EFFECTS OF THYROID INHIBITION AND REARING TEMPERATURE ON ZEBRAFISH DEVELOPMENT

By

Masha Reider

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Chair: Victoria Connaughton, Ph.D.

Maria Gomez, Ph.D.

Bryan Fantie, Ph.D.

Dean of the College of Arts and Sciences

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ABSTRACT

Zebrafish development is a complex process that is highly dependent on the thyroid system. The purpose of this study was to examine the effects of thyroid hormone deprivation and rearing temperature on embryonic development. Embryos were exposed to different concentrations of methimazole (MMI), a known thyroid inhibitor, and maintained at either 28°C or 31°C. Changes in growth were assessed by determining changes in 4 parameters: eye size, inter-eye distance, body length, and spinal curvature. Initial experiments tested the effects of three MMI concentrations (0.1mM, 0.2mM, and 0.3mM) at three developmental ages (50, 60, and 72 hours postfertilization; hpf). Results from this study identified 60-72hpf as a critical developmental window for thyroid inhibitor sensitivity and 0.3mM as the MMI concentration that gave the most consistent results. Subsequent experiments exposed embryos to 0.3mM and subsampled hourly between 60 and 72hpf. Results of morphological measurements indicated a significant temperature effect for all growth parameters examined in treated fish ($p < 0.01$). Our study also shows that the embryonic thyroid system is functioning and capable of feedback mechanisms between 66-68 hpf. Further, we found that MMI significantly affected the development of retinal layers, where MMI exposure caused significant decreases in cell and synaptic layers at 72 hpf.
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CHAPTER 1
INTRODUCTION

General Embryonic Zebrafish Development

Zebrafish development, as in other vertebrates, proceeds through specific stages that occur at known times after fertilization. As a result, developmental age can be represented as either time post fertilization (i.e., hours post fertilization; hpf) or developmental stage (i.e., blastula, epiboly, etc.). A seminal paper by Kimmel (1995) was instrumental in the understanding and successful staging of zebrafish embryonic development. Zebrafish embryos are transparent and develop externally, allowing clear access and visibility to the embryos. While each healthy embryo experiences similar developmental events, there is great variability of developmental rate, even within a single clutch or clonal line (Streisinger et al., 1981). Therefore, staging based on hours following fertilization is not a precise measure of developmental age, and morphological criteria must be used in order to accurately stage each embryo. The presented stages were used to go beyond discussing zebrafish embryos as a point in time; rather, accurate staging can provide information about the events occurring during the developmental continuum (Kimmel, 1995).

The first stage of embryonic development is called the zygote period, which lasts until the first cleavage. The zygote stage, or single cell stage, marks the beginning of the blastodisc and vegetal cytoplasmic separation that continues into the cleavage period. The
cleavage period lasts until about 2 ¼ hpf, with cells dividing at approximately 15-minute intervals and ending with 64 total blastomeres.

Next is the blastula period, marked by the midblastula transition, a period of asynchronous cell lengthening. At this time, the yolk syncytial layer (YSL), a structure found exclusively in teleosts, is formed and begins to divide and enlarge. Epiboly also begins during the blastula stage and is characterized by the thinning and covering of the YSL and blastodisc over the yolk. This continues through the gastrulation stage until the yolk is fully covered (Kimmel, 1995).

The gastrula stage, beginning at ~5 ½ hpf, continues epiboly, as well as several cellular and molecular events, including involution, leading to formation of the primary germ layers (epiblast and hypoblast) and the embryonic axis. The cells of the epiblast later become the epidermis and CNS, whereas the hypoblast cells become the mesoderm and endoderm (Kimmel, 1995). Towards the end of gastrulation at ~10 hpf, the first part of the CNS can be seen as a thickening of the dorsal epiblast, eventually becoming the neural plate. Gastrulation is complete following the formation of the tail bud at the caudal part of the embryo and the end of epiboly, when the yolk plug is entirely covered by the blastoderm (Kimmel, 1995).

Next is the segmentation period, which lasts from ~10-24 hpf, on average. At this time, several significant morphological events occur: the embryo lengthens, somites develop, the primary organs become more developed, and the tail bud grows and extends away from the body of the embryo (Kimmel, 1995). At this time, the anterior part of the neural plate becomes thickened and is considered the brain rudiment. The formation of the neural tube in zebrafish occurs by a process called secondary neurulation, where the
neural keel forms first and becomes a cylinder that hollows out to form the neural tube. Optic and otic primordia, lens placode, and the primary neurons form during this period.

The pharyngula period occurs from ~24-48 hpf. The bilateral embryo has a formed notochord with a rapidly maturing nervous system during this period. The brain matures into a five-lobed structure, the body keeps lengthening, and the fins begin to form. At this time, the circulatory system is mature, such that the heart begins to beat and the chambers are distinguishable and functional.

Next is the hatching period between 48-72 hpf, where the chorion that engulfs the embryo is detached and the tail straightens outward. Hatching is somewhat random, since embryos that hatch earlier are no more developmentally mature than unhatched embryos. During this stage, organ morphogenesis does not proceed at the rapid rate seen in earlier stages. However, the pectoral fins continue to develop quickly and begin to receive blood flow. Lastly, the mouth develops and rotates anteriorly during this time, and jaw cartilage continues to form.

**Retina**

The retina is the part of the central nervous system that relays messages from the eye to the brain in order to transform visual information into an integrated perception. The retina is located in the back of the eye along the inner surface (Figure 1) and connects the eyes to the brain, allowing visual processing to occur (Purves et al., 2008). There are seven major retinal cell types in most vertebrate retinas: rods, cones, horizontal, ganglion, bipolar, amacrine, Muller glial, and interplexiform cells, which are unique to
fish. These cells can be further subdivided into more distinct classes due to morphological and physiological differences among them.

Figure 1: Anatomy of the zebrafish eye. Retina is marked by the pale pink colored layers, with the optic nerve (OpN) extending through the layers and out of the eye. Figure includes cornea (Co), annular ligament (AL), iris (Ir), ciliary region (CR), retinal margin (CGZ), retinal pigmented epithelium (RPE), choroid, sclera, optic nerve (OpN). Taken from Vihtelic et al. (2005).

These seven types of retinal cells are arranged into three layers: the inner nuclear layer (INL), ganglion cell layer (GCL), and outer nuclear layer (ONL). The inner plexiform layer (IPL) is a synaptic layer that connects the INL to the GCL. Similarly, the
outer plexiform layer (OPL) connects the INL to the ONL (Dowling, 1987). Photoreceptor somas can be found in the ONL, whereas the somas of the four other retinal cells, excluding ganglion cells, can be found within the INL. Bipolar cells are considered the input neurons to the IPL, and ganglion cells the output neurons of the IPL, as well as the whole retina. Muller cells can be found throughout the retina extending vertically between the inner and outer limiting membranes (Dowling, 1987). In zebrafish, the order of cells arising during embryonic development of the eye is ganglion, amacrine, bipolar, horizontal, Muller, rods, then cones (Evans & Fernald, 1990).

Zebrafish Eye Development

The processes involved in zebrafish eye development take ~72 hours, beginning at ~14 hpf. Using light and transmission electron microscopy, Schmitt and Dowling (1994, 1999) observed and documented eye development in the zebrafish. The formation of the adult retina begins with the smooth and bulging cells of the lateral optic primordium giving rise to the neural retina (Schmitt & Dowling, 1994). During the invagination period, retinal cells die through apoptosis at random times and locations, and the neural retinal cells become thinner and more elongated in the area bordering the lens. At ~30 hpf, the first signs of ganglion cell axons comprising the optic nerve can be seen, which at 48 hpf reach the optic tectum (Burrill and Easter, 1994).

The retina consists of three to four layers of undifferentiated cells at 32-34 hpf (Schmitt & Dowling, 1999). Overall, 24-36 hpf is the period of rapid proliferation due to the presence of many clusters of dividing cells along the optic lumen of the retina, and at 36 hpf, the retina has grown to ~60 μm wide (vitreal-scleral direction) and 6 layers thick.
One aspect of teleost eye development that differs from other vertebrates is the eye rotation that occurs from 24-36 hpf. This rotation occurs such that the eye takes a position lateral and parallel to the brain, the choroid fissure moves ventrally, and the posterior groove moves dorsally (Schmitt & Dowling, 1994). Ganglion cell differentiation increases dramatically at 36-40 hpf through the temporal retina.

Lamination throughout both ventral and dorsal retinas occurs at 50 hpf, and three distinct layers can now be observed: INL, ONL, and the GCL, which are separated by the IPL and OPL (Schmitt & Dowling, 1999). The GCL is about 12 µm wide and consists of 2-3 rows of ganglion cells along the vitreal border of the retina. The inner plexiform layer (IPL) that separates the GCL and inner nuclear layer (INL) is about 6 µm across and consists of some nuclei thought to be amacrine cells (Schmitt and Dowling, 1999), which are interneurons that control input to the GCL as well as to bipolar cells (Masland, 2001). During this time, the INL is about 25 µm wide, with a couple of amacrine cell layers and three layers of neuroblasts (Schmitt & Dowling, 1999). The OPL, separating the INL and ONL, is about 2 µm wide, and the ONL about 8 µm wide, consisting of several small nuclei presumed to be those of rods and cones. At this point, however, it is impossible to tell the difference between the two photoreceptors.

The ventronasal patch, positioned nasally to the optic nerve and adjacent to the choroid fissure, contains rapidly differentiating photoreceptor cells, as well as amacrine, horizontal, and bipolar cell types, when comparing rate of overall differentiation in other areas. This region is known as the ‘initiator of differentiation of the retinal cells and photoreceptors’, as differentiation begins in the patch and then spreads nasally, dorsally, and temporally. This may be due to the optic stalk extending nasally from the ventral
retina, causing a displacement of cells into this ventronasal patch (Schmitt & Dowling, 1999).

At 60 hpf, the retina is about 70 µm wide, indicating a slower rate of growth compared to the previous rate of rapid development. Simultaneously, the IPL has grown slightly to about 8 µm and contains new nuclei that are predicted to be axons of bipolar cells (Dowling, 1987). Within the INL, new layers of bipolar cells can be seen, and the INL has also grown from 8 µm to 30 µm wide. The size of rods and cones continues to increase, and several organelles, such as rough and smooth endoplasmic reticulum, ribosomes, and mitochondria, can now be seen within the pigment epithelium. At this time, the OPL and ONL are both distinct and well structured regions within the central retina.

Based on synaptic ribbons found in the IPL and OPL at 70 hpf, it can be inferred that the vertical pathways between photoreceptors, ganglion cells, and bipolar cells have formed. At this time, the eye cup has grown to about 235 µm in diameter and retinal differentiation has occurred across the entire retina such that the ventronasal patch is no longer significantly more advanced than all other areas. By 74 hpf, photoreceptor differentiation has increased and the retina has grown to 80 µm wide. At this time, the optic nerve diameter is ~14 µm and the IPL is ~12-14 µm wide (vitreal-scleral direction). Outer segments of cones within the temporal area are arranged into three layers. This “area temporalis”, located anteriorly in the temporal retina, later becomes the region with high acuity visual abilities. However, rods are infrequently seen in the temporal region and are more common in the ventral retina. By 80 hpf, the optic cup has become a
sensitive and functional part of the eye that is able to respond to light (Easter & Nicola, 1996).

**Thyroid Hormones**

The two thyroid hormones (Figure 2), thyroxine (T4) and triiodothyronine (T3), are synthesized in the thyroid gland, located near the trachea in mammals and near the ventral aorta in fishes. Thyroid hormones (THs) are involved in metabolism, growth, the rate of protein synthesis, and development of the respiratory, cardiovascular, muscular, gastrointestinal, and central nervous systems (Romer & Parsons, 1977; Yadav, 2004). Thyroid hormones elicit a direct effect in many systems, though they also play a secondary role by supporting other hormones in their actions (Hadley, 1992). In many teleosts, thyroid follicles can be found in various secondary locations, including the kidney, liver, ovary, and brain (Wendelaar & Bonga, 1993; Janz, 2000).

![Chemical Structures](http://www.precisionnutrition.com/all-about-thyroid)

**Figure 2:** Thyroxine and Triiodothyronine Chemical Structures. These two structures are identical except for the number of iodine groups attached to the tyrosine rings: Thyroxine has an extra iodine group on the 5’ end of the first tyrosine ring. Taken from [http://www.precisionnutrition.com/all-about-thyroid](http://www.precisionnutrition.com/all-about-thyroid).
Thyroid hormones require iodine, which is consumed in the diet and taken up by the thyroid gland. The oxidation of iodine molecules and the iodination of many tyrosines on the glycoprotein thyroglobulin are catalyzed by the enzyme thyroid peroxidase. The two products of this oxidation are monooiodotyrosine (MIT) and diiodotyrosine (DIT). Thyroxine (T4) is formed when two diiodotyrosines combine, whereas T3, the biologically active thyroid hormone, is formed when one monooiodotyrosine molecule joins one diiodotyrosine molecule (Hadley, 1992). Thus, T4 consists of 4 iodine molecules bound to tyrosine, while T3 molecules contain 3 iodine molecules (Figure 2). Iodination is required for T3 and T4 production, and thus it is necessary for animals to have iodine in their diets (Romer & Parsons, 1977). In fish, the gills have an iodine pump that absorbs sufficient iodine found in both fresh water and salt water, and iodine can be further obtained from fish food (Blanton & Specker, 2007).

The mammalian thyroid gland is composed of follicles, principal cells, and C cells (Figures 3 and 4). Follicles are spherical structures that contain colloid, a gelatinous substance that stores thyroglobulin and contains the tyrosine that is later required for T3 and T4 formation. Thyroglobulin cells are also responsible for storing the complete thyroid hormone products prior to release into the bloodstream (Yadav, 2004). Follicular epithelial cells, also known as principal cells, surround the follicles and rest on the basal lamina. These cells control thyroglobulin synthesis, TH release, and iodination. In mammals, the parafollicular cells, or C cells, can be found either within the follicular epithelium or in the gaps between follicles (Yadav, 2004). In teleosts, the thyroid gland contains colloid, but no C-cells (Kardong, 2009).
Figure 3: Cells of the Mammalian Thyroid Gland. Image of colloid, follicles, follicular epithelium cells (principal cells), and parafollicular cells, which can be found within the epithelium or between follicles. Taken from Charlotte L. Ownby (2002).

Figure 4: Image of Vertebrate Thyroid Cells. Colloid, follicular, and parafollicular cells in teleosts. Teleost thyroglobulins are stored in the colloid. Taken from http://people.upei.ca/bate/html/notesonthyroidfunction.html
Thyroid function is regulated by the anterior pituitary gland, which releases thyroid-stimulating hormone (TSH) per the direction of the thyrotropin-releasing hormone (TRH) from the hypothalamus (Figure 5). TSH increases blood flow throughout the thyroid gland and increases the rate of thyroglobulin release, subsequently decreasing the amount of colloid within the follicles (Yadav, 2004). Feedback mechanisms within the thyroid system are crucial for normal development and for maintaining homeostasis. When circulating thyroxine levels are too high, TSH production decreases due to the negative feedback that contributes to the prevention of excess TH levels (Cooke, King, & van der Molen, 1988).

Figure 5: Negative Feedback in the Thyroid System. Feedback (-) is seen on two levels: circulating thyroid hormone levels feedback (1) to the pituitary inhibiting further TSH release and (2) to the hypothalamus inhibiting TRH release. Taken from bestpractice.bmj.com
Because of their lipid solubility, thyroid hormones travel through the blood via thyroid binding carrier proteins, which include transthyretin, albumin, thyroxine-binding globulin, and lipoproteins (Schreiber & Richardson, 1997; Babin, 1992). Direct binding of T3 or T4 onto their intracellular receptors affects the expression of particular genes by binding to sequences of DNA located on the target gene’s regulatory region, called thyroid hormone response elements, which then enhance or limit the amount of gene expression. THs therefore induce an altered cellular response upon binding to their nuclear receptors in target organs (Yen and Chin, 1994). These receptors are part of a superfamily of nuclear receptors that include receptors for retinoids, melatonin, vitamin D-3, and steroid hormones (Power et al., 2001). In zebrafish, there are two types of thyroid hormone receptors: TRα and TRβ (Essner et al., 1997; Liu et al., 2000).

**Thyroid Hormone Effects in Vertebrates**

THs play a role in the differentiation of cells and overall growth of primary organs in vertebrates ranging from fish to humans (Oppenheimer et al., 1995). Studies in flounder and *Xenopus laevis* have shown that both TH and thyroid receptors (TRs) are required to control the reorganization of all major tissues (Shi et al., 1996; Yamano & Miwa, 1998). Flounder metamorphosis is accompanied by an increase in T3, T4, TRα, and TRβ, which then decrease in concentration after metamorphosis has occurred (Yamana & Miwa, 1998). TH inhibitors block metamorphosis, which is initiated by a surge of T4 and ends when T4 levels are reduced (Blanton & Specker, 2007), indicating a causal relationship between thyroxine concentrations and developmental stage (Schreiber & Specker, 1998). Further, studies on the clawed frog indicate retinal growth can be
stimulated when T4 is injected into the eye, leading to increased cell proliferation (Beach & Jacobson, 1979).

In rats, T4 accelerates development of the brain by increasing the amount of DNA, RNA, and protein synthesis (Balazs et al., 1971). A study conducted to test the effects of T4 on rat retina found that six days after birth, rats treated with thyroxine had thicker INL and ONL measurements compared to controls (Macaione, DiGiorgio, Nicotina, & Lentile, 1984). At twelve days, treated rats resembled twenty-day-old control rats due to the increased rate of maturation seen following thyroxine treatment.

**Thyroid Hormone Effects in Zebrafish**

High levels of maternal THs are passed on to oocytes during maturation of the ovaries and subsequently to the embryo through the yolk-sac (Power et al., 2001; Tagawa & Hirano, 1990). Researchers suggest that high levels of THs in the mother are necessary for the growth and development of their offspring (Brown et al., 1989; Ayson & Lam, 1993). Methimazole, the thyroid hormone inhibitor used in these experiments, belongs to the thionamides group of chemical compounds and works by blocking the iodine oxidation such that MIT and DIT do not bond to form T3 or T4 (Capen, 1997; Engler et al., 1982). However, MMI treated embryos retain access to maternal supply and thyroid effects observed in early development occur through functional thyroid receptors.

For several decades, THs have been thought to play a role in the neural development and gene expression of zebrafish (Lyall, 1957), though the exact mechanisms of action are still unknown. Previous studies have identified the first thyroid follicle at 55hpf, followed by gradual development of the gland until an independent and
functioning embryonic thyroid system is present (Alt et al., 2006). While the thyroid gland is not mature until later in development, several thyroid hormone receptors (TRs) are expressed as early as the mid-blastula stage of development (~ 3 hpf) (Essner et al., 1997). Despite a lack of embryonic thyroid hormones at this early stage, THs from the mother are found in extremely high levels in the yolk sac when THs are considered crucial (Lam, 1994).

It has been previously established that when zebrafish are given high doses of thyroid hormone inhibitors such as MMI, many debilitating developmental defects are observed (Liu & Chan, 2002). For example, hatching is delayed, and body length and head cartilage growth is severely stunted (Elsalini & Rohr, 2003; Liu & Chan, 2002). THs also have significant effects on the development of the pectoral and pelvic fins, scale formation, and the striped pattern associated with adult zebrafish (Liu & Chan, 2002; Brown, 1997). Most recently, PTU, a common thyroid disruptor similar to MMI, was found to decrease eye size in zebrafish (Li et al., 2012).

**Standard Temperature Conditions**

Optimal conditions for zebrafish development and maintenance have been established in the literature (Westerfield, 2000; Lawrence, 2007). Zebrafish, like all teleosts, are poikilothermic, with their body temperature depending on external temperature. Previous reports have identified rearing temperature as a critical aspect of zebrafish growth and development. While zebrafish can reportedly sustain temperatures between 6–41.7°C, optimal temperature conditions have been identified to be between 28–29°C, and researchers most frequently cite 28.5°C as the temperature used to maintain
both embryonic and adult zebrafish (Lawrence, 2007). It has been found that embryos reared in higher temperatures (33°C) developed at an accelerated rate when compared to those reared in the standard 28.5°C (Kimmel, 1995). While this acceleration was significant, no other studies have examined temperature effects in larval development.

**Significance of Proposed Research**

While studies often cite 72 hpf, hatching, and/or yolk-sac resorption as the time of larval thyroid maturity, no study has determined a more precise window of thyroid development (Power et al., 2001; Alt et al., 2006; Wendl et al., 2002; Lam, 1994). Hatching ages and the time of yolk-sac resorption can be extremely variable (hatching ranges from 48-72hpf at 28.5°C), even within a single clutch raised at the same temperature, suggesting that using these events as markers for various stages of thyroid development can be problematic (Kimmel, 1995). Further, temperature and thyroid interactions during zebrafish development have not been assessed, although both have been shown to be critical factors in normal growth and development.

Zebrafish are an excellent model for genetic and developmental experiments. Zebrafish are often used as a tool to study the eye: their eyes are large and develop quickly, and the seven major retinal cells can be seen as early as 3 days post fertilization (Neumann, 2001). Zebrafish are particularly useful because their embryogenesis, neurogenesis, and eye formation have been previously established (Kimmel et al., 1995; Schmitt & Dowling, 1994; Schmitt & Dowling, 1999).

The measurements used in this study were eye size and body length, both of which increase with age in normal development (Schmitt & Dowling, 1999; Kimmel,
Spinal curvature was also measured based on previous reports that showed treatment with thyroid inhibitors led to deformed, overly curved larval spines, as compared to the straight spines of control fish (Shi et al., 2008). Lastly, inter-eye distance was assessed because it has been found that exposure to some toxic substances, such as ethanol, can cause inter-eye distances to be larger than controls (Bilotta et al., 2004). The cause of this is unknown, but may be a result of abnormal brain development. Reports in rats showed that ethanol exposure decreased thyroid levels and TH mRNA, so perhaps a decrease in thyroid hormones is the cause of the ethanol induced phenotype (Hannigan & Bellisario, 1990; Scott et al., 1998).

While several studies from the last few years have assessed the effects of MMI and other thyroid inhibitors on development, these studies have used extremely high doses of inhibitors (up to 1.5 mM MMI), resulting in very low hatching rates. The reported concentrations of thyroid disruptors in water supplies are relatively low (Yan et al., 2012; Brechner et al., 2000; Jugan et al., 2009; Weyer & Riley, 2001; Shi et al., 2011), but still significant enough to induce developmental defects (Brown, 1997). Consequently, this study aimed to examine the effect of lower, environmentally-relevant doses on development.

The purpose of this study was to determine the contribution of the thyroid system to general development, as well as development of the retina. Our hypothesis was that inhibition of endogenous thyroid production would significantly reduce overall growth, resulting in body deformation and thinner retinas. Findings from this study will help us better understand the time of origin of the embryonic thyroid system, and to assess whether various structures require thyroid hormone action during development.
The objectives were to:

1) Determine changes in growth as a result of chronic exposure to 0.1-0.3 mM MMI until 50 hpf, 60 hpf, and 72 hpf. These timepoints were selected because they encompass development of the thyroid gland and formation of the eye and retina.

2) More specifically identify a critical period of thyroid development and sensitivity on growth between 60-72hpf. This age range was selected based on the results from objective #1.

3) Examine the interaction between rearing temperature and thyroid development in both treated and untreated larvae.
CHAPTER 2

METHODS

Animal Maintenance

Experimental embryos were obtained by spawning zebrafish adults at American University in the Fish Facility throughout 2011-2012. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at American University.

Prior to breeding, adult males were separated from adult females for at least three days. Spawning of adults was accomplished by placing eight to fifteen adults, both male and female, into a breeding chamber with a mesh bottom, where adults were left to mate over night. The following morning, ~60-90 minutes after light onset, fertilized eggs that have fallen through the mesh to the bottom of the tank were collected. Fertilized eggs were staged, cleaned, and maintained at 28-31°C water in a common beaker until they were placed in their designated experimental conditions at the selected time points. Petri dishes and beakers in water baths were used to house the treatment embryos during their development. All zebrafish were euthanized with 0.02% tricaine (MS-222) prior to analysis at 72hpf.

Selected Time Points

For Experiment 1, embryos were collected at 50, 60, and 72 hpf. These time points represent the development of a wide range of structures and cell types related to both the eye and the retina, as well as a time of overall morphological development.
Additionally, these timepoints are consistent with critical periods in the development of thyroid follicles and embryonic thyroid supply: at 50hpf, thyroid follicles have not developed, though genes coding for thyroid development are expressed at this time (Rohr & Concha, 2000). 50 hpf was used as a control, because we did not expect to see MMI effects at this time. At 60hpf, the first thyroid follicles can be identified, but it is unclear if the embryonic thyroid system is independent of maternal supply, and at 72hpf, the embryonic thyroid system is reportedly fully mature (Alt et al., 2006).
Based on the results from Experiment 1 (see below), Experiment 2 was designed to subsample larvae hourly between 60-72hpf in order to see if a critical period, where increased sensitivity to MMI (indicating the need for a functioning thyroid system), could be identified. Additionally, this second experiment reared larvae at both 28°C and 31°C to identify any possible interactions between temperature and thyroid inhibition as well as to determine temperature effects on overall development.

**Experimental Treatments**

MMI doses as low as 0.1 can induce developmental defects (Brown, 1997). We chose to use 0.1 mM, 0.2 mM, and 0.3mM of MMI as the low, medium, and high dose treatments. MMI is stable in solution for at least 72 hours (Brown, 2011; Pickford, 2011), so stock solutions were mixed immediately prior to use, and the same solution was used for the duration of the 72-hour experimental period. Deer Park and AHAB system water were used as control solution.

**Experiment 1**

Fertilized eggs, aged 0-1 hpf, were placed in one of four beakers containing the different experimental concentrations of MMI. For each treatment, 20-40 embryos were randomly placed in each of the four conditions, with two replicate beakers per treatment (n=8 beakers). At each experimental timepoint, embryos were removed from beakers and transferred to petri dishes containing control/system water (Figure 7). All experimental containers were randomly distributed between the water baths in order to control for
location and/or lighting effects within the lab. Experiments were performed at 31°C to match the temperature within the AHAB rearing system.

At 50 and 60hpf, larvae (n=63 for the two timepoints) were removed from treatment conditions and reared in petri dishes until 72 hpf, when all larvae were euthanized in a 0.02% tricaine solution for 2-3 minutes. Larvae remained in the tricaine solution for 2-3 minutes after movement had stopped and they were unresponsive to tactile stimulation (squirts of water), indicating death. Once sacrificed, animals were fixed in 4% paraformaldehyde.

Figure 7: Experimental design. This image represents the experimental design from spawning of adults to subsampling larvae at the relevant timepoints.
Experiment 2

In these experiments, larvae were subsampled hourly between 60-72hpf. Each hour, larvae (n = 5-10) were removed from MMI treatment, transferred to control (0 mM MMI) water and allowed to develop until 72hpf, at which time they were euthanized in 0.02% tricaine solution and preserved in 4% paraformaldehyde for later analysis.

Results from Experiment 1 indicated that 0.3 mM MMI provided the most consistent results. The low dose (0.1mM MMI) did not consistently produce significant effects, and changes in growth following exposure to 0.2mM MMI were highly variable. Thus, we chose to use 0.3mM MMI as our experimental condition in Experiment 2.

Two rearing temperatures were used: 28°C and 31°C. The lower temperature is the standard laboratory rearing temperature for zebrafish larvae (Kimmel, 1995; Lawrence, 2007), and the higher temperature, although within the range of acceptable temperatures for zebrafish, is at the high end of this range. Given that, similar to thyroid hormones, temperature can increase metabolic rate, we were interested in determining if there was an interactive effect between rearing temperature and thyroid disruption.

Retinal Layers

To examine retinal development, fixed embryos (n >5 per treatment) were equilibrated overnight in a 30% sucrose solution prior to cryostat sectioning. Each embryo was sectioned (20 µm thick) beginning caudal to the head region and moving anteriorly. All sections were stored at -80°C until needed. Sections were mounted on silanated glass slides and stained with the nucleic stain 4',6-diamidino-2-phenylindole (DAPI) prior to fluorescent microscopy.
Measurements and Analysis

To examine larval development, preserved embryos (n = 50-100 per treatment) were examined using a stereomicroscope (Olympus SXZ16), and retinal layers were analyzed using an Olympus BX61 compound microscope with motorized stage. An Olympus DP72 color camera was used to take pictures of larvae. Metamorph software was used to capture the photographs, and Image J for the measurements.

Inter-eye distance was measured as the left-right length between the most anterior point of each eye. Eye size was measured as the length from the most anterior to the most posterior part of the eye. Body length was measured as the most anterior part of the head to the posterior portion of the tail. Spinal curvature, a measure of body deformation, was determined by measuring the angle of dorsi-flexion of the body (Figure 8). Both spinal curvature and body length were calculated using the J Image angle tool, which can calculate the sum length of, or angle between, two lines (see figure 8). Retinal layers were measured through the midline of the section in samples with clearly defined layering (figure 9). Only sections with either an optic nerve or the largest lens cross-section, indicating the central retina, were used for analysis.
Figure 8: Morphological measurements of zebrafish larvae
Representative image (bottom) and accompanying sketch (top) to illustrate how morphological measurements were made from 72hpf zebrafish larvae. (a) Eye size (b) Inter-eye distance (c) spinal curvature (d) body length. A = anterior, P = posterior, D = dorsal, V = ventral.
Figure 9: Retinal layer measurements.
The below image represents a low dose MMI-treated larvae fixed at 72 hpf. Labels indicate retinal layers through the central retina, where measurements were obtained.

Each measurement for both internal and external morphology was performed twice and averaged to decrease error. Univariate ANOVA analyses were performed in order to compare measurements for a single parameter among treatments. T-tests were performed for comparisons made between treatment or temperature effects in the 72 hpf larvae. Statistics were performed using SPSS (version 20), with an alpha level of 0.05.
CHAPTER 3

RESULTS

Experiment 1

Larvae reared in low (0.1mM), medium (0.2mM) or high (0.3mM) concentrations of MMI displayed comparable survival levels. Overall mortality was low (<1-3%) within the experimental containers.

Eye size

There was a main effect of MMI concentration on the development of eye size (N=83), $p < 0.05$, as well as a significant interaction between age and MMI dose, $p < 0.001$. For all three MMI treatments, the length of the eye increased linearly as a function of time, indicating a lack of exposure effect. However, a dramatic decrease of eye size was seen in the high MMI condition between larvae removed from treatment at 60 hpf (196.5 $\mu$m +/- 7.87) and larvae removed at 72 hpf (201.6 $\mu$m +/- 8.4), $p < 0.01$ (Figure 10). We interpret this reduction to reflect sensitivity to MMI, consistent with the development of the embryonic thyroid axis. Comparison results from larvae exposed to high dose MMI with pooled results from larvae in the control, low, and medium doses were performed to further assess trends in the data. A significant difference between the high MMI concentration and the lower dose group was found ($p < 0.001$), with the high dose having significantly smaller eyes (196.50 $\mu$m +/- 7.87) than the combined control, low, and medium doses (204.7 $\mu$m +/- 5.75).
Figure 10: Experiment 1 Eye Size Results. Effects of low, medium, and high doses of MMI on eye size in larvae removed from treatment at 50-72hpf. Comparing data at 72hpf shows that larvae exposed to lower doses of MMI (short and long dashed lines) had larger eyes than control larvae, and that high dose MMI treatment led to the lowest eye size measurements. Values presented are means (± SE).

Spinal curvature

When zebrafish larvae hatch, their bodies straighten, with little/no curvature in the spine. However, MMI treated larvae often developed a pronounced curve (dorsiflexion). Dorsiflexion of the spine resulted in a smaller angle, determined by measuring the degree of curvature of the head and tail, reflecting deformation as a result of MMI treatment. To quantify this curvature, measurements of the angle formed by the curvature were made for treated larvae, and compared to angles of larvae reared in control conditions (n = 26; 159.68° ± 7.85) (see Methods, Figure 9).
The interaction between age and MMI concentration was found not to be significant for spinal curvature (N=82), \( p = 0.062 \). However, interesting trends in the data were noted (Figure 11). First, MMI concentration was a significant factor in the development of the spine \( (p = 0.001) \), as was age \( (p < 0.001) \). In embryos exposed to MMI until 50 hpf, the spinal curvatures appeared equal within all MMI treatment groups \( (159.84^\circ +/- 7.29) \) and were similar to control values. However, there were marked differences in spinal curvature noted across treatments in larvae exposed to MMI until 60 hpf. In particular, spinal curvature or dorsal flexion of the tail, increased from control to low, low to medium, and medium to high treatment groups. Larvae within the control or low dose groups until 72 hpf had similar spinal curvatures, but a significant difference could be seen between control/low \( (155.99^\circ +/- 6.51) \) and medium/high doses \( (144.92^\circ +/- 6.01) \), \( p < 0.001 \).
Figure 11: Experiment 1 Spinal Curvature Results. Effects of low, medium, and high doses of MMI on spinal curvature. Larvae exposed to MMI until 50 hpf or 60 hpf did not have significant curvature of their spines, though there is a clear trend, whereas larvae exposed to MMI until 72 hpf showed increases in spinal curvature with increasing MMI concentration. At this timepoint, the straightest spines (largest values) were observed in larvae in the control group. Measurements from larvae in the high/medium MMI and low/control MMI treatments were visibly grouped, and t-tests indicated that these differences were significant, $p < 0.001$. Values presented are means +/- SE.

Inter-eye distance

A significant interaction between age and concentration was observed for inter-eye distance (N=56), $p = 0.001$. Additionally, there was a main effect of concentration, $p < 0.01$. However, no significance was observed during our presumed critical period between 60-72 hpf (Figure 12). Instead, the majority of effects accounting for the
significant results can be seen in comparisons of the high dose in larvae removed from treatment at 50hpf and larvae removed at 72hpf. Interestingly, no significant differences were seen at 72hpf between the doses for this parameter.

Figure 12: Experiment 1 Inter-eye Distance Results. Effects of low, medium, and high doses of MMI on inter-eye distance. Significant differences were observed in larvae reared in different MMI treatments until 50 hpf, with the smallest distance observed in larvae exposed to the medium MMI concentration. No significant differences were observed during 60-72 hpf, suggesting inter-eye distance is not sensitive to thyroid disruption at these later ages. Values are presented as means +/- SE.
Body length

MMI concentration was not a significant factor in overall growth, measured as an increase in length (body length, N=34). Additionally, there was no age by concentration interaction. Results indicate that body length stayed stable across treatments for our measured timepoints.

Figure 13: Experiment 1 Body Length Results. Effects of low, medium, and high doses of MMI on body length. No significant differences in body length were observed in larvae within any of the treatment groups. Values are presented as means +/- SE.
Retinal Layering at 31°C

Examination of DAPI-labeled retinal cryostat sections allowed retinal layers to be clearly distinguished and measured (vitreal-scleral dimension). DAPI labels DNA within cell nuclei, which are organized in a layered arrangement in the retina and alternate with non-labeled layers containing only neuronal processes.

There was a significant effect of MMI dose on the establishment of the ganglion cell layer (GCL) (N=17), p < 0.05. Control larvae (18.7 µm +/- 1.58) had a significantly thicker GCL than treated larvae (11.3 µm +/- 2.31). Similarly, MMI concentration affected inner plexiform layer (IPL) size (N=23), p < 0.05. For this parameter, control and low MMI larvae had a thicker IPL (7.82 µm +/- 0.55) compared to the larvae within the high and medium MMI treatment groups (6.04 µm +/- 0.82). MMI concentration also had a significant effect on inner nuclear layer (INL) thickness (N=18), p < 0.05. Similar to the GCL results, the measured thickness of the INL in control larvae (28.6 µm +/- 1.04) was greater than INL thickness in retinas of treated larvae (21.3 µm +/- 2.54). Outer plexiform layer (OPL) thickness, in contrast, was not significantly different in treated and control retinas. Example retinas can be seen in Figure 14.

The effect of age removed from MMI treatment was also assessed. In the high dose group, a significant difference in IPL thickness was observed in retinas of larvae removed from treatment at 60 hpf compared to larvae removed from treatment at 72 hpf, p < 0.05. Length of exposure to MMI also significantly affected GCL (p < 0.001) and INL (p < 0.001) thickness, with longer exposure times (i.e., larvae removed from treatment at 72 hpf) resulting in retinas with a thinner GCL and INL compared to retinas
of larvae removed from treatment at 60 hpf. However, MMI exposure did not significantly alter the thickness of the OPL regardless of concentration or exposure time.

Figure 14: Comparison of 72 hpf Control Retina vs 0.3 mM Treated Retina. (a) Control eye stained with DAPI at 72 hpf compared to (b) an eye from a larvae removed from high dose MMI treatment at 72 hpf. Differences in size can clearly be seen for the GCL and INL (blue lines). Retinal layers are labeled, with the OPL represented by an arrow.

Observations

While it was not formally analyzed, it consistently appeared that larvae reared in high MMI doses at both 28°C and 31°C hatched sooner than those in control water. This observation is contrary to previous findings that saw a delay in hatching following thyroid inhibition (Crane et al., 2006). However, the doses in that study were 1.5mM-15mM MMI as compared to the 0.3 mM used in this study. Further, hatching seemed to occur earlier in the high temperature group compared to the low temperature group.
Experiment 2

The previous experiment indicated that significant differences in growth resulting from thyroid disruption occur between 60-72hpf for two of our four measured parameters, as well as for retinal layer development. During this time frame, a wealth of developmental events occur, including maturation of the larval thyroid system and morphological development. We were interested in examining more specific effects of thyroid inhibition, and thus a second set of experiments was performed to determine more precisely when during this time the larvae are most sensitive to thyroid disruption.

In addition, temperature was also assessed in order to compare development and MMI sensitivity at the standard rearing temperature of 28°C, and at 31°C, a safe upper limit of the rearing temperature range. Presented below are the unpooled results from the raw data, with representative graphs from data pooled every two hours.

General effect of temperature on growth of untreated larvae

In general, growth was faster at the higher rearing temperature (Figure 15). For all four measurements, values taken from control larvae reared at 31°C were larger than the corresponding measurement made from larvae reared at 28°C. Factorial ANOVA analyses found significant temperature effects between untreated larvae were observed for two parameters: body length (p < 0.001) and inter-eye distance (N=24, p = 0.012).

The effects of MMI treatment on embryos reared at 28°C

To examine the effect of MMI under standard rearing conditions, data collected from treated larvae reared at 28°C were compared. Total body length measured at 72 hpf
averaged ~2-2.1 mm in larvae exposed to MMI until 60hpf (Figure 15a). Body length
decreased, though not statistically significantly, in larvae treated with MMI until 62-
66hpf, with the lowest body length measured following MMI exposure until 66hpf. We
interpret this reduction to reflect maximal sensitivity to MMI, consistent with the
development of the embryonic/larval thyroid gland. Body length development in larvae
treated for the full 72 hpf was similar to controls.

Inter-eye distance showed a trend similar to body length. Initial distance
measurements taken from larvae treated until 60hpf were 180-190 µm. This measurement
was consistent in larvae collected through 66hpf. Larvae removed from MMI treatment at
68hpf show a sharp reduction in inter-eye distance, which measured ~170 µm at this time
(Figure 15b). Larvae treated until 70 or 72hpf had a larger inter-eye distance, with values
comparable to controls at 72hpf and those measured at the initial timepoint.

Eye size generally decreased as a result of MMI treatment, with an increase of eye
sizes observed between 68-70hpf (Figure 15c). Measurements from larvae removed from
treatment at 60hpf averaged 205-210µm, while mean eye size at 72hpf was significantly
below control values, measuring at ~195 µm.

Spinal curvature appeared comparable in larvae removed from MMI treatment at
the various timepoints, averaging at ~140°, significantly different from untreated larvae.
A rapid increase in mean curvature was observed in larvae removed from MMI treatment
at 66hpf (~130°). This age is consistent with body length and inter-eye size data.
The effects of MMI treatment on larvae reared at 31°C

Measurements of body length, inter-eye distance, and spinal curvature showed greater variability in data obtained from the low temperature group compared to the high temperature group, which provided more consistent measurements throughout the timepoints. Total body length of treated larvae remained comparable for all timepoints (between 2.1-2.15 mm). However, a decrease in measurements was observed in larvae exposed to MMI until 68 hpf, similar to larvae reared in 28°C. A slight increase of measurements was observed in larvae exposed to MMI until 70-72 hpf, and final measurements obtained at 72 hpf were comparable to untreated larvae. Inter-eye sizes were similar across all collection times, with a mean distance of 200-210 µm. There was a reduction in distance observed in larvae removed from treatment at 66 hpf, followed by an increase of measurements comparable to previous timepoints. Similarly, average spinal curvature values in treated larvae reared at 31°C were consistent across the larval ages tested (measuring ~155°). Control larvae at 72 hpf measured at ~160°, while treated animals at this time measured ~145°. Finally, a decrease in mean eye size was observed in larvae removed from treatment at 68 hpf, and the mean value was increased at subsequent removal times until the lowest measurements were seen at the final subsampling period.

Comparisons between these findings at 31°C and results obtained from Experiment 1 revealed that eye size results for 60 and 72 hpf were comparable for treated fish in both experiments. However, control values were slightly higher in Experiment 2. This may reflect the much larger sample size used in the second experiment, or natural differential growth seen in zebrafish development. Spinal curvature data was similar
across experiments for both control and treated larvae, as was body length data. However, inter-eye distance data revealed that Experiment 2 yielded data on average larger by \( \sim 10 \) \( \mu \text{m} \) for treated and untreated animals.

**Comparison of MMI effects between high and low rearing temperature groups**

MMI effects for both temperature groups in larvae examined at 72 hpf were significant \( (p < 0.05) \) for eye size \( (N=254) \), inter-eye distance \( (N=203) \), and spinal curvature \( (N=223) \). The combined effect of temperature and MMI treatment on zebrafish embryonic growth and development can be seen in all of our parameters. Inter-eye distance measurements were consistently and significantly larger for all larval ages examined in the higher temperature group. Significant differences were observed at 72hpf, with greater distances measured in larvae reared at the higher temperature \( (p < 0.001; \text{Figure 15d}) \). Body length measurements \( (N=192) \) provided similar trends such that length measurements for larvae reared in the higher temperature group were consistently higher than measurements obtained from the low temperature group from larvae removed from treatment at the 62-72 hpf timepoints \( (\text{Figure 15b}) \). Mean eye size measurements were also higher in larvae reared at 31\(^{\circ}\)C at all treatment ages. By 72hpf, eye size was significantly larger in treated animals reared at 31\(^{\circ}\)C compared to the 28\(^{\circ}\)C group \( (p < 0.01; \text{Figure 15a}) \).

External examination of the larvae did not identify any gross anatomical defects in treated animals reared at either temperature, though bodies were somewhat deformed, as indicated by spinal curvature. Hatched, control larvae at 72hpf had nearly straight spines with the angle of curvature measuring \( \sim 160^\circ \), while treated larvae developed
spines with ~145° spines. Spinal curvature of treated larvae reared at 31°C were straighter than the curvature measured from larvae in the 28°C group (Figure 15d), with control larvae reared at both temperatures having significantly straighter spines than treated larvae.

Examination of each growth parameter individually indicates that the timing of the maximal sensitivity to MMI was different between larvae reared in the high and low temperature groups. These differences were most clearly seen in eye size and spinal curvature measurements. For both of these parameters, the point of maximal sensitivity in the 28°C group occurred when larvae were exposed to MMI until 66 hpf, 2 hours earlier than observed at 31°C. In both cases, however, the reductions in mean measurements were followed by an increase in length that more closely resembled control data. These data suggest that larvae exposed to MMI for longer than 66-68hpf display an increase in eye size and spinal curvature (indicating ‘recovery’). Similarly, mean inter-eye distance measurements were significantly reduced in treated larvae removed from MMI between 66-68 hpf. However, for this measurement, reduced distance was observed in larvae exposed until 66 hpf and reared at 31°C, whereas the reduction was observed in larvae exposed to MMI until 68 hpf and reared at 28°C. These timepoints are opposite to those found for the eye size and spinal curvature measurements. Body length measurements, however, suggest that maximal sensitivity to MMI is comparable for both temperature groups (with the greatest decrease in size observed in larvae removed from treatment at 68 hpf).
c. 

MMI/Temperature Effects and Eye Size

<table>
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<th>Temp</th>
<th>28</th>
<th>31</th>
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Eye Size (um)

Age Removed from MMI (hpf)

Error Bars: +/- 1 SE

d. 

MMI/Temperature Effects and Spinal Curvature

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<th>Temp</th>
<th>28</th>
<th>31</th>
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Spinal Curvature (degrees)

Age Removed from MMI (hpf)

Error Bars: +/- 1 SE
Figure 15: Experiment 2 Results for MMI and Temperature Effects on Development of Eye Size, Inter-eye Distance, Body Length, and Spinal Curvature.

Representative graphs showing the effects of MMI exposure and temperature on the development of body length, inter-eye distance, eye size, and spinal curvature in larval zebrafish. Embryos were exposed to MMI at either 28°C or 31°C starting ~0-1hpf. Embryos were removed from MMI treatment and transferred to control water hourly between 60-72 hpf. All larvae were collected and fixed at 72hpf for later measurement. Data in the graphs (mean +/- standard error) are presented in two-hour blocks. Significant temperature effects were found for all parameters ($p < 0.01$), and temperature by treatment interactions are clearly evident for inter-eye distance and spinal curvature. T-tests at 72 hpf found significant temperature effects for control body length ($p < 0.05$) and inter-eye distance ($p < 0.05$), but not eye size and spinal curvature.
CHAPTER 4

DISCUSSION

This study reveals that the visual system, spine, and body are most sensitive to thyroid inhibition between 66-68hpf, indicating that the embryonic thyroid system is functional during this window, and that the measured parameters are dependent on THs for their normal development (Li et al., 2012; Shi et al., 2008; Crane et al., 2006). This time is earlier than has been previously reported (Brown, 1997). We also found a significant effect of temperature in both treated and untreated fish, and that 0.3 mM MMI is a nonfatal, low dose that is sufficient to cause morphological defects. Lastly, we report a recovery of measurements to near control values following prolonged thyroid inhibition for all measured parameters.

Experiment 1

This study assessed low, medium, and high doses of MMI at 50, 60, and 72 hpf in 31°C temperature solution on growth, measured as changes in eye size, body length, spinal curvature, and inter-eye distance. These parameters are reported throughout the literature as quantitative ways to examine growth and/or to determine effects of embryonic/larval exposure to various compounds (Brown, 1997; Elsalini & Rohr, 2003; Liu & Chan, 2002). In general, the differences in MMI effects were significant in larvae exposed to MMI until either 60 hpf or 72hpf, suggesting that the embryonic thyroid system develops between these ages and becomes sensitive to thyroid inhibition.
Zebrafish eyes begin to rapidly develop at ~28 hpf and continue until 4 dpf, when visual behavior is mature such that the larvae can catch moving prey (Easter & Nicola, 1996). Overall, larvae exposed to the high dose (0.3mM MMI) had significantly smaller eyes than larvae exposed to either the medium (0.2mM) or low (0.1mM) doses, indicating a dose dependent response to embryonic thyroid inhibition on eye size. While not formally assessed, it appeared that the eye developed normally externally, with the exception of some cases exhibiting misshapen lens formation. Significant differences in retinal layers based on age removed from high dose treatment was found for the IPL and INL between 60 hpf and 72 hpf, a critical time consistent with the other findings of this study. The lack of growth of the cells within these layers may be what accounts for the decrease in overall external eye size associated with high dose MMI exposure, though it is unclear whether this is due to smaller cells or a decrease of total cells within the layers.

Control larvae had straight spines with very little dorsiflexion of the tail. Similarly, larvae exposed to MMI until 50 or 60 hpf showed no significant effects of MMI on spinal curvature. However, embryos exposed to MMI until 72hpf displayed a dose-dependent response to MMI. Larvae reared in the high MMI dose were most deformed and with the greatest curvature noted, whereas larvae exposed to the medium and low MMI doses also had dorsally curved spines, though the curvature was not as pronounced as in the high concentration group. These findings suggest that thyroid hormones are necessary during this time to form a straight spine that allows the larvae to swim efficiently. While swimming behavior was not assessed, it is predicted that fish with highly curved spines (e.g. 100-130°) would have more difficulty swimming than those with straight or only slightly curved spines. No study to our knowledge has
assessed swimming behavior of larvae with curved or deformed spines, and this would be an interesting next step.

Inter-eye distance showed no significant effects of MMI concentration when larvae were removed from treatment at either 60hpf or 72hpf. This parameter was used to assess findings of ethanol-induced changes to inter-eye distance in zebrafish (Bilotta et al., 2004) and examine the link between toxins and thyroid hormones. Though our results in Experiment 1 could reflect a need for higher doses of MMI to see an effect, or that eye development is not a thyroid-dependent process, we do not think these are likely. Results from Experiment 2 clearly showed that thyroid disruption can alter inter-eye distance, suggesting that the ages studied in Experiment 1 do not provide enough timepoints to see an effect. Significance in this parameter from the second set of experiments is in-line with published reports of toxins, such as ethanol, depleting thyroid hormones (Hannigan & Bellisario, 1990; Scott et al., 1998).

One standard way to examine growth in fish larvae is to measure body length, measured from the anterior end of the snout to the posterior end of the tail. Body length data showed that while there was no dose-dependent response, treated fish did have significantly shorter bodies than control fish at 72 hpf. This finding, consistent with previous reports using compounds similar to MMI (Shi et al., 2008), suggests that embryonic thyroid hormones play a role in overall growth of the body, and are sensitive to even the lowest concentration of MMI (0.1mM) throughout development. While differences in body length may have no functional consequences in a lab setting, it is possible that shorter larvae will be at a disadvantage in the wild where they must escape predators and attract mates.
No significant effects of MMI were seen at 50hpf. This was expected as the first thyroid follicles in zebrafish are not present until ~55hpf (Alt et al. 2006). At this time, however, larvae are able to obtain thyroid hormones from the maternal supply in the yolk, a process that has been previously shown to not be affected by MMI exposure (Crane et al., 2006). However, given that our parameters were significantly affected by MMI exposure between 60-72 hpf, we can infer that the embryonic thyroid gland is fully formed, functional, and producing THs during this time, and that inhibition of their thyroid hormone synthesis leads to abnormal development. These results support previous reports that suggest THs have a great range of effects in growth and development, as shown in other vertebrates (Beach & Jacobson, 1979; Kelly, Turner, & Reh, 1995; Cheng, Gan, & Flamarique, 2009; Kawakami, Yokoi, Kumai, & Ohta, 2008).

**Experiment 2**

Between 60-72 hpf, organogenesis continues in zebrafish, with brain development, mouth and jaw cartilage development, and the establishment of the pectoral fins (Kimmel, 1995). Our goal for the second set of experiments was to determine if there was a critical period within this timeframe during which the larval thyroid gland is maximally sensitive to MMI. If true, the critical period would identify specific age(s) that growth of a given parameter requires thyroid hormones for normal development. We performed these experiments at two temperatures in order to assess changes in MMI sensitivity due to rearing temperature. Thyroid hormones increase metabolic rate as does an increase in temperature; however, it is not known how these two may interact.
In general, there was greater variability in measurements observed in larvae reared at 28°C compared to measurements from the 31°C group. However, a significant effect of temperature on both development and MMI sensitivity was observed in larvae removed from treatment between 60-72 hpf, with the high temperature group having higher values (indicative of accelerated development) in both treated and untreated fish. The finding that larvae reared at 28°C showed maximal MMI effects before larvae reared at 31°C for eye size and spinal curvature was surprising given that higher temperatures increase metabolism and overall developmental rate (Kimmel, 1995). However, inter-eye distance measurements were most sensitive to MMI treatment earlier in larvae reared at 31°C than in larvae reared in 28°C, and both temperature groups produced maximal decreases in body length at the same age (68 hpf). This differential sensitivity to MMI likely reflects the continuing development of the various parameters during 60-72 hpf, with some parameters requiring thyroid hormones for their development slightly earlier than others.

Analysis of untreated larvae at 72hpf revealed that temperature had a significant effect on only two of our measurements: inter-eye distance and body length. Eye size and spinal curvature were not affected by temperature for 72 hpf untreated larvae. While this may suggest that temperature effects do not exist for the development of these two parameters, additional time points would have to be examined. Because 28°C and 31°C are both within the safe range of rearing temperatures for zebrafish embryos, the finding that temperature has such a wide range of significant effects in both treated and untreated fish suggests the importance of temperature as a control factor in all experiments involving zebrafish embryos and thyroid development.
Interestingly, the highly significant effects of rearing temperature in treated fish suggest thyroid and temperature interactions in early development. The interactions between temperature and MMI sensitivity may indicate that thyroid inhibition is temperature sensitive, such that larvae reared in higher temperatures may require exposure to a higher MMI concentration to see similar effects as larvae reared at lower temperatures. It is therefore possible that our MMI effects using 0.3 mM are not entirely due to a difference in developmental rates; rather, the high temperature group may show decreased overall sensitivity to MMI inhibition with maximal sensitivity occurring later. Physiological reasons for a seemingly protective function of higher rearing temperatures remain unclear. However, warmer water may possibly obtain more iodine, which can help counteract the MMI effects (Endler et al., 1982).

For all measurements, the reduced values observed in larvae removed from treatment at 66-68 hpf were followed by an increase in measurements at later ages. We interpret this finding to mean that larvae removed from MMI treatment at 70-72hpf have decreased sensitivity to MMI exposure and thyroid disruption. This suggests that MMI effects are somehow reversed following a more prolonged exposure, possibly reflecting a compensatory feedback mechanism that leads to a re-instatement of endogenous thyroid hormone levels (Manchado et al., 2008). This possibility is strengthened by the finding that the TSH gene is expressed as early as 42 hpf (Herzog et al., 2003) and can potentially participate in feedback at this age. However, given the action of MMI, continued feedback and stimulation of the thyroid gland when MMI is present should not lead to an increase in hormone release. Goiters would be expected under these conditions,
and have been observed in later ages using a compound similar to methimazole (Schmidt & Braunbeck, 2011).

We propose that the ‘recovery’ in growth observed in larvae exposed to MMI for a longer (>70hpf) duration involves embryonic feedback triggering the compensatory actions by prolactin, which has been shown to have a significant effect on morphological development in zebrafish (Zhu et al., 2007). Prolactin is a hormone released from the anterior pituitary in vertebrates. Prolactin and its receptors are present earlier than the development of the pituitary gland and are thus thought to be involved in early development through maternal supply (Nguyen et al., 2008). While the relationship between prolactin and thyroid hormones is not entirely clear in zebrafish, it is has been suggested that the two systems do communicate in other species, including *Xenopus laevis* (Tata et al., 1991). However, these studies suggest that prolactin has inhibitory effects on thyroid hormones, and does not address this relationship bi-directionally. It is therefore uncertain whether low embryonic thyroid hormones can upregulate prolactin to aid in morphological development.

**Future Perspectives**

Our experiments measured larvae at 72 hpf to allow for direct comparison between all groups and to guarantee that our sample would consist entirely of hatched larvae so spinal curvature and body length could be properly measured. While this design allowed us to determine when maximal effects of MMI were observed, the strategy does have limitations. In particular, we were not able to determine what the acute effects of MMI were at each removal time point. The present study would have been greatly...
supplemented with a closer look at 66-68 hpf critical window, and to expose embryos to MMI later than 0-1 hpf to guarantee a lack of early MMI effects.

Future studies should look at the feedback mechanisms of thyroid hormones in early development, as well as a more thorough study of the effects of thyroid inhibition on retinal layering. Future studies in the lab will examine the interaction(s) between thyroid and prolactin systems in early zebrafish development in order to better understand the possibly synergistic relationship between these two hormone systems.
REFERENCES


