UNCOVERING THE EFFECTS OF THYROID DISRUPTION ON IMMUNE FUNCTION AND DEVELOPMENT IN FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

By

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UNCOVERING THE EFFECTS OF THYROID DISRUPTION ON IMMUNE FUNCTION AND DEVELOPMENT IN FATHEAD MINNOW (*PIMEPHALES PROMELAS*)

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ABSTRACT

Previous studies showed that exposures to thyroid inhibitors during early stages of development lead to long-lasting alterations in disease resistance. Therefore, the goal of this project was to assess the effects of early life stages thyroid disruption on the maturation and function of immune cells using propylthiouracil (PTU)-exposed fathead minnow as a model system. The specific objectives of this study were to evaluate the impacts of early life stage PTU-exposure on 1) transcriptomic markers of lymphoid and myeloid cell development and 2) neutrophil migration. These objectives were accomplished by exposing fathead minnow embryos to 35 mg/L and 70 mg/L PTU for 10 days, while assessing immune cell development by measuring transcriptomic markers of maturation at 5, 7 and 10 days post fertilization (dpf) and evaluating neutrophils migration with a tail nicking assay at 10 dpf. There were no differences in transcriptomic markers for lymphoid cells between PTU and control groups. However, PTU-exposed larvae showed a decreased expression of v-myb gene and had less neutrophils at wound site compared to those of the control at days 7 and 10, indicating that early life thyroid disruption interfered with the normal development and function of these immune cells.

INTRODUCTION

Thyroid-disrupting compounds (TDCs) are increasingly prevalent in aquatic environments, arising from various sources such as wastewater treatment plants, drinking water, and agricultural facilities. Exposures to TDCs can cause adverse effects in organisms, including stunted growth, delayed development, and altered metabolism [1,2,3].

Traditionally, reductions in growth and body development were considered the primary negative consequences of thyroid hormone (TH) disruption; however, recent studies have suggested that thyroid disruption may also alter the immune system, reducing the ability of organisms to fight against disease [4,5]. In addition, a study from the Jeffries lab found that male fathead minnows exposed to polybrominated diphenylethers (BDE-47, an environmentally-relevant thyroid disruptor) during early life stages experienced alterations in their ability to fight and survive a bacterial infection, even one year after the exposure had ceased [6].

The mechanisms underlying this effect remain unclear; however, we hypothesize that exposures to thyroid disruptors during early life stages impair immune cell development and function. The goal of this project was to assess the effects of early life stage thyroid disruption on the maturation and function of immune cells using propylthiouracil-exposed (PTU) fathead minnow as a model system. The specific objectives of this study were to evaluate the impacts of early life stage PTU-exposure on 1) neutrophil migration and 2) transcriptomic markers of lymphoid and myeloid cell development. These objectives were accomplished by exposing fathead minnow embryos to 35 mg/L and 70 mg/L PTU for 10 days, while evaluating neutrophils migration with a tail nicking assay at 5, 7 and 10 days post fertilization (dpf) and assessing myeloid and lymphoid cell development by measuring transcriptomic markers of cell maturation at 10 dpf.

METHODS

General experimental design

Animal care procedures were approved by the Texas Christian University (TCU) Institutional Animal Care and Use Committee (protocol #18/01). Embryos used in the current study were produced from TCU minnow colony. Briefly, sexually-mature fathead minnow breeding pairs were housed in 30-L glass aerated aquaria filled with 27 L dechlorinated municipal water maintained at ~25.5°C ± 0.5°C and under a 16-hour light: 8-hour dark photoperiod. Each tank was outfitted with breeding structures in the form of PVC pipe cut in ½ lengthwise. Structures with eggs were collected and 540 embryos <32-cell stage were allocated to one of three groups: control, low PTU (35 mg/L) or high PTU (70 mg/L). Each group featured three replicates of 60 embryos each for a total of 180 embryos per group. Each replicate was housed in 1 L beakers kept in an incubator set to 27°C under aeration with 80% water change daily. Artemia *nuplii* were prepared for larval feeding by 24 hour incubation in 1 L 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 according to the number of larvae remaining in beakers as the experiment progresses (1.06 mg Artemia/larvae). Exposures persisted through 10 days post fertilization (dpf). At 5, 7, and 10 dpf, larvae from each group (3-4 per replicate) were subjected to a neutrophil migration assay, while 9 larvae (3 per replicate) were sampled for length assessment and gene expression analysis. At 10 dpf, 8 larvae from each group (2-3 per replicate) were collected for analysis of intrafollicular thyroxine (T4) content.

PTU exposures

PTU (Sigma Aldrich, Milwaukee, WI) stock solution of 350 mg/L was made daily by dissolving PTU powder in dechlorinated municipal water and mixing on a stir plate overnight at

26°C. The stock solution was then diluted to 70 mg/L for the high dose and 35 mg/L for the low dose exposure solution.

Confirmation of thyroid disruption

To confirm thyroid disruption by PTU, length, thyroid-related gene expression and intrafollicular T4 content were evaluated at 10 dpf. Larvae were euthanized with 0.3 g/L buffered MS-222 for length assessment. Those designated for gene expression analysis were flash frozen and stored at -80°C, while those used for assessment of T4 were placed in 4% PFA. The expression of two thyroid-related genes, deiodinase 2 (*di2*) which converts T4 to T3 and transthyretin (*ttr*) which transport thyroid hormone in the blood, was measured via qPCR as described under "Gene expression analysis" (*di2*, primer sequence F: AATTTTCGGATGTGGCAGAC and R: GCAGCAAACATCCTCTCCTC, annealing temperature: 54°C; *ttr*, primer sequence F: CTGGTGTGTATCGGGTGGAGTT and R: GCATGAGCTTCAAACACCACAT, annealing temperature: 60°C).

Intrafollicular T4 content was analyzed via an immunofluorescent assay (Thornton et al, 2018). Following fixation in 4% paraformaldehyde at 4°C overnight, larvae were washed in phosphate-buffered saline (PBS) three times for five minutes each, then progressively dehydrated through a series of washes in 25%, 50%, 75%, and 100% of MeOH/PBS. Larvae were then rehydrated with decreasing solutions of MeOH/PBS in the reverse order of the dehydration steps, washed with PBS (three times for five minutes each), depigmented (3% H_2O_2, 1% KOH in water) for 50 minutes, submerged in distilled water for five minutes (23°C), in 100% acetone for seven minutes (-20°C), and then in distilled water again for five minutes (23°C). Larvae were then washed with PBS two times, soaked in 0.1% collagenase solution for 15 minutes, in blocking buffer for 2-3 hours at RT, and incubated with primary antibody (1:500 concentration) for four

days at 4°C. Then, larvae were washed with blocking buffer six times for 30 minutes each, and incubated with secondary antibody (1:150 concentration) for four days at 4°C and washed with blocking buffer six times again like previous. Samples can be stored up to a few months at 4°C and must be in complete darkness to preserve fluorescence.

Neutrophil migration assay

Larvae were anesthetized with 0.1 g/L MS-222 for 7 and 10 dpf larvae and 0.2 g/L MS-222 for 5 dpf larvae. A small blade was used to make a straight cut across the tail (as illustrated by Figure 1), then larvae were returned to their original exposure solution for a 2-hour recovery period.

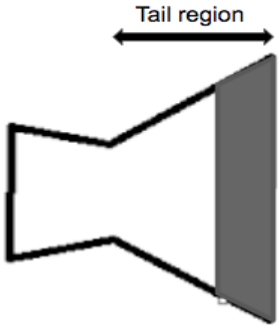

Figure 1. Tail nicking procedure. Grey area denoted the transected portion of the tail.

Following the recovery period, larvae were euthanized with 0.3 g/L buffered MS-222 and fixed in 4% formaldehyde for 2 hours at room temperature (RT). In the following steps, samples were put on a rocker during any waiting period. Larvae were rinsed in PBS three times for 5 minutes each, stained with Sudan Black for 20-30 minutes, washed in 70% Ethanol for 15 minutes and incubated for 1 hour. Larvae were then progressively rehydrated with 25%, 50%, 75% and 100% PBT in PBS for 10 minutes in each solution, depigmented (1% KOH/ 1% H_2O_2) for 2-3 hours depending on age, and progressively transferred through a series of glycerol/1% KOH (25%, 50%, 75%, 100%) for 40 minutes to 1 hour at RT or overnight at 4°C depending on age.

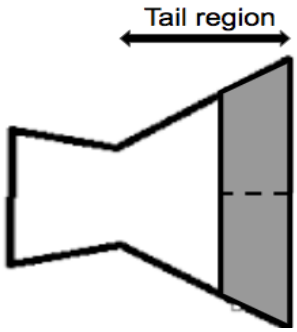

Figure 2. Grey area denotes the region set to count neutrophils. The dash line represents the measured distance from which the boundary is drawn.

Images of larvae were analyzed via *ImageJ* software using the following method: for 5 dpf larvae, at a distance of .716 mm from the midpoint of the edge of the tail, a straight line parallel to the cut of the wound was drawn (as illustrated in Figure 2). Neutrophils in the area between the ending of the tail and the drawn line was counted. For 7 and 10 dpf, the same .716/ total average length ratio was applied to find the appropriate distance to draw the line.

Immune cell development

To evaluate the development of lymphoid and myeloid cells, genes known to play key roles in the differentiation and maturation of each cell class were analyzed via qPCR as described in the "Gene expression analysis" section. Table 1 shows the target genes selected for analysis.

Gene	Function	Primer sequence	AT (ºC)
Lymphoid-related genes			
ikaros	Hematopoietic transcription factor essential for lymphoid differentiation	F: GGTTCACAAGCGAAGTCACA R: TGATGTGTCGGAGCAGGTTA	60
Recombination activating gene-1 *(rag1)*	Initiate V(D)J recombination for lymphocyte maturation	F: TCCTGAGAAGGCAGTGAGGT R: ATCCACAAACATGAGGCACA	60
Immunoglobulin Lambda Constant 3 (*IgLC3*)	Receptor on B lymphocytes	F: AGAGCAGAGCGGAGGAGTCT R: GGAACTGAACTGACGATCAGAA	60
Myeloid-related genes			
Avian Myeloblastosis Viral Oncogene homolog (*v-ymb*)	Hematopoietic transcription factor important for myeloid differentiation	F: CTGATGCTTCCCAACACAGA R: CTTCAGAGGGAATCGTCTGC	54
CCAAT/ binding protein alpha (*cebpa*)	Transcription factor important for the differentiation of myeloid progenitors	F: GGAGCAAGCAAACCTCTACG R: TCAATGTAGGCGCTGATGTC	60
Myeloperoxidase (*mpo*)	Peroxidase enzyme in granulocytes	F: CCGGGAGACCTTTCATTCTA R: TGAGTGTTTGCCCCATGTAG	60

Table 1. *Immune development genes.*

Gene expression analysis

Whole larvae, sampled at 10 dpf, were homogenized using a QSonica sonicator, and total RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit following manufacturer instructions. Total RNA content and purity of the samples were then measured using the NanoDrop 1000 spectrophotometer, and RNA was considered sufficiently pure if the 260/280 absorbance ratio was ≥ 1.8. RNA was converted to cDNA using the Quantabio cDNA synthesis kit. The synthesis reactions contained 2 µL of qScript cDNA Supermix and 0.1 µg of total RNA diluted into 8 µL of nuclease free water. Reactions were carried out in a TC100 thermocycler

(Bio-Rad) with a program of 5 min at 25° followed by 30 min at 42° and 5 min at 85 °C. The resulting cDNA was diluted with 30 µL of reverse transcriptase buffer. All qPCR reactions contained 0.4 µL of cDNA, 4.3 µL of nuclease free water, 0.3 µL of 10 µM primer mix and 5 µL PerfeCta SYBR Green FastMix (Quantabio) and were run in triplicate on a 96-well plate. Reactions were completed using a CFX Connect real-time PCR machine with a cycling program that consisted of an activation step (95 °C for 30 sec), 40 cycles of denaturing (95 °C for 10 sec) and an annealing step (primer specific temperature for 15 sec).

Gene expression was quantified using a standard curve, and each target gene was normalized to the geometric mean of ribosomal protein (*l8)* and acidic ribosomal phosphoprotein (*arp)* which are two common reference genes (*l8*, primer sequences: F: GCCCATGTCAAGCACAGAAAA, R: ACGGAAAACCACCTTAGCCAG, annealing temperature: 63.8°C; *arp*, primer sequence F: CTGAACATCTCGCCCTTCTC, R: GACACACACTGGCGATGTTC, annealing temperature: 60°C). The reference gene was not differentially expressed between groups.

Statistical analysis

Statistical analysis was conducted using JMP software. Differences between treatment groups were assessed by a one-way analysis of variance (ANOVA). When there were significant differences, post-hoc analysis with a Tukey's test was used to determine significant differences between groups. In cases of unequal variances, the Wilcoxon test was conducted, followed by nonparametric comparisons using the Steel-Dwass method. For all tests, statistical significance was set at $\alpha = 0.05$.

RESULTS

Confirmation of thyroid disruption

Throughout the exposure, no significant differences were found in length measurements between control and PTU-exposed fish at 5, 7 or 10 dpf (ANOVA, $p > 0.1$ for all time points; Figure 1). At 10 dpf, significant differences in mean *ttr* expression were detected between control and PTU-exposed larvae at 10 dpf, while none were detected in mean *di2* expression (*ttr*, Wilcoxon test, $p < 0.01$; *di2*, ANOVA, $p > 0.05$; Figure 2). Both the high-PTU and low-PTU groups had decreased *ttr* expression compared to the control.

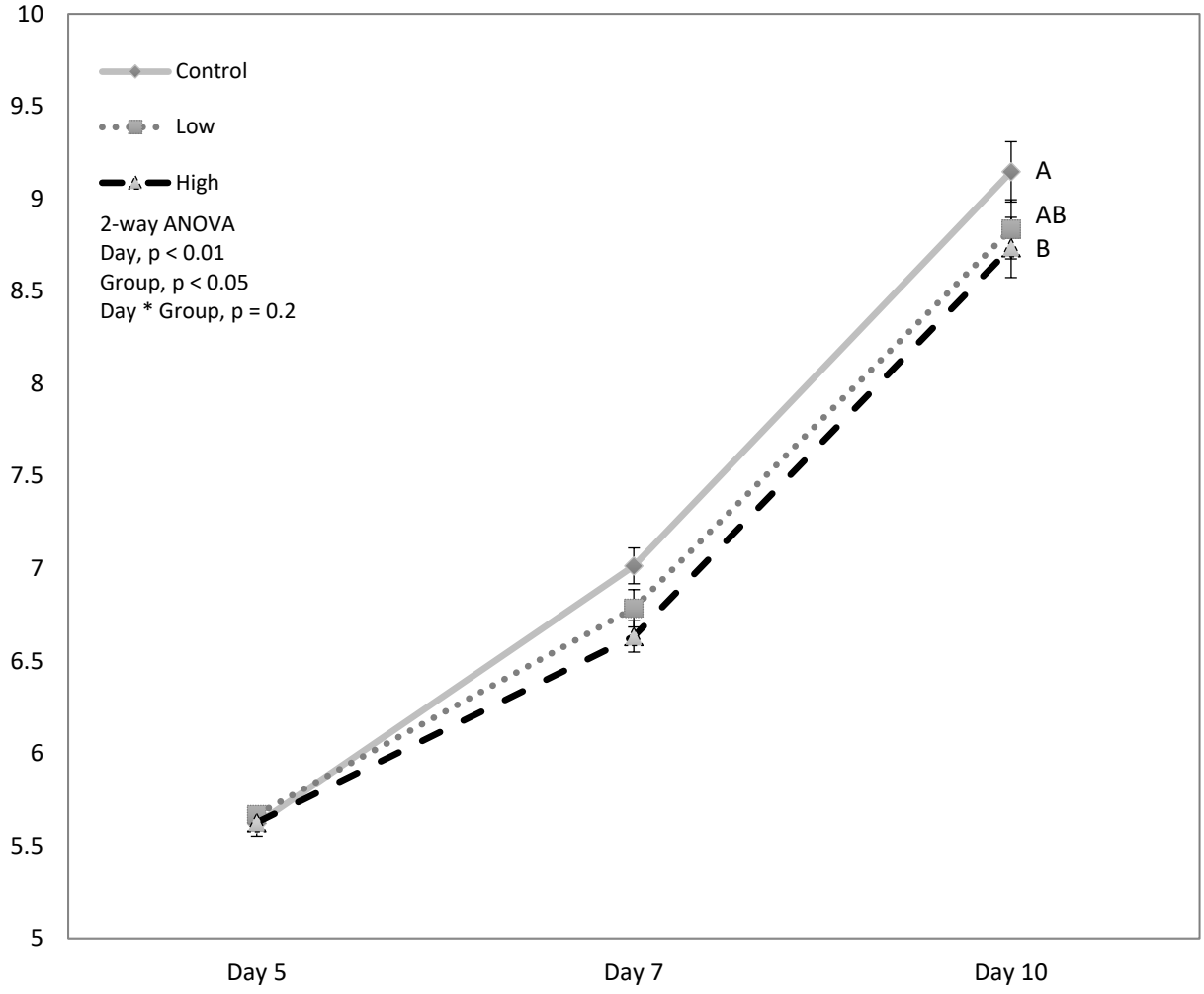

Figure 3. Mean length of fathead minnows in control, low-PTU (35 mg/L), and high-PTU (70 mg/L) groups at day 5, 7 and 10. Different letters indicate significant differences between groups. n = 9 per group.

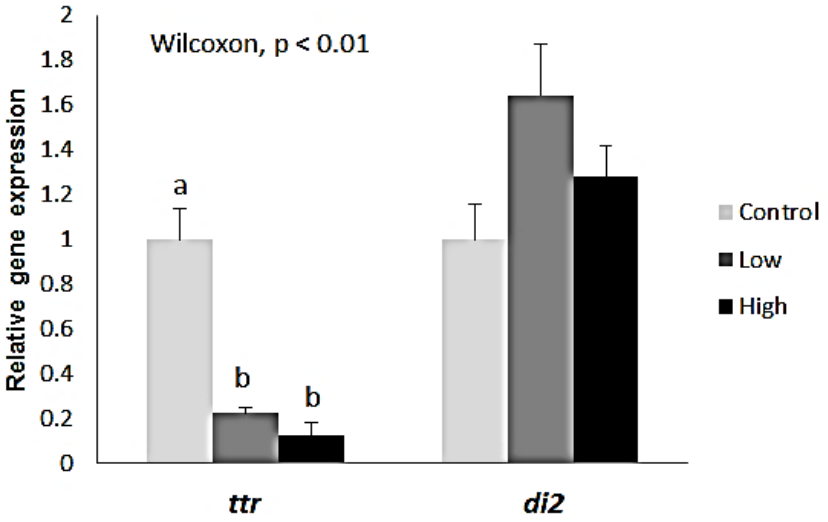

Figure 4. Mean expression of *transthyretin (ttr) and deiodinase 2 (di2)* in day 10 fathead minnows in control, low-PTU (35 mg/L), and high-PTU (70 mg/L) groups. Different letters indicate significant differences between groups. n = 9 per group.

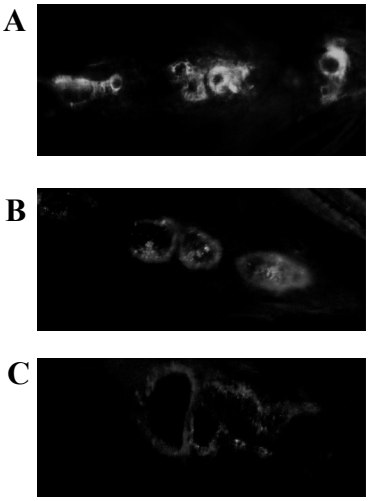

Figure 5. Representative photo of fluorescently labeled T4 in day 10 fathead minnows in **A)** control, **B)** low-PTU (35 mg/L), and **C)** high-PTU (70 mg/L) groups.

Neutrophil migration assay

During the exposure, the number of neutrophils recruited to wound site was significantly different between control and PTU-exposed larvae at 7 and 10 dpf, but not at 5 dpf (Wilcoxon test, $p < 0.05$ for day 7, $p < 0.01$ for day 10, $p > 0.4$ for day 5; Figure 3). Specifically, larvae in the low-PTU group had significantly less neutrophils migrated to wound site compared to those in the control group at 7 dpf. However, at 10 dpf, both the low-PTU and high-PTU groups had significantly less neutrophils at wound site compared to the control.

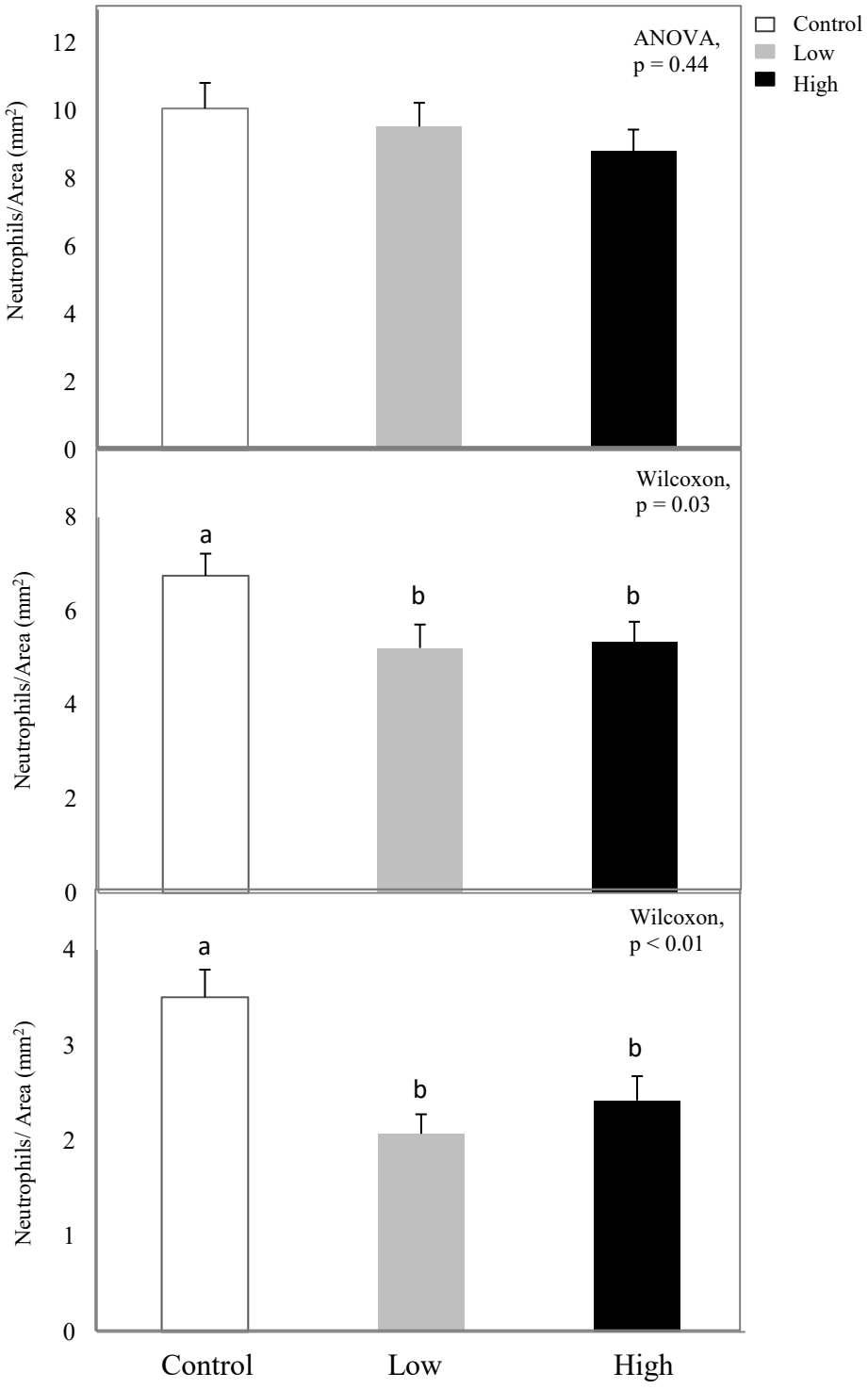

Figure 6. Neutrophils count per area in fathead minnows at **A)** 5, **B)** 7 and **C)** 10 dpf. Different letters denote significant differences between groups. n = 22-27 per group.

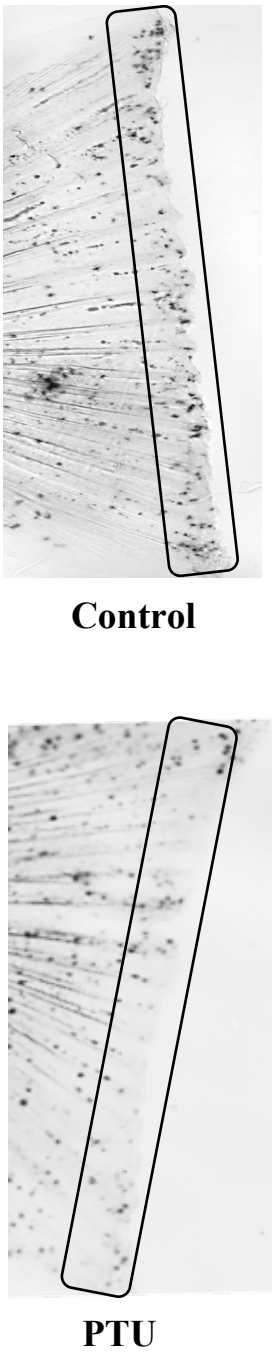

Figure 7. Representative photos of Sudan black stained neutrophils at tail wound in control and PTU-exposed larvae.

Lymphoid-related gene expression

There was no significance difference in the expression of *ikaros*, *rag1* and *IgLC3* between control and PTU groups at 10 dpf (Table 2).

	Control	Low	High
Ikaros	0.61 + 0.09	0.56 + 0.06	0.56 + 0.04
Rag1	0.62 + 0.06	0.63 + 0.12	0.70 + 0.08
IgLC3	0.44 + 0.07	0.47 + 0.07	0.46 + 0.07

Table 2. *Relative mean expression of ikaros, recombination activating gene 1 (rag1) and and immunoglobulin lambda constant region 3 (IgLC3) in control, low-PTU (35 mg/L) and high-PTU (70 mg/L) groups at 10 dpf.*

Myeloid-related gene expression

No significance difference was detected in the expression of *cebpa* and *mpo* between control and PTU groups at 10 dpf (Figure 8). However, the expression of *v-myb* gene is significantly different between control and PTU-exposed larvae (ANOVA, $p < 0.01$, Figure 8). Specifically, larvae in the high-PTU group experienced a decreased in *v-myb* expression.

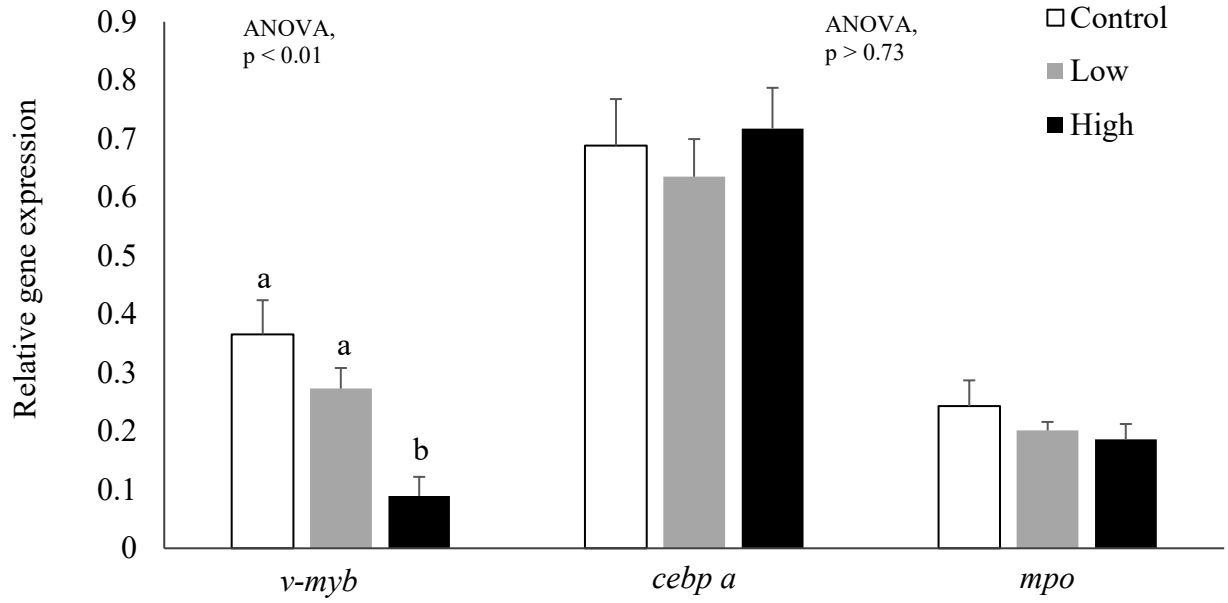

Figure 8. Mean relative gene expression of avian myeloblastosis viral oncogene homolog (*v-myb*), CCAAT/ binding protein alpha (*cebpa*), and myeloperoxidase (*mpo*) in fathead minnows in control, low-PTU (35 mg/L) and high-PTU (70 mg/L) groups at 10 dpf. Different letters denote significant differences between groups.

DISCUSSION

Confirmation of thyroid disruption

As thyroid hormones (THs) play a vital role in proper growth and development of organisms, decreases in mass and length are often used as markers for thyroid disruption. However, in this experiment, no alterations in growth were observed following PTU exposure. It is possible that the lack of growth alterations stems from the short exposure period of 10 days. Therefore, the expression of two genes known to be affected by thyroid disruption, *deiodinase 2 (di2)* and *transthyretin (ttr)*, were measured. We found that larvae in both PTU groups had significantly reduced expression of *ttr* compared to those in the control, which is consistent with previous studies (Bruns 2017, Path 2016). On the other hand, though there was almost a 2-fold difference between the high-PTU and control group, this study did not find significant upregulation of *di2* in PTU-exposed fish compared to the control as seen in previous studies (Bruns 2017, Path 2016). However, fluorescence labeling of intrafollicular T4 content indicated that TH synthesis had been inhibited in PTU-exposed larvae demonstrated due to the decreased fluorescence in these larvae compared to the control.

Immune cell development and function

The result of this study indicated that exposure to thyroid disrupting compounds during early life stages affected immune development as seen in the reduced expression of the *v-myb* gene. The *c-myb* proto-oncogene is the founding member of the *myb* family of transcription factors and *v-myb* is its retroviral homolog. It is highly expressed in hematopoietic cells, which develop progressively to generate multipotent common myeloid progenitor (CMP) cells, which can differentiate into either megakaryocyte-erythrocyte progenitor (MEP) or granulocyte-monocyte progenitor (GMP) cells [7]. GMPs in turn can give rise to cells that terminally

differentiate into granulocytes such as neutrophils or monocytes such as macrophages. *C-myb* is required for self-renewal and multi-lineage differentiation of HSCs as well as the proper commitment, normal maturation and differentiation of CMP and GMP progenitor cells [9]. Specifically, *c-myb* is required for the commitment to the granulocytic development for early myeloid progenitor cells. A study by Lieu and Reddy (2012) found that adult mice with a *c-myb* deletion has substantial reductions in CMP, GMP and MEP myeloid progenitor cells, resulting in a decrease of all peripheral blood cells, including neutrophils, basophils, monocytes and platelets [10]. Moreover, such observed role of *c-myb* in hematopoiesis is conserved across species as another study found a similar effect of *c-myb* disruption in zebrafish [11]. In this study, mutant *c-myb* fish demonstrated a state of anemia and immunodeficiency, with few detectable markers associated with the myelo-monocytic lineage (*spi1* and *l-plastin*), and a complete absence of granulocytic marker (*mpx*).

Regarding TH effects on hematopoiesis, other studies have found that TH can influence the clonogenicity of CD34+ hematopoietic stem cells (HSC) [12, 13]. Kawa et al (2010) found that levels of TH can affect the expression of TH receptor in HSC, with low levels of TH lead to a decreased in TH receptor expression in HSC [13]. In addition, clonogenic potential of HSC stimulated with growth factors for granulocyte–macrophage colony-forming units was lower in hypothyroid and hyperthyroid patients compared to that of euthyroid control subjects in the study. The authors postulated that the decreased number of colonies might be associated with the high percentage of apoptotic cells and significantly reduced expression of anti-apoptotic genes, both of which were observed in the HSCs of these patients. The result also correlated with the observed tendency of reduced amounts of white blood cells in patients with thyroid disorders compared with the healthy controls in that study.

Based on the discussion above, we hypothesized the reduced expression in *v-myb* might have led to alterations in immune function as shown in the decreased number of neutrophils at wound site in this study. Such a decrease could have harmful effects on the optimal health of organisms since neutrophils are an important component of the first line of defense against pathogens. Particularly, neutrophils contributed to wound closing, angiogenesis, and muscle injury repair [14]. Several studies found that neutrophils depletion resulted in more tissue debris following injury, decreased neuron regeneration, aggravated mucosal damage and delayed the wound healing process [14]. Moreover, other studies in human patients also indicated a relationship between thyroid disorders and neutrophil status. A study by Kyritsi (2015) found that among 218 patients with neutropenia, 95 (43.6%) had thyroid disorders, particularly 23.4% had Hashimoto thyroiditis (HT), 4.1%, Grave's disease (GD), 8.2% nontoxic multinodular goiter (NTMG), 5% subclinical hypothyroidism [15]. In another study, neutrophils showed a reduced ability to kill S. aurerus bacteria in 9 out of 17 hypothyroid patients compared with healthy controls [16]. The authors hypothesized that hypothyroidism could have slowed down the maturation of granulocytes, which led to a decreased turnover rate, resulting in old cells with a reduced capacity to kill bacteria [16]. In terms of other actions of neutrophils, a study by Hrycek et al (1993) found that neutrophils from blood of 30 women with primary hypothyroidism show an increased adherence to fiber while have decreased spontaneous migration compared to those of 20 healthy control women [17].

Overall, findings from other studies are consistent with the results of this study. Altered TH levels resulting from exposure to PTU led reduced the expression of *v-myb*, which could have disrupted the normal proliferation and differentiation of HSC and led to impaired maturation and migration of neutrophils. Such impairment disrupted the optimal protection

against invading pathogen as well as the normal healing process of organisms. More information is needed to determine whether the reduced number of neutrophils at wound site is a result of lacking mature neutrophils or their compromised migration ability.

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