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The Effects of Acute Urban Mixture Exposure on the T cells of Fathead Minnows (Pimephales promelas)

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The Effects of Acute Urban Mixture Exposure on the T cells of Fathead Minnows

(*Pimephales promelas*)

by

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A Thesis

Submitted to the Graduate Faculty of

St. Cloud State University

in Partial Fulfillment of the Requirements

for the Degree

Master of Science in

Biology–Cell and Molecular Biology

December, 2018

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Abstract

Contaminants of emerging concern (CECs), including pharmaceuticals, personal care products, industrial, and agricultural byproducts, have a myriad of effects on aquatic organisms. Numerous endpoints were studied in fathead minnow, which is a native species in the Great Lakes tributaries, and a standard model for aquatic toxicology research. It is critical to investigate the effects of urban CECs on the adaptive immune system due to its importance as a defense system in vertebrates. However, there are rare investigations into the effects of CECs on the adaptive immune system of fathead minnows. Generally, T cells and their major subpopulations, T helper and T cytotoxic cells, are important cell types in the adaptive immune system. Zeta-chain associated protein kinase 70 (zap70), cluster of differentiation 4-1 (CD4-1), and eomesodermin homolog A (eomesa) are T cell-, T helper- and T cytotoxic-selective proteins, respectively. In order to study the effects of urban CECs exposure on the adaptive immune system, adult male fathead minnows were exposed to eight CECs commonly detected in surface waters of the Great Lakes tributaries, either singularly or in a mixture. After a 96-hour exposure to CECs, splenic mRNA abundance for *zap70, CD4-1 and eomesa* were analyzed using quantitative RT-PCR. Of the four compouds hypothesized to increase the abundance of T cell-specific mRNA in the spleens of fathead minnows, the exposures to desvenlafaxine, sulfamethoxazole, triclosan and urban mixture were found to cause an increase. Furthermore, fluoranthene and metformin, of the four hypothesized urban CEC exposures, induced a decrease in the abundance of T cell-specific mRNA. Further research is needed to evaluate the effects of chronic and multigenerational exposures on the T cells of fathead minnows.

Acknowledgements

For my wife and my family. Without them I would not be the person I am today.

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Chapter 1: Introduction

Compounds of emerging concern (CEC) have been the focus of toxicology research in recent years because of their ubiquitous presence in the environment and emerging concern regarding potential deleterious effects on organisms (EPA, 2008). Such compounds include: persistent organic pollutants, pharmaceuticals, personal-care products, endocrine-disrupting compounds, and nanomaterials (Ankley et al., 2008). Just because CECs are coined as emerging does not mean that they have not been present in the environment for a significant amount of time. More recent and advanced detection methods have allowed for more thorough evaluation of environmental contamination with CECs (Raghav, Eden, Mitchell, Witte, & Polle, 2013). Environmental burden with CECs has been caused by wastewater treatment plant effluent, storm water, industrial run-off, and agricultural and urban run-off (Ankley et al., 2008; Elliott et al., 2017; Raghav et al., 2013). CECs can be found in waterways such as rivers, lakes, streams, and tributaries.

Over the last couple of years, the United States Geological Survey (USGS), in conjunction with the United States Fish and Wildlife Service (USFWS), had collected and analyzed 500 water samples from tributaries and waterways around the Great Lakes area, and subjected the data to a two-way cluster analysis. Analysis revealed the presence of many CECs, which were then subcategorized based on land use, where the samples was retrieved, and a chemical class. Compounds identified in ≥30% of water samples collected from the Great Lakes tributaries were compiled and separated into mixtures, composed of CECs from multiple chemical classes (Elliott et al., 2017). Based on the land use that compounds were found, artificial agricultural and urban mixtures of CECs were assembled. The urban mixture contained

eight compounds: desvenlafaxine (a serotonin-norepinephrine reuptake inhibitor used in the treatment of depression and other psychological disorders (Deecher et al., 2006)), fexofenadine (an antihistaminic medication used for allergy treatment (Amon, Amon, & Gibbs, 2000)), fluoranthene (a coal tar and petroleum asphalt byproduct (Kargar, Matin, Matin, & Buyukisik, 2017)), ibuprofen (a non-steroidal anti-inflammatory drug used in the treatment of inflammation and for pain relief (Dill et al., 2010)), metformin (a medication used in the treatment of type II diabetes to reduce blood glucose levels (Nair, Diehl, Wiseman, Farr, & Perrillo, 2004)), methyl-1H-benzotriazole (a component of aircraft deicing solution as well as an anticorrosive (Zeng, Sherry, & Bols, 2016)), and sulfamethoxazole and triclosan (antibiotics used to treat microbial infection (Choquet-Kastylevsky, Santolaria, Tedone, Aujoulat, & Descotes, 2001; Heath et al., 1999)).

CECs have widespread effects when introduced into waterways, which can be seen at multiple trophic levels; however, the most common target of aquatic toxicology research is fish because of their role as a trophic indicator and their presence across the world. Aquatic toxicology research in the United States has favored the use of fathead minnows (*Pimephales promelas)* as a model species (Ankley & Villeneuve, 2006). Fathead minnows are considered habitat generalists. This means that they can adapt to almost any environmental condition, such as modulation in pH, salinity, water quality, oxygen saturation, or temperature oscillation (Aadland, 2015). In addition, the biological endpoints of fathead minnows have been extensively studied for quite some time (Ankley & Villeneuve, 2006); the Environmental Protection Agency has studied CECs exposures in fathead minnows since the mid 1970's, and

have continued to use them because of their convenience as a model organism (Mattson, Arthur, & Walkbridge, 1976).

Considering that fish are subjected to mixtures of compounds in the environment and that combinations of CECs can have numerous effects on exposed fish, it is imperative to determine not only the effects of individual compounds, but also their complex mixtures. The effects of CECs have been studied on multiple biological endpoints, such as secondary sex characteristics (Shappell, Feifarek, Rearick, Bartell, & Schoenfuss, 2018), blood chemistry (Elliott et al., 2017; Shappell et al., 2018), histological analysis of varied tissue types (Elliott et al., 2017; Shappell et al., 2018; Thornton, Path, Nystrom, Venables, & Sellin Jeffries, 2016), morphometric indices (Elliott et al., 2017; Shappell et al., 2018; Thornton et al., 2016), reproductive success (Thornton et al., 2016), fecundity (Thornton et al., 2016), nest defense (Lehto, 2016), predatory avoidance (Lehto, 2016; McGee et al., 2009), and gene expression of multiple genes, such as (vitellogenin, estrogen receptor 1 and 2, retinol binding protein, aromatase, anti-Mullerian hormone, and hydroxysteroid dehydrogenase, to name a few (Garcia-Reyero et al., 2009; Thornton et al., 2016; Vidal-Dorsch et al., 2013)). However, there are no sufficient data about the effects of CECs on the immune system of fathead minnows.

The immune system is responsible for protecting an organism from invasive pathogens and exogenous compounds. The anterior kidney, thymus, and spleen are the three organs implicated in the production, maturation, and maintenance of the cells of teleost fish immunity (Kum & Sekki, 2011). Considering that fish, unlike tetrapods, do not possess bone marrow, a major site for hematopoiesis, or lymph nodes, responsible for mounting an immune response (Kum & Sekki, 2011), the anterior kidney takes over a majority of immunological functions,

such as hematopoiesis (Sunyer, 2013), antigen processing, presentation, and memory immune

cell generation (Kum & Sekki, 2011; Uribe, Folch, Enriquez, & Moran, 2011). Thymic function

in teleost fishes, similar to mammalian, allows for T cell maturation, and a release of only

immunocompetent cells into circulation (Bowden, Cook, & Rombout, 2005; Kum & Sekki,

2011). Splenic function in teleost fishes, as compared to tetrapods, has been conserved, allowing

for blood-borne pathogen clearance, antigenic presentation (Kum & Sekki, 2011), antigen

capture, and also functions as a center for hematopoiesis (Ali, Rexroad, Thorgaard, Yao, &

Salem, 2014).

The immune system can be broken into two branches: innate and adaptive immunity.

The innate portion of the immune system functions as the primary, non-specific defensive

mechanism, which confronts invasive pathogens, process and present them in order to activate

the adaptive immunity. The adaptive portion of the immune system functions as the secondary

defensive mechanism, which specifically confront invasive pathogens and creates immunological

memory. The adaptive immune system is subdivided into two main branches that include

cellular immunity (T cells) and humoral immunity (B cells) (Bonilla & Oettgen, 2010). Teleost

fishes are one of the first vertebrates to possess an adaptive immune system similar to that of

mammals, apart from some departures (Sunyer, 2013).

T cells are identified as the cells responsible for the initiation and maintenance of the

specific immune responses within an organism. In humans, mice, and chickens the amount of

time required for an initiation of the adaptive immune response is five to six days post initial

exposure to a pathogen (Miao et al., 2010). Within ectothermic organisms, such as fish,

activation periods can be modified as a result of environmental conditions. Decreases in

temperature result in a suppression of immune responses (Avtalion, Wojdani, Malik, Shahrabani, & Duczyminer, 1973; R. K. Liu & Walford, 1972) due to a slowing of cellular activity. T cells require an activation in order to function. Activation of naïve T cells is accomplished by the presentation of a processed, specific antigen, by a professional antigen presenting cell (APC), such as dendritic cell, macrophage or B cell, to the T cell receptor (TCR), in the context of major histocompatibility complex (MHC) class I or II molecules. Since TCRs exhibit low affinity for their ligands, co-receptors are required for stabilization of the MHC-TCR complex (Stone, Chervin, & Kranz, 2009). Upon activation, T cells progress to an effector stage. Based on T cell markers (receptors), and the type of secreted cytokines, they can be categorized into two main populations: T helper (Th) cells and cytotoxic T lymphocytes (CTL). Th cells are identified by the presence of a CD4 co-receptor, while CTLs express a CD8 co-receptor. Th cells can be further subcategorized based on the types of cytokines they secrete into, to name a few, Th1, Th2, Th17, and regulatory T (Treg) cells. The specific function of Th cells is broad in the sense that there is no adaptive immune function that can occur without interaction with Th cells. However, specificity depends on the cytokine profile that is present at the time of activation, and Th cells can differentiate into the subset that is required at that time for potentiation of an immune response (Alberts et al., 2002). Th1 and Th17 cells promote inflammation and activation of CTLs and cells of the innate immune system, such as macrophages. Th2 cells promote the activation of B cells to produce antibodies (Zhenhu Li, Zhang, & Sun, 2011); Tregs regulate immune responses, while CTLs act as direct killers of infected or cancerous cells.

B cells are identified by the presence of surface receptors called B cell receptors (BCR), which are membrane-bound antibody molecules (Karger, 2009). B cells, when activated to an

effector cell, referred to as plasma cells, is able to secrete a variety of antibodies, each of which has a particular biological function and a specific antigen affinity (Ye, Kaattari, Ma, & Kaattari, 2013). The antibody repertoire of B cells of higher vertebrates, such as mammals, is extensively developed and diverse, including immunoglobulin (Ig) G, IgA, IgM, IgE, and IgD (Karger, 2009). However, all teleost fishes possess a limited repertoire of immunoglobulin isotypes that are more limited due to a lower affinity and wider polymeric secretion (Sunyer, 2013), allowing B cells to secrete only IgM, IgD, and IgT (Ye et al., 2013), sometimes referred to as IgZ in different fishes (Magadan, 2015).

Several research groups have investigated the effects of some CECs, such as phenanthrene (Shirmohammadi, Salamat, Ronagh, Movahedinia, & Hamidian, 2017), light cycle oil (Bado-Nilles et al., 2011), bisphenol A (BPA) (Qiu et al., 2016; Yaghoobi, Safahieh, Ronagh, Movahedinia, & Mousavi, 2017), nonylphenol (Herbert et al., 2009; Shelley, Osachoff, van Aggelen, Ross, & Kennedy, 2013; Shelley, Ross, & Kennedy, 2012; Shelley, Ross, Miller, Kaukinen, & Kennedy, 2012), and ethinylestrodiol (Gómez González et al., 2017) on multiple adaptive immune parameters of different fish species (Appendix II). BPA has been one of the most studied CEC. While various effects of BPA on immune responses have been observed, many of the reports are controversial. In general, BPA exposure in mice has been associated with modulation of Th1/Th2 cytokine and antibody production (Alizadeh et al., 2006; Goto et al., 2007; M. H. Lee et al., 2003; Yan, Takamoto, & Sugane, 2008; Yoshino et al., 2003, 2004; Youn et al., 2002). Similarly, dose-dependent adverse effects of BPA exposures on IgM secretion were observed in the liver of *Cyprinus carpio*, (Qiu et al., 2016). In addition, Yaghoobi et al. discovered a reduction of the percentage of lymphocyte populations in *Acanthopagrus latus*

following a chronic exposure to BPA at 1, 5, 10, and 50 µg/g for 21 days (2017), whereas an increased proliferation of lymphocytes was found in *Carassius auratus* by Yin et al. (Yin et al., 2007). To date, only one study revealed the effects of particular CECs on the adaptive immune system of fathead minnow, showing that tramadol and a mixture of selective serotonin reuptake inhibitors (comprised of fluoxetine, paroxetine, and venlafaxine) induced an increase in the proportion of T cells, while having no effect on B cell populations (Schoenfuss et al., 2016).

Certain markers could be used for identification of particular lymphocytes, either T or B cell-types. Zeta-chain associated protein kinase 70 (Zap70), cluster of differentiation 4-1 (CD4-1), and eomesodermin homolog a (Eomesa) are T cell-, T helper- and T cytotoxic-representative proteins, respectively. Pax5 is a B cell-selective protein, representative of this cell population.

Zap70 is a protein belonging to the tyrosine kinase family of enzymes, which becomes phosphorylated upon TCR activation (Chan, Iwashima, Turck, & Weiss, 1992). T cell development and function are dependent on the function of Zap70 that (Fischer et al., 2010) associates with TCRs across all T cell subpopulations. Zap70 has been identified in the spleens of *Oreochromis niloticus* (Gan, Wang, Zhou, & Lu, 2016), in the skin and mucosa of *Epinephelus coioides* (ZX Li et al., 2017)*,* and in the kidney of *Carassius auratus langs-dorfii* (Miyazawa et al., 2018).

In teleost fishes, there are a pair of homologs to the Cd4 co-receptor present on mammalian T cells, which are denoted as CD4-1 and CD4-2 (Toda et al., 2011). CD4-1 has been shown to have the homologous structural composition to mammalian CD4 co-receptors, with four extracellular Ig domains, a transmembrane portion, and a cytoplasmic tail with a p56[lck] binding site in mammals and teleost fishes. CD4-2 has been shown to have varied extracellular

Ig domains depending on the teleost species (Nakanishi, Shibasaki, & Matsuura, 2015; Toda et al., 2011). CD4-1 is important to be investigated in teleost fish, because of the structural and functional similarities to the mammalian CD4 co-receptor. Studies showed that T cells in teleost have the potential to express CD4-1 and CD4-2 simultaneously, whereas there is a single positive population of T cells that expresses CD4-2. These single positive cells are less proliferative and display a less diverse gamut of TCR (Takizawa et al., 2016). Therefore, the best candidate receptor to study Th cells in teleost would be CD4-1. Homolog variants of CD4 have been investigated in *Danio rerio* (Takizawa, Araki, Ito, Moritomo, & Nakanishi, 2007); however, as to date, CD4 has not been reported in fathead minnows.

Eomesa is a transcriptional factor, which acts as a master regulator for the differentiation of effector $CD8^+$ T cells into CTLs/memory $CD8^+$ T cells in mammals (Kumari, Bøgwald, & Dalmo, 2013; Takizawa et al., 2007). It is associated with mesoderm formation in vertebrates and expressed in the central nervous system as well.

Pax5 has been identified within teleost (*Oncorhyncus nerka, O. gorbuscha,* and *O. kisutch*) B cells during their development, from early B cell maturation to late B cell activation, but is absent in effector plasma cells (Zwollo, 2011). Pax5 expression at a progenitor stage causes a commitment to a B cell pathway in teleost fishes (Cobaleda, Schebesta, Delogu, & Busslinger, 2007). During embryonic development, Pax5 expression has been shown to be associated with not only humoral immunity regulation, but also neural development and spermatogenesis in humans and mice (Adams et al., 1992).

CECs, depending on their drug class, exhibit different mechanism of action. Considering the xenobiotic nature of CECs, one of the main proposed pathways include binding to aryl

hydrocarbon receptors (Ahr) (Gargaro, Pirro, Romani, & Zelante, 2016; L. P. Nguyen &

Bradfield, 2008; N. T. Nguyen, Hanieh, Nakahama, & Kishimoto, 2013; Prigent et al., 2014;

Quintana, 2012; Stevens, Mezrich, & Bradfield, 2009; Zeng et al., 2016). In addition, endocrine

disruption, through binding to estrogen receptors, has been extensively studied in fish (Garcia-

Reyero et al., 2009; X. Liu, Ji, & Choi, 2012; Yurino et al., 2004). CECs might affect adaptive

immune cells by both mechanisms. However, as many CECs are small molecules, their hapten

properties have to be taken into consideration. Thus, the adaptive immune cells can be affected

(activated) through their TCRs if CECs act as haptens.

Ahr, also known as dioxin receptors, are a group of nuclear hormone receptor family

present within the cytosol (Gargaro et al., 2016). AHR function to maintain cellular

homeostasis. Within immune cells, homeostasis is accomplished by regulation of proliferation

and differentiation, gene regulation, cellular motility, inflammation, and cytokine release (N. T.

Nguyen et al., 2013). Expression levels of Ahr within lymphocytes are lower than in other cell

types. Studies have shown that of the different subpopulations of T cells, Th17 and Treg cells

express Ahr in humans (Prigent et al., 2014) as well as that Ahr plays an important role in their

activation and differentiation in humans and a variety of animal species (Gargaro et al., 2016;

Quintana, 2012).

Beyond Ahr, there are two other pathways of stimulation/suppression within T cells.

Estrogen receptors (ESR) also belong to a nuclear hormone receptor family, and act as the

ligand-dependent transcription factors (Parikh, Rajendran, Su, Lopez, & Sar, 1987). There are

two subtypes of ESRs; ESR1 (formerly ERα) and ESR2 (formerly ERβ). CD4$^+$ T cells express

more ESR1 than ESR2, whereas CD8$^+$ T cells have lower expression of both in humans (Khan

& Ansar Ahmed, 2016). Estradiol-17β suppressed proinflammatory subpopulation differentiation of T cells (Lelu et al., 2011). The xenoestrogen BPA, binds to ESR2 as an agonist, and ESR1 with dual agonistic and antagonistic effects (Rogers, Metz, & Yong, 2013). Differential effects of BPA on different T cell subpopulations have been observed, inducing a potentiation of Th function through increased secretion of interferon-γ and IL-4, and a decrease of Treg cell population (Rogers et al., 2013).

Haptens are small molecules that alone are not able to cause an immune response; however, when bound to a carrier protein, the hapten-carrier complex can become a potent immunogen. Xenobiotic compounds, such as pharmaceuticals, can act as haptens, causing a heterogeneous immune response due to a nonspecific binding to proteins (Pichler, 2002). Responses can vary between T cell and B cell activation, depending on the immunogenicity of the compound.

The aim of this study was to identify the effects of acute exposures to complex urban mixture, and its individual components on the T cells of adaptive immune system of the fathead minnow. In order to study T cells in the fathead minnow, the most suitable lymphoid organ was chosen, and the research tools, including PCR primers for studying expression of T cell-related genes and functional T cell assay were developed.

Based on previously observed immunosuppression expressed either by reduced responsiveness or reduction in the number of T cells, it was hypothesized that exposure to fexofenadine, fluoranthene, ibuprofen, and metformin would induce a decrease in the abundance of T cell-specific mRNA in the spleens of adult male fathead minnows. Since desvenlafaxine, methyl-1H-benzotrazole, nonylphenol, sulfamethoxazole, and triclosan were previously observed

to cause an autoimmune or hypersensitivity reaction, it was hypothesized that these compounds

would increase the abundance of T cell-specific mRNA in the spleens of adult male fathead

minnows.

Chapter 2: Material and Methods

2.1 Animals

The male fathead minnows (*Pimephales promelas*) were obtained from a laboratory fish supplier (Environmental Consulting and Testing, Superior, WI, USA) at 6-month of age, when fish is sexual mature. Fish were maintained in the Aquatic Toxicology Laboratory at Saint Cloud State University (SCSU) in a continuous-flow system with a 16 h: 8 h day-night cycle. Eight % (w/v) of Tricaine mesylate (MS-222) (Sigma-Aldrich, Saint Louis, MO, USA), buffered with sodium bicarbonate, was used to anesthetize minnows prior to dissections. Handling and experimental procedures with minnows have been approved by the SCSU Institutional Animal Care and Use Committee (Protocol #8-82).

2.2 Experimental Design

Based on the findings described by Elliot et al., 2017, the urban mixture and its relevant eight individual compounds were selected for analysis. CECs classified as urban compounds are important because of the heavy industrial/urban environment influence surrounding the Great Lakes tributaries. Five groups of fish per particular CEC exposure, containing six animals per group, were exposed at different concentrations for 96-hours using a flow through system. Each exposure consisted of a control, ultra-low, low, medium, and high concentration CEC. Control fish were exposed to SCSU well water. Medium treatment fish were exposed to environmentally relevant concentrations of compounds (Appendix I) (Elliott et al., 2017), whereas low and ultra-low treatment fish were exposed to concentrations ten times less and ten times less than low treatment concentrations than environmental concentrations, respectively. High treatment fish were exposed to concentrations ten times higher than environmental concentrations.

2.3 Collection of Lymphatic Organs for RNA Extraction

Anesthetized minnows were dissected by creating an incision from the cloaca to the gill plates along the ventral surface of the minnow. An opening was created in the side of the abdominal cavity to allow for greater visibility of internal organs. Spleen and anterior kidney were removed and immediately preserved with ice-cold RNAlater (Thermo-Fisher Scientific, Waltham, MA, USA). Tissues were incubated at 4˚C overnight and stored at -80˚C.

2.4 RNA Extraction

Total RNA was extracted according to a manufacture's protocol for miRNA easy kit (Qiagen, Hilden, Germany) using regents of an acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method (Green & Sambrook, 2014) and a total RNA isolation system (Promega, Madison, WI, USA). Tissues preserved with RNAlater were homogenized with APGC lysis reagent, which consists of 47.6% water-saturated acidic phenol, 4 M guanidinium thiocyanate, 0.025 M sodium citrate (pH 7.0), 0.5% (w/v) sodium N-lauroyl sarcosine, 0.1 M sodium acetate (pH 4.0), 0.049 M β-mercaptoethanol, in a 1.5 mL microcentrifuge tube with a 6-mm stainless bead using Mixer Mill MM 400 (Retsch, Haan, Germany) for two minutes at 40 Hz. A homogenate was mixed with chloroform (Thermo Fisher Scientific, Waltham, MA, USA) for a phase separation, and an aqueous phase of the homogenate was purified using a silica membrane mini spin column (Epoch, Missouri City, TX, USA). RNA was eluted with 30-50 μL of nuclease-free water, and stored at -80˚C.

2.5 RNA Quality Check

RNA quantity and quality was evaluated using NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA) and an agarose gel electrophoresis (Green & Sambrook,

2014). In order to eliminate genomic DNA contamination in RNA, RNA was treated with

DNase-I using TURBO DNA-free kit according to the manufacture's protocol (Thermo Fisher

Scientific).

To confirm that there is no genomic DNA contamination in RNA, the DNase treated

RNA samples were subjected to quantitative polymerase chain reaction (qPCR) with a primer

pair for genomic DNA (Table 1) as described below. All samples were maintained to have the

genomic DNA contamination less than 10^2 copies/µL.

2.6 cDNA Synthesis

RNA was adjusted to a certain concentration with nuclease-free water, dependent on the

lowest concentration of the cohort of samples. RNA was reverse-transcribed into cDNA using

High-Capacity cDNA Reverse Transcription kit with RNase-inhibitor (Thermo Fisher Scientific)

according to the manufacture's protocol. The cDNA was diluted at 1:5 ratio with TE buffer, and

stored at -20˚C.

2.7 PCR Primer Design

Based on currently annotated genes available on the published fathead minnow genome

through the Society of Environmental Toxicology and Chemistry site (Society of Environmental

Toxicology and Chemistry, 2018), cluster of differentiation 4-1 *(cd4)*, eomesodermin homolog a

(eomesa), zinc-associated protein kinase 70 *(zap70)*, and paired box 5 *(pax5)* were selected as

gene targets to represent the particular cells of the adaptive immune system, such as Th cells,

CTL, T cells in general, and B cells, respectively. Using the Primer-Blast ("Primer-BLAST,"

n.d.), a primer pair was designed to include a greater than 1000 base pairs of intron(s) between

primers to avoid amplification of genomic DNA contamination within the sample. Primers were

obtained from Eurofins Genomics (Louisville, KY, United States).

Three internal control genes were used, such as ribosomal protein L8 (*rpl8*),

hypoxanthine phosphoribosyltransferase 1 (*hprt1*), and TATA box binding protein (*tbp*) (Table

1). Internal control genes were selected as a result of their use in previous studies (Cox, 2016),

availability of sequence in fathead minnow genome, and considering that their varied uses in

cellular function. Rpl8, Hprt1 and Tbp are involved in a protein synthesis, nucleoside

metabolism and a transcriptional initiation, respectively (Filby & Tyler, 2007).

Table 1. Primers for quantitative PCR. Genome positions were retrieved from published fathead minnow genome (Burns et al., 2016).

Genes	Forward Primer	Forward Position	Reverse Primer	Reverse Position	Annealing Temp.	Genome Position
cd4	CTTTATGTTGG CCAAAGGGT	651	TGTTCCATTGTC ACTTCAGG	793	64˚C	JNCD01047724.1:1..3117 (- strand)
eomesa	TTCGGGAAACT GAAGCTAAC	1060	CAGGGAAAGTG AAGGTTTGT	1231	62˚C	JNCD01002199.1:27969.. 32930 (+ strand)
foxl2	TTAACGTAAAG GCTTGACC	569	CTCATGCCGTTG TAAGAGTT	668	64˚C	JNCD01000174.1:183..10 58 (+ strand)
hprt1	ATCTGTCCACA CTCACAGGA	507	TCCTCTTCACCA GCAAACTG	647	64˚C	JNCD01023078.1:380..18 859 (+ strand)
rpl8	CCCACAATCCT GAGACCAAG	375	TTGTCAATACG ACCACCACC	473	64˚C	JNCD01006776.1:50145.. 53627 (- strand)
tbp	CATTCGATTAG AGGGCCTGG	792	CCTGGGAAATA ACTCTGGTTCA	861	62˚C	JNCD01008442.1:132079. .133844 (- strand)
zap70	GGAAAGCAAA CAGATTGACG	1140	CATCTCCATCAC CAACATGA	1275	64˚C	JNCD01003797.1:17888.. 27258 (+ strand)

2.8 Optimization of qPCR and Preparation of Standard Samples

The optical density of each primer were measured using NanoDrop, and primers pairs were mixed together at a concentration of 1.0 µM with autoclaved ultrapure water using a web program, Oligo Calc (Kibbe, 2007). To optimize an annealing temperature for each primer pair, qPCR was run using an extra cDNA with a temperature gradient from 60-72˚C. The optimal annealing temperatures of *cd4*, *eomesa*, and *zap70* are 64˚C, 62˚C, and 64˚C, respectively (Table 1).

A PCR product of each primer pair was cloned and sequenced using pGEM-T easy vector system according to the manufacture's protocol (Promega, Madison, WI, USA). Plasmid DNA was isolated using Wizard® Plus SV Minipreps DNA Purification System according to the manufacturer's protocol (Promega). The isolated plasmid DNA was quantified using NanoDrop, and a concentration of each DNA was adjusted. Plasmid DNA was analyzed using Sanger cycle sequencing by Eurofins Genomics (Louisville, KY, USA), and sequences of PCR products were confirmed using BLAST search on NCBI website.

Plasmid DNA was purified with a polyethylene glycol precipitation solution, 1.6 M NaCl and 13% (w/v) polyethylene glycol (PEG8000) in H_2O (Sigma-Aldrich). Transforming units of plasmid DNA concentration from ng/µL to copies/µL was done by using equation 1:

$$\left(\frac{copy}{\mu L}\right) = (\frac{ng}{\mu L})/\ [(insert\ basepairs + vector\ basepairs) \times 660]\ \times 6.022 \times 10^{14}$$

Plasmid DNA was serially diluted with a dilution buffer (5 ng/µL tRNA in TE buffer) from 10^8 to 10^1 copy/µL, and stored at -20˚C. They were used as standard samples to quantify mRNA abundances in tested samples.

Quantitative PCR was conducted on CFX 96 touch real time PCR detection system (Bio-Rad, Hercules, CA, USA) using a homemade SYBR green master mix, which consist of 10 mM Tris-HCl, 50 mM KCl, 3 mM MgCl$_2$, 0.01% Tween 20, 0.8% glycerol, 40000-fold dilution of SYBR Green I (Thermo-Fischer Scientific), 200 µM dNTPs, 10 pmol of each primer, 2 µL of diluted cDNA, and 0.075 uL of 5 U/uL Ampli Taq Gold DNA polymerase (Crump, Werry, Veldhoen, Van Aggelen, & Helbing, 2002). Reaction mixture was incubated at optimal annealing temperature (Table 1). Reactions were run in triplicate as follows: 1.) 95.0˚C for 5 minutes, 2.) 95˚C for 15 seconds, 3.) optimal annealing temperature for 45 seconds, 4.) repeat to number two 39 times, 5.) 95˚C for 10 seconds, and 6.) melting curve from 65˚C to 95˚C.

2.9 Statistical Analysis of qPCR Data

Data for each target gene qPCR was normalized by the most stable internal control gene selected by NormFinder (Andersen et al., 2004). All statistical analyses were conducted using JMP Pro version 13.2.0 (SAS Institute, Cary, NC, USA). Normalized data was tested for normality and then analyzed based on distribution. Normally distributed data was analyzed using a one-way ANOVA followed by a Dunnett's multiple comparison. Nonparametric data was analyzed using a Kruskal-Wallis test followed by Steel's multiple comparison.

2.10 Lymphocyte Isolation

Lymphocytes were isolated from fathead minnow spleens, according to the original protocol described by Palic (2005). Spleens were removed from 10 fathead minnows, placed in Hanks' balanced salt solution without Ca, Mg, and phenol red (HBSS) (Mediatech Inc., Manassas, VA, USA) using a tissue grinder with six to seven gentle pestle strokes. Splenocyte suspension was filtered through a 40 µm filter (Biologix, Lenexa, KS, USA), centrifuged at 250

x *g* for 15 minutes, resuspended in HBSS, and laid atop of Lymphoprep with a density of 1.077-1.080 g/mL (Mediatech Inc.). Initial attempts at isolating lymphocytes were unsuccessful due to poor visualization of cell interface in Lymphoprep density gradient. Thus, the splenocytes were resuspended in HBSS without Ca, Mg, but with phenol red, before their addition to Lymphoprep. After centrifugation, cells (lymphocytes) were removed from the upper layer of the gradient and suspended in HBSS. Isolated lymphocytes were counted using the upper left quadrant (ULQ) and the lower right quadrant (LRQ) of a hemocytometer. The total number of cells per volume was obtained by equation 2:

$$\frac{ULQ + LRQ}{hemocytometer\ factor} \times \frac{dilutin\ factor}{\#\ of\ quardants\ counted} = \# \times 10^6 cells/mL$$

2.11 Lymphocyte Proliferation Assay

In order to study lymphocyte function, a proliferation assay was performed. Mitogens, such as concanavalin A (Con A) and lipopolysaccharide (LPS), have been used for analysis of T and B cell function, respectively. Isolated fathead minnow's lymphocytes were diluted at 8 x 10^6/mL in L15 (Thermo Fisher Scientific) or RPMI 1640 medium (Thermo Fisher Scientific). Complete medium consists of either L15 or RPMI 1640 with addition of 10% or 5% fetal bovine serum (Sigma-Aldrich). Lymphocytes were seeded at a concentration of 4 x 10^5 cells in 50 µL per well in ~~on~~ a 96-well-plate (Greiner Bio-One, Monroe, NC, USA). ConA (Sigma-Aldrich) was added in appropriate concentrations (varied in different experiments). Particular wells contained only medium, or cells with addition of medium only (no ConA). All group of wells containing medium only, cells only, and cells with addition of ConA were cultured in triplicates. Cells cultured with RPMI or L15 complete medium were incubated for 72 to 120 hours in the incubator (New Brunswick Scientific, Edison, NJ, USA) at 25°C with 5% CO_2, or at 23-26°C at

ambient percentage of CO_2, respectively. After incubation period, 10 µL of Alamar blue (VWR, Radnor, PA, USA) was added to each well and incubated, depending on the type of complete media, under the previously described incubation conditions for 5 hours. Absorbance of each well was measured at 570 nm by a 96-well-plate reader (Remel Elx800, Thermo Fischer Scientific).

2.12 Cytospin and Cytochemistry

Isolated lymphocytes, diluted to 1 x 10^6 cells/mL in L15 complete medium, were spun in a cytospin (Thermo Shandon Cytospin 3, Marshall Scientific, Hampton, NH, USA) for five minutes at 500 rpm, in order to quantify lymphocyte purity in an isolate. Post cytospin, slides were stained with hematoxylin and eosin (H&E) (MilliporeSigma, Saint Louis, MO, USA) following manufacturer's instructions.   Lymphocytes were identified based on their characteristic morphology and quantified by counting the number of lymphocytes among the 100 cells on the slide.

Chapter 3: Results

Prior to evaluating of the effects of CECs on the T cell-specific genes of fathead minnows, the optimal lymphoid organ had to be selected, based on the relative mRNA abundances of the genes of interest in these organs. Spleens and kidneys of fathead minnows were used for comparison. Relative mRNA abundance of *cd4* (p = 0.0191), *eomes* (p = 0.0209), and *zap70* (p = 0.0200) was higher in the spleens compared to kidneys of fathead minnows (Figure 1). The most stable internal control genes were *rpl8* and *hprt1*. In addition to mRNA abundance, the percentages of lymphocytes were studied in the single-cell suspensions obtained from spleens and kidneys of fathead minnows. Figure 2 shows that only 18.8 ± 12.8 % of lymphocytes were observed in the cytospin preparation of kidney cells, in comparison to 58.1 ± 7.6 % of lymphocytes present in the splenic suspension ($P = 2.965 \times 10^{-13}$). Figure 3 shows a representative lymphocyte, as a round cell with a characteristic big nucleus that fills nearly entirety of the cell volume, surrounded by a small amount of cytoplasm. Thus, initial studies of T cell-specific gene expression, as well as lymphocyte content and morphology, confirmed the spleen as a lymphoid organ of choice for studying CEC's effects on T cell lymphocytes.
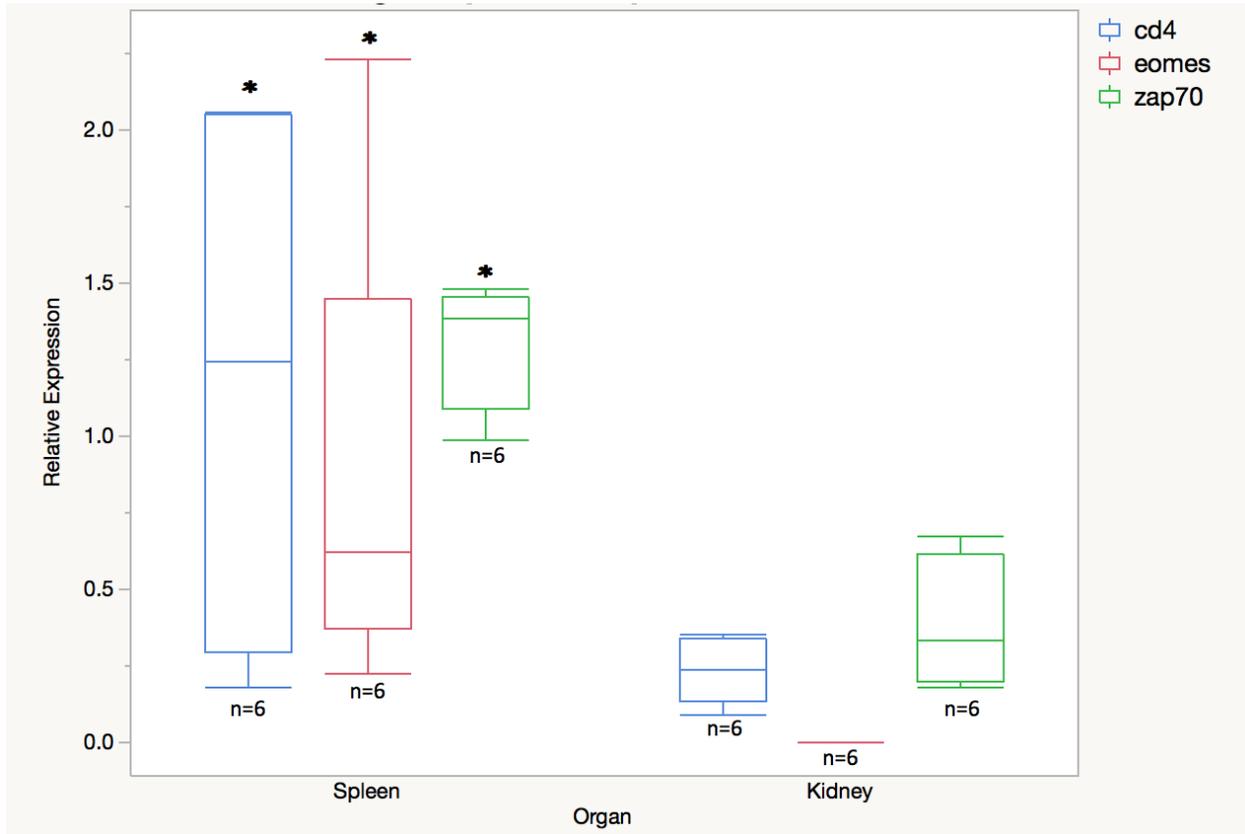
Figure 1. Relative mRNA abundances of T cell-specific genes between spleen and kidney of fathead minnows detected by quantitative RT-PCR and normalized to the expression of the most stable internal control mRNA abundance (s). Statistical difference indicated by asterisk above expression level (*P < 0.05).
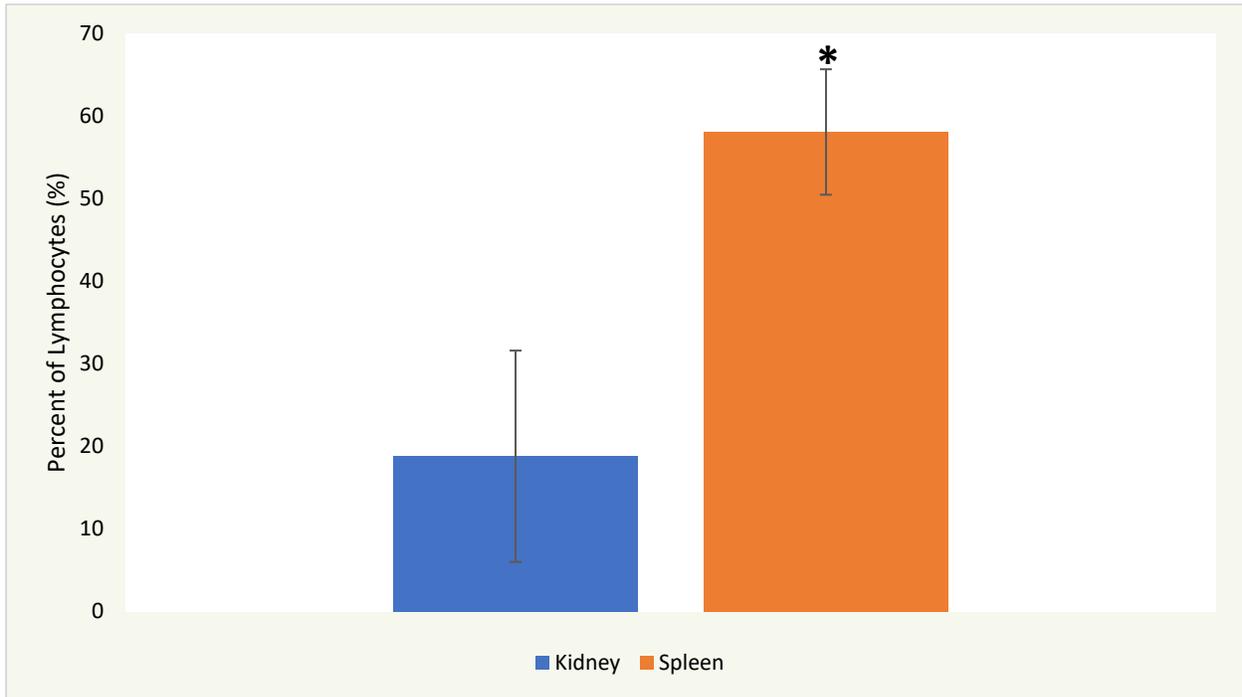
Figure 2. Percent of lymphocytes present in the spleens (n = 27) and kidneys (n = 10) of fathead minnows. Data presented as mean±SD. Statistical difference indicated by asterisk (*P < 0.05).
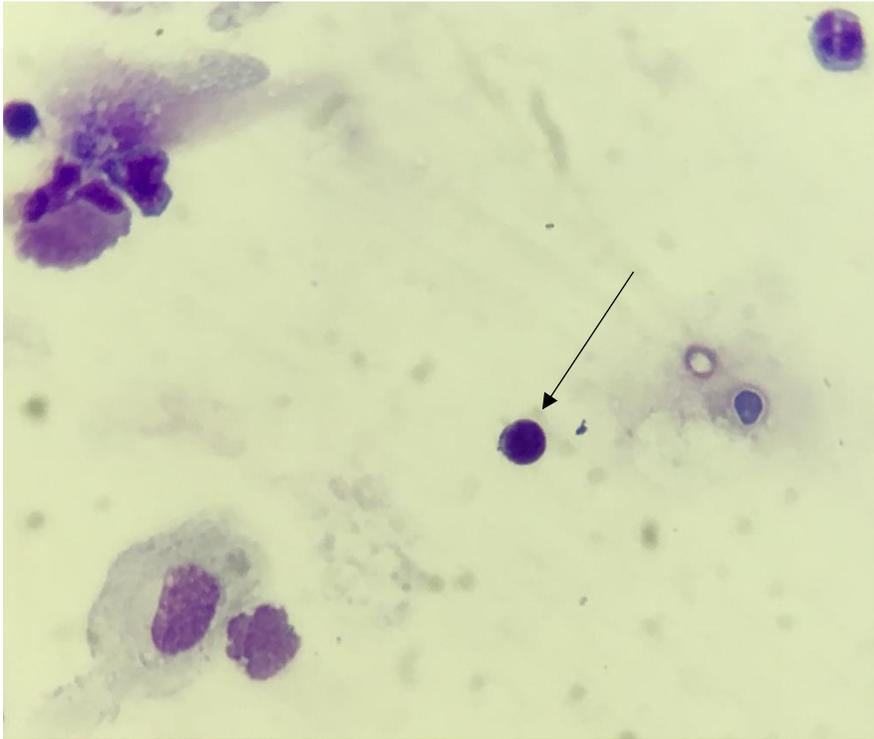
Figure 3. A representative photo of a fathead minnow lymphocyte. A cytospin preparation of cells obtained from the spleen of fathead minnow was stained with Hemacolor stain. Lymphocyte indicated by black arrow; 100x magnification.

Abundance of T cell-specific mRNA *cd4* (p = 0.0327), *eomes* (p < 0.0001), and *zap70* (p = 0.0256) were higher in desvenlafaxine-exposed minnow at the high treatment group as compared to control (Figure 4). No other difference was observed across the other treatment groups as compared to control. The most stable internal control genes were *hprt1* and *tbp*.

Figure 4. Relative abundances of T cell-specific mRNA in the spleens of fathead minnows exposed to desvelafaxine detected by quantitative RT-PCR and normalized mRNA abundance of the most stable internal control gene(s). Statistical difference indicated by asterisk above expression level (*$P < 0.05$).
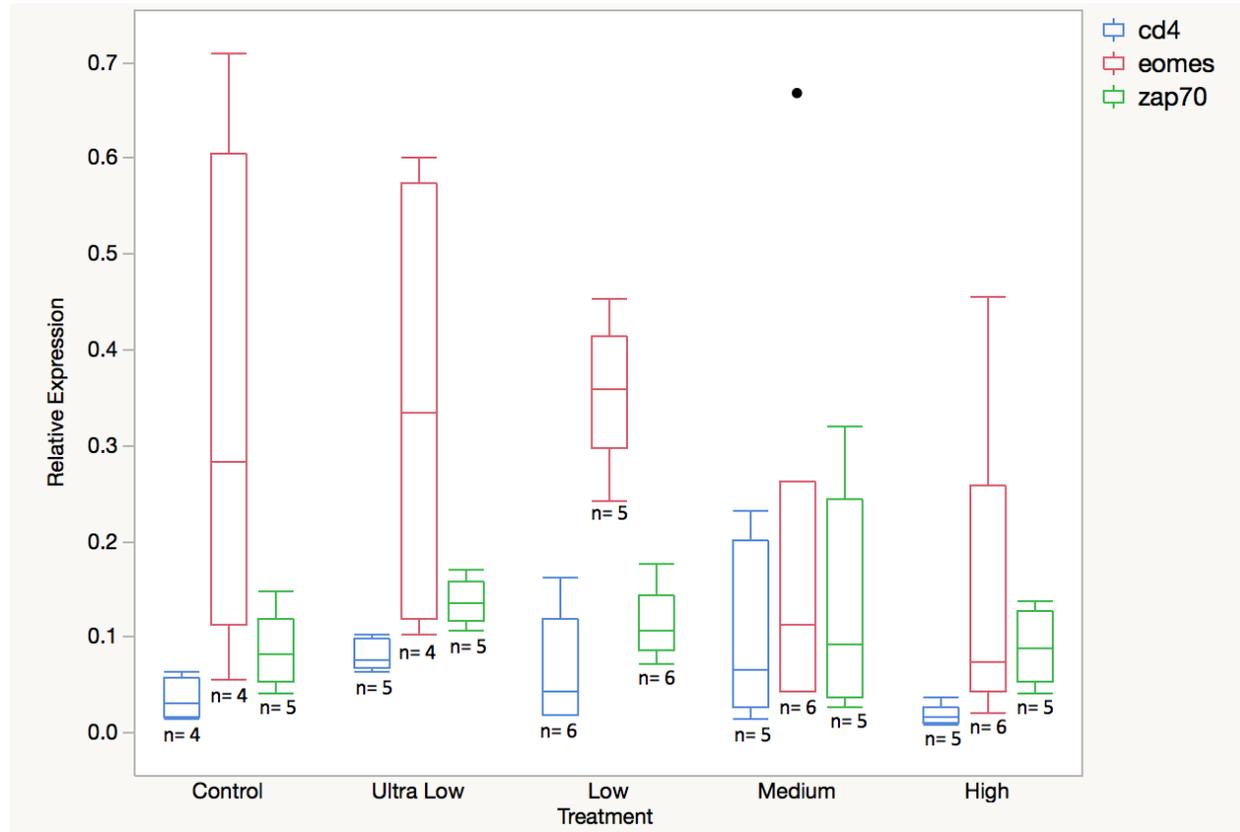
Figure 5. Relative abundances of T cell-specific mRNA in the spleens of fathead minnows exposed to fexofenadine detected by quantitative RT-PCR and normalized to mRNA abundance of the most stable internal control gene(s).

Expression of all three T cell-specific genes displayed no difference across the treatment groups of fathead minnows exposed to fexofenadine (Figure 5). The most stable internal control genes were *rpl8* and *hprt1*.

Figure 6. Relative abundances of T cell-specific mRNA in the spleens of fathead minnows exposed to fluoranthene detected by quantitative RT-PCR and normalized to mRNA abundance of the most stable internal control gene(s). Statistical difference indicated by asterisk above expression level (*$P < 0.05$).

Expression of *zap70* decreased for fathead minnows exposed to fluoranthene at medium ($p = 0.0217$) and high ($p < 0.0001$) concentrations as compared to control (Figure 6). The most stable internal control genes were *hprt1* and *tbp*.

Figure 7. Relative abundances of T cell-specific mRNA in the spleens of fathead minnows exposed to ibuprofen detected by quantitative RT-PCR and normalized to mRNA abundance of the most stable internal control gene(s). Statistical difference indicated by asterisk above expression level (*P < 0.05).

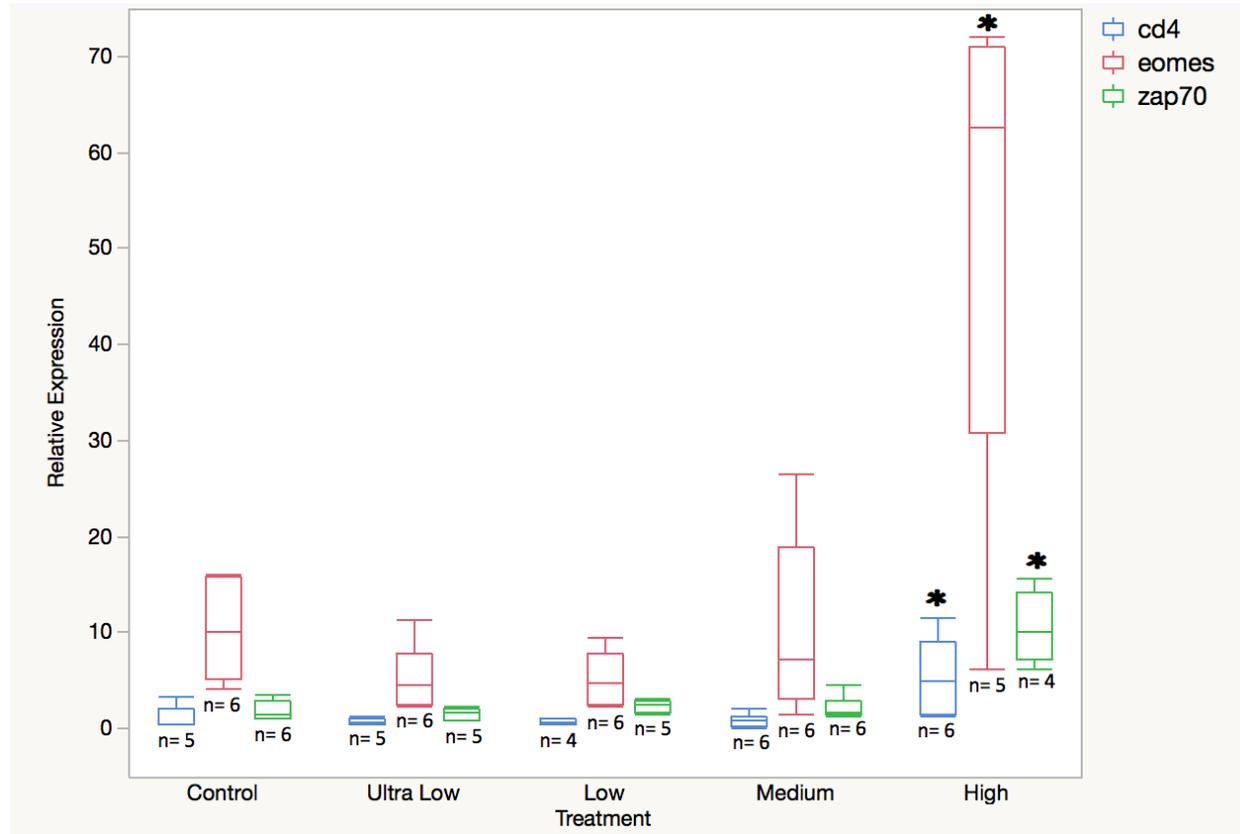Expression of all three T cell-specific genes displayed no difference across the treatment groups of fathead minnows exposed to ibuprofen (Figure 7). Trends indicate a decrease in the expression of *cd4, eomes,* and *zap70* at medium concentration as compared to control, but there was no statistical difference that was observed. The most stable internal control genes were *hprt1* and *tbp.*
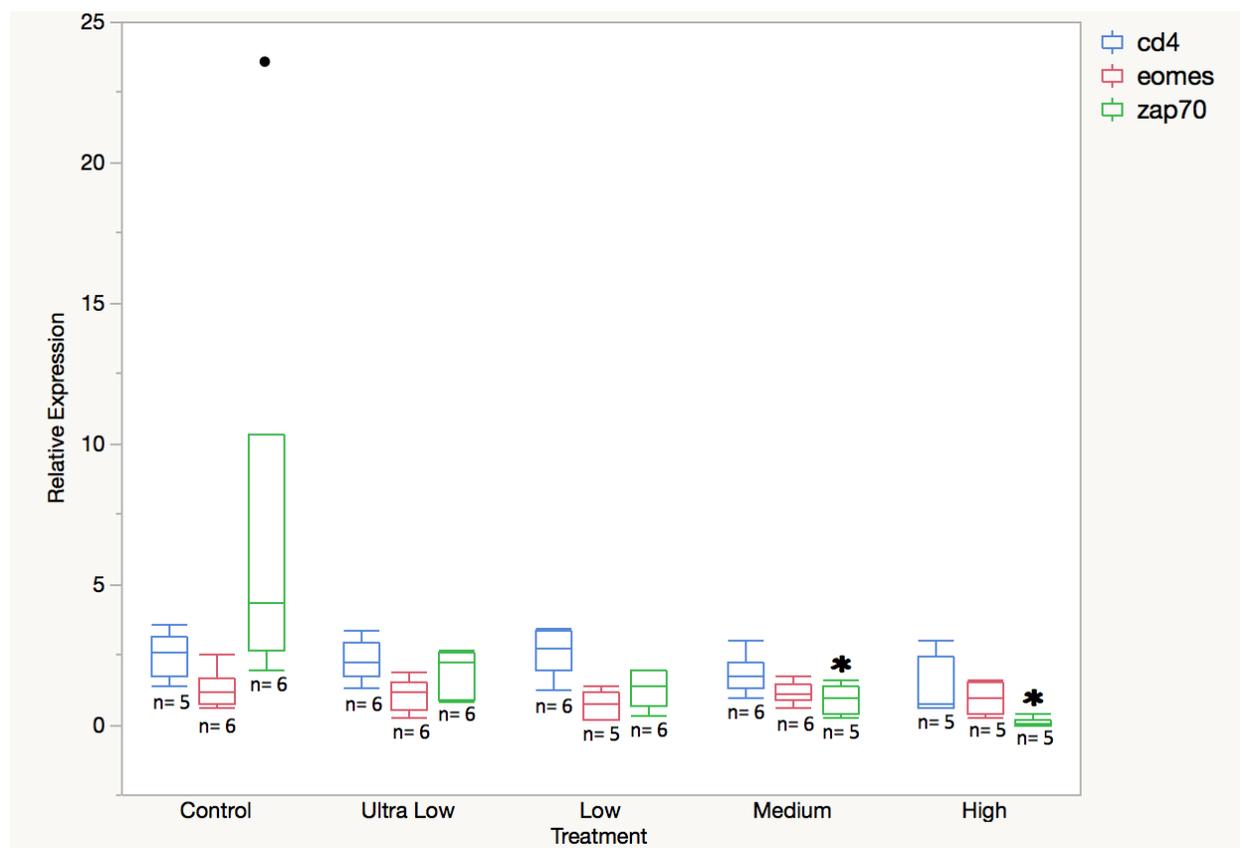
Figure 8. Relative abundances of T cell-specific mRNA in the spleens of fathead minnows exposed to metformin detected by quantitative RT-PCR and normalized mRNA abundance of the most stable internal control gene(s). Statistical difference indicated by asterisk above expression level ($*P < 0.05$).

Expression of *eomes* (p = 0.0178) in the spleen of fathead minnows exposed to low concentrations of metformin was significantly decreased as compared to control (Figure 8). Expression of *eomes* at ultra-low concentrations displayed a decrease that approached significant levels (p = 0.0525), with no other differences observed across the different treatment groups or genes. The most stable internal control genes were *rpl8* and *tbp*.
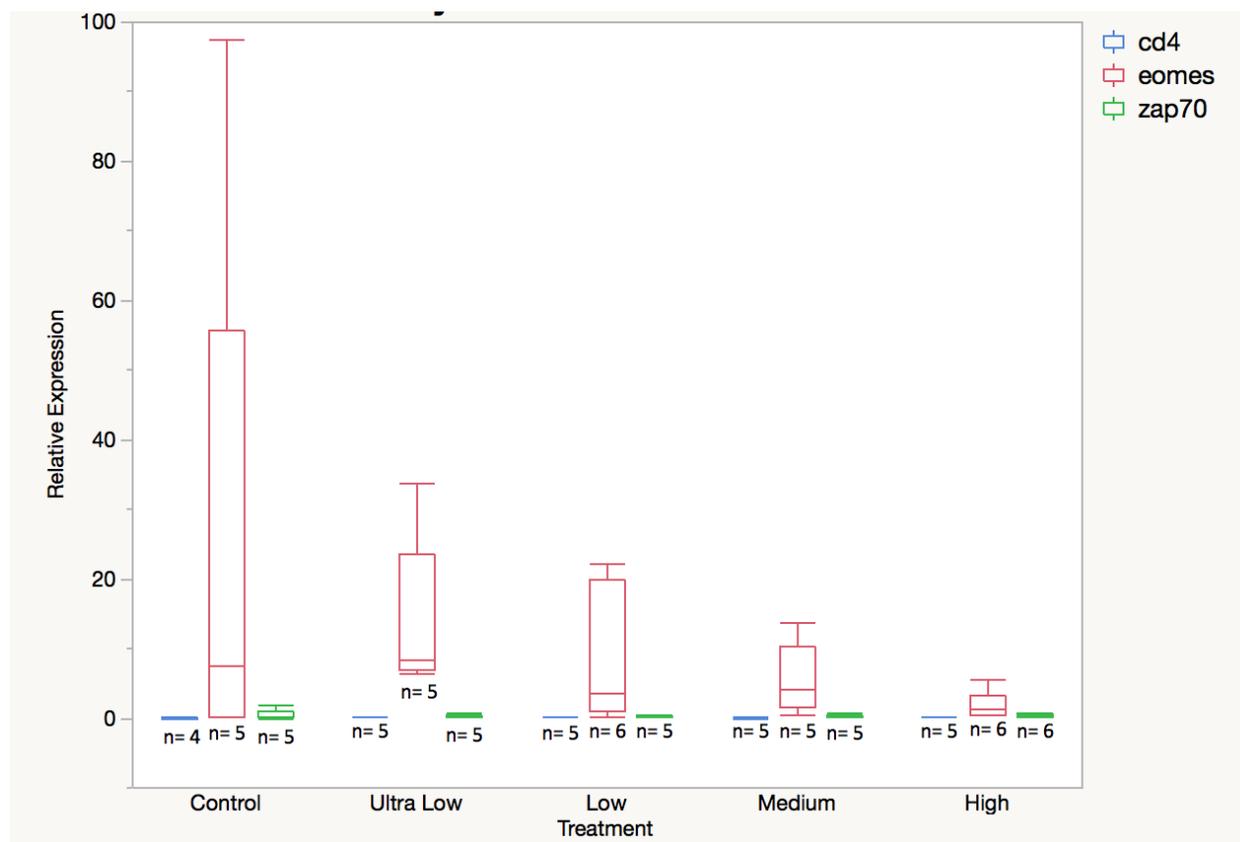
Figure 9. Relative abundances of T cell-specific mRNA in the spleens of fathead minnows exposed to methyl-1H-benzotriazole detected by quantitative RT-PCR and normalized to mRNA abundance of the most stable internal control gene(s).

Expression of all three T cell-specific genes displayed no difference across the treatment groups of fathead minnows exposed to methyl-1H-benzotriazole (M1HB) (Figure 9). The most stable internal control genes were *rpl8* and *hprt1*.
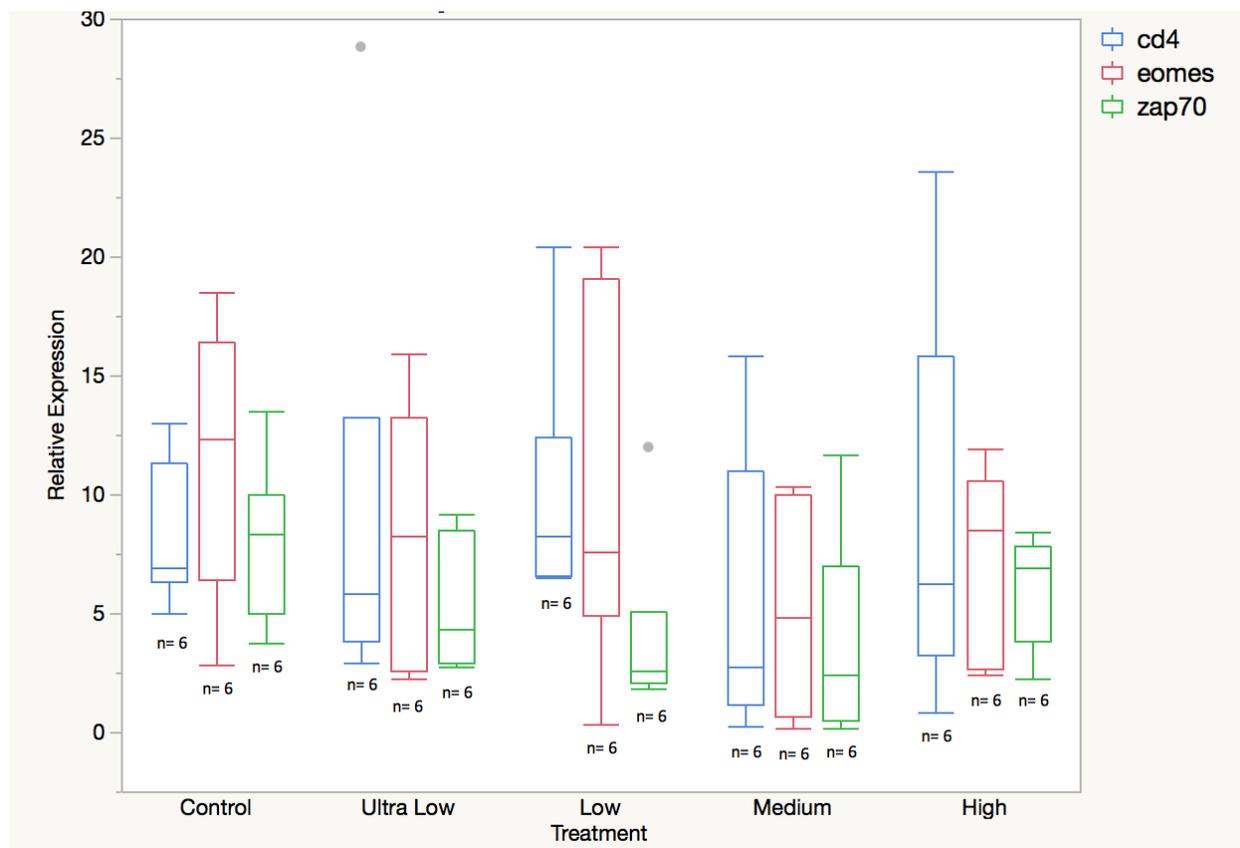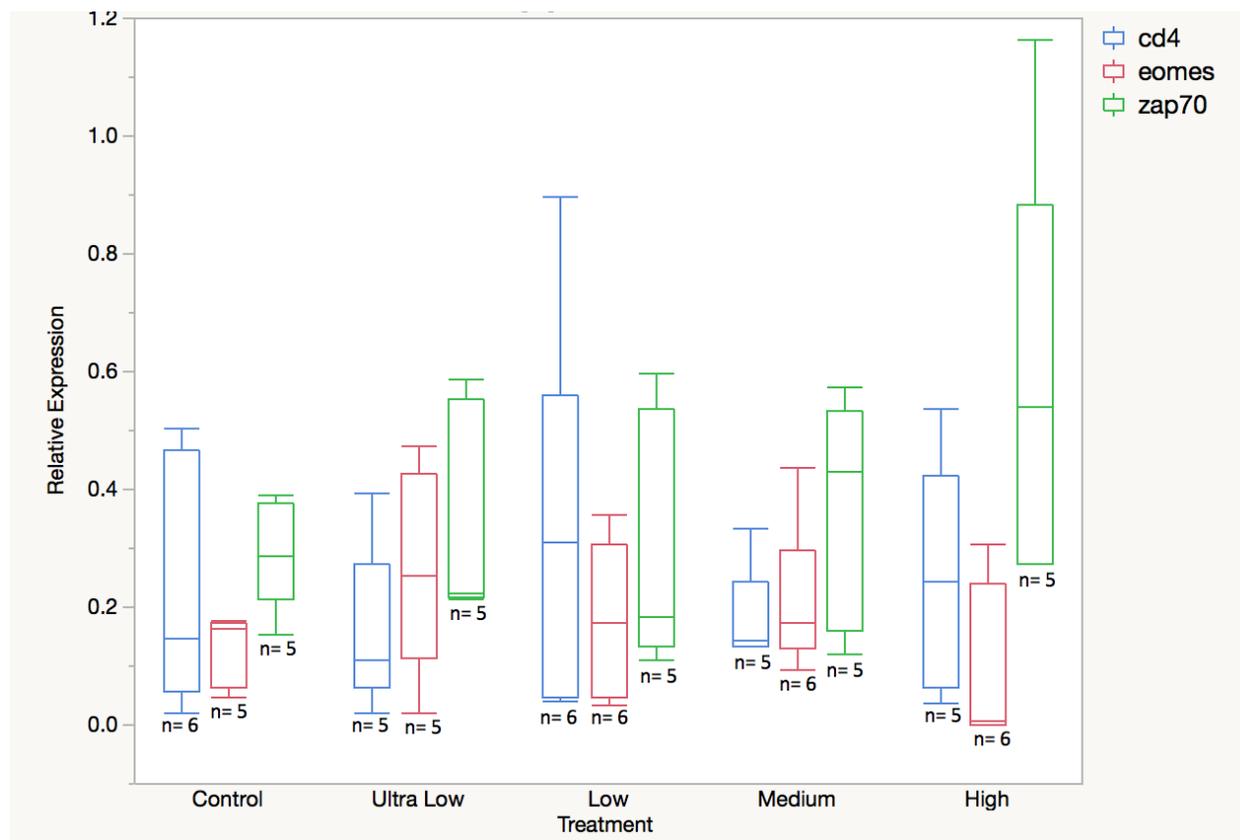
Figure 10. Relative abundances of T cell-specific genes in the spleens of fathead minnows exposed to nonylphenol detected by quantitative RT-PCR and normalized to mRNA abundance of the most stable internal control gene(s).

Expression of all three T cell-specific genes displayed no difference across the treatment groups of fathead minnows exposed to nonylphenol (Figure 10). The most stable internal control genes were *rpl8* and *hprt1*.
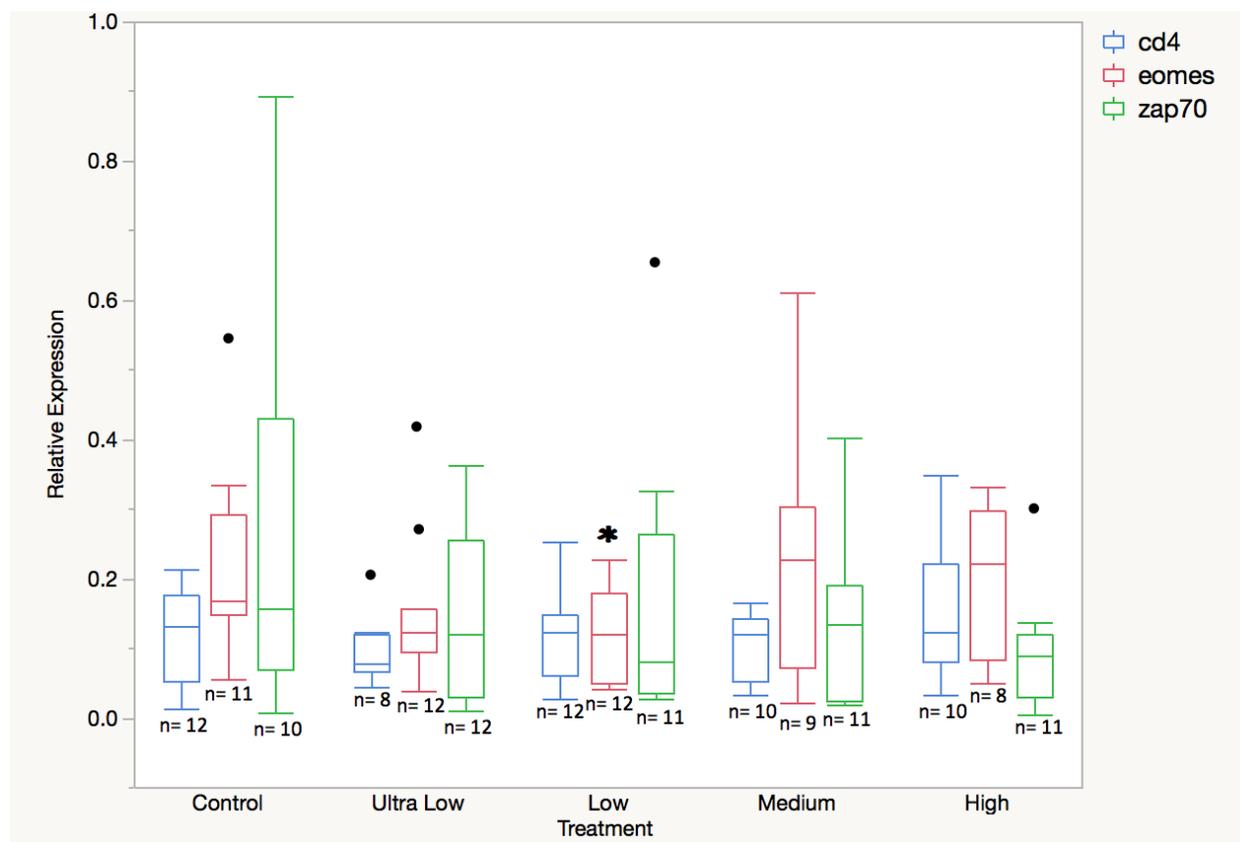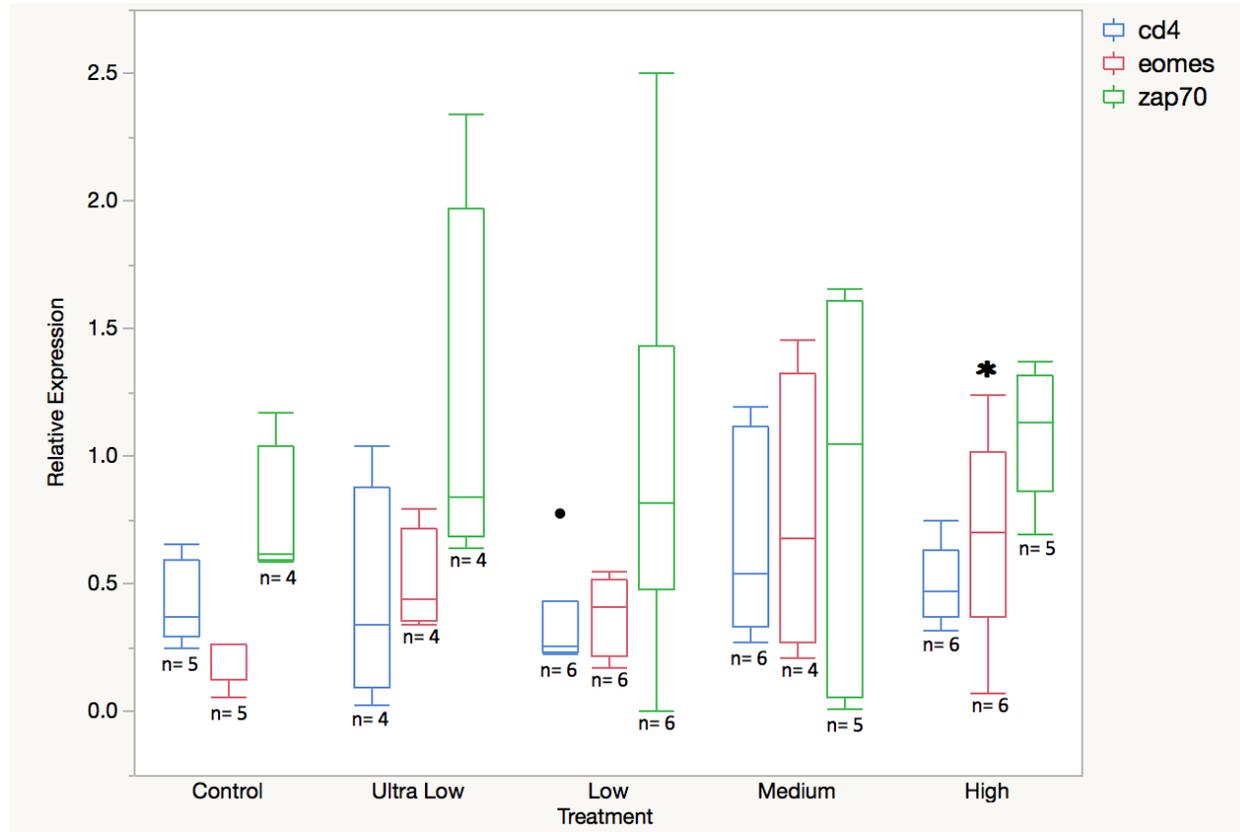
Figure 11. Relative abundances of T cell-specific mRNA in the spleens of fathead minnows exposed to sulfamethoxazole detected by quantitative RT-PCR and normalized to mRNA abundance of the most stable internal control gene(s). Statistical difference indicated by asterisk above expression level (*P < 0.05).

Expression of *eomes* (p = 0.0435), detected in the spleens of fish exposed to high concentration of sulfamethoxazole was significantly increased compared to control (Figure 11). The most stable internal control genes were *hprt1* and *tbp.*
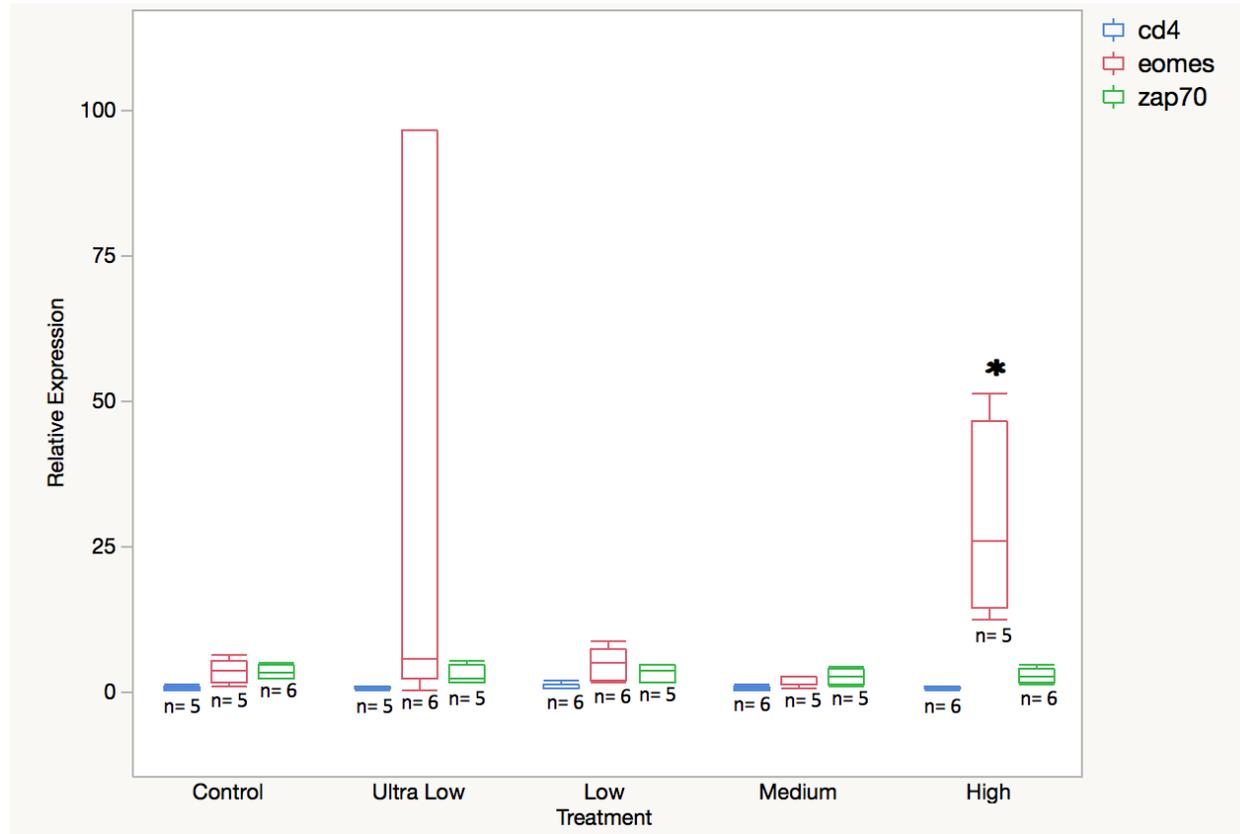
Figure 12. Relative abundances of T cell-specific mRNA in the spleens of fathead minnows exposed to triclosan detected by quantitative RT-PCR and normalized to mRNA abundance of the most stable internal control gene(s). Statistical difference indicated by asterisk above expression level (*$P < 0.05$).

Expression of *eomes* significantly increased ($p = 0.0270$) in the spleen of fish exposed to high concentration of triclosan compared to control (Figure 12). The most stable internal control gene was *tbp*.
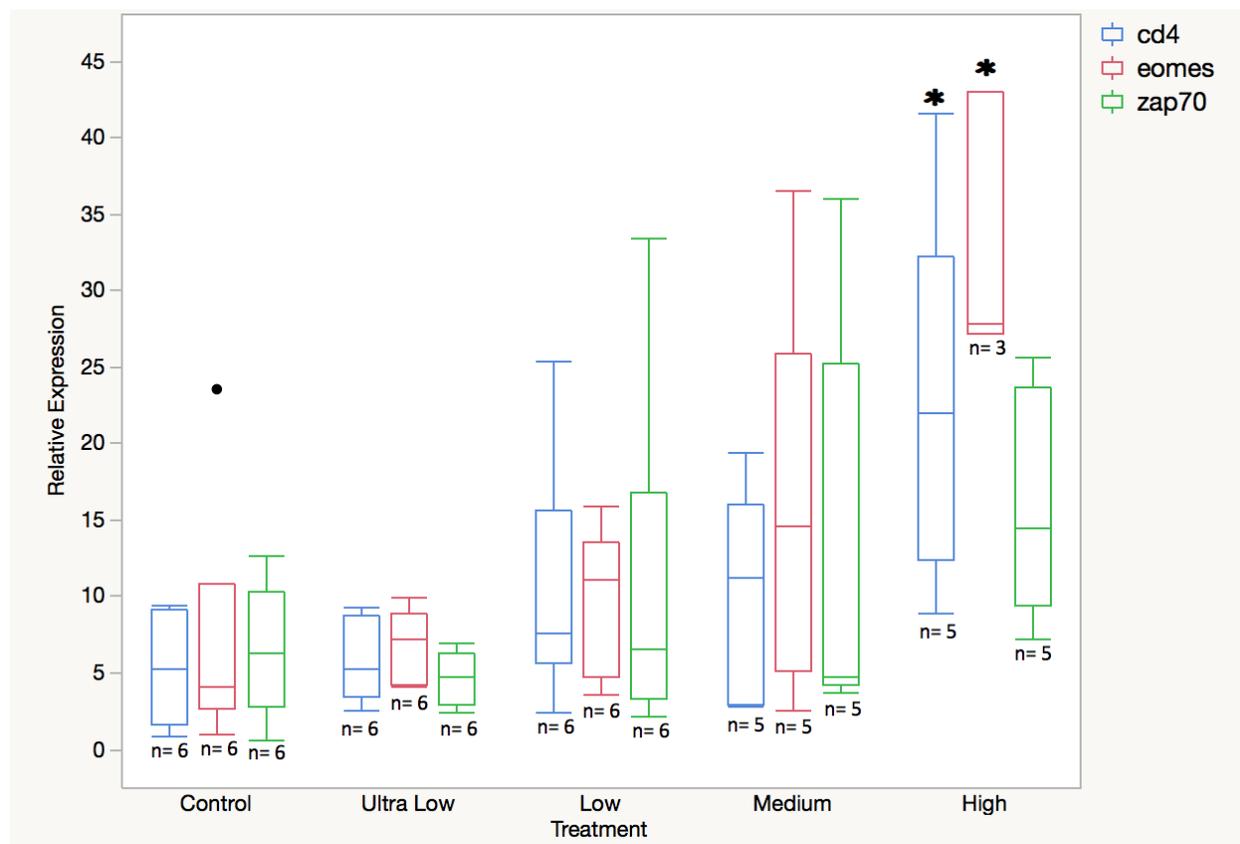
Figure 13. Relative abundances of T cell-specific mRNA in the spleens of fathead minnows exposed to urban mixture detected by quantitative RT-PCR and normalized to mRNA abundance of the most stable internal control gene(s). Statistical difference indicated by asterisk above expression level (*$P < 0.05$).

A significant increase of expression of *cd4* (p = 0.0231) and *eomes* (p = 0.0118) was observed in fathead minnows exposed to high concentration of urban mixture compared to control (Figure 13). There was a trend of increase in expression of *zap70* observed in the high-dose exposure group. The most stable internal control genes were *hprt1* and *tbp*.
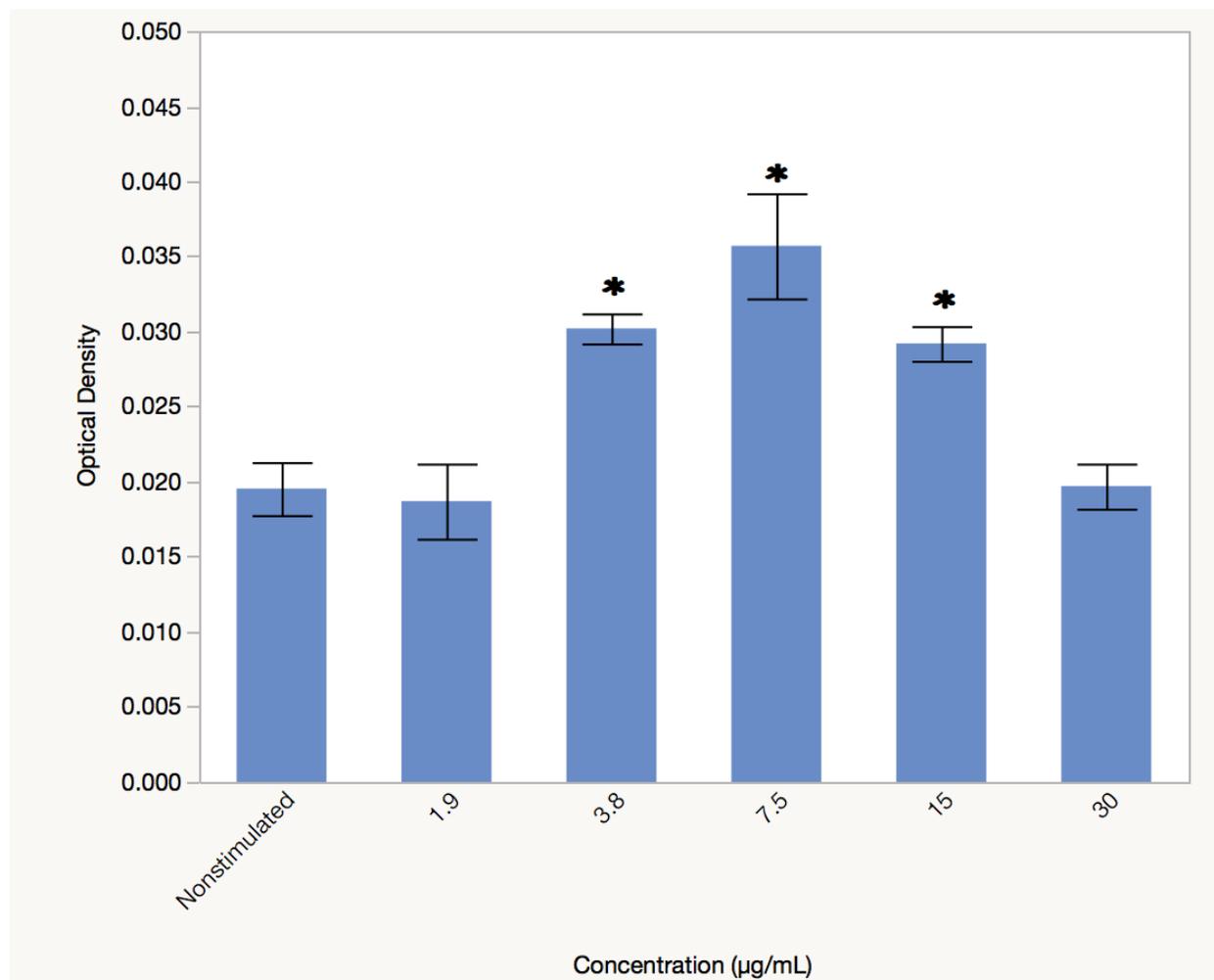
Figure 14. T cell proliferation in the culture of fathead minnow splenic cells induced by concanavalin A (ConA) at serial concentrations. Cells were cultured for 5 days, Alamar Blue reagent added and optical density read by spectrophotometer at 570 nm. Data presented as mean±SE of the triplicate. Statistical difference compared to nonstimulated culture is indicated by asterisk (*$P < 0.05$).

In order to evaluate T cell function of fathead minnows, it was intended to establish an *in vitro* T cell proliferation assay by using isolated splenic T cells stimulated by a mitogen. Attempts were made to establish a T cell proliferation assay from a spleen of an individual fish; three million cells were required for a single triplicate of non-stimulated and Con A-stimulated cells. Whereas cell counts, obtained prior the isolation from 10 pooled spleens, and averaged per

fish, were 12.3 ± 5.2 x 10^6 cells, only 2.7 ± 0.4 x 10^6 cells per fish were obtained post

lymphoprep gradient isolation. Beyond the cell numbers, a minimum of three fish were required

for visualization of the cells in the gradient for proper isolation. Thus, further experiments

utilized lymphocytes obtained from 10 pooled fathead minnow's spleens post isolation on the

lymphocyte gradient. Several culturing variables, including cell numbers, culture media, culture

environment requirements (temperature, CO_2 percentage, culture additives) as well as different

mitogens (phytohemagglutinin and concanavalin A (ConA)) were tested. Overall, 12

experiments were performed in which these variables were evaluated for obtaining a T cell

response after the stimulation. Figure 14 shows the best (desired) results obtained by culturing

5.0 x 10^6 cells/mL in the RPMI medium enriched with 5 % fetal bovine serum (FBS) at a

temperature of 25˚C, with addition of different concentrations of mitogen ConA. A statistically

significant increase in T cell proliferation, presented by optical density, was observed in T cell

cultures exposed to 3.8 µg/mL (p = 0.0057), 7.5 µg/mL (p = 0.0007), and 15 µg/mL (p = 0.0104)

of Con A, compared to control unstimulated cells. These results suggested that those

concentrations of ConA might be optimal for induction of T cell proliferation. While occasional

proliferation was observed several times post Con A stimulation in the experiments that

followed, a reliable, reproducible results were not obtained in our experiments by using the same

concentrations of ConA under the same conditions. In conclusion, the reproducibility of

promising preliminary results, as well as the number of fish required to obtain adequate cell

quantity, were limiting factors that precluded us from further usage of proliferation assays in this

fish model.

Chapter 4: Discussion

In the study at hand, the optimal lymphoid organ for T cells-specific gene expression in fathead minnow was identified, based on significantly higher mRNA abundances of T cell-specific genes as well as higher percentage of lymphocytes, obtained in the spleen in comparison to kidney. These results are in an agreement with previously published data found in other species that lymphocytes are the predominant immune cell type in the spleen (Ali et al., 2014; Castro et al., 2013; Fechner, Julliard, O'Driscoll, & Mezrich, 2015; Koppang et al., 2010; Nakanishi et al., 2015; Uribe et al., 2011). Overall, our data showed significant increases in relative mRNA abundances of some of our target T-cell-associated genes in the spleens of fathead minnows exposed to particular concentrations of desvenlafaxine (*cd4, eomes, and zap70*), sulfamethoxazole (*eomes*), and triclosan (*eomes*) (Table 2). Interestingly, only exposure to the highest concentration of desvenlafaxine induced a significant increase in mRNA abundance of all three genes of interest - *cd4*, *eomes*, and *zap70*. Decreases in relative gene expressions were observed at some concentration levels of exposure in fluoranthene (*zap70*) and metformin (*eomes*) treatment groups. However, there was no change in relative expression of *cd4*, *eomes* or *zap70* in fexofenadine, ibuprofen, methyl-1H-benzotriazole, and nonylphenol exposure groups. The urban CEC mixture caused an increase in *cd4* and *eomes* expression at the highest exposure concentration. The initial hypothesis regarding increases in mRNA abundance of T cell-specific genes in the spleens of adult male fathead minnows has been confirmed for exposures to particular concentrations of following urban CECs, desvenlafaxine, sulfamethoxazole, triclosan and the urban mixture. In addition, the initial hypothesis that certain

urban CECs would decrease the abundance of T cell-specific mRNA was confirmed in the case

of fluoranthene and metformin exposures.

Table 2. Summary of results on the expression of T cell-specific genes obtained by acute exposure to the individual urban CECs and their mixture.

	cd4-1	*eomes-a*	*zap70*
Desvenlafaxine	↑ (H)	↑ (H)	↑ (H)
Fexofenadine			
Fluoranthene			↓ (M, H)
Ibuprofen			
Metformin		↓ (L)	
M1HB			
Nonylphenol			
Sulfamethoxazole		↑ (H)	
Triclosan		↑ (H)	
Urban Mixture	↑ (H)	↑ (H)	

4.1 Desvenlafaxine

Desvenlafaxine is an antidepressant which acts as a selective serotonin and

norepinephrine reuptake inhibitor (SNRI); the major action is to increase the amount of free

serotonin and norepinephrine within the synapses. Following exposure of fathead minnows to

high-dose desvenlafaxine, the spleens exhibited an increase in the mRNA abundances of all

studied genes - *cd4, eomes,* and *zap70*. There were no other significant changes observed in

mRNA abundance across the other treatment groups as compared to control (Figure 4).

The effects of selective serotonin reuptake inhibitors (SSRI) and SNRIs on T cells have shown conflicting results. Gobin, Steendam, Denys, and Deforce categorize SSRIs as an immunosuppressant, acting on T cells as an anti-proliferative or as a pro-apoptotic compound in mammals (2014). However, post treatment with venlafaxine, Basterzi et al. found a decrease in CD45, a T cell activator, with no change in CD4$^+$ or CD8$^+$ cell populations as confirmed by flow cytometric analysis in humans (2010). Other studies, that have investigated the effects of venlafaxine on the gene expression profile of lymphocytes of humans, found 31 genes highly expressed and 26 transcripts as less abundant (Kálmán et al., 2005). Of those, specific genes associated with cell survival, signal transduction, and metabolism showed differential expression. These conflicting results may be due to the fact that venlafaxine acts as a SSRI at low-dose and an SNRI at high-dose administration (Medsker, Forno, Simhan, Juan, & Sciences, 2016). The only research that has been conducted on fathead minnows T cells in reference to SSRIs/SNRIs was a 21-day exposure, which described an increase in the T cell population, evaluated by flow cytometric analysis (Schoenfuss et al., 2016).

The acute exposure to desvenlafaxine in our study showed an increase in all three genes of interest at the highest concentration. Overall, whereas several studies showed effects of SNRIs/SSRIs on T cells after a long exposure/treatment window ranging from 21 to 60 days (Basterzi et al., 2010; Gobin et al., 2014; Kálmán et al., 2005; Medsker et al., 2016; Schoenfuss et al., 2016), our acute exposure to desvenlafaxine showed an increase in all three T cell genes. The effect observed may be due to an increase in the T cell population as seen in Schoenfuss et al. (Schoenfuss et al., 2016), or due to an increase in gene expression of signal transduction-associated genes of elderly humans as seen in Kálmán et al (Kálmán et al., 2005). In either case,

an increase in the number of T cells or an increase in the abundance of mRNA present will cause

an excitation of the immune system. An excitatory response can lead to an increased risk of

autoimmune or allergic reactions to develop in the minnows. Excitatory events being produced

without the presence of a pathogen leads to ineffective energy usage and can take away from

other areas of energy expenditure, such as mating or growth.

4.2 Fexofenadine

There were no significant differences were seen in the expression of any T cell genes

across fexofenadine treatment groups in our study (Figure 5). However, literature findings

suggest a reduction of CD4+ T cell levels and impairment of their function when exposed to

fexofenadine.

Fexofenadine, being an anti-histamine medication commonly used to combat seasonal

allergies, acts to decrease the histamine response of basophils and mast cells, which are cells that

store and release histamine in mammals and teleost fishes (Mulero, Sepulcre, Meseguer, Garcia-

Ayala, & Mulero, 2007; Odaka, Suetake, Maeda, & Miyadai, 2018).

T cells are shown to react to histamine by shifting towards a Th2-type (Martino, Rocchi,

Escelsior, & Fornaro, 2012), which allows for the potentiation of histamine reactions through the

activation of B cells and their antibody production in mice and humans. Fexofenadine acts as a

histamine receptor antagonist (Aventis Pharmaceuticals, 2005), blocking the action of histamine

on tissues and cells systemically. Studies have found that mice treated with fexofenadine

showed a decrease in the mRNA expression levels of IL-4 and IL-5 (Ashenager, Grgela,

Aragane, & Kawada, 2007; Matsubara et al., 2017), which are hallmark cytokines of Th2

subpopulation. Furthermore, CD4$^+$ T cell levels were reported to be decreased in mice treated

with fexofenadine with little to no change in the presence of CD8^{+} T cell levels (Matsubara et al., 2017). To corroborate Matusbara et al., marked reductions in the cytokine release of T cells subjected to fexofenadine treatment were recorded for IL-4, IL-5, and IL-10 following a seven day exposure in a BALB/cAJcl mouse model (Gelfand, Cui, Takeda, Kanehiro, & Joetham, 2002, 2003).

4.3 Fluoranthene

Following exposure to fluoranthene, expression of *zap70* was significantly decreased at medium concentration and further decreased at high concentration as compared to control (Figure 6).

Fluoranthene is a polycyclic aromatic hydrocarbon that has the potential to act through AHRs with high affinity (Esser & Rannug, 2015), thereby altering the course of adaptive immune responses in mice and humans. Expression level of AHR within lymphocytes is lower than in other cell populations. Studies have shown that CD4^{+} T cells express AHR, but certain subpopulations express them much higher than others: those being Th17 and Treg subpopulations in mice (Fechner et al., 2015). Interestingly, our results, while clearly showing a decreased zap70 expression, only showed a trend of decreased *cd4* expression in the spleens of fathead minnows exposed to medium/high concentration of fluoranthene. If fluoranthene acts on Th17 and Tregs through AHR receptor, that entire CD4+ T cell population would be affected.

Boule, Burke, Jin, and Lawrence found a reduction in the responsiveness of viral-specific CD8^{+} T cells as well as an increase in the Treg cell population post exposure to fluoranthene (2018). The increase in Treg cell populations acted as a control or cap to the other cell subpopulations thereby decreasing their activity or deactivating them. The increase in Treg cells

could explain the reduction in abundance of *zap70* that was observed post exposure to fluoranthene with a tapering of the other subpopulations and their activity. Besides cell-to-cell interactions, a compound with relatively similar structure compared to fluoranthene, benzo[k]fluoranthene, induced decreased cellularity of both the thymus and spleen of Balb/c mice, with the reduction following a dose-dependent manner through AHR (Won Jeon et al., 2005). The reduced number of cells was seen in $CD4^+$ and $CD8^+$ population of splenic T cells, and in $CD4^+CD8^+$, $CD4^+CD8^-$, and $CD4^-CD8^+$ thymic T cells in mice (Won Jeon et al., 2005). Deceased abundance of *zap70* would indicate a state of immune suppression across the total T cell population. A suppressed state would leave the organism vulnerable to infection or development of cancer.

4.4 Ibuprofen

Following exposure to ibuprofen, no significant differences were seen in the expression of any genes of interest across treatment groups. Trends indicated a decrease in the expression of *cd4, eomes,* and *zap70* at the medium concentration as compared to control, with no statistical significance reached (Figure 7).

Ibuprofen is a nonsteroidal anti-inflammatory drug that provides relief of pain and inflammation by acting as an non-specific inhibitor of cyclooxygenase (Dill et al., 2010), which leads to the production of prostaglandins. This effect is conserved across species as suggested by the read-across hypothesis (Patel et al., 2016). Patel and colleagues tested their hypothesis specifically in fathead minnows with a conclusion that as long as the molecular pathways are conserved between species, the effects seen in one species should be observed in fathead minnows.

Ibuprofen is believed to suppress T cell function (proliferation), expression of activating surface receptors such as CD25 and CD71 as well as to decrease the production of proinflammatory cytokines such as IL-2, IFNγ, and TNFα (Iniguez, Punzon, & Fresno, 1999; H.-J. Kim, Lee, Im, Kim, & Lee, 2010). Whereas ibuprofen-induced inhibition of T cell proliferation seemed to occur via p38 MAP kinase inhibition (Paccani, Boncristiano, Ulivieri, & Milco, 2001) Paccani et al found that upstream TCR-associated signal transduction, which involves zap70 and lck, was unaltered (Paccani et al., 2005). Based on this data, it is not likely that ibuprofen would affect *zap70* expression in our experimental system. Thus, our data are in line with Paccani et al. (2005) finding.

4.5 Metformin

Following exposure to metformin, mRNA abundances of *eomes* was decreased at low treatment concentration as compared to control. No other differences were observed across treatment groups (Figure 8).

Metformin acts as an anti-diabetic drug to reduce the amount of free-floating glucose. Metformin changes the metabolic activity of cells via activation of 5' AMP-activated protein kinase (AMPK) (Eikawa et al., 2015; Kunisada et al., 2017; Shin et al., 2013; Watanabe et al., 2016). AMPK inhibits an immune activator, mammalian target of rapamycin (mTOR) (Araki et al., 2009). Metformin treatment has been shown to mimic the action of rapamycin, which acts as an immunosuppressant able to stop the immune system-provoked rejection of transplanted organs in mammals (Cox & Zajac, 2010).

Until recently, metformin was used exclusively for the treatment of type II diabetes mellitus; however, there have been shifts in the usage of metformin because of realized immune

interactions that the drug exhibits. Metformin has shown effective in regulation of CD8+ T cells and Tregs in mice (Cox & Zajac, 2010; Eikawa et al., 2015; Kunisada et al., 2017). During a virally infected or tumor state, CD8$^+$ T cells and their effectors, CTLs, are useful for clearance of infected/damaged cells. Treg cells regulate the cell populations present within a microenvironment by suppression of their action. Metformin has been shown to cause the shift from an effector CTL stage to the memory stage (Cox & Zajac, 2010), while simultaneously causing an increase in the tumor infiltrating potential of effector CD8$^+$ T cells in mice (Eikawa et al., 2015). The expression of eomes has been shown to increase in response to cells shifting from an effector or naïve phenotype to a memory phenotype in mice and many different teleost fishes (Araki et al., 2009; Cox & Zajac, 2010; Rao, Li, Odunsi, & Shrikant, 2010). The decrease in eomes mRNA levels at low and ultra-low treatment concentrations of metformin observed in our study is in contrast to literature statements. However, one should take into consideration high dosage of metformin (Cox & Zajac, 2010; Kaech & Cui, 2012), which caused eomes increase used in previous studies in comparison to ours. A trend of increase in eomes RNA expression post medium- and high-dose metformin exposure was observed in our study as well. Decreased eomes would indicate a state of immune suppression leaving an organism vulnerable to infection, specifically in the case of eomes, for viral infection.

4.6 Methyl-1H-benzotriazole (M1HB)

Following exposure to M1HB, no significant differences were seen in mRNA abundances of tested genes across treatment groups (Figure 9).

M1HB is used as an anti-corrosive/anti-icing agent used on aircraft industry. In respect to the immune system, little research has been conducted on M1HB. Kim et al. found that

benzotriazole acts to re-activate HIV-1 virus through the inhibition of SUMOylation of STAT5 within the nucleus, thereby stopping the nuclear export of STAT5, which causes the continual transcription of virally integrated genetic material (2017). With the increase in nuclear localized STAT5, it can be assumed that cellular transcription, controlled by STAT5, would increase as well. For example, since expression of STAT5 is required for the maintenance of $CD8^+$ T cell effector phenotype (Tripathi et al., 2010), one might speculate that an increased transcription of CD8+ T cell-associated molecules would be observed post exposure to M1HB. However, an increase in CD8+ T cell-associated mRNA was not a case observed in our study.

4.7 Nonylphenol

Following exposure to nonylphenol, no significant effect was seen in mRNA abundance of tested gene of interest across treatment groups (Figure 10).

Nonylphenol is the biproduct of surfactant degradation commonly found in waterways. Several studies observed immunosuppressive effect of nonylphenol on T cells in a context of reduced proliferation (function). Thus, nonylphenol addition (10^{-4} M) *in vitro* inhibited Con A-induced proliferation of T cells of mice (J. Lee, Han, Park, & Moon, 2017). The exposure of juvenile *O. mykiss* to nonylphenol of 1 ug/L for 54 days resulted in the reduced stimulatory response of T and B cells when stimulated with PHA and LPS, respectively (Herbert et al., 2009). In contrast, the exposure of isolated mouse lymphocytes to 1 ug/L and 5 ug/L nonylphenol for four days resulted in the increase in IL-4 secretion from $CD4^+$ T cells (M. H. Lee et al., 2003). Regardless the final outcome, these studies indicated solely functional changes in T cells exposed to nonylphenol. Nonylphenol has been also shown to induce immunosuppression by differentiation of Treg cells through modulation of dendritic cells of mice

(Bruhs et al., 2015). In the context of these literature reviews, one would not expect to observe changes on a T cell population level. Even if nonylphenol specifically induces the Treg population increase, a change in such a small subpopulation of T cell pool would be hard to detect.

4.8 Sulfamethoxazole

Following exposure to sulfamethoxazole, mRNA abundance of *eomes* was increased at high treatment concentrations as compared to control (Figure 11).

Sulfamethoxazole is an anti-microbial compound that falls into a class of compounds known as sulfonamides. The immunogenicity of sulfamethoxazole, as a free-floating compound, is negligible. However, the immunogenicity of the sulfonamide increases profoundly when bound to a protein carrier as a hapten. Many studies support the theory of sulfamethoxazole acting through the hapten-carrier model (Burkhart et al., 2002; Castrejon et al., 2010; Depta et al., 2004; Pichler, 2002; Schnyder et al., 2000b; Watkins & Pichler, 2013). Interaction and stimulation of T cells via a hapten reaction occurs by covalent binding of sulfamethoxazole to an endogenous protein, then a presentation in the context of MHC-class II molecule on APC to a T cell in humans and mice (Burkhart et al., 2002; Pichler, 2002).

Previous studies have shown varied T cell proliferative responses to sulfamethoxazole treatment. An increase in the percentage of CD8^{+} T cells of humans was observed by Hertl, Jugert, & Merk (1995), whereas increase in CD4^{+} T cells was observed by Schnyder et al. following treatment with sulfamethoxazole (2000a). Our study is in agreement with Hertl, Jugert, & Merk (1995) results. The significant increase in *eomes* expression can be explained by an increase in the number of CD8^{+} T cells present in the spleen following sulfamethoxazole

exposure. Increases in CD8^{+} T cell-associated mRNA abundance can indicate an immune dysregulation or prolonged inflammatory process, which increases the mortality of an organism through over-stimulation and immune exhaustion leaving the organism susceptible to infection.

4.9 Triclosan

Following exposure to triclosan, expression of *eomes* was increased at high treatment concentrations as compared to control (Figure 12).

Triclosan is an antimicrobial additive that has been thought to play a role in the increased cases of allergies across the United States (Rees Clayton, Todd, Dowd, & Aiello, 2011). Research indicated a depressed Th2 cytokine profile present in topically treated mice, associated with blockage of specific toll-like receptors by triclosan in mice (Marshall et al., 2017). Triclosan initiated a toll-like receptor 4-mediated activation of the MAPK/NFκB/AP-1 pathways, leading to the promotion of proinflammatory cytokine production and CD8^{+} T effector cell differentiation in mice (Molteni, Gemma, & Rossetti, 2016). This observed increase in CD8^{+} T cells is in line with increased mRNA abundance of *eomes* obtained in our study following treatment with triclosan. Increases in CD8^{+} T cell-associated mRNA abundance can indicate an immune dysregulation or prolonged inflammatory process, which increases the mortality of an organism through over-stimulation and immune exhaustion leaving the organism vulnerable to infection.

4.10 Urban Mixture

Following exposure to the urban mixture, expression of *cd4* and *eomes* increased at high treatment concentrations compared to control (Figure 13).

The results of chemical interactions are hard to predict, especially when complex chemical mixtures are considered. Complex mixtures can have a myriad of effects dependent on the concentrations and biological interactions that can occur. Complex disparate action of chemicals can result in stronger effect, whether that be additive, synergistic, or a potentiated response (Binderup et al., 2003). Explaining the exact molecular mechanism that the mixture could have is far too elaborate to anticipate, however, the result of our study clearly shows an increase in the expression of *cd4* and *eomes*. This could be a result of increased T cell numbers because of their proliferation, suggesting a "false" immune response induced by exposure to the urban CEC mixture, instead of to a particular pathogen/antigen (Rehberger, Werner, Hitzfeld, Segner, & Baumann, 2017).

4.11 T cell Proliferation Assay

The function of T cells, e.g. their proliferation, should be ideally evaluated in addition to their gene expression. Several conditions and parameters were tested in an attempt to establish the assay for evaluation of T cell proliferation. The RPMI and L15 culture media were used to allow for incubation of the cells with and without the need for the infusion of CO_2 into the ambience of the incubator. Incubation temperatures were tested between the range of 18˚C to 27˚C , with an emphasis placed at 25˚C and 27˚C, as temperature ranging from 22˚C to 27˚C has been described as the most permissive range for proliferation of T cells stimulated by mitogen ConA in *Ictaluraus punctatus* (Bly, Cuchens, & Clem, 1986). Cell concentrations were adjusted from $4x10^5$ cells/well to $5x10^5$ cells/well to $1x10^6$ cells/well. The optimal results were obtained with a concentration of $5x10^5$ cells/well, confirming previously published data in *I. punctatus* and *O. mykiss* (Bly et al., 1986; Tillitt, Giesy, & Fromm, 1988). Concentrations of fetal bovine

serum (FBS) were adjusted at five and ten percent, with optimal concentration being identified at five percent. Literature cites the use of up to 15% with positive results (Yin et al., 2007); however, the majority of studies used five percent FBS (Bly et al., 1986; Marsden, Vaughan, Foster, & Secombes, 1996; Tillitt et al., 1988). The optimal Con A concentration, described for induction of T cell proliferation in different fishes, such as *O. mykiss* (Tillitt et al., 1988), *C. auratus* (Yin et al., 2007), and *I. punctatus* (Bly et al., 1986),was 10 ug/mL, 30 ug/mL, and 25 ug/mL, respectively. After manipulating experimental conditions including cell number, culturing conditions, and mitogen used for situation, there were instances of successful stimulation with ConA. Furthermore, there were no results that were reliable or reproduceable under the identical experimental conditions. As a result, the assay could not be used on this animal model.

4.12 Adverse Outcome Pathways

All the discussion points thus far have bobbled between the realms of toxicology, molecular biology, cell biology, and immunology. Now is the time to consider what all the results mean at a cellular and a molecular level, as well as at an organismal and population level.

Changes in gene expression can have a plethora of potential effects on an exposed organism and population. Reduction in the gene expression of any of the discussed T cell genes of interest, could put organisms at risk for infection by pathogen due to the induction of an immunosuppressed state (Rehberger et al., 2017). As mentioned previously, immunosuppression can lead to increased risk for infection, but it can also increase the risk for development of neoplasms. An increase in the gene expression of the T cell-specific genes of interest could also lead to an autoimmune reactions, immune exhaustion, neoplastic development, or

hypersensitivity reaction. Autoimmune and hypersensitivity reactions lead to major consequences to an organism resulting in endocrine dysfunction, neuromuscular/coordination dysfunction, *etc*. Further investigations with longer exposures are required to elucidate adverse outcomes associated with the adaptive immune system.

4.13 Experimental Weaknesses

Considering that the approach of this study was entirely based on the use of targeted PCR, and not high-throughput PCR, there are some experimental limitations applied without the use of an immunophenotypic evaluation or a functional test to aid in the elucidation of genetic changes. Based on these limitations, there are two conclusions that were equally as likely in the event of observed differences within a treatment group. First, there was a change in the cell population present within the organism, meaning that there may be an increase/decrease in the number of cells present due to proliferation, migration, or apoptotic mechanism, thereby changing the sheer number of transcripts present within a treatment group. Second, there was no change in the number of cells present, however, there was a change in the expression via upregulation or downregulation of the particular gene or genes that were being investigated.

4.14 Future Direction

Future endeavors into this area of study should consider five possible avenues: 1.) chronic exposure, 2.) multigenerational exposure; 3.) immunophenotypic characterization of fish immune cells, 4.) reaction to infection, and 5.) allogeneic graft rejection competency post exposure to the same CECs and their mixture.

Keeping in mind that the adaptive immune system is slow in its response (five to seven days), a chronic exposure would be able to observe more pronounced effects on the adaptive

immune system, specifically T cells that this study was probably unable to fully elucidate with the acute exposures.

A multigenerational study would potentially allow for more of a pronounced effect on T cells to be observed as well as the potential for developmental changes to occur that may have untold effects on the immune system of exposed fish and their offspring.

A functional and immunophenotypic evaluation of fish adaptive immune system by flow cytometry should be performed. While there is information that can be gathered from targeted genetic work, the insight that it sheds is limited by the nature of the test performed. Without functional data and immunophenotypic evidence to support genetic changes, multiple possibilities are equally likely. Functional and immunophenotyping techniques would allow for more effective evaluation of results; however, with a lack of research tools for a fathead minnow fish model, this is not as likely to occur at this time.

Evaluation of the immune system is classically thought of as the ability for an organism's immune system to respond to perturbation, whether that be by viral, bacterial, fungal, or parasitic infection. By infecting chemically exposed fish to a pathogen, and evaluating immune parameters during and post infection, the effect of a chemical on the immune system can be evaluated more effectively.

Finally, performing an allograft transplantation on chemically exposed fish would once again allow for evaluation of immune competency following exposure. Immunocompetent organisms would normally reject an allograft or xenograft. This rejection is mediated by T cells. If a transplanted tissue is accepted by chemically treated fish, when control fish reject the graft, it can be assumed that the treatment induced some sort of immunosuppressive state within the

organism. If a transplanted tissue is rejected faster/more aggressively by chemically treated fish, it can be assumed that the treatment induced an immunostimulatory state within the organism (Rehberger et al., 2017).

Chapter 5: Conclusion

Acute exposure to several urban CECs affected molecular targets associated with T cell adaptive immunity of fathead minnows. Acute exposure to desvenlafaxine, sulfamethoxazole, triclosan, and an urban mixture resulted in an increase in the abundance of T cell-specific mRNA in the spleens of adult male fathead minnows, which might induce an adverse outcome such as autoimmunity or hypersensitivity. In contrast, exposure to fluoranthene and metformin resulted in a decrease in the abundance of T cell-specific mRNA in the spleens of adult male fathead minnows, which might induce a state of immunosuppression with a consequent susceptibility to infection or neoplasia. Further research is needed to understand the effects of chronic and multigenerational exposure on the T cells of fathead minnows.

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IMER_PRODUCT_MIN=70&PRIMER_PRODUCT_MAX=200&PRIMER_NUM_RETU
RN=30&PRIMER_MIN_TM=57.0&PRIMER_OPT_TM=58&PRIMER_MAX_TM=60&P
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Appendix A. List of Compounds Found to be in >30% of Water Samples Collected from the Great Lakes Tributaries and their Environmentally Relevant Concentrations

Compound Name	Chemical Concentration (ng/L)
4-Nonylphenol	3710
5-methyl-1H-benzotriazole	6680
Atrazine	400
Bisphenol A	600
Bromacil	120
Desvenlafaxine	583
Estrone	24
Fexofenadine	1000
Hexahydrohexamethyl Cyclopentabenzopyran	2180
Metformin	1210
Metolachlor	170
N,N-Diethyl-m-toluamide	1600
Sulfamethoxazole	559
Tris(2-butoxyethyl) phosphate	21000
2,4,6 trinitrotoluene	1500
2,4-Dinitroanisole	1000
2,6-diamino-3,5-dinitropyrazine-1-oxide	1000
2,4,6-Trinitro-3-bromoanisole	1000
Fluoranthene	0.1
Imidacloprid	140
Triclosan	0.5
Ibuprofen	440

Appendix B. Summary of Current Literature Describing Adaptive Immunity in Teleost Fishes

Fish	Organ	Cell Type	Compound	Reference
Oncorhynchus mykiss	Blood and Spleen	B and T cells	Sewage water	(Erlangung, 2003)
Acanthopagrus latus	Blood and Head Kidney	Melanomacrophagic center cells	Phenanthrene	(Shirmohammadi et al., 2017)
Dicentrarcus labrax	Blood and Spleen	Mononucleated leukocytes	Light Cycle Oil (fluoranthene)	(Bado-Nilles et al., 2011)
Sparus aurata	Plasma	B cell	Sex-steroid hormones	(Cuesta et al., 2007)
Acanthopagrus latus	Blood	Lymphocytes	BPA	(Yaghoobi et al., 2017)
Cyprinus carpio	Serum	B cells	BPA	(Qiu et al., 2016)
Oncorhynchus mykiss	Head Kidney	B and T cells	Nonylphenol	(Herbert et al., 2009)
Oncorhynchus mykiss	Blood	Peripheral Blood Leukocytes and B cells	Nonylphenol and Atrazine	(Shelley, Ross, Miller, et al., 2012)
Oncorhynchus mykiss	Blood	T and B cells	Nonylphenol	(Shelley et al., 2013)
Oncorhynchus mykiss	Blood	B cells	Nonylphenol	(Shelley, Ross, & Kennedy, 2012)
Sparus aurata L.	Head Kidney	B and T cells	Temoxifen	(Bleda, 2017)
Sparus aurata L.	Peritoneum	B cells	17α-Ethynylestrodiol	(Gómez González et al., 2017)