.

Richardson, S.J., Bradley, A.J., Duan, W., Wettenhall, R.E., Harms, P.J., Babon, J.J., Southwell, B.R., Nicol, S., Donnellan, S.C. and Schreiber, G. 1994. Evolution of marsupial and other vertebrate thyroxine-binding plasma proteins. *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*. **266**: R1359–1370.

Richardson, S.J., Monk, J.A., Shepherdley, C.A., Ebbesson, L.O.E., Sin, F., Power, D.M., Frappell, P.B. Kohrle, J. and Renfree, M.B. 2005. Developmentally regulated thyroid hormone distributer proteins in marsupials, a reptile and fish. *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*. **288**: R1264-R1272.

Ring, P., Bjorkman, U., and Ekholm, R. 1987. Localization of the incorporation of 3H-galactose and 3H-sialic acid into thyroglobulin in relation to the block of intracellular transport induced by monensin: Studies with isolated porcine thyroid follicles. *Cell and Tissue Research.* **250**: 149–156.

Romare, P. 2010. Growth of larval and juvenile perch: the importance of diet and fish density. *Journal of Fish Biology.* **56:** 876–889.

Ronyai, A. 1999. Effect of food ration and water temperature on growth of juvenile Siberian Sturgeon (*Acipenser baeri* Brandt). *Journal of Applied Ichthyology*. **15**(4): 344-345.

Rutland, B.E., Nachreiner, R.F., and Kruger, J.M. 2009. Optimal testing for thyroid hormone concentration after treatment with methimazole in healthy and hyperthyroid cats. *Journal of Veterinary Internal Medicine*. **23**: 1025-1030.

Rybnikar, J., Prokes, M., Mares, J., and Cilicek, M. 2011. Early development and growth of sterlet (*Acipenser ruthenus*) in the Czech Republic. A*CTA Universitatis Agriculturae et Viculturae Medelianae Brunensis*. **26**(5): 217-226.

Salvatore, G. 1969. Thyroid hormone biosynthesis in agnatha and protochordata. *General and Comparative Endocrinology Supplement*. **2**: 535-552.

Samaranayaka, M. 1977. Role of Acetylcholine in Organophosphate-Induced Release of Adipokinetic Hormone in Locust *Schistocerca Gregaria*. *Pesticide Biochemistry Physiology*. **7(3)**: 283-288.

Santos, C.R. and Power, D.M. 1999. Identification of transthyretin in fish (*Sparus aurata*): cDNA cloning and characterisation. *Endocrinology*. **140**: 2430–2433.

Santos, C.R.A, Anjos, L. and Power, D.M. 2002. Transthyretin in fish: stat of the art. *Clinical Chemistry and Laboratory Medicine*. **40**(12): 1244-1249.

Saxena, P.K. and K. Mani. 1988. Effects of safe concentrations of some pesticides on thyroid in the freshwater murrel, *Channa punctatus*: a histopathological study. *Environmental Pollution*. **55**: 97-105.

Schnitzler, J.G., Koutrakis, E., Siebert, U., Thomé, J.P., and Das, K. 2008. Effects of persistent organic pollutants on the thyroid function of the European Sea Bass (*Dicentrarchus labrax*) from the Aegean sea, is it an endocrine disruption. *Marine Pollution Bulletin*. **56**: 1755–1764.

Scott, W.B. and Crossman, E.J. 1973. Freshwater fishes of Canada. *Bulletin Fisheries Research Board of Canada*. **184**: 1-966.

Shafi, M., Wang, Y., Wang, W., Muhammad, F., Qi, J. and Zhang, Q. 2012. Isolation and characterization of thyroid hormone receptors (TRα and TRβ) in Black Rock Fish, *Sebastes schlegelii*. *Pakistan Journal of Zoology*. **44(5)**: 1215-1223.

Sharma, P. and Patino, R. 2013. Regulation of gonadal sex ratios and pubertal development by the thyroid endocrine system in Zebrafish (D*anio rerio*). *General and Comparative Endocrinology*. **184**: 111-119.

Shi, X., Liu, C., Wu, G., and Zhou, B. 2009. Waterborne exposure to PFOS causes disruption of the hypothalamus-pituitary-thyroid axis in Zebrafish larvae. *Chemosphere*. **77**: 1010-1018.

Sinha, N., Lal, B., and T.P. Singh. 1991. Pesticides induced changes in circulating thyroid hormones in the freshwater catfish *Clarias batrachus*. *Comparative Biochemistry and Physiology*. **100(1/2)**: 107-110.

Specker, J. L., Brown, C. L. and Bern, H. A. 1992. Asynchrony of changes in tissue and plasma thyroid hormones during the parr–smolt transformation of Coho Salmon. *General and Comparative Endocrinology.* **88:** 397– 405.

Suzuki, S., Gorbman, A, Rolland, M., Montfort, M-F. and Lissitzky, S. 1975. Thyroglobulins of cyclostomes and an elasmobranch. *General and Comparative Endocrinology*. **26**: 59-69.

Szczezepkowski, M., Szczezepkowska, B., and Piotrowska, I. 2011. Impact of higher stocking density of juvenile, Atlantic Sturgeon, *Acipenser oxyrinchus* Mitchill, on fish growth, oxygen consumption, and ammonia excretion. *Archives of Polish Fisheries.* **19**(2): 59-67.

Szkudlinski, M.W., Fremont, V., Ronin, C., and Weintraub, B.D. 2002. Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationships. *Physiology Review*. **82**(2): 473-502.

Tachihara, K., El Zibdeh, M.L., Ishimatsu, A., and Tagawa, M. 1997. Improved seed production of Goldstriped Amberjack *Seriola lalandi* under hatchery conditions by injection of triiodothyronine (T3) to broodstock fish. *Journal of the World Aquatic Society*. **28**: 34–44.

Tagawa, M., Tanaka, M., Matsumoto, S. and Hirano, T. 1990. Thyroid hormones in eggs and various freshwater, marine and diadromous teleosts and their changes during egg development. *Fish Physiology and Biochemistry*. **8**(6): 515-520.

Tanaka, M. 1971. Studies on the structure and function of the digestive system in teleost larvae-III. Development of the digestive system during postlarval stage. *Japanese Journal of Ichthyology*. **18**: 164-174.

Tanaka, M., Tanangonan, J.B., Tagawa, M., de Jesus, E.G., Nishida, H., Isaka, M., Kimura, R. and Hirano, T. 1995. Development of the pituitary, thyroid and interrenal glands and applications of endocrinology to the improved rearing of marine fish larvae. *Aquaculture.* **135**: 111-126.

Taurog, A. 2000. Hormone Synthesis: Thyroid Iodine Metabolism. In: The Thyroid: A Fundamental and Clinical Text, 8th ed., L.E. Braver- man and R.D. Utiger, eds., pp 61–85. Lippincott-Raven, Philadelphia.

Tian, H., Ru, S., Wang, W., and Bing, X. 2010. Effects of monocrotophos on the reproductive axis in the female goldfish (*Carassius auratus). Comparative Biochemistry and Physiology*. **152C**(1): 107-113.

Thangavel, P., Sumathiral, K., Karthikeyan, S., and Ramaswamy, M. 2005. Endocrine response of the freshwater teleost *Sarotherodon mossambicus* (Peters) to dimecron exposure. *Chemosphere*. 61: 1083-1092.

Tomy, G.T., Palace, V.P., Pleskach, K., Ismail, N., Oswald, T., Danell, R., Wautier, K., and Evans, B. 2007. Dietary exposure of juvenile Rainbow Trout (*Oncorhynchus mykiss*) to 1,2-bis(2,4,6-tribromophenoxy)ethane: bioaccumulation parameters, biochemical effects, and metabolism. *Environmental Science and Technology*. **41**: 4913-4918.

Tripathi, G. and Shasmal, J. 2010. Reparation of chlorpyrifos-induced impairment by thyroxine and vitamin C in fish. *Ecotoxicology and Environmental Safety*. **73**(6): 1397-1401.

Van der Guyten, S., Mol, K.A., Pluymers, W., Kuhn, E.R. and Darras, V.M. 1998. Changes in plasma T_3 fasting/refeeding in tilapia (*Oreochromis niloticus*) are mainly regulated through changes in hepatic type II iodothyronine deiodinase. *Fish Physiology and Biochemistry*. **19**: 135-143.

Van der Guyten, S., Toguyeni, A., Baroiller, J.F., Fauconneau, B., Fostier, A., Sanders, J.P., Visser, T.J., Kuhn. E.R., and Darras, V.M. 2001. Hypothyroidism induces type I iodothyronine deiodinase expression in tilapia liver. *General and Comparative Endocrinology*. **124**: 333-342.

Vayre, L., Sabourin, J.C., Caillou, B., Ducreux, M., Schlumberger, M., Bidart, J.M. 1999. Immunohistochemical analysis of Na+/I− symporter distribution in human extra-thyroidal tissues. *European Journal of Endocrinology*. **141**: 382–386.

Viswanath, G., Chatterjee, S., Dabral, S., Nanguneri, S.R., Divya, G., and Roy, P. 2010. Anti-androgenic endocrine disrupting activities of chlorpyrifos and piperophos. *Journal of Steroid Biochemistry and Molecular Biology.* **120**(1): 22-29.

Wendelaar-Bonga, S.E. 1993. Endocrinology. In: The Physiology of Fishes, D. H. Evans, ed., pp. 469–502. CRC Press, Boca Raton, FL.

Weirich, R. T., Schwartz, H. L. and Oppenheimer, J. H. 1987. An analysis of the interrelationships of nuclear and plasma triiodothyronine in the Sea Lamprey, Lake Trout, and rat: Evolutionary considerations. *Endocrinology.* **120:** 664 – 677.

Wiggs, A.J. 1971. Seasonal changes in the extrathyroidal distribution of iodide in the teleost fish the burbot, *Lota Iota. Canadian Journal of Zoology.* **49**: 1505-11.

Wikstrom, L., Johansson, C., Salto, C., Barlow, C., Campos-Barros, A., Baas, F., Forrest, D., Thoren, P., and Vennstrom, B. 1998. Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor α1. *The EMBO Journal*. **17**: 455-461.

Williams, J.E., Johnson, J.E., Hendrickson, D.A., Contrerasbalderas, S., Williams, J.D., Navarromendoza, M., Mcallister, D.E., and Deacon, J.E. 1989. Fishes of North-America Endangered, Threatened, Or of Special Concern. *Fisheries*. **14**(6): 2-20.

Yamano, K., Miwa, S., 1998. Differential gene expression of thyroid hormone receptor α and β in fish development. *General and Comparative Endocrinology*. **109**: 75-85.

Yamano, K. 2005. The role of thyroid hormone in fish development with reference to aquaculture. *Japan Agriculture Research Quarterly*. **39**(3): 161-168.

Yoshiura, Y., Sohn, Y.C., Munakata, A., Kobayashi, M., and Aida, K. 1999. Molecular cloning of the cDNA encoding the beta subunit of thyrotropin and regulation of its gene expression by thyroid hormones in the goldfish, *Carassius auratus*. *Fish Physiology and Biochemistry*. **21**(3): 201-210.

Youson, J.H., Plisetskaya, E.M. and Leatherland, J.F. 1994. Concentrations of insulin and thyroid hormones in the serum of landlocked Sea Lampreys (*Petromyzon marinus*) of three larval year classes, larvae exposed to two temperature regimes, and both during and after metamorphosis. *General and Comparative Endocrinology*. **94**: 294–304.

Youson, J.H., Manzon, R.G., Peck, B.J. and Holmes, J.A. 1997. Effecs of exogenous thyroxine (T_4) and triiodothyronine (T_3) levels in immediately premetamorphic Sea Lampreys, *Petromyzon marinus*. *The Journal of Experimental Zoology*. **279**: 145-155.

Yu, L., Lam, J.C.W., Guo, Y., Wu, R.S.S., Lam, P.K.S., and B. Zhou. 2011. Parental transfer of polybrominated diphenyl ethers (PBDEs) and thyroid endocrine disruption in Zebrafish. *Environmental Science and Technology*. **45**: 10652-10659.

Zandieh-Doulabi, B., Schiphorst, M.P., Kalsbeek, A., Wiersinga, W.M., and Bakker, O. 2004. Hyper and hypothyroidism change the expression and diurnal variation of thyroid hormone receptor isoforms in rat liver without major changes in the zonal distribution. *Molecular and Cellular Endocrinology*. **219**: 69-75.

Zoeller, R.T., Tan, S.W., and Tyl, R.W. 2007. General background on the hypothalamic-pituitary-thyroid (HPT) axis. *Critical Reviews in Toxicology*. **37**: 11-53.

Zubair, S., Peake, S.J., Hare, J.F. and Anderson, W.G. 2012. The effect of temperature and substrate on the development of the cortisol stress response in the Lake Sturgeon, *Acipenser fulvescens*, Rafinesque (1817). *Environmental Biology of Fishes.* 93: 577-587.