IDENTIFYING SENSITIVE ENDPOINTS OF THYROID DISRUPTION IN THE FATHEAD MINNOW AFTER EXPOSURE TO PROPYLTHIOURACIL

by

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ABSTRACT

A growing body of evidence suggests that endocrine disrupting compounds present in the environment have the ability to impair thyroid function, which may consequently interfere with essential processes such as growth and development. Therefore, it is important to test new and existing chemicals for their ability to disrupt thyroid function. Testing the effects of these compounds on aquatic organisms is necessary due to the pervasive nature of thyroid disrupting chemicals in the aquatic environment. The fathead minnow (*Pimephales promelas*) is frequently used to study acute toxicity, but recently studies have used this species to screen chemicals for endocrine disrupting capabilities. If fathead minnows are to be used in this capacity, we must first determine how known thyroid disruptors affect this species. As such, the goal this study is to expose developing fathead minnows to a low, medium, or high dose of propylthiouracil (PTU; a known thyroid inhibitor) for 35 days, and determine the effects of this compound on a variety of endpoints. Specifically, we measured markers of growth, morphology, and thyroid related gene expression. Fish exposed to the highest dose of PTU experienced significant reductions in mass, length, as well as skin pigmentation compared to the control. Additionally, the high dose PTU exposed fish exhibited significant alterations in the expression of four of ten thyroid related genes evaluated (*trβ, ttr, di2, di3*). The results of this study suggest that metrics of growth, pigmentation, and gene expression are able to indicate thyroid disruption in the developing fathead minnow.

INTRODUCTION

The thyroid gland plays an integral role in the endocrine system by producing thyroid hormones, thyroxine (T4) and triiodothyronine (T3), to mediate important biological processes including metabolism, growth, and development. Ontogeny, the process of a zygote maturing into an adult organism, is suggested to be highly dependent and sensitive to thyroid hormones in a variety of organisms, thus maintaining proper levels of thyroid hormones is imperative during this critical developmental stage (Power et al. 2001). The hypothalamic-pituitary-thyroid (HPT) axis functions to detect and respond to fluctuating thyroid hormone levels to maintain vertebrate homeostasis, however, if this communication system is disrupted, the organisms may suffer from impaired growth alongside other detrimental effects.

There is an increasing concern associated with the environmental presence of endocrine disrupting compounds (EDCs), which are defined as "exogenous agents that interfere with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (Kavilock et al.1996). Since the thyroid is an essential component of the endocrine system, studying the effects of such compounds on the HPT axis is necessary. This is especially true for developing organisms, because the thyroid system has been shown to be responsible for regulating fundamental morphological and physiological processes in mammals and amphibians during early life stages, and these early life stages are considered particularly vulnerable to disruption (Power et al. 2001; Boas et al. 2006).

EDCs are ubiquitous in nature, arising from various point and nonpoint sources such as wastewater treatment plants, drinking water, sewage discharge, and agricultural facilities (Kavilock et al. 1996). Due to various routes of chemical exposure, a wide variety of organisms

are at risk to EDC exposure, including mammals and aquatic vertebrates. Manmade chemicals, such as perchlorate, PCBs and PBDEs, are examples of environmental contaminants that have demonstrated the ability to reduced circulating levels of thyroid hormones in a variety of animals at various developmental stages, including humans (Boas et al. 2006). While studies have demonstrated the capabilities of EDCs to impair the HPT axis in mammals, they are not always practical model organisms. For this reason, small aquatic organisms such as Xenopus laevis, fathead minnow (Pimephales promelas), zebrafish (Danio rerio), Japanese medaka (Oryzias latipes) and other higher-order vertebrates function as surrogate models for mammals (Ankley et al. 2004.)

Researchers have developed endocrine disruption assays using small fish species, like the fathead minnow, due to the high conservation of the thyroid axis across vertebrates (Power 2001; Blanton 2007). The anatomy of the thyroid system in fish differs slightly from the majority of mammals because thyroid follicles are arranged as diffuse clusters as opposed to a single localized structure. However, the essential components of the thyroid system, such as the chemical structure thyroid hormones and their receptors are identical, which allows researchers to extrapolate how EDCs affect thyroid function across vertebrate species (Power et al. 2011). It is environmentally relevant to use fish in this capacity considering endocrine disrupting compounds are commonly leached into bodies of water by mechanisms discussed previously. However, there has been a lack of research dedicated to how thyroid disruption manifests in these species (Crane et al. 2004). Therefore, it is necessary to elucidate markers of thyroid disruption in commonly used aquatic model organisms used to screen EDCs.

The goal of the present study is to determine sensitive, yet practical endpoints indicative of thyroid disruption in fathead minnow (Pimphales promelas). Specifically, the objective of this

study is to identify growth, morphometric, and genomic markers reflecting thyroid disruption in the fathead minnow after exposure to a propylthiouracil (PTU), a known thyroid inhibitor, at various doses.

MATERIALS AND METHODS

Egg Collection and Treatment Assortment

Fathead minnows purchased from Aquatic Ecosystem (Hampton, NH) were housed in 30 L tanks and arranged in breeding pairs. Each tank was filled with 27 L dechlorinated water maintained at approximately 26°C and exposed to a 16-hour light: 8-hour dark photoperiod. Eggs were harvested from tank breeding structures daily and then placed into 1L beakers filled with dechlorinated water under aeration. Beakers of embryos were kept in an incubation chamber maintained at approximately 28°C. Beakers were cleaned each day by 80% water changes for fungus growth prevention and undeveloped embryo removal. During this time, eggs were monitored for hatching. Larvae were ensured to be less than 24 hours old by euthanizing hatched larvae if there were not enough present to initiate a replicate, while unhatched eggs were kept. Once a sufficient number (n=104) of robust appearing larvae were obtained for a replicate, they were randomly assorted into the four treatment groups, resulting in 26 fathead minnows per group. Measures to avoid cross contamination of solutions were taken while allocating larvae to experimental groups.

General Experimental Design

Newly hatched fathead minnow larvae (>24 hours old) were randomly allocated into four treatment groups as follows: unexposed (control), low-dose PTU, medium-dose PTU, and high-dose PTU-exposed fish. Each group consisted of three replicates of 104 larvae each for a total of

26 larvae per group. Exposures persisted for 35 days. On days 7, 21, and 35, larvae were randomly sampled from each treatment group for morphometric and growth assessment. On day 35, sampled larvae were kept for gene expression analysis.

Solution Preparation

Propylthiouracil (PTU) was purchased from Sigma Aldrich (St. Louis, MO) and a 750 mg/L PTU stock solution was prepared daily. This stock solution was diluted with dechlorinated water to yield 10, 35 and 70 mg/L exposure solutions for the low, medium, and high doses of PTU, respectively. All solutions were warmed to temperatures of approximately 26°C before 80% solution changes took place in the beakers housing the larvae.

Animal Husbandry

Beakers of larvae containing 1L of the appropriate treatment solution were placed in a warm water bath at approximately 26 °C and exposed to a 16 h light: 8 h dark photoperiod. *Artemia* nuplii were prepared for larval feeding by 24 hour incubation in 100mL of 35 g/L of aerated salt water under light. Twice daily, hatched *Artemia* were collected, rinsed with dechlorinated water, and wet weight was measured prior to feeding in amounts specified by Table 1. Brine shrimp were suspended in 8 mL of water, and each beaker received 1 mL. Beaker solution changes of 800mL were performed daily to removed excess food and waste. During this time, larvae survival counts and observed abnormalities were recorded.

Table 1. Amount of *Artemia* fed to larvae per replicate based on days post hatch (dph) and number of larvae present in each beaker.

Dph	Artemia suspended in water (mg/8 mL)
1-7	0.221
8-12	0.730
13-16	1.459
17-20	1.938
21-28	1.836
29-35	2.387

Assessment of Growth and Pigmentation

On day 7, 21, and 35 of the experiment, seven larvae were randomly sampled from each replicate of each treatment group. Following larval euthanization with a lethal dose of tricane mesylate (0.3g/L MS-222), their mass and length were recorded and photographs were taken. A pigmentation scoring system was developed due to the noticeable decrease in pigmentation development in PTU exposed fish. Fish were given a pigmentation rating of 0-3, based on their photographs taken after sampling, where 0 signified a lack of pigmentation denoted by visible viscera, giving the fish a translucent quality, while a score of 3 indicated developed pigmentation, particularly focusing on the anterior dorsal aspect of the fish, and no indication of visible internal organs.

Gene Expression Analysis

For gene expression analysis, liver and brain tissues were extracted from 35-day-old larvae. All larval tissues intended for gene expression analysis kept on dry ice until transported to the -80°C freezer. Brain and liver tissues were removed from -80°C freezer for tissue homogenization, using the QSonica tissue sonicator. After homogenization, RNA was extracted from samples using the Maxewell 16 LEV SimplyRNA purification Kit (Promega, Madison, WI). The Nanodrop 1000 (Thermoscientific) performed quantification of total RNA and verified the purity of the RNA from each sample. Transcription of RNA to cDNA was accomplished using the iScript CDNA synthesis kit (Bio-Rad, Hercules, CA). Quantification of gene expression was achieved with CFX qPCR detection system (bio-Rad) by running qPCR reactions with SsoAdvanced Sybr Green as a fluorescent marker. Ribosomal protein L8 was used as a reference gene to normalize the expression of selected thyroid related genes. RNA processing, cDNA synthesis and qPCR was carried out per methods published by Sellin Jeffries et al. 2015.

Table 2. Target genes analyzed in the present study and their respective function.

Target Gene	Function
Ribosomal Protein L8 (*l8*)	Reference/ housekeeping gene
Thyroid Receptors (*trα, trβ*)	Thyroid hormones bind to this receptor
Transthyrettin *(ttr)*	Thyroid hormone binding protein in the blood
Deiodinase 1 *(di1)*	Activates/ inactives thyroid hormone
Deiodinase 2 *(di2)*	Converts inactive thyroid hormone to active thyroid hormone
Deiodinase 3 *(di3)*	Degrades active thyroid hormone to a metabolite for excretion
Growth Hormone Receptor *(ghr)*	Binds to growth hormone
Insulin-like Growth Factor 1 *(igf1)*	Production stimulated by growth hormone
Thyroid Stimulating Hormone *(tshβ)*	Stimulates the release of thyroid hormone from the thyroid gland
Brain Transcription Element Binding Protein *(bteb)*	T3-driven transcription factor influencing the growth of neurons

Table 3. Primer sequence and specified annealing temperature of thyroid-related genes chosen for qPCR analysis in the present study.

Gene	Primer Sequence (5' to 3')	Annealing Temp (°C)
l8 (Kolok et al. 2007)	Forward: GCCCATGTCAAGCACAGAAAA Reverse: ACGGAAAACCACCTTAGCCAG	63.8
trα (Kolok et al. 2007)	Forward: CCATCACACGACTTGTGGAC Reverse: TAAGGTCAGCGTCTCGCTCT	62
trβ (EF191016)	Forward: CAGTGAGTCCGTGGAAGACA Reverse: CAGGTGATGCATCGGTAATG	61
ttr (Chen et al. 2011)	Forward: CTGGTGTGTATCGGGTGGAGTT Reverse: GCATGAGCTTCAAACACCACAT	61
di1 (KF042854)	Forward: ACGGACAGAAAACGAGCATT Reverse: TGAGGAAATCTGCCACATCA	61
di2 (KF042854)	Forward: AATTTTCGGATGTGGCAGAC Reverse: GCAGCAAACATCCTCTCCTC	61
di3 (KF042854)	Forward: TAATGAAGATGCGGGAAAGC Reverse: CGCCGTTTTAAAGAAGTCCA	60
igf1	Forward: CAACGGCACACGGACATC Reverse: CCTCGGCTTGAGTTCTTCTG	60
tshβ (Lema et al. 2008)	Forward: GGTGCAGCCTCTCTGAACCA Reverse: CTTCTGCTTCTCCAGGGACAGT	61
bteb (Lema et al. 2008)	Forward: CAAACCGGCGTAAAGGAAAA Reverse: CATGCAGTCTGTCACAGTTCCA	54

Statistical Analysis

Statistical analysis was conducted using JMP 11.2 software. Differences between treatment groups were measured by a one-way analysis of variance (ANOVA), with alpha set at 0.05. Post-hoc analysis was run with a Dunnet's test if variances were not statistically different between groups. If variances were found to be statistically different between groups as indicated by Wilcoxin/Welch's test, a Steel nonparametric test was performed to compare each group to the control.

RESULTS

Growth

Larval mass and length were not significantly decreased after 7 days of chemical exposure in any treatment group (Figure 1 and 2, ANOVA, $p=0.09$ for mass; $p=0.27$ for length). The 21 and 35 day old larvae exposed to the highest concentration of PTU exhibited significantly reduced measurements of mean mass and length compared to the control (Figures 1 and 2, ANOVA, $p<0.01$ at both time periods).

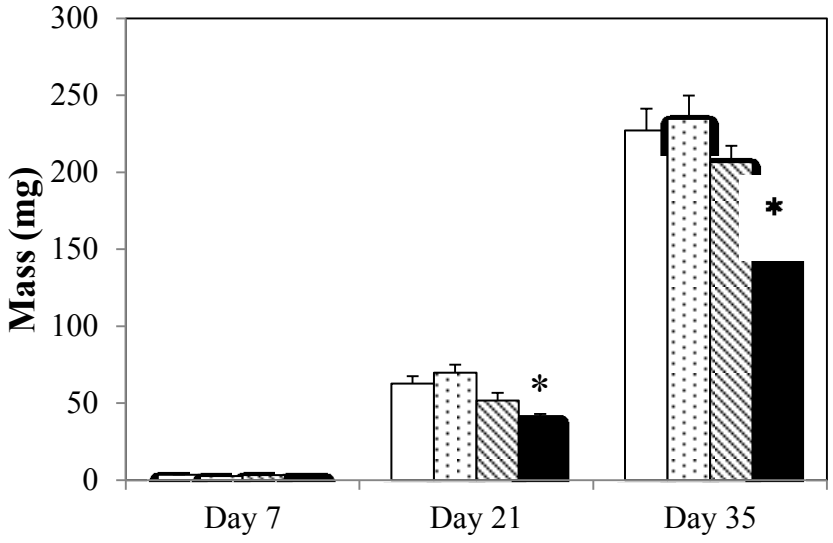


Figure 1. Mean mass of fathead minnow larvae after 7, 21, or 35 days of PTU exposure, respectively. * indicates significant differences from control (evaluated by one-way ANOVA). Error bars indicate standard error.

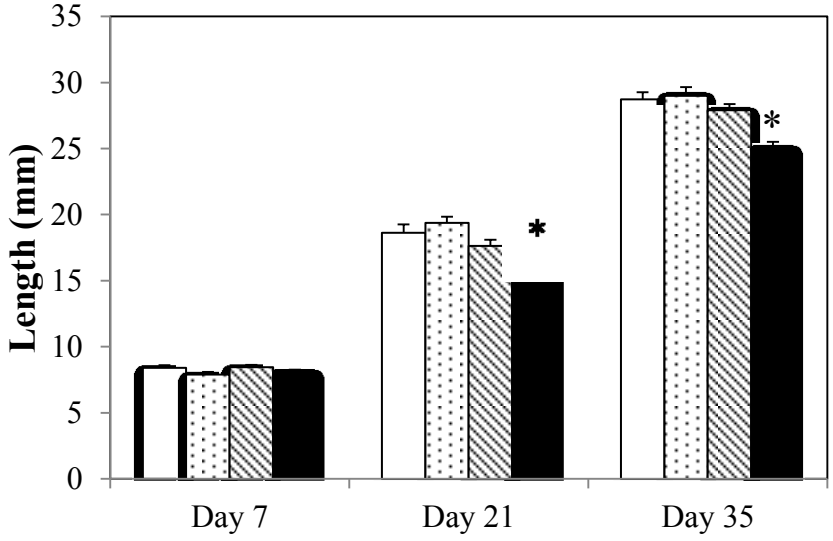


Figure 2. Mean length of fathead minnow larvae after 7, 21, or 35 days of PTU exposure, respectively. * indicates significant differences from control (evaluated by one-way ANOVA). Error bars indicate standard error.

Pigmentation Score

Mean pigmentation scores were significantly reduced in both medium and high PTU exposed groups after 21 and 35 days post hatch (Figure 3, ANOVA, p= 0.01). Although the low PTU group did not exhibit significant decreases in pigmentation compared to the control, a dose-dependent decrease in pigmentation scores was observed. Larvae sampled at day 7 were not scored for pigmentation because of apparent translucency and underdeveloped pigmentation across all groups at this sampling period.

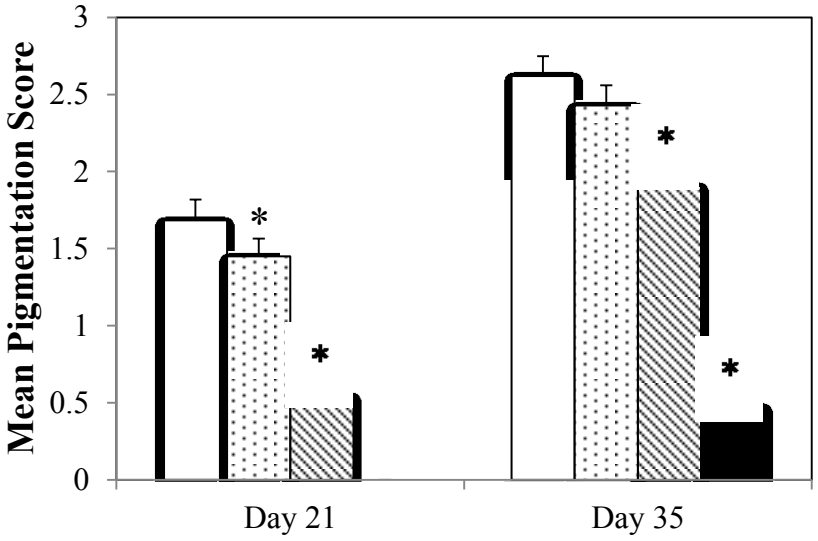
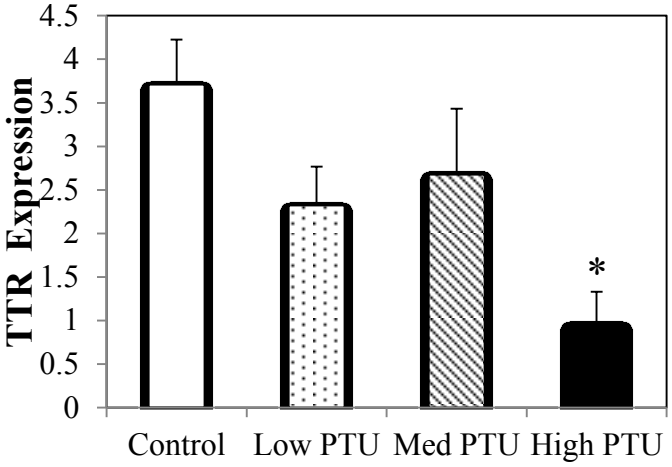
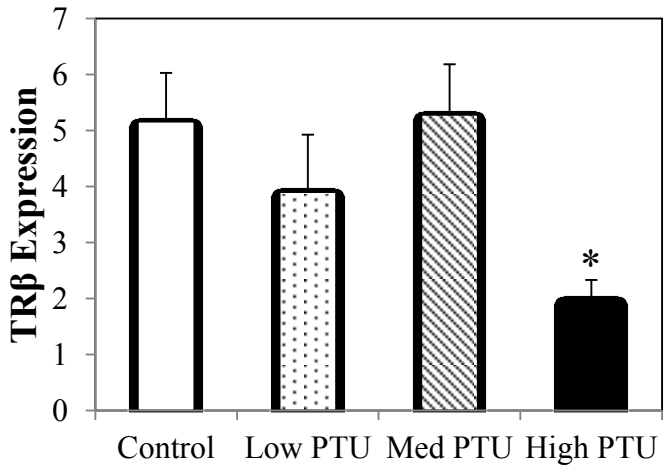


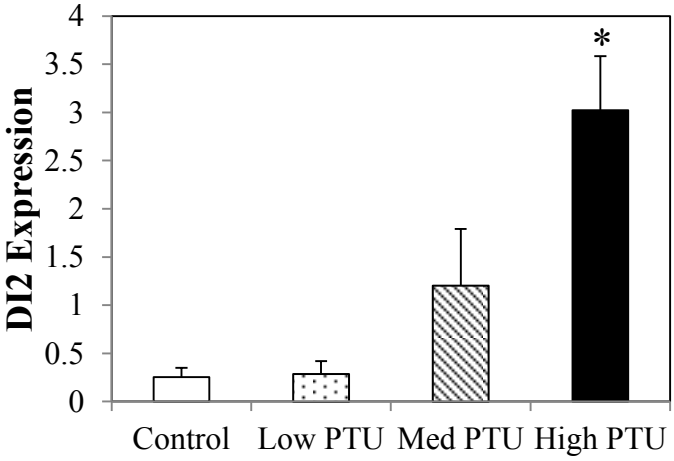
Figure 3. Mean Pigmentation scores of 21 and 35 day old fathead minnow larvae after PTU exposure. * indicates significant differences from control (evaluated by one-way ANOVA). Error bars indicate standard error.

Gene Expression Data

PTU exposed fish did not experience alterations of genes *trα*, *di1*, *ghr*, or *igf1* in the liver, nor in *tshβ* or *bteb* in the brain (Table 4. ANOVA, $p \geq 0.17$). Growth hormone expression was measured in the brain, but could not be quantified due to low expression levels in all groups. Gene expression was significantly reduced in *ttr*, *trβ*, and *di3*, while expression of *di2* was significantly upregulated in the group exposed to the highest dose of PTU (Figure 4, ANOVA, $p<0.01$; $p=0.01$; $p=0.03$; $p=0.04$).


TTR Expression
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
Low PTU
Med PTU
High PTU


TRβ Expression
7
6
5
4
3
2
1
0
*
Control
Low PTU
Med PTU
High PTU


DI2 Expression
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
Low PTU
Med PTU
High PTU

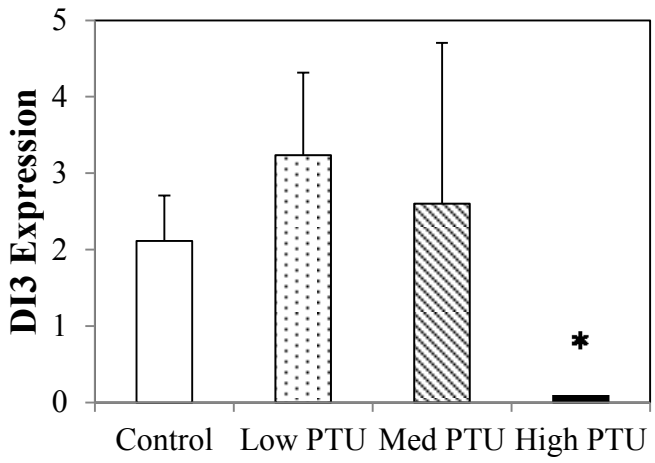


Figure 4. Relative gene expression of transthyrettin (*ttr*), thyroid hormone receptor β (*trβ*), deiodinase II (*di2*) and deiodinase III (*di3*). (*) indicates significant differences from control (evaluated by one-way ANOVA). Error bars indicate standard error.

	trα	*di1*	*ghr*	*igf1*	*tshβ*	*bteb*
Control	(5.12±1.12)	(2.47±0.23)	(3.09±0.66)	(0.32±0.09)	(2.89±0.63)	(2.14±0.62)
Low PTU	(3.95±0.63)	(2.43±0.44)	(3.21±0.47)	(0.27±0.09)	(3.93±0.67)	(2.67±0.44)
Medium PTU	(4.99±1.12)	(3.43±0.86)	(4.46±0.64)	(0.35±0.21)	(3.98±0.69)	(3.69±0.79)
High PTU	(2.84±0.89)	(2.13±0.33)	(3.46±0.81)	(0.22±0.06)	(2.53±0.30)	(3.29±0.49)

Table 4. Relative gene expression of thyroid hormone receptor α (*trα*), deiodinase 1 (*di1*), growth hormone receptor (*ghr*), insulin-like growth factor-1 (*igf1*), thyroid stimulating hormone β (*tshβ*), and brain transcription element binding protein (*bteb*) from control (evaluated by one-way ANOVA). (Mean ± standard error).

DISCUSSION

The primary objective of this study was to determine if markers of growth, physical appearance, and gene expression are sensitive indicators of thyroid disruption in developing fathead minnows to develop this species as a model for the screening of chemicals for thyroid disrupting abilities. Alterations in all three endpoints indicate that the selected metrics were able to indicate a disrupted thyroid state in the juvenile fathead minnow induced by PTU exposure.

Growth metrics as Indicators of Thyroid Disruption

The PTU exposed larvae experienced a reduction in markers of growth, specifically in regards to mass and length after 21 and 35 days. Since this was only observed in the fish exposed to the highest concentration of PTU, this indicates only a substantial decrease in thyroid hormone levels during development in the fathead minnow has the ability to retard growth and development after a prolonged period, while modest inhibition of thyroid hormone production may not result in significant reductions of growth. No significant differences in mass or length were observed between groups at day 7 implying that experiments lasting longer than 7 days are required to assess for growth inhibition due to chemical exposure. Previous studies have obtained similar results of decreased in a variety of model organisms exposed to various thyroid disruptors. For example, zebrafish larvae exposed to a different goitrogen, methimazole (MMI), experienced no significant decreases in length after 14 days, but had reported significantly lower length measurements after 28 days when compared to the control (Lam et al.2005). Likewise, juvenile Japenese flounder (*Paralichthys olivaceus*) had significant reductions in length and mass after 25 days PCB exposure, and the developing fathead minnow exhibited reductions in mass and length after a 28 day ammonium perchlorate exposure (Lam et al.2005; Dong et al. 2014 ;Crane et al. 2005). This suggests alteration in mass in length in fish during early life stages

after chemical exposure is a suitable metric to point to thyroid disrupting abilities. However, other metrics should be explored in conjunction to growth for further evidence of a chemical's thyroid disrupting effects.

Morphometrics

Lack of pigmentation was observed over the course of the experiment among PTU-exposed groups and thus a pigmentation scoring system was developed to quantify the extent of pigmentation development in each individual larvae sampled. The results of the mean pigmentation scores showed significant reductions in both medium and high dose exposed PTU larvae after 21 and 35 days. This indicates pigmentation was a sensitive endpoint due to its ability to detect thyroid disruption at a lower concentration of PTU and can be indicated after 3 weeks versus 5 weeks of exposure, demonstrating the practicality of this endpoint. Furthermore, decreases in pigmentation are not an occurrence exclusive to PTU exposure or to fathead minnow thyroid disruption as indicated by a number of previous studies noting similar alterations in pigmentation in various aquatic organisms after exposure to various thyroid-disrupting agents. For example, developing fathead minnow larvae exposed to perchlorate and zebrafish larvae exposed to MMI, both for 28 days, were also reported to have abnormal pigmentation and scale formation (Crane et al. 2005; Lam et al. 2005). However, most of these studies did not quantify differences in pigmentations, yet abnormalities in pigmentation and delayed scale formation were reported nonetheless. This demonstrates the ability of other thyroid-disrupting agents to induce pigmentation alterations during development in various model organisms.

Thyroid Gene Expression

It has proposed that thyroid hormones auto-induce of expression of thyroid hormone receptors (Lui et al. 2002). Our results suggest this to be true, as the fish exposed to the highest dose of PTU experienced a significant down regulated of TRβ, while TRα expression was not significantly different from the control. It has been noted by previous studies that TRα is affected to a lesser extent than TRβ expression in fish exposed to thyroid disruptors other than PTU, such as amiodarone and MMI. This could be due to lower levels of TRα expression occurring during early life stages in fish. This is reasonable because levels TRβ expression has been shown to be comparatively higher than TRα expression during early embryonic development and therefore differences in TRα expression as a result of thyroid disruption will likely be a less sensitive endpoint (Lui et al. 2002.)

Transthyrettin (TTR) is a thyroid hormone binding protein present in blood plasma in vertebrates and is thought to regulate thyroid hormone (TH) homeostasis. It is believed to accomplish this by rendering thyroid hormones biologically inactive when TTR is bound to TH in the blood, and conversely, when TH is unbound to proteins in the blood, it is considered biologically active to diffuse into target cells and elicit a response (Morgado et al. 2006). TTR is relatively unstudied in fish, although previous studies have shown TTR binds to TH in teleost fish suggesting its role as a thyroid hormone binding protein (THBP). Morgado et al. 2006 sought to uncover the relationship of TH levels and TTR levels in the developing sea breams (*Spirdae*) exposed to MMI, which functions as a thyroid inhibitor through a similar mechanism as PTU. This species exhibited reduced expression of TTR levels in the liver, where TTR is largely produced and secreted, although plasma TTR levels were statistically higher in MMI, T3, and T4 treated fish compared to control fish (Morgado et al. 2006). Another study exposed the Chinese rare minnow (*Gobiocypris rarus*) to exogenous T3 and found TTR gene expression was

unchanged after 21 days in larvae and in adults (Li et al. 2011). Similarly, adult minnows exposed to T3 did not exhibited alterations in TTR expression (Morgado et. al 2006). Taken together, the mechanism that TTR functions in relation to thyroid hormone levels remains unclear, and thus, its expression may not be the best indicator of thyroid disruption in fish.

In addition to PTU being a known inhibitor of TPO, a key enzyme involved in thyroid hormone synthesis, it also functions as an inhibitor of deiodinase 1, an important enzyme that converts TH to become biologically active or inactive. However, in this study we did not observe any alterations in the expression of DI1 in the liver. A possible explanation for this is the relative importance of DI1 in fish is less than in mammals given that studies suggest fish mostly rely on deiodinase 2 (DI2) to produce biologically active T3 from T4 via outer rind deiodination in peripheral tissues (Rijntjes et al. 2013; Walpita et al. 2009). We observed a significant increase in DI2 after the 35-day PTU exposure. This is reasonable because we would expect a prolonged PTU exposure to reduced levels of thyroid hormone and as a result the body would want to convert T4 to T3, the more potent thyroid hormone, to compensate for this loss. Walpita et al. 2009 explored how a knockout DI2 in zebrafish embryos would affect development and found pigmentation development to be significantly reduced compared to the control. It is interesting to note that when T3 was administered to the KO DI2 fish, normal development of pigmentation was restored, showing the importance of adequate T3 for proper pigmentation in fish, which seemed to be largely influenced by DI2, according to this study. Additionally, pigmentation was not restored to the level of the control after T4 administration in the KO fish. In regards to gene expression alterations in this study, TR α and β expression was quantified and observed TRα expression wasn't significantly different from the control, while TRβ was significantly reduced at 31 hours post fertilization, a similar finding to TRβ gene expression in our study as discussed

previously (Walpita et al. 2009). We also noted significant decreases in DI3 expression in PTU-exposed larvae. By reducing DI3, the body is targeting less thyroid hormone for metabolic excretion by the liver, thus retaining more thyroid hormone as an attempt to regulate homeostatic thyroid hormone levels. Taken together, previous literature suggests that DI1 and DI3 expression in fish may give the most variable alterations in expression, yet DI2 expression in the liver has been relatively consistent. This was also observed in the Japanese flounder after exposure to Aroclor 1254, a PCB, which caused a consistent upregulation of DI2 expression compared to the control, while DI1 and DI3 expression gave variable results in regards to the time point it was measured, the dose of PCB that was administered, and the tissue it was measured in (Dong et al. 2014). Differences in DI expression in a variety of aquatic model organisms after exposure to anti-thyroid contaminants has been observed and can likely be explained by the time point at which expression was quantified, differences in the mechanism of the thyroid disruptor, and tissue or species specific differences in the response.

Conclusion

Alterations in endpoints of growth, morphology, and thyroid-related gene expression were sufficient to detect for chemically induced thyroid disruption by PTU in the developing fathead minnow. However, we found these metrics differed in sensitivity to thyroid disruption. Pigmentation appeared to be the most sensitive marker for thyroid disruption in developing fish, while growth also seemed to be affected in the majority of cases. In respects to gene expression, it has been suggested that DI2 plays an integral role in T3 formation in developing fish and which influences both pigmentation and TRβ expression, and is also a good metric for thyroid disruption, although it as not as feasible to measure as metrics of pigmentation and growth.

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