Exploring the Transcriptional Changes Underlying Altered Reproductive Behavior Following Early-Life-Stage Thyroid Disruption in Fathead Minnows

by

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Abstract:

Thyroid disrupting compounds are ubiquitous in every-day life and are commonly found in aquatic environments where they have the potential to impact aquatic species. These compounds can inhibit thyroid hormone synthesis and are most well-known for leading to delayed development and altered metamorphosis. However, a recent study by Bryant (2021) found that fathead minnows exposed to propylthiouracil (PTU, a known thyroid disrupting compound) during development experienced altered reproductive behavior upon maturation with significant reductions in competition, courtship and nest care behaviors. The mechanisms linking thyroid disruption to altered reproduction behavior remain unknown; thus, the principal objective of this study was to determine how the expression of genes known to play a role in sexual differentiation of the brain and neural development are impacted by early-life stage thyroid disruption. In the present study, a subset of fish from the same group of fish utilized in the Bryant (2021) study were used to analyze the expression of aromatase, estrogen receptor α, estrogen receptor β, androgen receptor, tyrosine hydroxylase, dopamine receptor 2 and basic transcription element binding protein in the brain. All three of the genes involved in neurogenesis, *tyh, dr2,* and *bteb*, exhibited a significant increase in expression in the high exposure group (70 mg PTU/L) compared to the control group. While these gene expression changes suggest alterations in neural development, the sample population did not exhibit altered feeding behavior or C-start response (both indicative of neural development in general); thus, it is unlikely that the observed alterations in reproductive behavior can be attributed to such gene expression changes. Of the genes involved in sexual differentiation and sex steroid hormone signaling, only *erα* demonstrated a significant increase in expression in the high exposure group

(70 mg PTU/L) compared to the control group indicating that altered estrogen signaling could be linked to the observed alterations in reproductive behavior.

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Introduction:

Commonly used chemicals such as flame retardants used in consumer goods, antimicrobial compounds used in personal care products and plasticizers have been shown to possess thyroid disrupting activity (Lema et al. 2008, Yang and Chan 2015). Given their widespread use, these thyroid disruptors can end up in aquatic systems where aquatic organisms such as fish may be exposed. Though thyroid disruptors can act through a variety of mechanisms of action, those that inhibit thyroid hormone synthesis are of particular interest. Exposure to such thyroid disruptors may reduce circulating levels of thyroid hormones often through alterations in the activity of enzymes involved in thyroid hormone synthesis (Baumann et al. 2016). Because thyroid hormones play key roles in the growth and development of young organisms (Deal and Volkoff 2020), exposures to thyroid disruptors are most often associated with delayed hatch (Lee et al. 2019), delayed/altered metamorphosis (Wei et al. 2018) and reduced growth (Yu et al. 2020).

More recent studies have begun to investigate the impacts of early life stage exposures to thyroid disruptors on reproduction and behavior. Studies by both Bruns (2017) and Seeman (2018) investigated the potential effects of developmental thyroid disruption on reproduction in fathead minnows. Bruns (2017) sought to evaluate the effect of the model thyroid hormone synthesis inhibitor, PTU, on reproductive function through the evaluation of fecundity (i.e., the number of eggs laid per day), clutch size and spawning frequency. In this study, newly-hatched larvae were divided into three groups, exposed to 0, 35 or 70 mg PTU/L for 43 days, transferred to clean water and raised to sexual maturity (~164 days post hatch) at which time a 21-day breeding study was performed. The results of the breeding assay revealed that compared to the control group, the high-PTU exposure group demonstrated significant decreases in fecundity, clutch size, and number of clutches. Bruns (2017) formed three hypotheses to explain these

results: delayed development, gonadal dysfunction, or improper male reproductive behavior. The first hypothesis was ruled out due to a lack of size difference between the control and high-PTU exposure groups once the fish reached sexual maturity. Similarly, there was no evidence to support the second hypothesis as there were no observed alterations in sperm motility or in gonadosomatic index (a measure of gonad mass relative to body size). As for the third hypothesis, a significant decrease in the spawning frequency of high-PTU fish was noted suggesting that male behaviors related to courtship may have been impacted.

Seeman (2018) sought to further investigate the possible cause of PTU-induced decreases in reproductive output in fathead minnows. Following early-life stage PTU exposures at concentrations of 0, 35 or 70 mg/L followed by a grow-out period in clean water, a breeding assay was carried out to determine if decreased reproductive output was due to changes in male or female reproductive performance. Using breeding pairs of 1) control male x control female, 2) control male x PTU-exposed female, 3) PTU-exposed male x control female and 4) PTU-exposed male x PTU-exposed female, the sex-specific impact of developmental thyroid disruption was determined. The breeding assay revealed that fecundity and the number of clutches produced by pairs of fish was significantly reduced when PTU-exposed males were paired with either control or PTU-exposed females. However, when PTU-exposed females were paired with control males, there were no significant effects of either parameter. These results indicate that the exposure history of the male, but not the female, was responsible for the observed PTU-induced alterations in reproductive output. This result indirectly supports the hypothesis that improper male behavior is responsible for decreased reproductive output following developmental thyroid disruption.

To directly test the hypothesis that PTU-induced alterations in reproductive output stem from alterations in male behavior, Bryant (2021) sought to determine if developmental thyroid disruption by PTU resulted in altered male reproductive behavior. In this study, newly-hatched fathead minnow larvae were divided into three groups, exposed to either 0, 35 or 70 mg PTU/L for 39 days, transferred to clean water and grown to sexual maturity at which point a reproductive behavior assay was performed. The assay findings indicated male fathead minnows exposed to PTU exhibited a significant reduction in competition, courtship, and nest care behaviors in comparison to the control male group (Bryant 2021). Similar results were found by Bernhardt (2008), demonstrating reduced completion of behaviors essential to reproduction in threespine stickleback (*Gasterosteus aculeatus*) following developmental thyroid disruption by perchlorate, which, like PTU, inhibits thyroid hormone synthesis. These findings further support the hypothesis that early-life-stage thyroid disruption reduces reproductive output by altering male reproductive behavior.

Though these previous studies demonstrated that early-life stage thyroid disruption results in alterations in male reproductive behavior, the underlying cause of these changes is unknown. The present study aimed to provide insight into the source of the observed behavioral alterations through identification of the PTU-induced molecular changes in the brains of fathead minnows following early-life stage thyroid disruption. The principal objective of this study was to determine how the expression of genes known to play a role in sexual differentiation of the brain, mating behavior and neural development are impacted by early-life stage thyroid disruption. To accomplish this, a subset of fish from the Bryant (2021) study, which were confirmed to show alterations in reproductive behavior, were used to analyze the expression of genes associated with sex steroid hormone signaling and sexual differentiation of the brain

including aromatase (*arom*), estrogen receptors α and β (*erα* & *erβ*), and androgen receptor (*ar*). The expression of genes known to play key roles in neurogenesis, specifically tyrosine hydroxylase (*tyh*), dopamine receptor 2 (*dr2*) and basic transcription element binding protein (*bteb*) were also evaluated.

Methods:

General Animal Care & Exposure Methods

Detailed animal husbandry and exposure methods used in this study are as described by Bryant 2021. Fish were randomly divided into control (0mg/L PTU), low (35 mg/L PTU), or high (70 mg/L PTU) groups upon hatch and were exposed to PTU through 39 dph. At 39 dph, 20 fish from each exposure group were euthanized using buffered MS-222 (0.3 g/L). This subset of 60 total fish were used in the present study to assess the expression of genes associated with sexual differentiation, behavior, and neuronal development as described below. This subset of fish was taken from a larger group that were used for the evaluation of thyroid disruption and reproductive behavior. Analysis of this larger group revealed that fish exposed to PTU experienced thyroid disruption as demonstrated via gene expression analysis, immunohistochemistry staining and morphometric analysis at 7 dph (Bryant 2021). In addition, those exposed to the high concentration of PTU experiences alterations in male reproductive behavior as indicated by the breeding assay described in Bryant (2021).

RNA Isolation and quantification

The brains of the 60 fish sampled at 39 dph were removed, flash-frozen and maintained at -80°C until RNA extraction. From the set of 20 brain samples per group, 16 were randomly selected to use for gene expression analysis. The Maxwell 16 LEV Simply RNA Tissue Kit was used to isolate RNA from the sample according to the manufacturer instructions. Samples were

homogenized in a manufacturer-supplied homogenization solution with a QSonica Sonicator. Total RNA present in the sample and sample purity were quantified using the NanoDrop 1000 spectrophotometer. RNA content was considered to be sufficiently pure for further gene expression analysis if the 260/280 ratio and 230/280 ratio were both above 1.8. All 48 samples met this purity criteria and were used in gene expression analysis.

cDNA synthesis

Total RNA was converted to cDNA using the Quantabio cDNA synthesis kit and TC100 Thermocycler (Bio-Rad). RNA samples were diluted to a concentration of 13.4 ng/μL using nuclease free water and 8 μL of the diluted RNA was combined with 2 μL of QuantaBio cDNA super mix. Samples were placed in thermocycler and run with the QBio DNA protocol using a heat cycle setting of 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. Following cDNA synthesis, 10 μL of cDNA was combined with 30 μL reverse transcription buffer and this diluted cDNA was used in subsequent quantitative polymerase chain reactions (qPCR).

qPCR

To carry out the qPCR reactions needed to quantify gene expression, 14.33 μL nuclease-free water, 16.67 μL qBio SYBR Green and 1 μL primer (0.3 μM) for the gene of interest were combined with 1.33 μL of the cDNA sample. Of this mixture,10 μL was pipetted into the PCR plate well in triplicate. With 48 samples in total for each gene analyzed, two PCR plates had to be used. To account for any differences between runs, internal controls were included. Samples were run in a CFX Real-Time System (Bio-Rad) under a heat setting of 95°C for 30 seconds, followed by 40 cycle of 95°C for 10 seconds, and the primer annealing temperature (shown in Table 1) for 15 seconds. The following genes were measured: *arom*, *ar*, *erα*, *erβ*, *tyh*, *dr2* and

bteb. These genes are involved in the physiological processes of sex differentiation of the brain (*arom*, *ar*, *erα*, *erβ*) and neural development (*tyh*, *dr2*, *bteb*).

Table 1: List of genes used for expression analysis, primer sequence (forward and reverse sequences) and annealing temperature used.

Gene	Primer Sequence (5' → 3')	Annealing Temp
Aromatase (*arom*)	F: tgctgacacatgcagaaaaactc	51°C
	R: cagctctccgtggctctga	
Androgen receptor (*ar*)	F: gtttccgtaacctgcatgtgg	60°C
	R: cgcgcattagcgttcttgta	
Estrogen receptor α (*erα*)	F: cggtgtgcagtgactatgct	60°C
	R: ctcttcctgcggtttctgtc	
Estrogen receptor β (*erβ*)	F: cgttttggcataaccatgtg	62°C
	R: tgctgtcagacttccgaatg	
Tyrosine Hydroxylase (*tyh*)	F: ttggtttcccagaaaaatcg	60°C
	R: cgtttcctgtaaacagggtca	
Dopamine receptor 2 (*dr2*)	F: atcttgaggtggtgggtgaa	54°C
	R: atcgatgctgatggcacata	
Basic transcription element binding protein (*bteb*)	F: caaaccggcgtaaaggaaaa	54°C
	R:catgcagtctgtcacagttcca	

Statistical Analysis

Results were analyzed in JMP 11 software. Differences in gene expression between groups was analyzed using a one-way analysis of variance (ANOVA). If a significant difference

in expression was detected, means were compared using a Tukey test. In all tests, a p-value of 0.05 was used to evaluate significance.

Results:

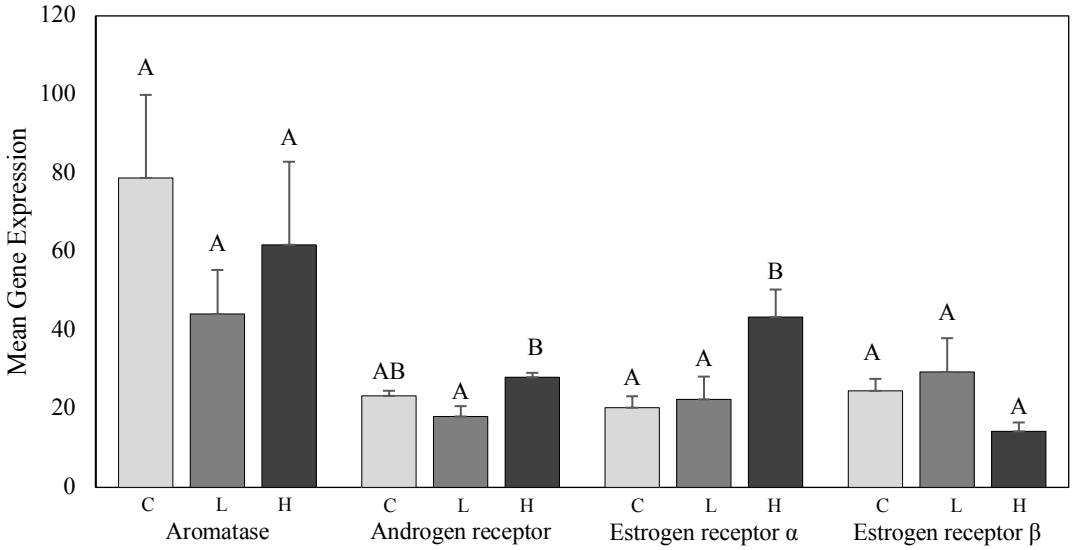

Figure 2: Mean gene expression of genes involved in sexual differentiation in the brains of fathead minnows sampled at 39 dph. Light grey indicates the control group (C, 0 mg/L PTU), dark grey indicates the low exposure group (L, 35 mg/L PTU), and black indicates the high exposure group (H, 70 mg/L PTU). Standard error is indicated by error bars, significant differences are indicated by different letters, n = 16 for each group.

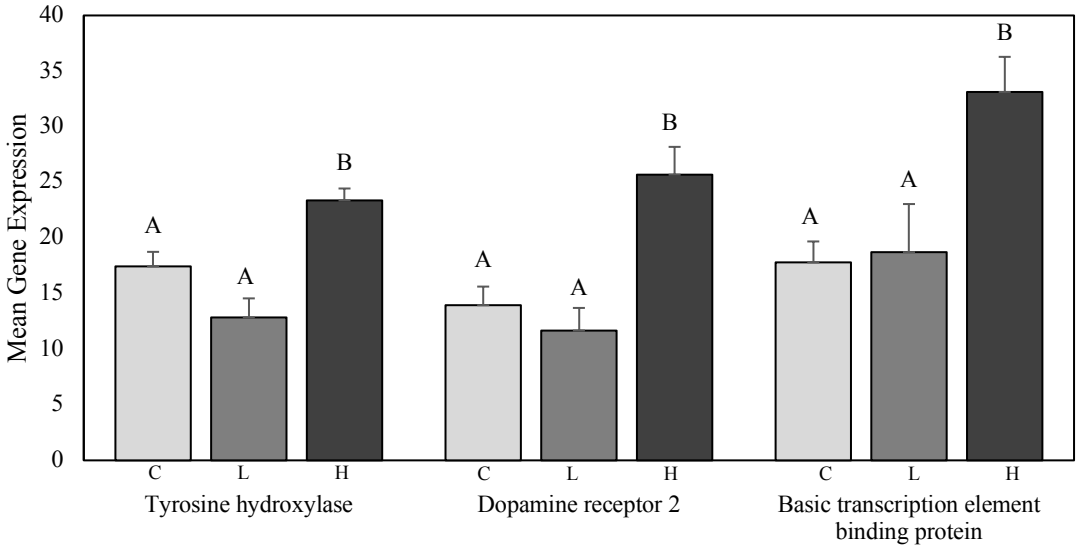

Figure 3: Mean gene expression of genes involved in neuronal development (*bteb*) and behavior (*tyh, dr2*) in the brains of fathead minnows sampled at 39 dph. Light grey indicates the control group (C, 0 mg/L PTU), dark grey indicates the low exposure group (L, 35 mg/L PTU), and black indicates the high exposure group (H, 70 mg/L PTU). Standard error is indicated by error bars, significant differences are indicated by different letters, n = 16 for each group.

The results of the gene expression analysis are shown in figures 2 and 3. Both *arom* (ANOVA, $p = 0.41$) and *erβ* (ANOVA, $p = 0.16$) did not show a significant difference in expression between groups. Significant increases in *erα* (ANOVA, $p < 0.01$), *tyh* (ANOVA, $p < 0.01$), *dr2* (ANOVA, $p < 0.01$) and *bteb* (ANOVA, $p < 0.01$) expression were detected. In all cases, fish from the high exposure group (70 mg/L PTU) had significantly higher expression than both the control (0 mg/L PTU) and low (35 mg/L PTU) exposure groups. A significant increase in *ar* (ANOVA, $p < 0.01$) expression was detected in the high (70 mg/L PTU) exposure group compared to the low (35 mg/L PTU) exposure group, however no significant difference was detected compared to the control group (0 mg/L PTU). Of the genes involved with sexual differentiation *erα* demonstrated a 2.1 fold increase in expression. Of the genes involved in

neural development and behavior, *tyh* demonstrated a 1.3 fold increase, *dr2* demonstrated a 1.8 fold increase and *bteb* demonstrated a 1.9 fold increase.

Discussion:

Genes *tyh*, *dr2* and *bteb* all demonstrated significant increases in expression in the high exposure group (70 mg PTU/L) compared to the control group. Tyrosine hydroxylase, the protein encoded by *tyh*, is an enzyme involved in the rate limiting step of catecholamine synthesis, which produces the hormones dopamine, norepinephrine and epinephrine (Kobayashi et al. 2012). Catecholamine activity has a variety of important physiological roles including regulation of motor activity, behavior and neurodevelopment (Filippi et al. 2010, Souoza and Tropepe 2011). With transient knock-down of *tyh* expression in larval zebrafish, Formella et al. (2012) showed that behavioral alterations can persist into adulthood, suggesting the importance of developmental catecholamine signaling. Following introduction to an unfamiliar area, significant reductions in both freezing behavior (movement <0.2 cm/s) and bottom-dwelling behavior (tendency to dive to bottom and gradually explore the top of a new area) were observed, indicating long-term behavioral impacts of developmental *tyh* suppression (Formella et al. 2012). Dopamine receptors including DR2 regulate the activity of protein kinase Akt which is critical in the processes of neural development such as neuronal migration and cell proliferation (Souza et al. 2011). Dopaminergic signaling functions to regulate behavior, neurotransmission and memory; thus, developmental alteration of dopamine signaling has the potential to induce lasting impacts (Liu et al. 2021). Following exposure to a DR2 antagonist during the larval period, zebrafish exhibited a significant increase in motility during period of darkness, demonstrating the ability of altered dopamine signaling to impact behavior (Oliveri and Levin 2019). Basic transcription element binding protein, the protein encoded by *bteb* is important in

the regulation of genes involved in neurite growth and development and in the brain (Cayrou et al. 2002). This was demonstrated by the findings of Morita et al. (2020) which showed that *bteb* knockout mice that displayed deficits in motor coordination, learning and memory. These processes are all dependent on proper development of the cerebellum and hippocampus, indicating that *bteb* elimination may cause neuronal disruption in these areas (Morita et al. 2020).

Given the observed changes in the expression of these genes, changes in behavior may be expected. To evaluate behavior, Bryant (2021) assessed C-start response, feeding behavior, and reproductive behaviors in a subset of fish from the same population of fish used in this study. Bryant (2021) found a significant reduction in competition, courtship and nest care behaviors in fish from the high (70 mg PTU/L) group compared to the control group indicating improper reproductive behavior. The feeding assay did not demonstrate any significant changes in feeding behavior in the PTU exposure groups compared to the control (Bryant 2021). Similarly, the C-start assay did not reveal significant differences in responses, indicating that neurological function was intact (Bryant 2021). The C-start response is indicative of the predator escape response which, in addition to feeding behavior, may be altered in response to neurological changes. McGee et al. (2009) found that manipulation of synaptic transmission by estrogens led to impairment of the C-start response and an overall decreased ability to escape a threat stimulus in fathead minnows. Additionally, the loss of dopaminergic neuron in the brain caused by exposure to the pesticide rotenone caused significant reduction in feeding behavior in Indian freshwater catfish (*Mystus cavasius*) (Badruzzaman 2021). The significant increases in *tyh*, *dr2*, and *bteb*, would lead us to believe that behavioral responses in PTU exposed fish could have been impaired. However, if the increase in expression of *tyh*, *dr2* and *bteb* were responsible for the behavioral changes seen by Bryant (2021), we would have expected not only to see changes

in reproductive behavior, but also in feeding behavior or C-start response. Thus, it is unlikely that changes in *tyh*, *dr2* and *bteb* expression can explain the observed changes in reproductive behavior. It is possible that thyroid disruption did in fact lead to a decrease in Tyh, Dr2, and Bteb protein levels within the fish, however their expression was increased to compensate for this decrease. This would explain the observed increase in expression and lack of behavioral alteration.

Several genes (*arom*, *ar*, *erβ*, and *erα*) known to be involved in sex steroid hormone signaling and sexual differentiation were measured in the present study. Androgen receptor, the protein encoded by gene *ar*, plays a key role in androgen signaling in zebrafish, being expressed from as early as 24 hpf through adulthood. Though important for development of both sexes, *ar* levels have been found to be increased in male developing male testis tissue, suggesting the role of *ar* in sexual development (Hossain et al. 2008). Encoded by gene *arom*, aromatase is an enzyme that is essential for sexual development and reproduction zebrafish (Tang et al. 2017). Through the creation of *arom* knockout zebrafish, Yin et al. (2017) determined that aromatase is needed for proper development of both males and females, indicating the importance of its expression during development. Protein ERβ, encoded by gene *erβ*, is important in estrogen signaling in males and females. Estrogen signaling has been found to be important in sexual and neuronal development, processes that are key in the proper behavioral functioning of zebrafish (Froehlicher et al. 2009). Though genes *ar*, *arom* and *erβ* are all involved in the process of sexual differentiation and development, their expression did not differ significantly from controls; therefore, the behavioral alterations observed cannot be attributed to differences in their expression.

As the only gene exhibiting a significant increase in expression in the present study, *erα* was of particular interest. Estrogens are crucial in the growth and differentiation of male and female reproductive organs and brain development. Estrogen activity is modulated through binding to specific estrogen receptors, including ERα, which can act as transcription factors for genes necessary for sexual development (Filby et al. 2005). Chen et al. (2018) found that zebrafish mutants lacking the ERα protein had premature ovarian degradation and decreased fertility at 180 dpf, indicating the importance of proper estrogen signaling on proper reproductive functioning.

An increase in *erα* expression may be indicative of altered estrogen signaling in the brain of PTU exposed fish. With altered estrogen signaling, altered reproductive behavior upon maturation would be expected. As previously mentioned, the reproductive behavior assay conducted by Bryant (2021) showed a significant reduction in competition, courtship and nest care behaviors by 71%, 73% and 75%, respectively in fathead minnows exposed to the high concentration of PTU (70 mg/L). These alterations in reproductive behavior indicate that the physiological pathways responsible for reproductive behavior had been altered following thyroid disruption. It is important to note that the present study focused on gene expression in juvenile fathead minnows following an early-life-stage exposure to PTU. Conclusions drawn following adult exposure to thyroid disruptors may not necessarily be applied to the developmental processes responsible for behavior. Despite the differences between developmental and adult pathways, previous studies have shown that altered estrogen signaling can lead to altered reproductive behavior in adults. Following exposure to exogeneous estrogen, adult male fathead minnows showed significantly decreased nest care and competition behaviors compared to control males, in addition to increased vitellogenin production and decreased 11-ketotestosterone

levels (Martinović et al. 2009). This finding that disrupted estrogen signaling can lead to impaired male reproductive behavior would support the hypothesis that the changes in *erα* expression in the present study may have led to the alterations in reproductive behavior. The observed significant increase in *erα* expression suggests that there was alteration in estrogen signaling in PTU-exposed fish that may have resulted in the observed changes in behavior.

The mechanism of action of estrogen signaling in the brain is complicated and variable, therefore further analysis is needed to understand the impact of transcriptional changes following thyroid disruption. Investigation of the role of sex steroid hormones and their receptors in the brain may provide a better understanding of how changes in this signaling can lead to behavioral alterations. Additionally, future study of the transcriptional changes specifically in the brains of juvenile male vs female fish might provide further insight into the sex-specific physiological changes leading to altered reproductive behavior.

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