DEVELOPMENTAL SENSITIVITY AND IMMUNOTOXICITY OF BENZO[A]PYRENE IN THE AMPHIBIAN *XENOPUS LAEVIS*

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ABSTRACT

The immune system is increasingly recognized as a vulnerable and sensitive target of contaminant exposure. Disruption of this critical system can lead to significant morbidity and/or mortality of organisms in contaminated environments. Amphibians can be simultaneously exposed to both pathogens and contaminants within aquatic habitats, making contaminant-induced immunotoxicity particularly relevant for this taxonomic group. Polycyclic aromatic hydrocarbons (PAHs) are a major group of environmental contaminants that widely occur as mixtures in aquatic environments, including amphibian habitats. Benzo[a]pyrene (B[a]P) is recognized as the most potent toxicant of PAH mixtures and is the most extensively studied PAH with demonstrated adverse effects on the immune system of many vertebrate species. Understanding how aquatic contaminants, such as B[a]P, can impact the immune system of amphibians is imperative since contaminant-induced immunotoxicity is hypothesized to exacerbate disease-driven amphibian population decline. Thus, the overall goal of this thesis was to gain a better understanding of developmental and contaminant-induced changes in the immune system of amphibians, and ultimately how exposure to B[a]P modulates the response to immune stimulation.

Specific research questions were addressed through four studies conducted within the broader goal of this thesis. The objective of the first study was to characterize the expression profiles of various pro-inflammatory cytokines in the model amphibian species, *Xenopus laevis*, throughout larval developmental stages and determine the impacts of thyroidal modulation on their expression. Results suggest that expression of two cytokines, tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) varied over development, increasing with developmental stage, while interferon-γ (IFN-γ) remained relatively unchanged. Exogenous manipulation of thyroidal status was successful, as demonstrated by an altered rate of development and thyroid gland histology; however, thyroidal modulation had negligible effects on basal cytokine expression. The dynamic nature of the tadpole immune system suggested that the immunotoxic impacts of environmental contaminants may depend on the stage of development at the time of exposure. Therefore, the second study aimed to identify the sensitive developmental phases for sub-lethal effects of B[a]P exposure in terms of the development (stage, weight and length) and changes in the expression of immune- and detoxification-related genes. Exposure to B[a]P at all life-stages tested (embryo-larval, pre-metamorphic, and pro-metamorphic tadpoles) resulted in

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CYP1A7 mRNA induction (43, 53, and 47-fold, respectively) as well as stage-specific modulation of pro-inflammatory cytokine expression. Exposure of pre-metamorphic tadpoles to B[a]P demonstrated the greatest effect on weight, length and stage of development. Taken together, these initial studies indicate that the unique development of the tadpole immune system influences their susceptibility to contaminant-induced immune modulation despite having biotransformation capacity across tadpole life-stages.

In the subsequent two studies, the ability of B[a]P to alter immune variables and impair a coordinated immune response was evaluated using an immune challenge model both *in vivo* and *in vitro*. Juvenile *X. laevis* were exposed to B[a]P for seven days and then immune stimulated with an injection of lipopolysaccharide (LPS). The highest concentration of B[a]P (350 μg/L) impaired the inflammatory response to LPS as indicated by an inability to induce granulocyte:lymphocyte (G:L) ratio or IL-1β mRNA expression, demonstrating that B[a]P can impair the inflammatory immune response to a simulated pathogen. Next, to assess the mechanisms underlying B[a]P-induced effects in amphibians, *X. laevis* kidney A6 epithelial cell line were used to evaluate the cellular response to B[a]P exposure and identify if B[a]P could directly alter the ability of these cells to respond to an immune stimulation. A time-course of B[a]P exposure showed the progression from up-regulation of biotransformation-related genes, to reactive oxygen species production, and ultimately to cytotoxic effects; pre-exposure to B[a]P did not appear to impair the ability of the A6 cells to mount an inflammatory response to LPS. Taken together, the immune challenge models showed that B[a]P exposure can alter immune variables and impact the ability of juvenile *X. laevis* to appropriately respond to immune stimulation and that B[a]P-induced biostransformation pathways and production of reactive oxygen species may play a role in this response.

Overall, the research presented in this thesis contributes to our understanding of developmental and contaminant-induced changes in the immune system of amphibians. This research provides a strong foundation for further study into the mechanisms underlying chemical-induced immunotoxicity in amphibians. The tools and approaches developed as part of this research could be used in screening chemicals for immunotoxic potential in amphibians and be expanded to evaluate immune responses of ecologically relevant species that are under real risk of compromised immune function and increased disease in contaminated environments.

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LIST OF ABBREVIATIONS

3-MC	3-methylcholanthrene
α-NF	α-naphthoflavone
AhR	Aryl hydrocarbon receptor
AIP	AH interacting protein
ANOVA	Analysis of variance
AMP	Antimicrobial peptide
ARNT	Aryl hydrocarbon nuclear translocator
ASTM	American society for testing and materials
ATRF	Aquatic toxicity research facility
B[a]P	Benzo[a]pyrene
Bd	*Batrachochytrium dendrobatidis*
BKA	Bacterial killing assay
cDNA	Complementary deoxyribonucleic acid
CCAC	Canadian council on animal care
CS	Corticosteroids
CSF-1	Colony-stimulating factor-1
CV	Coefficient of variation
CYP	Cytochrome P450
$DiOC_5(3)$	3,3′-dipentyloxacarbocyanine iodide
DMBA	7, 12-dimethylbenz[a]anthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
Dpi	Days post injection
EF-1α	Elongation factor-1α
EROD	Ethoxy-resorufin O-deethylase
EtOH	Ethanol
FBS	Fetal bovine serum
FETAX	Frog embryo teratogenesis assay-*Xenopus*
FSC	Forward scatter

FV3	Frog virus 3
G:L	Granulocyte:lymphocyte ratio
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic deoxyribonucleic acid
GSTM1	Glutathione S-transferase mu 1
H$_2$DCFDA	2',7'dichlorodihydrofluorescein diacetate
hCG	Human chorionic gonadotropin
hsp90	Heat shock protein 90
i.p.	Intraperitoneal
IFN-γ	Interferon-γ
IgM	Immunoglobulin M
IL-1β	Interleukin-1β
IQR	Interquartile range
IU	International units
IUCN	International union for the conservation of nature
LC$_{50}$	Lethal concentration 50
LD$_{50}$	Lethal dose 50
LPS	Lipopolysaccharide
LSI	Liver somatic index
mRNA	Messenger ribonucleic acid
MS-222	Tricaine methanesulfonate
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NF	Nieuwkoop and Faber
NoRT	No reverse transcriptase
NTC	No transcript control
OECD	Organisation for economic co-operation and development
PAH	Polycyclic aromatic hydrocarbons
PAMP	Pathogen associated molecular pattern
PBDE	Polybrominated diphenyl ethers
PBS	Phosphate-buffered saline

PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PHA	Phytohemagglutinin
PolyI:C	Polyinosinic:polycytidylic acid
qPCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SEM	Standard error of the mean
SSC	Side scatter
T_3	Triiodothyronine
T_4	Thyroxine
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TH	Thyroid hormone
TNFα	Tumor necrosis factor-α
TSH	Thyroid stimulating hormone
US EPA	United States environmental protection agency
UV	Ultraviolet
XRE	Xenobiotic response element

NOTE TO READERS

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate and Postdoctoral Studies guidelines for a manuscript-style thesis. Therefore, there is some repetition between the material presented in each chapter. Chapter 1 is a general introduction and literature review, including project goals and objectives. Chapter 6 contains a general discussion and overall conclusion. Chapters 2-5 are organized as manuscripts for publication in peer-reviewed scientific journals and a description of author contributions is provided in the preface for these chapters. Chapter 2 is published in *General and Comparative Endocrinology*, Chapter 4 is under review for publication in *Aquatic Toxicology*, and Chapters 3 and 5 are being prepared for submission to *Environmental Science and Pollution Research* and *Toxicology In Vitro*, respectively. References cited in each chapter are combined and listed in the References section of the thesis. Supporting information associated with the research chapters are presented in the Appendix section at the end of this thesis.

CHAPTER 1:
LITERATURE REVIEW

1.1. The immune system and immunotoxicity

The immune system is a complex biological system, primarily responsible for distinguishing between self and non-self through active surveillance and elimination of foreign and abnormal cells and structures from the body (Kaplan et al., 2013). Exposure to xenobiotics, such as environmental contaminants, may alter the immune system's normal function. Immunotoxicity is the study of such adverse effects on the immune system (Kaplan et al., 2013). Immune system responses can be imagined as a continuum where there is an expected range of responses to a stimulus (such as a pathogen). Immune modulation can occur through either an increase (*e.g.* autoimmunity or hypersensitivity) or decrease (*e.g.* immune-suppression) in the magnitude of the response. Aberrant responses on either end of the continuum can lead to significant morbidity and/or mortality.

Since the emergence of the discipline of immunotoxicology, the immune system has been identified as exceedingly sensitive to contaminants (Bols et al., 2001; Van Loveren et al., 2003). Traditional ecotoxicology evaluates contaminant-mediated changes at the population level using apical endpoints such as growth, reproduction, and survival. However, immunotoxicity can occur at lower concentrations than other adverse outcomes, indicating that immune-modulation is a distinct mechanism of action, rather than a side effect of general toxicity (Rehberger et al., 2017). In fact, sublethal impairment of the immune system can remain undetected until the organism is challenged with a pathogen (Brousseau et al., 2016). The functional consequence of impaired immunocompetence is observed if contaminant exposure impairs the ability of the host to resist pathogen infection, and thus increases host susceptibility to disease (Segner et al., 2012b).

Many factors contribute to the vulnerability of the immune system to contaminant insult. Immune organs are dispersed throughout the body, and immune cells exist within virtually every tissue, as well as in circulation, and this broad distribution increases the probability of exposure to contaminants (Ladics et al., 2005). Additionally, many types of immune cells have high metabolic demands due to their rapid proliferation and differentiation (Dean, 2006). High metabolic demand increases the susceptibility of immune cells to contaminants impacting cell energy metabolism, protein synthesis or replication (Dean, 2006). The immune system also relies on communication across immune cell types to mount coordinated responses as well as with the neuroendocrine system; therefore, contaminants that perturb the neuroendocrine system or

chemical messengers may have functional consequences on the immune system (Ladics and Woolhiser, 2006). Finally, all immune cells originate from hematopoietic stem cell populations in the bone marrow, making this tissue a vulnerable target for immunotoxicity (Kaplan et al., 2013). Damage to precursor cell populations can have devastating consequences for the ability of the organism to produce immune cells. The vulnerability of this vital, protective system coupled with increasing contamination of our global ecosystems, highlights the need to understand the consequences of contaminant stress on immune function and disease resistance in exposed organisms.

1.1.1. Aquatic contaminants and disease susceptibility

Inherent abiotic properties of aquatic environments can facilitate the transmission of pathogens. The water serves as a transmission medium that can aid the movement of disease vectors in three-dimensional space, thus increasing local dispersal (Johnson and Paull, 2011). Furthermore, warm and moist conditions can extend the infective stage of some pathogens and prevent desiccation (Ariel et al., 2009; Engering et al., 2013; Martel et al., 2013; Piotrowski et al., 2004). Together, these properties of aquatic systems promote the transmission of pathogens. Freshwater environments are particularly well suited to transmit pathogens due to the reliance of many species on this relatively rare habitat (< 0.08% of the landscape) for survival and reproduction (Dudgeon et al., 2006). As such, freshwater ecosystems increase the probability that susceptible hosts will encounter infectious stages of a pathogen (Johnson and Paull, 2011).

Many anthropogenic contaminants introduced to aquatic systems can affect immune defences and increase disease susceptibility in aquatic organisms. Contaminants with reported immunotoxic effects in aquatic vertebrates include specific types of pesticides (Forson and Storfer, 2006b; Gilbertson et al., 2003; Rohr et al., 2008), polybrominated diphenyl ethers (Cary et al., 2014), endocrine disrupting compounds (reviewed in Casanova-Nakayama et al., 2011), metals (reviewed in Zelikoff, 1993), and polycyclic aromatic hydrocarbons (PAHs) (Carlson et al., 2004a; Martini et al., 2012; McNeill et al., 2012). Contaminants can directly interact with components of the immune system to cause immune impairment (Rehberger et al., 2017). For instance, contaminants can impair immunocompetence through increased apoptosis of immune cells, reducing immune functions such as oxidative burst activity, and interfering with signalling pathways (Bols et al., 2001; Casanova-Nakayama et al., 2011; Köllner et al., 2002; Reynaud and

Deschaux, 2006). Indirect impairment of the immune system can occur by energetic trade-offs and induction of a stress response. Up-regulation of costly biotransformation processes upon exposure to contaminants may leave the organism with less energy for immune defences (Segner et al., 2012b). Similarly, increased endogenous glucocorticoid levels due to contaminant exposure indirectly cause immune impairment through natural suppression of cytokine and antibody production, as well as decreased T cell proliferation (reviewed in Blaustein et al., 2012). By directly or indirectly impairing the immune system, contaminants can alter pathogen-host dynamics, and thus disease outcomes.

Pathogens are a natural part of all ecosystems, and freshwater habitats are among the most anthropogenically disturbed in the world; thus, pathogens and contaminants often co-occur in the aquatic environment (Dudgeon et al., 2006; Johnson and Paull, 2011). Many field studies have demonstrated that contaminant-pathogen interactions coincide with disease outbreaks. For example, dietary exposure to polychlorinated biphenyls (PCBs) suppress critical immune functions (*e.g.* phagocytosis, respiratory burst, T-lymphocyte function, lymphocyte signaling, and lymphocyte counts) in harbor seals (*Phoca vitulina*), which may contribute to the severity of phocine distemper virus outbreaks in Europe and North America (de Swart et al., 1996; Mos et al., 2006). Increased occurrence of external opportunistic infections (*e.g.* fin rot and fin erosion) are also reported in fish exposed to contaminants, such as oil-sands affected water (McNeill et al., 2012; van den Heuvel et al., 2000), pulp and paper mill effluents (Lindesjöö and Thulin, 1990), PCBs and metal contamination (McCain et al., 1992), and legacy persistent organic pollutants in the Puget Sound Basin (Arkoosh et al., 2001). Finally, a study by Rohr et al. (2008) found that atrazine exposure was the best predictor of trematode load in northern leopard frogs (*Lithobates pipiens*) collected from field sites. Taken together, these studies demonstrate that contaminants can increase the frequency and severity of disease outbreaks in aquatic ecosystems.

1.1.2. Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a prominent class of environmental contaminants with a basic structure that consists of two or more fused benzene rings in various conformations (Abdel-Shafy and Mansour, 2016). In general, PAHs have increased stability and hydrophobicity with greater molecular size and angularity (Abdel-Shafy and Mansour, 2016; Lawal, 2017). Environmental input of PAHs can be derived from both natural (*e.g.* forest fires

and volcanoes) and anthropogenic (*e.g.* burning fossil fuels and waste incineration) sources (Finlayson-Pitts and Pitts, 1997; Lawal, 2017; Samanta et al., 2002), with the majority of PAHs released as a result of anthropogenic activities (Lawal, 2017). The anthropogenic release of PAHs combined with their stability and global transport is the reason for their consistent and widespread distribution in the environment (Abdel-Shafy and Mansour, 2016; Bard, 1999).

Within aquatic systems, PAHs partition between the water and the sediment based on their physical and chemical properties. Although the water column and the sediment of aquatic systems can contain measurable concentrations of PAHs, the lipophilicity of these compounds results in the majority of PAHs being associated with the sediment or suspended particulate (Abdel-Shafy and Mansour, 2016). For example, water and sediment samples collected from the Yinma River Basin, China contained between 175 to 325 ng/L and 1000 to 5750 ng/g of total PAHs, respectively (Sun et al., 2017). These properties of PAHs, and thus their distribution within aquatic habitats, influence the primary routes of exposure for aquatic species.

While some PAHs have relatively low toxicity, others, such as benzo[a]pyrene (B[a]P), are compounds with cytotoxic, genotoxic, teratogenic, and carcinogenic effects (reviewed in Lawal, 2017; Reynaud and Deschaux, 2006; USEPA, 2014). In light of their considerable risk to biological systems, the US EPA has designated 16 PAHs as priority pollutants (USEPA, 2014). Additionally, PAHs are potent immunosuppressants with documented effects on cell-mediated immunity, humoral immunity, and host resistance in numerous vertebrate species (Ball and Truskewycz, 2013).

1.1.1.2. *Mechanisms of polycyclic aromatic hydrocarbon-induced immunotoxicity*

The mechanisms and consequences of PAH-induced immunotoxicity are well studied in mammals (reviewed in Abdel-Shafy and Mansour, 2016; Holladay and Smialowicz, 2000; White et al., 1994) and is garnering attention in aquatic vertebrates given increasing PAH contamination of aquatic environments (Desforges et al., 2016). Reynaud and Deschaux (2006) suggest that the mechanisms of PAH-induced immunosuppression are phylogenetically conserved from fish to mammals. Benzo[a]pyrene is regarded as the prototypical PAH and is the most extensively studied member of this group of contaminants (Gelboin, 1980) with studies in the late 1960s that first identified the inducible benzo[a]pyrene hydrolases (now known as cytochrome P450 enzymes), which are responsible for the metabolism of PAHs (reviewed in

Nebert et al., 2013). Currently, B[a]P is recognized as one of the most potent PAHs in terms of carcinogenicity and mutagenicity (Lawal, 2017; Miller and Ramos, 2001).

Although characterization of the metabolic activation pathway for B[a]P is most extensive in mammals, similar pathways exist in numerous aquatic vertebrates (Lavine et al., 2005; Reynaud and Deschaux, 2006). The aryl hydrocarbon receptor (AhR) is expressed in a variety of cell types, where it resides in the cytosol in its inactive form (Beischlag et al., 2008; Petrulis and Perdew, 2002). The inactive AhR exists in a complex with the molecular chaperone heat shock protein 90 (hsp90) and AH interacting protein (AIP) and the cochaperone p23 (Petrulis and Perdew, 2002). Following ligand binding, the AhR undergoes a conformational change that exposes a nuclear localization sequence on the N-terminus (Denison et al., 2011). Upon translocation to the nucleus, binding of the AhR to aryl hydrocarbon receptor nuclear translocator (ARNT) results in the displacement of the co-factors from the protein complex and formation of the heterodimeric ligand:AhR:ARNT complex (Hankinson, 1995). This heterodimeric complex is the high-affinity DNA-binding form, which recognizes a specific sequence in the promoter region of AhR responsive genes called the xenobiotic response element (XRE) (Beischlag et al., 2008; Hankinson, 1995). When the heterodimer binds to the XRE, DNA bending ensues leading to recruitment of coactivators and transcription factors and increased rates of transcription for genes containing the XRE sequence (Beischlag et al., 2008; Hankinson, 1995). The most commonly documented target gene with an XRE is the cytochrome P450 1A (CYP1A), which is a phase I enzyme that aids in the detoxification and elimination of the planar aromatic hydrocarbon ligand and its metabolites (Schrenk, 1998). However, CYP1A also promotes the production of reactive PAH metabolites and reactive oxygen species (ROS) responsible for carcinogenic, mutagenic and immunotoxic properties.

Numerous animal models demonstrate that CYP1A induction contributes to PAH-induced immunotoxicity. The results from knockout experiments in fish with the AhR and CYP1A inhibitor α-naphthoflavone (α-NF) indicate that immunotoxicity occurs due to metabolic activation by CYP1A (Carlson et al., 2004a; Reynaud et al., 2001). For example, intraperitoneal injection of B[a]P in Japanese medaka (*Oryzias latipes*) significantly decreased lymphocyte proliferation, superoxide generation by phagocytes and antibody-forming cells (Carlson et al., 2002). These results were reversed in the presence of α-NF, suggesting that the B[a]P-induced immunosuppression is dependent on the metabolism of the compound (Carlson et al., 2004a).

Furthermore, intraperitoneal injection of 3-methylcholanthrene (3-MC) in common carp (*Cyprinus carpio* L.) increased macrophage respiratory burst activity, which coincided with the peak induction of CYP1A and ethoxy-resorufin O-deethylase (EROD) activity (Reynaud et al., 2002). The ROS production returned to control levels when 3-MC was co-administered with α-NF, again, suggesting that the immunotoxicity was dependent on the metabolism of the PAH by CYP1A (Reynaud et al., 2002). In this study, the authors inferred that the ROS production resulted in immunosuppression, in part based on previous studies demonstrating that PAH-induced oxidative damage caused cell membrane disruption and apoptosis (Davila et al., 1995).

While activation of the AhR and subsequent induction of CYP1A plays a predominant role in PAH-induced immunotoxicity, PAHs can also directly disrupt intracellular calcium homeostasis and impact immune cells. A dose-dependent increase in intracellular calcium concentrations in both lymphocytes and phagocytes was observed in the common carp following exposure to 3-MC (Reynaud et al., 2001; 2003). Mammalian cell lines exposed to B[a]P and 7, 12-dimethylbenz[a]anthracene (DMBA) also exhibited altered calcium homeostasis (Burchiel et al., 1991; Tannheimer et al., 1999). Due to the importance of calcium in signal transduction, prolonged increases in intracellular calcium levels generally precede apoptosis and therefore has the potential to result in immunosuppression when specific immune cells are affected (Davila et al., 1995; Reynaud and Deschaux, 2006).

1.1.1.3. Amphibians exposure to polycyclic aromatic hydrocarbons in the environment

Amphibians that inhabit PAH-contaminated environments exhibit decreased growth, and survival, as well as delayed development (Marquis et al., 2006). Native amphibian populations can be exposed through a number of sources including run-off or aerial deposition PAHs from near-by roadways, industrial activities, oil spills (Bradford et al. 2010; Garrigues et al. 2004). In Canada for example, several studies have examined amphibian species inhabiting the Athabasca oil sands region, where PAHs have been measured at high concentrations in the sediment of wetlands surrounding oil sands extraction facilities (Bilodeau, 2017; Hersikorn and Smits, 2011; Melvin and Trudeau, 2012). Wood frog (*Lithobates sylvaticus*) tadpoles collected near the Athabasca oil sands region had accumulated body burdens over the Canadian Interim Sediment Quality Guidelines for freshwater sediments (Bilodeau, 2017). In another study, wood frog tadpoles caged in a reclaimed wetland had an decreased ratio of triiodothyronine (T_3) to

thyroxine (T_4) and delayed metamorphosis (Hersikorn and Smits, 2011). Taken together, these studies indicate that PAHs can adversely affect amphibians within ecologically relevant exposure scenarios.

Contact with various PAH contaminated environmental media contributes to uptake in amphibians. Dietary uptake and dermal contact with contaminated sediment during feeding and burrowing behaviours account for the majority of PAH exposure in amphibians due to the partitioning of these contaminants into the sediment (Bilodeau, 2017; Linder et al., 2010). Although only a small fraction of PAHs naturally reside in the water column, amphibians can also absorb PAHs from contaminated water through dermal and respiratory surfaces (Bilodeau, 2017). Amphibians can remain in contact with contaminated media (*e.g.* soil, sediment or water) throughout their life-cycle, and the propensity for these contaminants to bioaccumulate can lead to high body burdens. In fact, body burdens in amphibians can exceed environmental concentrations. For example, newt larvae exposed to radiolabeled B[a]P were 200 times more radioactive than the surrounding water within 12 h of exposure (Grinfield et al., 1986). Similarly, newts exposed to sediment containing radiolabeled phenanthrene, pyrene or B[a]P at concentrations ranging from for 2.26 to 3.57 ng/kg had whole body concentrations of 2200, 1450, and 155 µg/kg wet weight, respectively after 24 h of exposure (Garrigues et al., 2004; Sparling, 2010a).

Uptake of PAHs is followed by either incorporation into lipid-rich tissues, bioactivation, or elimination through metabolism and/or excretory pathways (Ball and Truskewycz, 2013; Newman, 2010). Regardless of the route of exposure, accumulated PAHs in amphibians are thought to be metabolized similarly when compared to mammals (Ball and Truskewycz, 2013). This hypothesis is primarily based on the presence of similar components of the metabolic pathway, namely, two isoforms of AhR and ARNT, as well as functional members of the CYP family identified in amphibians (Lavine et al., 2005). Specifially, the African clawed frog (*Xenopus laevis*) is known to express two CYP1A paralogs: CYP1A6 and CYP1A7 and both CYP1As are inducible by TCDD and other AhR ligands (Lavine et al., 2005). However, frog CYP1As are remarkably less responsive to induction by typical AhR ligands (including PAHs), which reflects their relative insensitivity to their toxic effects compared to mammals. For example, the median lethal dose (LD_{50}) for bullfrog tadpoles and adults exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is reported to be greater than 500 µg/kg (Beatty et al.,

1976). Additionally, Jung and Walker (1997) found no effects on mortality at body burdens of TCDD as high as 5.66 and 19.33 μg/kg in American toad eggs and tadpoles, respectively, and 73.72 μg/kg in green frog eggs. Furthermore, concentrations of 200 μg/L of TCDD did not significantly increase mortality in *Xenopus laevis* (Mima, 1997). These results are in sharp contrast to LD_{50} of TCDD for early life-stage fish: 360 ng/kg for red seabream (Yamauchi et al., 2006), 127 ng/kg for brook trout (Johnson et al., 1998), 539 ng/kg for fathead minnow (Elonen et al., 1998). Although amphibians are clearly more resistant to lethality induced by some AhR ligands compared to other taxa, sublethal endpoints such as time to metamorphosis and morphological indices tend to be more sensitive. For instance, while exposure to 30 μg/L TCDD had little effect on survival western chorus frogs (*Pseudacris triseriata*), 3 μg/L TCDD resulted in decreased length and increased time to metamorphosis (Collier et al., 2008). Therefore, assessment of sub-lethal and chronic impacts of PAHs, such as carcinogenicity, genotoxicity, endocrine disruption and immunotoxicity may be more relevant to amphibian populations.

Historically, toxicity studies and ecological risk assessments have overlooked amphibians, and results from fish studies are often extrapolated to amphibians due to the lack of data. However, the results of meta-analyses comparing the relative sensitivity of these taxa remain inconclusive as to whether fish are appropriate surrogates for amphibians. Birge et al. (2010) compared acute early life-stage toxicity data for amphibians to results from similar tests in rainbow trout and found that amphibians were more sensitive for 49% of compounds. In contrast, Weltje et al. (2013) found that fish and amphibian toxicity data were highly correlated and that amphibians were more sensitive than fish in only 7% of studies. As evidenced by the vast differences in lethality of AhR ligands between fish and amphibians, extrapolating findings from the sub-lethal impact of PAHs studies from fish to amphibians may or may not be accurate.

Very few studies have assessed sub-lethal effects of B[a]P on amphibians, and even fewer have evaluated potential mechanisms and consequences of B[a]P-induced immune modulation in this taxon. Martini et al. (2012) found that *X. laevis* tadpoles exposed to B[a]P displayed altered interleukin-1β (IL-1β) and heat shock protein 70 mRNA expression and they suggested that these endpoints could be used as indicators of B[a]P-induced immunotoxicity (Martini et al., 2012). Exposure to B[a]P also increased mast cell number in the liver of adult *Physalaemus cuvieri* and *Leptodactylus fuscus* (Fanali et al., 2018). These cells contribute to inflammatory processes by releasing of histamines, leukotrienes, proteases and eicosanoids into

the tissues (Shakoory et al., 2004). Furthermore, phagocytic activity and efficiency as well as the cellularity and viability of spleen cells in *X. tropicalis* are sensitive indicators of B[a]P and triclosan co-exposure but not exposure to B[a]P alone (Regnault et al., 2016). Taken together, these studies suggest that B[a]P may have adverse effects on components of the amphibian immune system but do not address possible implications for altered immunocompetence and disease susceptibility. For example, Japanese medaka (*Oryzias latipes*) exposed to 0.5 µg/L and 5 µg/L B[a]P at three different life-stages (embryonic, larval, and juvenile stages) had increased viral loads and decreased survival when challenged with betanodavirus (Pannetier et al., 2019).

1.2. Amphibian population status, disease and immunity

1.2.1. Current status of amphibian populations

The International Union for the Conservation of Nature (IUCN) Red List of Threatened Species lists 2,135 of a total 6,609 species of amphibians globally as extinct or at some level of risk (IUCN, 2017). Among the various taxa, amphibians are more threatened and rapidly declining than either birds or mammals (Stuart et al., 2004). Amphibian populations likely began declining earlier in the 20th century, but the magnitude and scope were not recognized until the 1980s (Collins and Storfer, 2003). Without long-term data sets, it is difficult to distinguish between large annual variations and true population declines (Pechmann et al., 1991). Nearly 23% of amphibians species have insufficient long-term monitoring data to assess their level of risk (IUCN, 2017). Thus, as research efforts intensify and data is generated for data-deficient species, more populations are likely to be identified as declining.

The value of amphibians to ecosystems and humans provide compelling reasons for their conservation. Amphibians are often cited as effective and sensitive "bioindicators" of environmental change due to their permeable skin, climate-sensitive breeding cycles, potentially high rates of contaminant bioaccumulation, and the fact that their unique life-history renders them susceptible to environmental stress in either the aquatic or terrestrial life-stages (Blaustein and Bancroft, 2007). Amphibians have diverse and significant roles in ecosystem services. Evidence suggests that the loss of amphibians from aquatic ecosystems can alter nutrient cycling, primary production, algal community structure, and food chains (Hocking and Babbit, 2014; Whiles et al., 2006). Amphibians are vital predator and prey species in the ecosystem, and their numbers and biomass can, in some habitats, exceed that of all other vertebrates (Burton and

Likens, 1975). In terms of human medicine, amphibian skin secretions contain novel bioactive compounds with analgesic and anti-microbial properties (Gomes et al., 2007). Collectively, these characteristics highlight that declining amphibian populations could have serious repercussions for the entire ecosystem as well as humans.

Amphibian declines have been attributed to a range of threats. The major threats to amphibian populations include habitat destruction, invasive species, ultraviolet (UV) radiation, climate change, environmental contaminants and diseases (Blaustein et al., 2012; Rollins-Smith and Smits, 2005). The factors responsible for the declines may vary between regions, species, life-stages and even populations of the same species (Blaustein and Bancroft, 2007). Furthermore, synergies between multiple factors likely accelerate the rate of these declines now and in the future (Hof et al. 2011).

1.2.2. Pathogens and disease susceptibility in amphibians

Pathogens can have dramatic impacts on amphibian populations and increasing reports of disease outbreaks around the world have drawn attention to this potential driving factor in population declines. Amphibians are hosts for numerous microparasites including viruses, bacteria and fungi, as well as macroparasites such as nematodes, flatworms and mites (Densmore and Green, 2007). These pathogens can infect amphibians at various stages of development, and disease outbreaks have been recorded in pristine and anthropogenically-disturbed habitats (Carey et al., 1999). Of primary concern are emerging infectious diseases, which are defined as pathogenic agents recently isolated from a new host, increased in geographic range or prevalence, or genetically distinct from other known species of the pathogen (Daszak et al., 2003; 2000; Engering et al., 2013). Chytrid fungus and ranaviruses are considered emerging pathogens that have caused the greatest devastation to amphibian populations worldwide (Chinchar, 2002; Daszak et al., 2003; 2000).

Of the fungi in the class Chytridiomycetes, only two members are known to infect vertebrates; *Batrachochytrium salamandrivorans* (Bsal) primarily infects salamanders, whereas *Batrachochytrium dendrobatidis* (Bd) infects amphibians more broadly (James et al., 2015). These fungi cause the disease chytridiomycosis, which is responsible for mortality events on every continent that contains amphibians (Fisher et al., 2009). Furthermore, there is increasing evidence that chytridiomycosis is directly related to amphibian population declines, extirpations

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and extinctions events around the world (reviewed in Daszak et al., 2003; Fisher et al., 2009). Although these fungal pathogens have a dramatic impact on amphibian biodiversity, the host-pathogen-environmental interactions involved in these disease outbreaks are poorly understood.

Members of the *Ranavirus* genus have been reported in every continent, elevation and latitude containing amphibians and can infect most families of Anurans and Caudates (Carey et al., 2003; Daszak et al., 2003). Ranaviruses are widely distributed and associated with amphibian mortality events around the world (reviewed in Duffus et al., 2015; Gray et al., 2009). Die-off events are characterized by rapid onset and high mortality, generally occurring in late tadpole life-stages or newly metamorphed individuals during the mid-to-late summer months (Brunner et al., 2015). These characteristics are exemplified by a recent report from Maine, USA, where more than 200,000 wood frog tadpoles died in less than 24 h, resulting in the complete loss of an age class (Wheelwright et al., 2014).

1.2.3. Ontogeny of immunity

Similar to most other vertebrates, amphibians possess both innate and adaptive branches of the immune system (Carey et al., 1999) and immune defences present in early life-stages are considered immature as compared to those in adults (Cramp and Franklin, 2018). However, primary and secondary lymphoid organs in amphibians do not necessarily fulfill the same roles as they do in mammals. Adult amphibians have both thymus and bone marrow, while tadpoles lack bone marrow and although bone marrow begins to appear late in ontogeny, it is not involved in the hematopoietic system until completed metamorphosis (Colombo et al., 2015). The bone marrow present in adult amphibians is not the site of B-cell differentiation – it is less specialized and supports the differentiation of neutrophils and macrophage precursors (Colombo et al., 2015; Grayfer and Robert, 2013). B-lymphocyte differentiation occurs in the spleen and the tadpole liver (Hadji-Azimi et al., 1990). Secondary lymphoid organs allow for antigen recognition and subsequent antigen-driven proliferation of T- and B-lymphocytes. Amphibian secondary lymphoid organs are the spleen and the gut-associated lymphoid tissue (GALT) (Colombo et al., 2015).

During metamorphosis, there are drastic changes in physiology, external morphology, organ/tissue organization and molecular signalling across many organ systems, including the

immune system. Throughout development, different components of the immune system are being remodelled, removed, replaced or expanded. This complex process is well characterized in *X. laevis* (Robert and Ohta, 2009) and depicted in Figure 1.1. The cellular components and overall function of the amphibian immune system are transient over development, and this is reflected in the ontogeny of critical immune organs such as the thymus, spleen and liver. Generally, as immune organs develop there is an increase in function beginning in pre-metamorphosis (according to Nieuwkoop and Faber; NF stage 47 or 48) until the climax of metamorphosis (NF stage 60), followed by a striking decrease of larval T-cells and B-cells. During the involution of these immune organs at the climax of metamorphosis, there is a 90-95% loss of lymphocytes in the thymus, 40% loss in the liver and 50% loss in the spleen (Flajnik et al., 1987; Rollins-Smith, 1998). This developmental pattern results in a natural period of immune suppression between pro-metamorphosis and completed metamorphosis. This immune suppression is postulated to be a consequence of the removal of non-essential lymphocytes. Loss of these lymphocytes likely represents a protective mechanism evoked during tadpole development to prevent the destruction of newly synthesized adult-specific molecules (Rollins-Smith, 1998). As metamorphosis nears completion, lymphocyte population expansion occurs within the immune system as the amphibians enter the adult form. Furthermore, as the primary and secondary lymphoid organs increase in function, there is a decreased reliance on the innate immune system for pathogen defence with increasing development and activation of the adaptive immune system.

The major hormones that drive metamorphosis are intimately connected to the development of the immune system. For example, immune system function increases from embryo to tadpole pro-metamorphosis and then sharply declines as corticosteroid hormone concentrations increase during metamorphosis (Flajnik et al. 1987). The naturally elevated levels of corticosteroid hormone during metamorphosis results in apoptosis of most larval lymphocytes (Barker et al., 1997; Rollins-Smith, 1998). Similarly, thyroid hormone (TH) also plays an integral role in the morphological, physiological and immunological changes occurring during metamorphosis. Thyroid hormones are ultimately responsible for tissue remodelling and cellular apoptosis (*e.g.* limb development, tail resorption and intestinal reorganization). However, THs are also involved in immune system development. Suppression of TH production in tadpoles (via exposure to sodium perchlorate) results in a decreased proliferation of T- and B-lymphocytes

(Rollins-Smith and Blair, 1990), potentially compromising development and function of the adaptive immune system. Thyroid hormones are critical for normal metamorphosis and adaptive
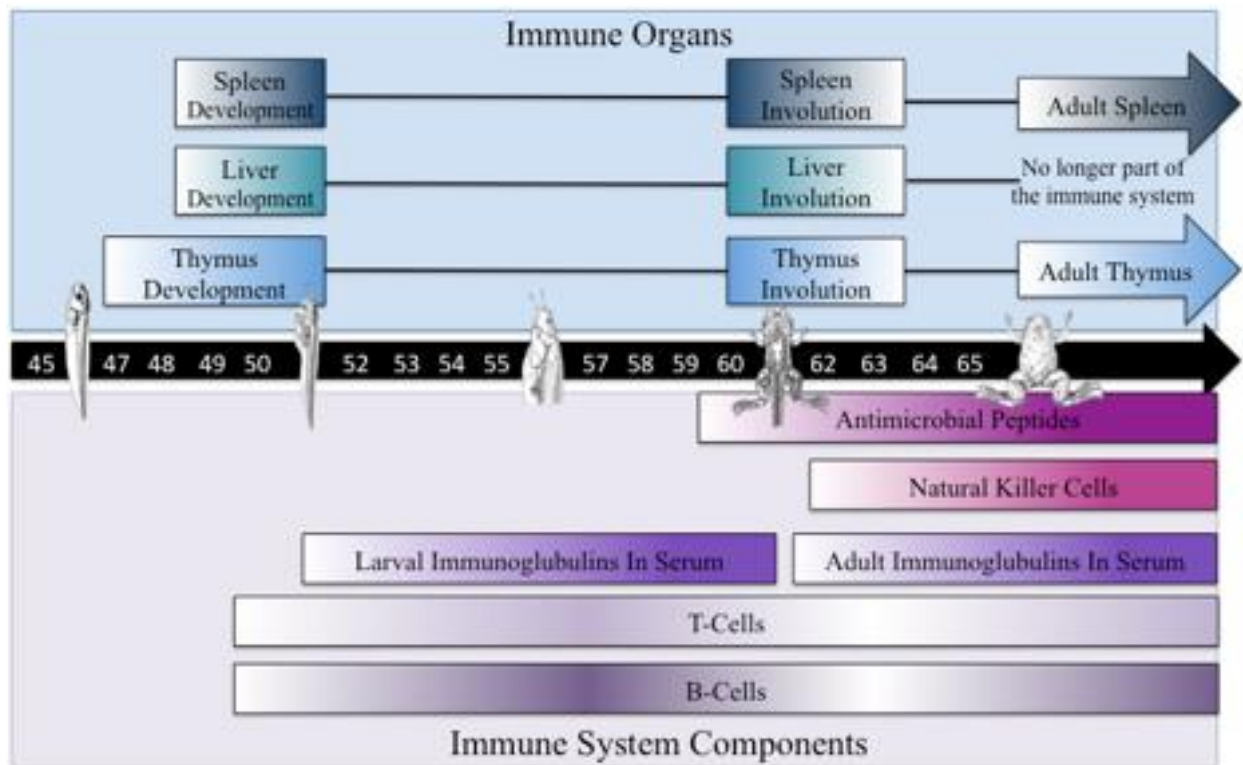
Figure 1.1. Summary of the development of key immune organs and components of the immune system in *Xenopus laevis*. Gradients in colour represent increases or decreases in function or cell number.

immune system development; however, the relationship between thyroid homeostasis and innate immune system development is not well understood. Due to the reliance of early tadpole life-stage on the innate immune system, studies designed to obtain a better understanding of the factors impacting its development are warranted.

1.2.4. Disease susceptibility across life-stages

The dynamic nature of amphibian development leads to different maturation states of the immune system across life-stages, which has implications for resistance to disease. Immune function gradually increases after the natural period of immune suppression, but full functionality may not be acquired until a year post-metamorphosis (Rollins-Smith, 1998). The comparatively immunodeficient larval and juvenile life-stages may be at greater risk of infection due to the immaturity of the immune system (Rollins-Smith, 2016). The higher incidence of disease in amphibians during the embryonic, larval and juvenile life-stages (Gray et al., 2009; Miller et al., 2011) exemplifies the compromised immune responses in these life-stages. In a study by Gantress et al. (2003a), infection of *X. laevis* adults with ranavirus resulted in approximately 10% mortality, whereas similar infections in NF stage 55-56 tadpoles results in > 50% mortality. These stage-specific differences in susceptibility are attributed to reduced and delayed innate immune responses in the tadpole life-stage (De Jesús Andino et al., 2012). Although the stage of development at the time of ranavirus infection influences the probability of survival, what is considered the most sensitive stage varies by species (Haislip et al., 2011). Stage-specific susceptibility to a pathogen was also reported in Pacific chorus frogs (*Pseudacris regilla*), where trematode infection during pre-limb and early limb development resulted in increased mortality and malformation as compared to infection during completed limb development and adults (Johnson et al., 2011). It is important to note that the majority of studies examining the effects of pathogens (and other environmental stressors) on amphibians often evaluate a single stage of development, and some then use results to extrapolate responses across amphibian life-stages. However, the limited number of studies that have examined different life-stages suggest that such extrapolations should be done cautiously, if at all. These extrapolations are further complicated by the addition of multiple stressors (*e.g.* contaminants), which may also alter immune function and disease susceptibility in a stage-specific manner.

1.3. Challenges and opportunities in assessing immunotoxicity in amphibians

The immune system is present in some form (*e.g.* as chemical mediators, barrier epithelia, or immune cells) in most tissues and organs. Regardless of the route of uptake, these immune system components can be exposed to contaminants, and together with a pathogen challenge, this exposure may result in levels of morbidity and mortality greater than either stressor alone. In addition, changes in components of the immune system generally precede such adverse outcomes and can be used as early indicators in the evaluation of altered immunocompetence. For humans and mammals, there are established frameworks for immunotoxicology risk assessment, including structured experimental approaches and assays (Holsapple, 2005). No comparable framework is available for assessing immunotoxicity in non-mammalian vertebrates, although a piscine tiered immunotesting strategy was recently proposed. Segner et al. (2012b) recommended that in fish, immunological assessment should include evaluation of cellular and humoral components of immune defences, as well as the structure of lymphoid tissues and organs, and immune challenge assays. The rationale for studying different levels of biological organization and immune system integration and their use (or lack of) in amphibian immunotoxicity studies is discussed below.

Selecting appropriate immune-related endpoints for evaluating contaminant-induced immunotoxicity in amphibians poses many challenges. First, although not specific to amphibians, the immunocompetence of an organism is more than the sum of its parts. Measuring a single endpoint is insufficient to evaluate the immunocompetence of the whole organism due to the capacity of the immune system to compensate for some perturbances (Segner et al., 2012b). Second, methods are limited for most aquatic species and depend on the availability of tools or protocols. Endpoints that require sampling blood or removal of specific immune organs (such as the spleen) are difficult due to the small size of many amphibians, particularly larval stages. Development of clones, antibodies, and sequence information for *X. laevis* and *X. tropicalis* through immunological research (De Jesús Andino et al., 2012; Morales et al., 2010; Robert and Ohta, 2009) has facilitated assessment of immunotoxic effects but these tools are usually specific to these laboratory model species and are not effective for ecologically relevant species.

There are a limited number of assays available for assessing the status of the amphibian immune system. Leukocytes, or white blood cells, are an essential part of the immune system and there are five different cell types: lymphocytes, neutrophils, eosinophils, monocytes and

basophils (Allender and Fry, 2008; Davis et al., 2010; 2008). Changes in the proportion of various types of leukocytes can be an indication of stress as well as altered immune function in aquatic species (Davis et al., 2008). In amphibians, changes in leukocyte profiles have been used to evaluate the immune response following exposure to anesthetics (Sears et al., 2013), the stress hormone corticosterone (Burraco et al., 2017), and when infected with *Batrachochytrium dendrobatidis* (Woodhams et al., 2007). The bacterial killing assay (BKA) is reflective of the capacity of blood to impede a potential pathogen, the most common one used being *Escherichia coli*, via phagocytes, antibodies, lysozymes and complement (Millet et al., 2007; Venesky et al., 2012). In southern leopard frog tadpoles, the BKA was used to determine the immunological consequences of dietary protein restriction (Venesky et al., 2012). The phytohemagglutinin (PHA) challenge is a delayed-type hypersensitivity response that utilizes a lectin to stimulate localized inflammation and reflects an organism's capacity to mount an immune response. When PHA is injected subcutaneously, it results in a non-specific immune challenge and the localized inflammation is a direct result of infiltration by T-cells and the recruitment and activation of cytokines, macrophages, natural killer cells and cytotoxic T-cells (Demas et al., 2011). By quantifying the level of inflammation that occurs at the site of PHA injection (*e.g.* by measuring skin thickness before and after injection) it is possible to determine the relative immunocompetence of an individual; greater inflammation indicates a stronger T cell-mediated immune response (Vinkler et al., 2010). The PHA assay has been performed to assess the effects of environmental stressors (*e.g.* pathogen, dietary, cortisol, organochlorine pesticides) on immunocompetence in larval (Venesky et al., 2012) and adult (de Assis et al., 2015; Fites et al., 2014; Gilbertson et al., 2003) amphibians. Lymphocyte proliferation is an essential response to a pathogen challenge, and impairment of this function by a contaminant is used as an indication of immunotoxicity (Demas et al., 2011). *In vitro* lymphocyte proliferation assays following *in vivo* exposure to a contaminant has previously been used to assess the consequences of exposure to atrazine, metribuzin, aldicarb, endosulfan, lindane and dieldrin in leopard frogs (Christin et al., 2003). Finally, immune organ size and histology are also used in amphibian studies to provide information on the targets of immunotoxic contaminants, and the relationship between organ damage and observed adverse outcomes. Examination of the size and histopathology of the liver, kidney, spleen, and/or skin, have been used to visualize damage caused by uranium tailings ponds and B[a]P exposure or infectious agents such as ranavirus and chytrid fungus in

amphibians (Grayfer et al., 2014b; Longcore et al., 1999; Marques et al., 2009; Regnault et al., 2014). Overall, there are a few options for measuring immune responses in amphibian species, and tissue/body size requirements that prevent many of these assays from being used on small species and/or early life-stage amphibians.

An additional consideration when designing studies that assess the amphibian immune system is justifying the stage of development at the time of exposure and/or the endpoints used in relation to that stage of development. For instance, anti-microbial peptides do not appear until NF stage 59 so attempting to assess contaminant-induced changes in this endpoint before this stage of development would be a fruitless endeavour (Reilly et al., 1994). Parameters chosen to assess contaminant-induced immunotoxicity must consider the dynamic development of the amphibian immune system.

Immunological methods and approaches utilized for small-bodied fish can be adapted and used to further immunotoxicological research in amphibians. Immune-related endpoints commonly evaluated in various fish species include expression of immune-related genes, somatic indices of immune organs as well as organ histology, characterizing leukocyte populations, quantifying humoral factors from the innate (lysozymes, C-reactive proteins, complement) and the adaptive (immunoglobulin M; IgM) immune systems, as well as functional assays such as phagocytic activity, proliferative responses and oxidative burst assays (reviewed in Köllner et al., 2002; Rehberger et al., 2017; Segner et al., 2012b). Despite this diverse list of immunological assays available for fish, the body of literature for this taxa has been criticized for a bias towards examining immunosuppression without considering the possibility of allergic or autoimmune reactions, preferential assessment of the innate over the adaptive immune system, not providing a rationale for the immune endpoints chosen, and an inability to relate changes in immune endpoints to whole organism immunocompetence (Rehberger et al., 2017; Segner et al., 2012b). Host resistance assays are often performed by first exposing the organism to a contaminant, followed by activation with a pathogen or pathogen-associated molecular pattern (*e.g.* lipopolysaccharide; LPS) (Köllner et al., 2002; Segner et al., 2012b); therefore, the assay can be used to inform on whether a contaminant exposure impairs the ability of the host to mount an immune response and resist infection. For example, exposure of rainbow trout to 17β-estradiol under resting immune conditions showed no impact on the expression of members of the complement system relative to the control treatment (Wenger et al., 2011). Impairment of the

immune response by 17β-estradiol was only observable upon challenge of the immune system with a bacteria (Wenger et al., 2011); thus, the immunotoxicity of the compound would have been underestimated without the host resistance assay. Furthermore, the host resistance assay also confirmed that the rainbow trout exposed to 17β-estradiol were more likely to succumb to the bacterial infection (Wenger et al., 2011). Currently, most immunotoxicological studies with amphibians focus on 1) measuring immune-related endpoints following contaminant exposure (Gilbertson et al., 2003; Martini et al., 2012) or 2) attempting to correlate contaminant exposure in their natural habitat to pathogen load/mortality (Rohr et al., 2008). Although these studies are essential in the overall understanding of contaminant-induced changes in immune-related parameters, they do not assess the function of the immune system or changes in disease susceptibility. Therefore, host resistance assays would be a valuable tool to add to the repertoire of standardized amphibian immune assays.

1.4. Project rationale and research objectives

Understanding how aquatic contaminants influence immune parameters and immunocompetence in amphibians is critical since immune impairment by contaminant exposure is hypothesized to exacerbate disease-driven amphibian population decline (Blaustein et al., 2012). Furthermore, immunotoxicity is increasingly recognized and a specific mechanism of action for many contaminants present in amphibian habitats. Despite this increasing awareness, risk assessments involving aquatic species often overlook immunotoxicity due to the complexity of the immune system and/or the lack of information relating immune parameters to observed immunocompetence in these organisms (Segner et al., 2012b).

The overall goal of my thesis research was to gain a better understanding of developmental and contaminant-induced changes in the immune system of amphibians, and ultimately how exposure modulates the response to immune stimulation. Research was focused on establishing novel endpoints to evaluate immune status in larval and juvenile amphibians, determining how short-term, waterborne exposure to B[a]P influences immune parameters and the ability of amphibians to respond to immune stimulation, and identifying potential mechanisms of B[a]P immunotoxicity using a *Xenopus*-specific cell line. Studies were conducted with the standard laboratory amphibian test species, *Xenopus laevis*, because (1) it has been used as a model for studies of immunity (Robert, 2016; Robert and Ohta, 2009) as well as of

immunotoxicity (Christin et al., 2004; De Jesús Andino et al., 2017; Sifkarovski et al., 2014) in amphibians, (2) hormone-induced breeding can provide embryos year-round, and (3) the time it takes for development from early larval stages to metamorphic climax and early juvenile life stage is relatively short. The immunotoxicant and environmental contaminant, B[a]P, was chosen for both *in vivo* and *in vitro* exposures as the immunotoxicity of B[a]P has been extensively studied in mammalian systems (White et al., 1994) and B[a]P exposure is also reported to suppress innate, humoral, and cell-mediated immunity in fish (Carlson et al., 2004a; 2002; Phalen et al., 2014).

The specific objectives of this thesis research were as follows:

1) Determine whether rising thyroid hormone levels during amphibian larval development play a role in regulating the development of the innate immune system.

2) Identify the most sensitive larval developmental stage (embryo, pre-metamorphosis, or pro-metamorphosis) to short-term waterborne exposure to B[a]P.

3) Determine the ability of *Xenopus laevis* to respond to an immune stimulation through the activation of the immune system.

4) Determine if the ability to respond to immune stimulation in *Xenopus laevis* is compromised by previous short-term waterborne B[a]P exposure.

5) Identify molecular mechanisms by which B[a]P could impact the response to immune stimulation.

CHAPTER 2:
DEVELOPMENTAL EXPRESSION PROFILES AND THYROIDAL REGULATION OF CYTOKINES DURING METAMORPHOSIS IN THE AMPHIBIAN *XENOPUS LAEVIS*

Preface

The objective of Chapter 2 was to characterize the expression of key pro-inflammatory cytokines throughout amphibian development and determine the impacts of thyroidal modulation on their expression. Despite evidence that the transitioning immune system of tadpoles during metamorphosis is influenced by thyroid hormone, there have been no studies directly evaluating the importance of thyroidal status in cytokine expression during development. This study was conducted to provide better understanding of the regulation of immune genes during amphibian early life stages and to develop tools for assessing cytokine expression in evaluating mechanisms of immunomodulation in amphibians.

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Author contributions:

Melanie Gallant (University of Saskatchewan) helped design and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript

Natacha Hogan (University of Saskatchewan) provided scientific input and guidance on the design of the experiment, supervised Melanie Gallant, commented on and edited the chapter, and obtained funding for the research.

Abstract

Early life-stages of amphibians rely on the innate immune system for defence against pathogens. While thyroid hormones (TH) are critical for metamorphosis and later development of the adaptive immune system, the role of TH in innate immune system development is less clear. An integral part of the innate immune response are pro-inflammatory cytokines – effector molecules that allow communication between components of the immune system. The objective of this study was to characterize the expression of key pro-inflammatory cytokines, tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β) and interferon-γ (IFN-γ), throughout amphibian development and determine the impacts of thyroidal modulation on their expression. *Xenopus laevis* were sampled at various stages of development encompassing early embryogenesis to late pro-metamorphosis, and cytokine expression was measured by real-time PCR. Expression of TNFα and IL-1β varied over development, increasing with developmental stage, while IFN-γ remained relatively stable. Functionally athyroid, pre-metamorphic tadpoles were exposed to thyroxine (0.5 and 2 µg/L) or sodium perchlorate (125 and 500 µg/L) for seven days. Tadpoles demonstrated characteristic responses of advanced development with thyroxine exposure and delayed development and increased thyroid gland area and follicular cell height with sodium perchlorate exposure. Exposure to thyroxine for two days resulted in decreased expression of IL-1β in tadpole trunks. Sodium perchlorate had negligible effects on cytokine expression. Overall, these results demonstrate that cytokine transcript levels vary with stage of tadpole development but that their ontogenic regulation is not likely exclusively influenced by thyroid status. Understanding the direct and indirect effects of altered hormone status may provide insight into potential mechanisms of altered immune function during amphibian development.

2.1. Introduction

Amphibian metamorphosis is one of the most dramatic examples of thyroid hormone (TH)-dependent development among vertebrates. Metamorphosis is mediated by TH and is characterized by three distinctive stages: (1) pre-metamorphosis when tadpoles have no measurable circulating THs, (2) pro-metamorphosis when production of TH begins and stimulates hindlimb growth, and (3) metamorphic climax when most tissues are undergoing transformation, and a tadpole morphs into a frog (Brown and Cai, 2007; Shi, 2000). Corticosteroids (CS) also synergize with TH at target tissues to promote morphogenesis (Denver, 2009). In amphibians, these hormones have been studied mainly with regard to their classical function in development and metamorphosis. In contrast, there is scarce information about how thyroid status influences the development of the immune system in amphibians – although this relationship has been recognized in other vertebrates (De Vito et al., 2011; Quesada-García et al., 2014).

Amphibian embryos and tadpoles are free-living organisms that are exposed to numerous pathogens in their aquatic environment before their lymphoid organs have matured and adaptive immunity has developed (Flajnik et al., 1987). Components of both the innate and adaptive immune systems are present during tadpole development; however, the tadpole adaptive immune response is less developed relative to that of adults. For example, *Xenopus laevis* adults can reject skin allografts from individuals that only differ in their minor histocompatibility complexes, whereas tadpoles cannot (DiMarzo and Cohen, 1982). Given that tadpoles have an under-developed adaptive immune response, the innate immune system is of increased importance for defense against pathogens during these early life-stages. During early pro-metamorphosis, rising levels of circulating TH and CS are responsible for the tissue remodelling and cellular apoptosis of some larval immune features (Rollins-Smith et al., 1997) while driving development of the adaptive immune system, specifically the expansion of adult lymphocytes (Rollins-Smith and Blair, 1990). The action of TH on adaptive immune system development is supported by the fact that suppression of TH production in tadpoles (via exposure to sodium perchlorate) decreases the proliferation of T and B-lymphocytes (Rollins-Smith and Blair, 1990), which are critical for the adaptive immune response in adult frogs. Although tadpole and adult amphibians are immunocompetent, the possible immunomodulatory role of THs in the innate immune system of tadpoles remains unclear.

During larval development, the tadpole immune response primarily relies on the action of cytokines – effector molecules that allow communication between various components of the immune system distributed throughout the organism (Segner et al., 2012a). Pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα), interleukin-1 (IL-1) family and interferon gamma (IFN-γ) play a key role in the innate immune response. Tumor necrosis factor-α is among the first cytokines produced in an inflammatory response and is rapidly up-regulated in adult *X. laevis* in response to bacterial and viral infections (De Jesús Andino et al., 2012; Morales et al., 2010; Swain et al., 2008). IL-1β has been detected in *X. laevis* adults exposed to a simulated bacterial infection (Zou et al., 2000), and in adults and tadpoles exposed to Ranavirus (De Jesús Andino et al., 2012). Up-regulation of IL-1β is responsible for the acute phase response and the associated acute protein synthesis (reviewed in Kaiser et al., 2004). Finally, IFN-γ can reduce viral replication and is induced in *X. laevis* during viral infection (De Jesús Andino et al., 2012). Notably, IFN-γ can act synergistically with TNFα to inhibit viral gene expression to a greater extent than either cytokine alone (reviewed in Bartee et al., 2008). Cytokines are often assessed together to evaluate innate immunity, as this is a better indicator of the inflammatory response compared to the measurement of individual cytokines (Cheng et al., 2014; De Jesús Andino et al., 2012; Holopainen et al., 2012). Due to their various roles in the pro-inflammatory immune response, proper regulation of cytokines is imperative in mounting an effective and coordinated immune response. Changes in the expression of cytokines are used as indicators of immune dysregulation in adult and larval fish exposed to perfluorooctane sulfonate (Fang et al., 2013), benzo[a]pyrene (Hur et al., 2013) and cypermethrin (Jin et al., 2011), as well as in tadpoles exposed to benzo[a]pyrene (Martini et al., 2012). In fact, exposure to such chemicals and other thyroid disrupting compounds may have implications for disease susceptibility in fish as well as amphibians. Early tadpole exposure to polybrominated diphenyl ethers (PBDE), a known thyroid disrupting compound, results in long-term immunosuppression that is detectable in juvenile frogs, although the mechanism of action for PBDE immunotoxicity remains unclear (Cary et al., 2014). A better understanding of the regulation of immune genes during amphibian early life stages would provide a basis to use cytokine expression in evaluating mechanisms of immunomodulation in amphibians, particularly how environmentally-induced changes (*e.g.* by pathogens, chemicals, predation) in immune parameters translate into altered immune function.

Despite evidence that the transitioning immune system of tadpoles during metamorphosis is influenced by TH (reviewed in Rollins-Smith, 1998), there have been no studies directly evaluating the importance of thyroidal status in cytokine expression during development. The ability to stimulate or disrupt the process of metamorphosis with exogenous exposure to (anti)thyroidal compounds makes the tadpole an ideal model. In the present study, we used *X. laevis* as it is considered a standard species for non-mammalian immunological studies and there is significant knowledge regarding molecular mechanisms of TH-driven metamorphosis (Robert and Ohta, 2009). The first aim of this study was to characterize the expression of the pro-inflammatory cytokines, TNFα, IL-1β and IFN-γ, over development. Secondly, we manipulated the thyroid status of tadpoles using waterborne exposures to thyroxine (T_4) and sodium perchlorate to examine the potential role of TH in regulating the expression of these pro-inflammatory cytokines. Histopathology of the thyroid gland and developmental stage were used to confirm a positive response to treatment with the thyroid-modulating compounds.

2.2. Material and Methods

2.2.1. Breeding and rearing of *Xenopus laevis*

Sexually mature adult *X. laevis* were purchased from Xenopus 1 (Boreal Labs, St. Catharines, ON, Canada) and housed at $18 \pm 1°C$ water temperature and a 12 h light:12 h dark photoperiod in the Aquatic Toxicology Research Facility (ATRF) located in the Toxicology Centre, University of Saskatchewan, Saskatoon, SK. Three adult male and female pairs were acclimated to $22 \pm 1°C$ and mating was induced by injecting human chorionic gonadotropin (hCG, Sigma-Aldrich, St. Louis, MN, United States) into the dorsal lymph sac in males and females. A 25 IU priming dose of hCG dissolved in sterilized PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) was first injected into the dorsal lymph sacs of each individual with a second dose of hCG administered approximately 24 h later (males 250 IU hCG and females 500 IU; ASTM, 2012). Breeding pairs were placed in aquaria maintained at $22 \pm 1°C$ and left to spawn overnight. Adults were then separated and removed from the breeding tanks following egg deposition.

De-jellying embryos and embryo selection were done as described in "Standard guide for conducting the Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX)" (ASTM, 2012). Embryos from a combination of breeding pairs were used in each experiment to avoid a possible clutch

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effect in the obtained results. Up to 50 selected embryos were placed in each egg cup, a vertical PVC pipe with two holes cut below a 100 μm Teflon mesh insert, to incubate in facility water maintained at 22 ± 1°C for 4 days. At 5 days post fertilization, animals were transferred to 10 L aquaria and fed daily *ad libitum* commercially available Sera Micron (Sera, Heinsberg, Germany). A 16 h light: 8 h dark cycle was maintained where lights came on at 6h00. A 75% water change was performed daily and water quality was monitored (pH 7-7.5, dissolved oxygen > 90%, temperature = 20-22°C). The care and treatment of animals used in this study were approved by the University of Saskatchewan's Animal Research Ethics Board (protocol no. 20120062), and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

2.2.2. Sample collection for developmental profile

Embryos/tadpoles were randomly sampled at specific stages based on the *Xenopus* normal table of development (Nieuwkoop and Faber, 1956). Stages sampled represent early (NF stage 9) and late (NF stage 20) embryogenesis; early (NF stage 35), mid (NF stage 44) and late (NF stage 51) pre-metamorphosis and pro-metamorphosis (NF stage 56). All sampling for a specific stage was done on the same day.

Tadpoles were euthanized by immersing them in a buffered 0.1% tricaine methanesulfonate (MS-222) solution until movement had stopped for one minute. Due to limited amounts of tissue per individual, embryos (NF stages 9 and 20), as well as pre-metamorphic tadpoles (NF stages 35 and 46) were pooled (n = 5, 4, 3 and 3 per sample, respectively) to ensure sufficient material for RNA isolation. NF stage 56 tadpoles were sub-sectioned into head, trunk and tail by cross-sectioning between the eyes and the heart and immediately after the gut. mRNA expression was analyzed in the trunk, where the majority of the immune components are located. Eight samples (pooled embryos or trunk sections) were obtained from each stage of development for gene expression analysis.

2.2.3. Thyroxine and sodium perchlorate exposures

To assess the role of TH in modulating cytokine expression in early life stages of *X. laevis*, NF stage 51 tadpoles were exposed to either thyroxine (Sigma-Aldrich; L-Thyroxine sodium salt pentahydrate; T_4; 0, 0.5 μg/L [0.64 nM] and 2 μg/L [2.57 nM]) or sodium

perchlorate (Sigma-Aldrich; 0, 125 µg/L [1.02 µM] and 500 µg/L [4.08 µM]) for 7 days.

Tadpoles at NF stage 51 are considered functionally athyroid and precocious metamorphosis can be induced by exposure to exogenous T_4, whereas exposure to sodium perchlorate can delay metamorphosis by inhibiting TH production (OECD, 2007). The solvent control treatment for the T_4 exposure was 0.1 M NaOH (final solvent concentration 0.007% v/v) while sodium perchlorate was dissolved in ATRF facility water. Exposures were conducted in triplicate tanks with 7 L of water and 12 tadpoles/tank (T_4 exposure) or 16 tadpoles/tank (sodium perchlorate exposure). Daily water changes were performed during the exposures to ensure water quality (monitored daily; pH 7-7.5, dissolved oxygen > 90%, temperature = $21 \pm 0.5°C$), and tanks were subsequently re-dosed to maintain target nominal concentrations.

Sampling was conducted on days 2 and 7 of the exposure, with half of the tadpoles randomly sampled each day (T_4: 18 tadpoles/treatment/day; sodium perchlorate: 24 tadpoles/treatment/day). Tadpoles were euthanized, weighed (to the nearest 0.01 g), length taken (to the nearest 0.1 cm) and staged according to Nieuwkoop and Faber (1956). Individuals (n=10/treatment) were trimmed to trunk (as described in section 2.2), flash frozen and stored at -80°C until gene expression analysis. Three tadpoles from the solvent control treatment and high concentration groups were randomly selected from those sampled at the 7 d time-point and preserved in 10% neutral buffered formalin for histological analysis of the thyroid gland.

2.2.4. Gene expression analysis

Total mRNA was obtained using the Qiagen Rneasy Mini Kit as described by the manufacturer (Qiagen, Mississauga, ON, Canada). Tissues were disrupted using an Omni Bead Ruptor (Omni International, Kennesaw, GA, USA) at 40 Hz for 2 min. Isolated RNA was re-suspended in RNAse-free water, and DNAse treated using the Turbo DNA Free Kit (Ambion, Burlington, ON, Canada) as directed by the manufacturer. The RNA concentration was determined by NanoDrop-1000 spectrophotometer (Thermo Scientific, Waltham, MA, United States) and RNA quality was verified on a 1% agarose gel. Total cDNA was prepared from 1 µg of total RNA using the iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA, United States). Samples were diluted 10-fold prior to real-time polymerase chain reaction (qPCR).

The expression of individual gene targets was determined by qPCR on a CFX96 Real-time C1000 Thermal Cycler (Bio-Rad Laboratories). Gene-specific primers (Invitrogen,

Carlsbad, CA, United States) for targets IL-1β, TNFα and IFN-γ as well as elongation factor 1 α (EF-1α), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S rRNA, which were used as reference genes, were designed in-house or sourced from the literature (Table 2.1). Specificity of each primer set was verified by sequencing the single amplicon at Plant Biotechnology Institute of Canada (National Research Council, Saskatoon, Canada). Primer sets were optimized for the cDNA input resulting in minimal C_t, optimal annealing temperature and a single sequence-specific peak in the melt curve using a SYBR Green-based detection system. Each 20 µL qPCR reaction contained 1x SsoFast EvaGreen Supermix (Bio-Rad Laboratories), optimized concentrations of each primer set (Table 2.1) and 2 µL of diluted cDNA template. The thermal cycle program included an enzyme activation step at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C and 5 s at the gene-specific annealing temperature (Table 2.1); after the amplification phase, there was a denaturation at 95°C for 1 min, and then a melt curve analysis from 55°C to 95°C, increasing in 1°C increments every 30 s. In every qPCR assay, samples were run in duplicate with a no template control (Rnase-free water instead of cDNA template) and a no reverse transcriptase control (cDNA template for which water was added instead of reverse transcriptase enzyme). A standard curve was run on each qPCR plate, made from a pooled cDNA sample that was serially diluted to encompass a wide range of gene expression. Standard curves for all primer pairs had an amplification efficiency of 90-110% and an $R^2 > 0.98$. The relative standard curve method was used to interpolate relative mRNA abundance of target and reference genes within each sample. Duplicate data for each sample was averaged and normalized to the reference genes (developmental profile normalized to the geometric mean of EF-1α and 18S, thyroxine and sodium perchlorate exposures normalized to the geometric mean of GAPDH and EF-1α). Data are expressed as fold-change relative to NF stage 9 for the developmental profile and relative to solvent control treatment and water control treatment for the thyroxine and sodium perchlorate exposures, respectively.

Table 2.1. List of target genes and the specific forward and reverse primers used for transcript expression analysis by qPCR in *Xenopus laevis*.

Target	Primer	Sequence (5' to 3')	Accession numbers	Amplicon Length (bp)	Primer (mM)	Annealing Temp (°C)	References
IL-1β	Fwd Rev	GGCCTCAATGAAACCTCCAC AGGCAGATATCTCCCAGCAC	NM_001085605.1	232	0.4	60	This Chapter
IFN-γ	Fwd Rev	TGAGACAATCATATAGGCTGC AAGTATTTCCTCAGTTCTTCAG	JN634068.1	121	0.6	60	This Chapter
TNFα	Fwd Rev	TGTCAGGCAGGAAAGAAGCA CAGCAGAGAGCAAAGAGGATGGT	AB298595.1	203	0.4	62	(Sifkarovski et al., 2014)
18S	Fwd Rev	ATGGCCGTTCTTAGTTGGTG TATTGCTCGATCTCGTGTGG	X04025.1	151	0.2	62	(Yu et al., 2015)
GAPDH	Fwd Rev	GACATCAAGGCCGCCATTAAGACT AGATGGAGGAGTGAGTGTCACCAT	AF549496.1	130	0.4	58.4	(Sifkarovski et al., 2014)
EF-1α	Fwd Rev	GAGGGTAGTCTGAGAAGCTCTCCACG CCTGAATCACCCAGGCCAGATTGGTG	NM_001086133.1	221	0.4	60	This Chapter

Fwd, forward; Rev, reverse

2.2.5. Thyroid gland histology

Individuals (n=3) from both sodium perchlorate (water control treatment and 500 μg/L) and thyroxine exposures (solvent control treatment and 2 μg/L) were processed using a Sakura Tissue Tek VIP 6AI Tissue processor. Briefly, samples were step-wise dehydrated (70% ethanol (EtOH) for 30 min, 90% EtOH for 30 min, 100% EtOH for 45 min, 100% EtOH for 1 h 30 min and 100% EtOH for 1 h 30 min), cleared with xylene (30 min, 1 h and 1h 30 min) and embedded in paraplast wax (30 min, 30 min, 1 h and 2 h). Samples were cross-sectioned at a thickness of 5 μm through the thyroid gland. Sections were stained using regressive hematoxylin and eosin (Sigma Aldrich) and examined with the Zeiss Axiostar Plus microscope and imaged using AxioVision Rel. 4.8 Software (Carl Zeiss, Oberkochen, Germany). The thyroid glands were measured using methods similar to those used in Opitz et al. (2006). Briefly, for each tadpole, three sections were chosen that maximized the surface area of the gland. In order to determine the epithelial cell height, three follicles were chosen at random from each section and three random epithelial cells within each chosen follicle were measured. Therefore, the epithelial cell height of an individual tadpole is the result of 27 total measurements. The total surface area was also measured in these same sections by tracing around the entire thyroid gland and determining the surface area in mm^2. All histological measurements were taken using a masked (or blind) evaluation and analyzed using Image J Software (Bethesda, MD, United States).

2.2.6. Statistical analysis

Data were tested for normality using the Kolmogorov-Smirnov test on residuals and for homogeneity of variance using Levene's test. Data not meeting the assumptions of parametric statistics were log transformed. Gene expression data were analyzed using a one-way ANOVA, followed by a Tukey's post hoc test for the developmental profile and Dunnett's post hoc test for the exposures. For T_4 and sodium perchlorate exposures, individuals sampled after 2 or 7 d of exposure were analyzed separately, as differences in stage of development would be a confounding factor in the analysis. Data for tadpole weight and length were analyzed using a one-way ANOVA. If results were significant ($p < 0.05$), the Dunnett's post hoc test was used to evaluate significant differences between groups. Stage of development is categorical and therefore analysing differences in median stage of development across treatments was done using

the Jonckheere-Terpstra test. All analyses were conducted using SPSS version 22 (SPSS Inc, IBM, North Castle, NY, United States).

2.3. Results

2.3.1. Developmental profile

For the first experiment, developmental profiles of cytokines TNFα, IL-1β and IFN-γ were established from NF stage 9 until NF stage 56 in *X. laevis* (Figure 2.1). Expression of both TNFα and IL-1β varied over these developmental stages (Figure 2.1A and B). The TNFα was highly variable at NF stage 9; however, NF stage 9 exhibited the highest expression of TNFα with a subsequent decrease in expression at NF stage 20 and 35 and gradual increase through pre- and pro-metamorphosis (Figure 2.1A). For IL-1β, expression was lowest during embryogenesis and early pre-metamorphosis except for an increase at NF stage 20 and highest expression at late pre-metamorphosis and pro-metamorphosis (Figure 2.1B). In contrast, IFN-γ mRNA levels did not differ across the developmental stages examined (Figure 2.1C).
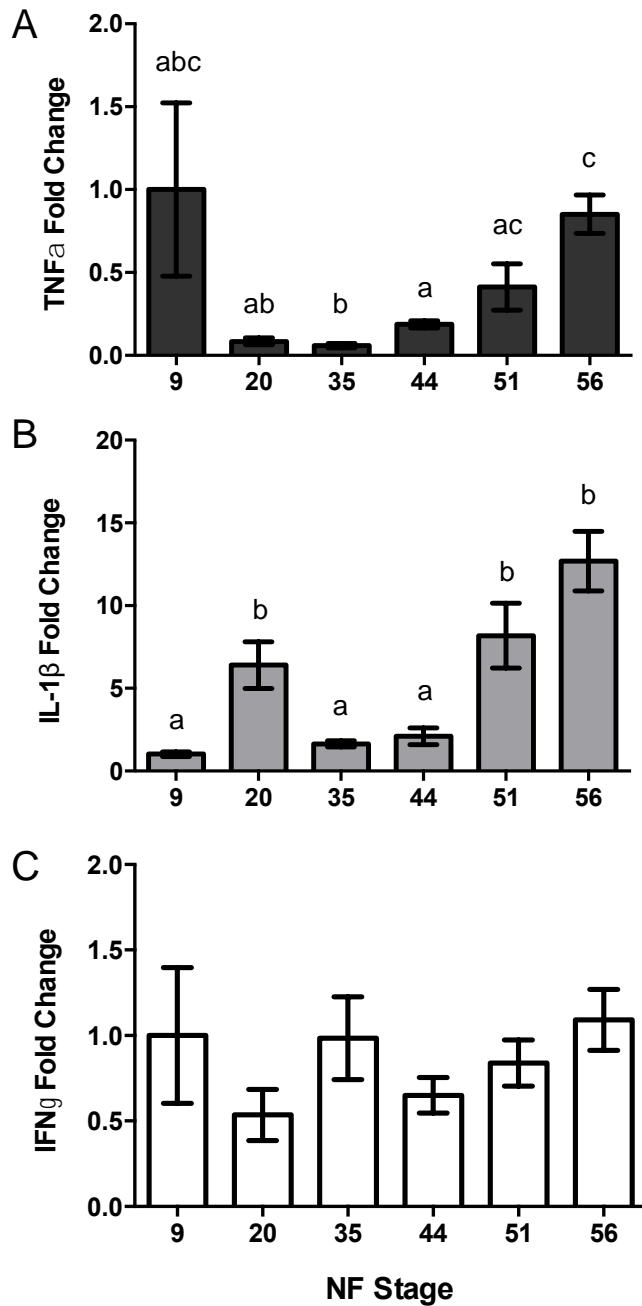
Figure 2.1. Developmental profiles of cytokines (A) TNFα, (B) IL-1β and (C) IFN-γ during *Xenopus laevis* embryogenesis and larval development. Transcript levels were measured in whole embryos and larvae (trunks) from NF stage 9 to NF stage 56. Data were normalized to the geometric mean of EF1α and 18S rRNA and expressed relative to NF stage 9. Bars represent the mean ± SEM (n = 6–8 individuals/NF stage). Different letters indicate statistically significant differences between stages (one-way ANOVA, Tukey's post-hoc test, $p < 0.05$). The scales of the y-axis vary between graphs.

2.3.2. Thyroxine exposure

Pre-metamorphic *X. laevis* exposed to both low and high concentrations (0.5 and 2 μg/L) of T_4 had increased stage of development after 2 and 7 d of exposure when compared to the solvent (NaOH) control group (Figure 2.2). There were no changes in the weight of the tadpoles, however, there was a decrease in the length of the tadpoles following 7 d exposure (Table 2.2). Transcript expression of TNFα and IFN-γ did not change with T_4 treatment after 2 d (Figure 2.3A and C) or 7 d (Figure 2.3D and F) of exposure. There was a significant decrease in IL-1β mRNA after 2 d of exposure to 0.5 and 2 μg/L T_4 when compared to the control group (Figure 2.3B) but no change in IL-1β after 7 d exposure (Figure 2.3E). After 7 d exposure to T_4, histological examination of the thyroid gland did not show visual differences between the solvent control group and 2 μg/L T_4 (Figure 2.4) or measured differences in the surface area of the gland (mean ± SEM = 27.77 ± 10.16 vs 22.70 ± 4.5 mm^2) or the epithelial cell height of the follicles (mean ± SEM = 9.87 ± 0.20 vs 9.04 ± 0.07 μm).
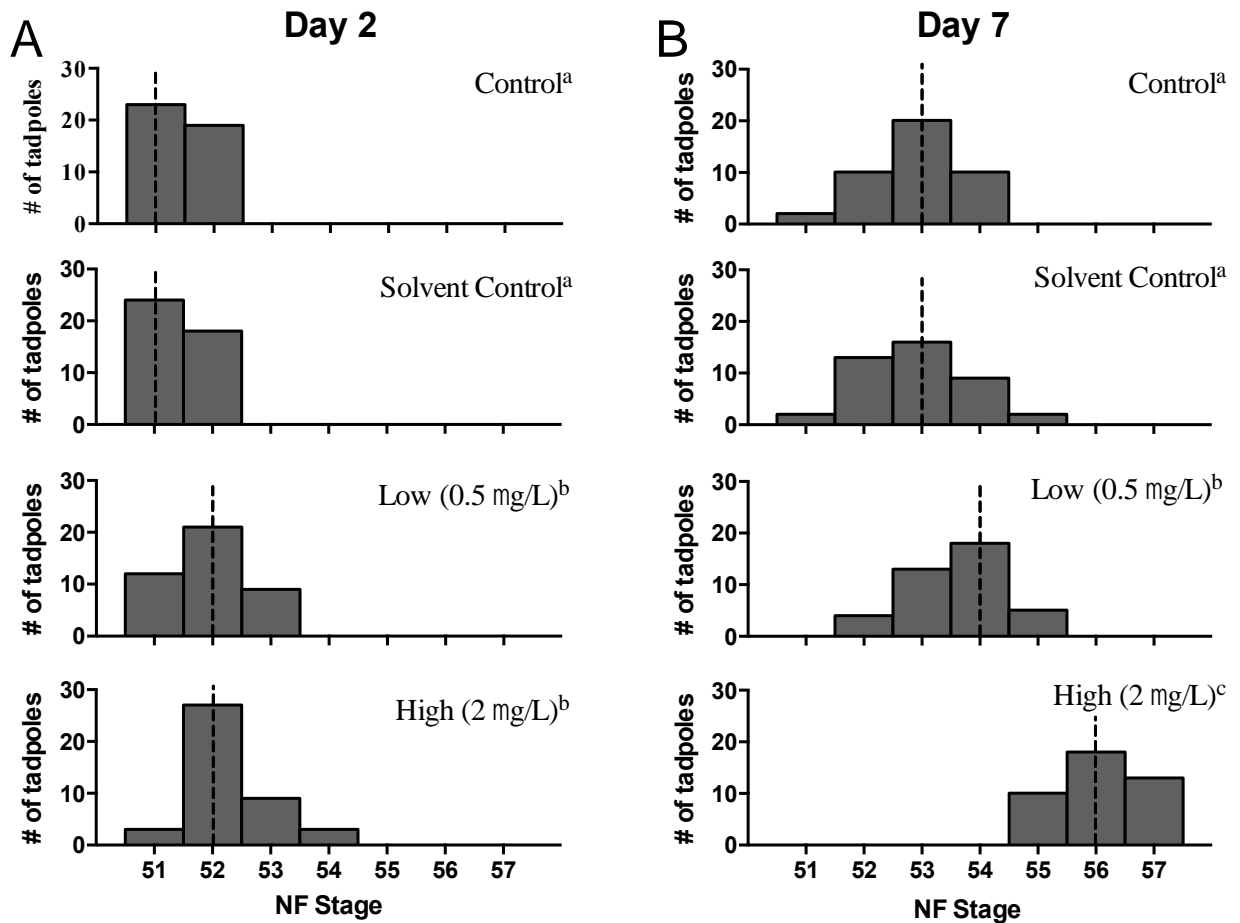
Figure 2.2. Frequency distribution of developmental stage of *Xenopus laevis* tadpoles following exposure to thyroxine (control, solvent control, 0.5 μg/L, 2 μg/L) for (A) 2 d or (B) 7 d. Data were analyzed for differences in median NF stage using the Jonckheere-Terpstra test. Different letters indicate statistically significant differences between treatments and vertical lines represent the median value in each treatment.

Table 2.2. Weight and length of *Xenopus laevis* tadpoles after 2 or 7 d of exposure to thyroxine (T_4) and sodium perchlorate.

	Day 2		Day 7	
	Weight (g)	Length (cm)	Weight (g)	Length (cm)
Thyroxine				
Control	0.19 ± 0.002	2.72 ± 0.05	0.36 ± 0.018	3.47 ± 0.08
Solvent control	0.18 ± 0.001	2.58 ± 0.03	0.37 ± 0.019	3.40 ± 0.08
Low (0.5 µg/L)	0.20 ± 0.003	2.64 ± 0.04	0.38 ± 0.018	3.40 ± 0.07
High (2 µg/L)	0.21 ± 0.004	2.59 ± 0.05	0.35 ± 0.017	$3.15 \pm 0.08^{*}$
Sodium Perchlorate				
Control	0.45 ± 0.037	3.71 ± 0.12	0.46 ± 0.045	4.38 ± 0.17
Low (125 µg/L)	0.47 ± 0.044	3.33 ± 0.15	0.43 ± 0.037	4.12 ± 0.13
High (500 µg/L)	0.37 ± 0.038	$3.05 \pm 0.12^{*}$	0.37 ± 0.032	3.93 ± 0.13

Data presented as mean $\pm$ SEM. The asterisk (*) indicates statistically significant differences between treatments and control (one-way ANOVA, Dunnett's post hoc test, $p < 0.05$). For each time point, n=18/treatment (thyroxine), n=24/treatment (perchlorate).
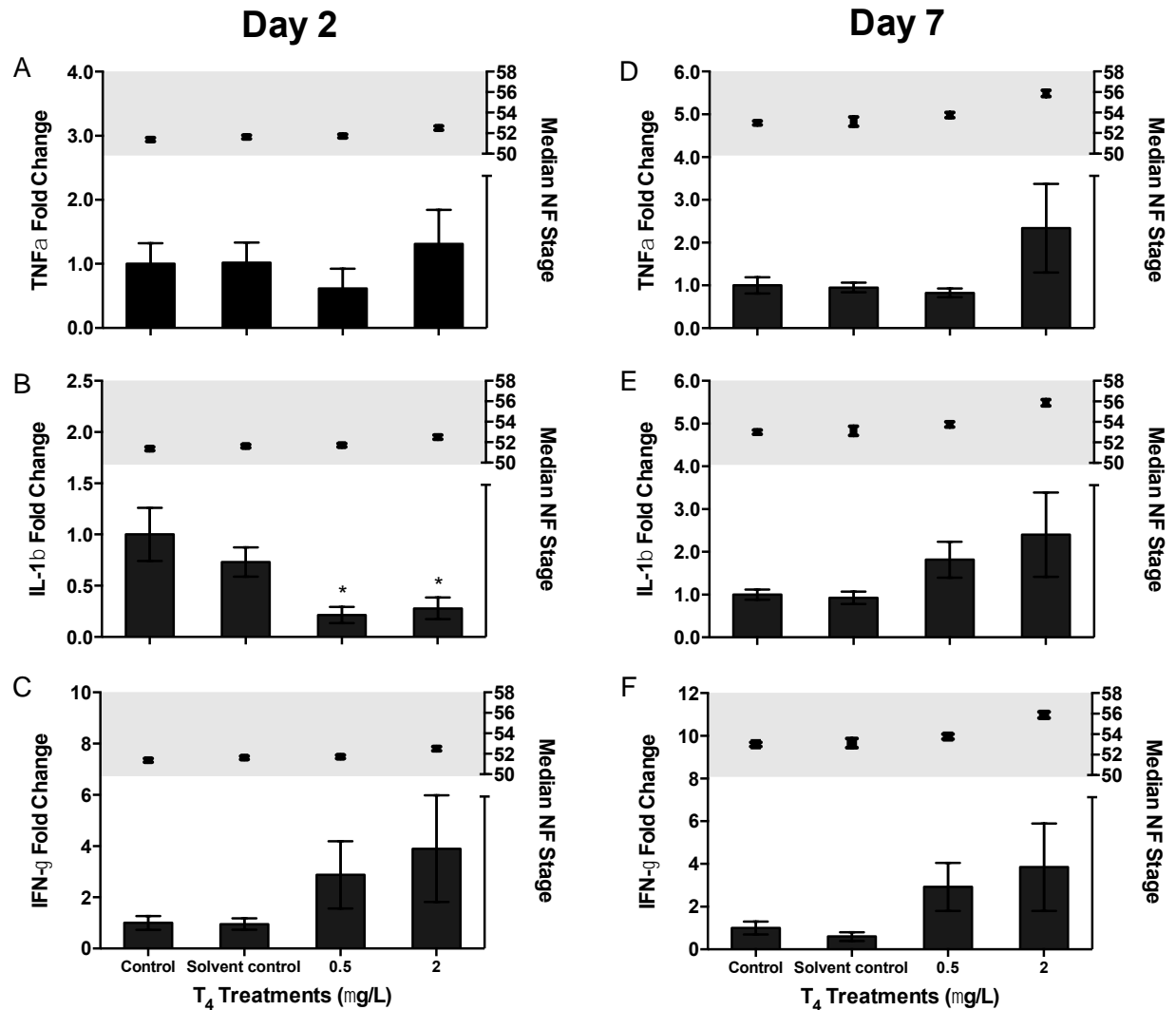
Figure 2.3. Effects of thyroxine (T_4) exposure on the expression of cytokines in pre-metamorphic *Xenopus laevis*. Tadpoles were exposed to low (0.5 µg/L) or high (2 µg/L) T_4 and sampled after 2 d or 7 d of exposure. Effects of T_4 on TNFα (A, D), IL-1β (B, E) IFN-γ (C, F) are presented. Data were normalized to the geometric mean of GAPDH and EF1α and expressed relative to the water control group. Bars represent the mean ± SEM (n = 6-8), and asterisks indicate statistically significant differences compared to control treatment ($p < 0.05$, one-way ANOVA, Dunnett's post-hoc test). Squares represent NF stage of development (Median ± IQR) for the sub-set of individuals specifically processed for gene expression analysis.
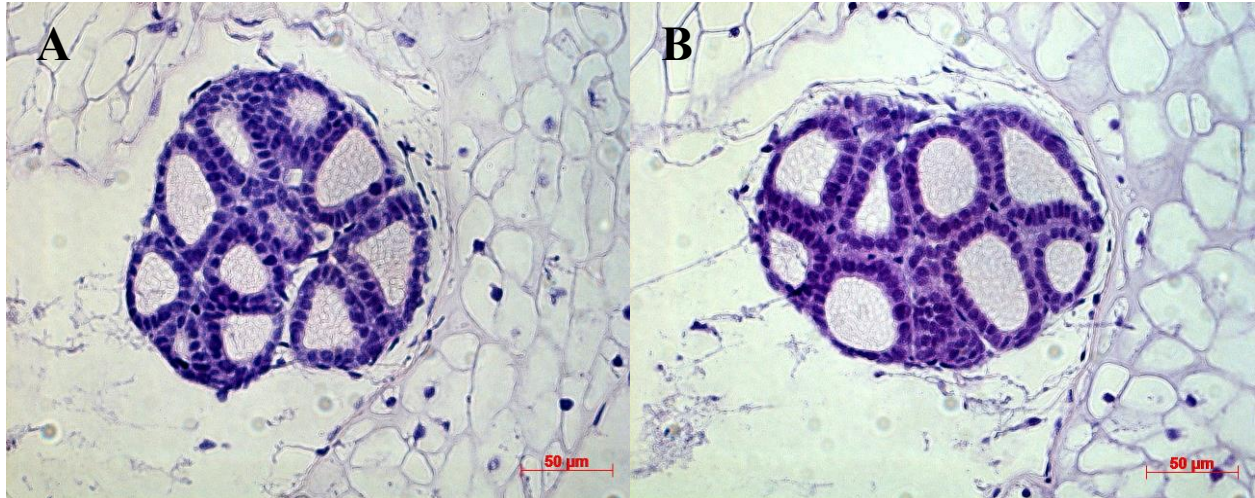
Figure 2.4. Representative thyroid glands of *Xenopus laevis* tadpoles exposed for 7 days to (A) solvent control and (B) high concentration (2 µg/L) of thyroxine (T$_4$) at 40x magnification.

2.3.3. Sodium perchlorate exposure

Pre-metamorphic *X. laevis* tadpoles exposed to the highest concentration (500 µg/L) of sodium perchlorate had decreased length after 2 d exposure when compared with the control group; a similar pattern was observed after 7 d exposure although non-significant (Table 2.2). There were no differences in stage of development (Figure 2.5) or weight (Table 2.2) of sodium perchlorate-exposed tadpoles compared to the control group. There was no change in TNFα mRNA following 2 d exposure to sodium perchlorate (Figure 2.6A) but a decrease following 7 d exposure to 125 µg/L (Figure 2.6D). There were no changes in the expression of IL-1β or IFN-γ after 2 d (Figure 2.6B and C) or 7 d (Figure 2.6E and F) of exposure to sodium perchlorate. The thyroid glands of tadpoles exposed to sodium perchlorate for 7 d were larger than the control group (Figure 2.7), with a 67% increase in epithelial cell height (mean $\pm$ SEM = 16.11 ± 2.04 vs 9.63 ± 0.16 µm) and 58% increase in the surface area (mean $\pm$ SEM = 45.05 ± 8.65 vs 28.51 ± 3.91 mm^2).
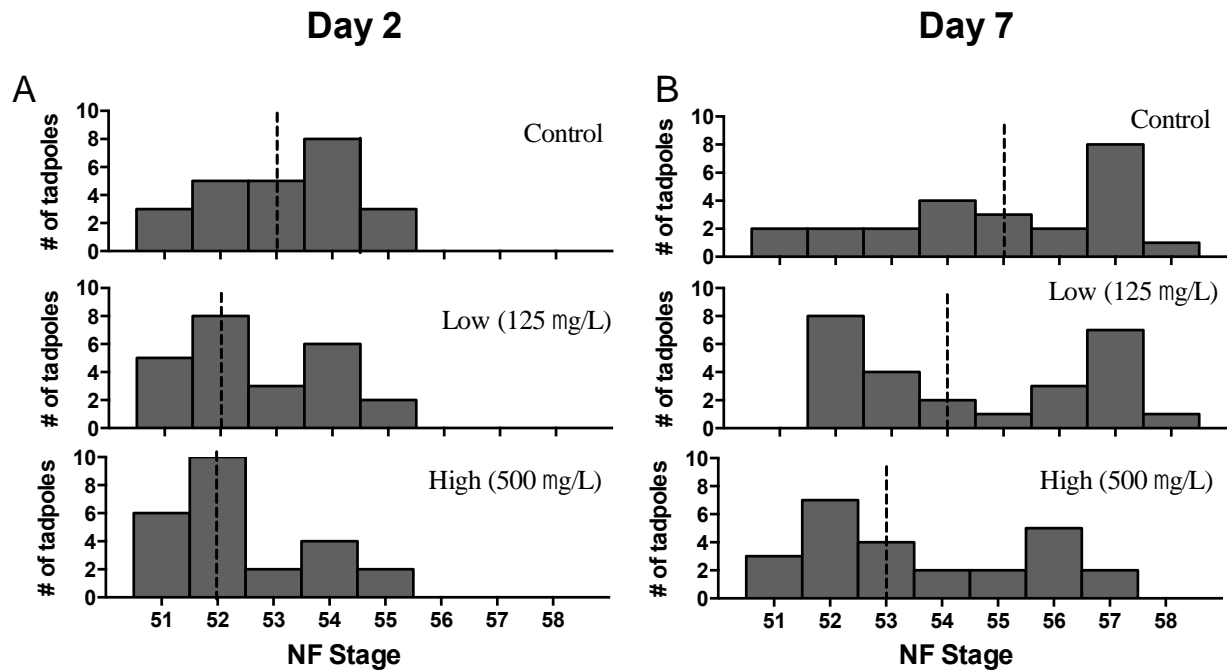
Figure 2.5. Frequency distribution of developmental stage of *Xenopus laevis* tadpoles following exposure to sodium perchlorate (control, 125 µg/L, 500 µg/L) for (A) 2 d and (B) 7 d. Data (n = 24 individuals/treatment) were analyzed using the Jonckheere-Terpstra test. Vertical lines represent the median value in each treatment.
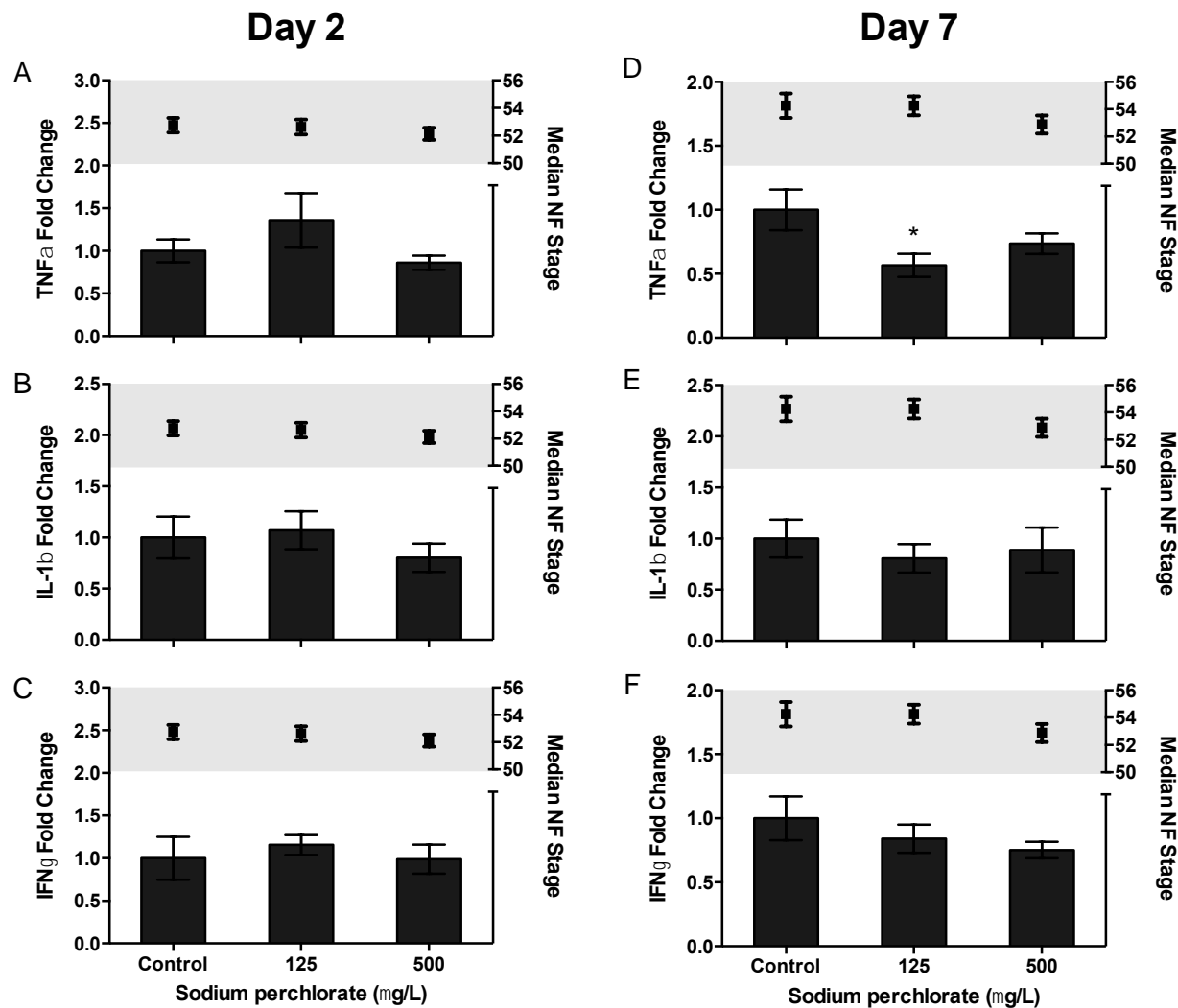
Figure 2.6. Effects of sodium perchlorate exposure on the expression of cytokines in pre-metamorphic *Xenopus laevis*. Tadpoles were exposed to low (125 μg/L) or high (500 μg/L) sodium perchlorate concentrations and sampled at 2 and 7 d of exposure. Effects of sodium perchlorate on TNFα (A, D), IL-1β (B, E) IFN-γ (C, F) are presented. Data were normalized to the geometric mean of GAPDH and EF1α and expressed relative to the control group. Bars represent the mean ± SEM (n = 6-8), and asterisks indicate statistically significant differences compared to control groups ($p < 0.05$, one-way ANOVA, Dunnett's post-hoc test). Squares represent NF stage of development (median ± IQR) for the sub-set of individuals specifically processed for gene expression analysis.
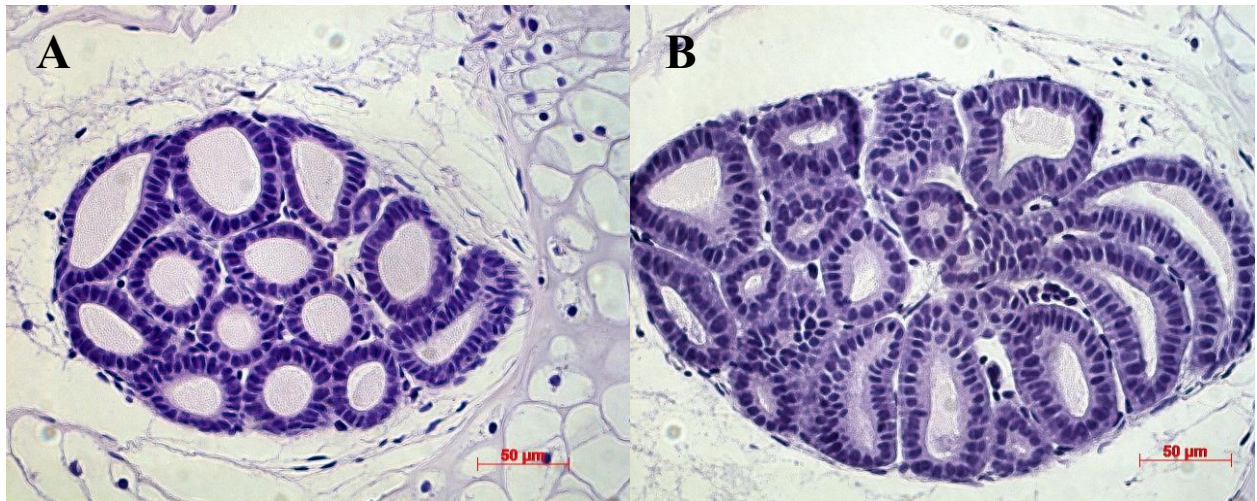
Figure 2.7. Histopathology of thyroid glands of *Xenopus laevis* tadpoles exposed for 7 days to (A) control and (B) high concentration (500 µg/L) sodium perchlorate at 40X magnification.

2.4. Discussion

Cytokines play an important role as mediators of both innate and adaptive immune responses in tadpoles during early stages of pathogen infection (Grayfer et al., 2015a; 2015b). In the present study, the expression profiles of cytokine mRNA in embryos and larval stages were analyzed for the first-time during amphibian ontogeny. Cytokines TNFα and IL-1β exhibit low expression during embryogenesis with increased expression through the latter part of metamorphosis while IFN-γ expression remains relatively stable. Exposure of functionally athyroid tadpoles did modulate the expression of these cytokines although not to the same magnitude or directionality as would be expected given their developmental profiles, suggesting that their ontogenic regulation is not likely exclusively influenced by thyroid status.

2.4.1. Immune genes are differentially expressed during amphibian larval development

The aim of the first experiment was to characterize the developmental expression profile of pro-inflammatory cytokines from early embryogenesis through late pro-metamorphosis. For TNFα, there was high initial (NF stage 9) expression with subsequent decrease during later embryogenesis and gradual increase through pre- and pro-metamorphosis. This pattern suggests maternal transfer of cytokine mRNA transcripts to the embryos; however, oviparous egg quality, and thus maternal transfer, can be affected by factors such as brood size, egg weight and body condition of the female (Brooks et al., 1997), which were not controlled for in this experiment. These potential variations in egg quality may account for the high variability at this stage of development. In *X. laevis* embryos, the first 12 synchronous cell divisions occur without transcription; this is possible due to the large amount of mRNA and proteins deposited into the egg by the female during oogenesis (Duval et al., 1990). Past NF stage 9, a number of simultaneous physiological changes occur including asynchronous cell division (Gerhart, 1980; Satoh, 1977), cellular motility (Satoh et al., 1976) and the onset of RNA synthesis (Bachvabova and Davidson, 1966). Therefore, high levels of TNFα before the onset of transcription (NF stage 9) may be explained by maternal transfer of these transcripts to the embryo. Additionally, overall mRNA is degraded 10 h post-fertilization (NF stage 10; Duval et al., 1990), which is reflected in the lowest expression of TNFα and IL-1β later embryogenesis and early pre-metamorphosis. At this point in ontogeny, tadpoles do not have functional immune organs and a very limited repertoire of immune cells, limiting the synthesis of new cytokine mRNA. The increase in TNFα

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and IL-1β mRNA expression at NF stage 51 coincides with the appearance of a functional spleen in tadpole development (Hadji-Azimi et al., 1990; Nieuwkoop and Faber, 1956) and the subsequent diversification of immune cell production (Pasquier, 1973; Rollins-Smith, 1998). The spleen is the primary site of B-cell differentiation and activation in tadpoles (Hadji-Azimi et al., 1990), which secrete cytokines, including TNFα and IL-1β (reviewed in Lund, 2008). It is important to note that expression of target genes during development was determined using pooled embryos/larvae or tadpole trunks. The small size of *X. laevis* tadpoles precluded the analysis of specific immune tissues. However, expression profiles of cytokines do provide insight into the developmental changes in expression in the whole organism.

In contrast to TNFα and IL-1β, IFN-γ exhibits a relatively consistent expression level throughout tadpole development. This may be due, in part, to the cell types that express each cytokine as TNFα and IL-1β are produced by both immune and non-immune cells (Stadnyk, 1994), while IFN-γ is almost exclusively produced by immune cells (Schroder et al., 2004). Thus, the lack of an observed change in IFN-γ across development could be a consequence of processing procedure; any changes in IFN-γ expression may be masked by the fact that we assessed expression in the whole organism, which included both immune and non-immune cells. Furthermore, both TNFα and IL-1β typically have high constitutive expression (Bird et al., 2002; Grayfer et al., 2012; 2008; Hirono et al., 2000), whereas basal expression of IFN-γ mRNA is lower and requires induction by an immune challenge (De Jesús Andino et al., 2012; Grayfer and Belosevic, 2009; Igawa et al., 2006). The IFN-γ plays a central role in coordinating the antiviral response (Boehm et al., 1997; Schroder et al., 2004) and stimulated production of IFN-γ, along with TNFα, results in a number of downstream pathways that synergistically prevent viral replication (Bartee et al., 2008).

2.4.2. Impacts of thyroidal modulation on cytokine expression

Postembryonic development in anuran amphibians represents an ideal model to study TH modulation of a particular pathway since metamorphosis can be induced or delayed by exposure to exogenous thyroid hormones or inhibitors of thyroid hormone synthesis, respectively. We evaluated morphometrics, developmental stage and thyroid gland histology to confirm that our exposures to T_4 and sodium perchlorate were successful in modulating the thyroid axis of pre-

metamorphic (NF stage 51) tadpoles. As expected, tadpoles exposed to T_4 exhibited advanced developmental stage after 2 and 7 d of exposure. This is a characteristic response and one of the main endpoints in the standardized amphibian metamorphosis assay to screen chemicals that potentially disrupt the thyroid hormone axis (OECD, 2007).

While developmental stage was accelerated by T_4, exposure to sodium perchlorate did not alter the rate of metamorphosis. Under control conditions (*i.e.* no exposure to thyroid modulating compounds), NF stage 51 tadpoles would normally progress through approximately two NF stages over seven days (Nieuwkoop and Faber, 1956). Since our exposure was only seven days, it was not enough time to discern a thyroid-inhibiting effect of perchlorate exposure at the level of changes in developmental stage. However, a dramatic increase in thyroid gland area and follicular cell height in perchlorate-exposed tadpoles was evidence that perchlorate was able to inhibit T_4 synthesis. Perchlorate inhibits the uptake of iodide, reducing synthesis of TH and increasing thyroid stimulating hormone (TSH) through negative feedback. Increased TSH acts on the thyroid gland and leads to the hypertrophy of the thyroid gland and epithelial cells (Capen, 1994). Histological effects of perchlorate exposure are among the most sensitive endpoints of thyroid disruption in amphibians (Tietge et al., 2005) and the magnitude of response exhibited in the present study is consistent with those reported in other studies with amphibians (Carr et al., 2015; Grim et al., 2009; Tietge et al., 2005), fish (Bradford et al., 2005; Petersen et al., 2015; Schmidt et al., 2012) and mammals (York et al., 2001; 2003).

With confirmed thyroid modulation in exposed tadpoles, we sought to determine the role of TH in regulating the expression of specific cytokines. Although statistically significant differences were observed following thyroidal modulation with either T_4 or sodium perchlorate, these changes were not drastic as would be expected if TH was primarily responsible for cytokine regulation. During amphibian larval development, THs act together with other hormones, such as corticosteroids, to advance the development of various physiological systems, including the immune system (Denver et al., 2002; Rollins-Smith et al., 1997; Rollins-Smith and Blair, 1993). In fact, the natural increase of corticosteroid hormones during metamorphosis plays a major role in the decrease of lymphocyte populations by apoptosis (Rollins-Smith et al., 1997; Rollins-Smith and Blair, 1993). Both in mammals and fish, cortisol can inhibit inflammatory mediators including cytokines (Castillo et al., 2009; Castro et al., 2011; Tort, 2011) and lead to the immunosuppressive consequences of stress. Just as TH alone is insufficient to complete

metamorphosis (Bonett et al., 2010), the timing and nature of immune system development during post-embryonic life stages is likely regulated by interactive and independent effects of many hormones that also drive metamorphosis.

The relationship between thyroid hormones and the immune system in aquatic vertebrates such as amphibians and fish has been studied mainly in context of comparative endocrinology and immunology (Harris and Bird, 2000; Rollins-Smith, 1998). However, such information is significant with regard to potential impacts of thyroid-disrupting chemicals present in the aquatic environment. Both lab and field studies have reported that the hypothalamus-pituitary-thyroid axis of amphibians can be disrupted by aquatic contaminants, such as perchlorate (Goleman et al., 2002a; 2002b; Rollins-Smith, 2001; Tietge et al., 2005), polychlorinated biphenyls (Gutleb et al., 2000), bisphenol A (Iwamuro et al., 2003), PBDEs (Balch et al., 2006), the insecticide methoxychlor (Fort et al., 2004a; 2004b) as well as exposure to municipal wastewater effluent (Sowers et al., 2009). Larval amphibians have highly restricted habitat ranges and are confined to the aquatic system where thyroid-disrupting contaminants and pathogens may be present simultaneously. This period of tadpole development is also characterized by dramatic changes in both thyroid status and immune system development, including expression of various cytokines as indicated by the present study. Although transcript expression of pro-inflammatory cytokines has been used as an indicator of potential immune dysfunction (Fang et al., 2013; Hur et al., 2013; Jin et al., 2011; Martini et al., 2012), this is not a functional endpoint. Altered cytokine expression may indicate a change in number or activity of immune cell types responsible for cytokine production, but such mechanisms still need to be elucidated in amphibians. Future work should consider whether thyroid disruption or other forms of endocrine disruption is capable of modulating immune function in amphibians and whether there are implications for disease susceptibility.

CHAPTER 3:

DEVELOPMENTAL TIMING OF BENZO[A]PYRENE EXPOSURE ALTERS MORPHOMETRICS AND EXPRESSION OF GENES RELATED TO IMMUNE AND BIOTRANSFORMATION PATHWAYS IN *XENOPUS LAEVIS*

Preface

The goal of the research presented in Chapter 3 of this thesis was to identify the most sensitive developmental stage (embryo, pre-metamorphosis, or pro-metamorphosis) to the developmental (stage, weight and length) and gene expression (immune and detoxification related genes) impacts of a short-term waterborne exposure to B[a]P. Despite the dynamic nature of tadpole development, there are few studies that compare the sensitivity of amphibians to contaminants across tadpole life-stages. Thus, this study was conducted to determine if all tadpole life-stages express detoxification-related genes and if there was differential developmental sensitivity to B[a]P-induced morphological changes and immunomodulation. The content of Chapter 3 is being prepared as a manuscript for submission to Environmental Science and Pollution Research under the joint authorship of Melanie Gallant and Natacha Hogan.

Author contributions:

Melanie Gallant (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Natacha Hogan (University of Saskatchewan) provided inspiration, scientific input and guidance on the design of the experiment, supervised Melanie Gallant, commented on and edited the manuscript, and obtained funding for the research.

Abstract

The amphibian immune system is dynamic over development – early embryo-larval life stages rely primarily on the innate immune system until late pro-metamorphosis at which time the adaptive immune system begins to increase in function. Therefore, the impacts of environmental contaminants on the immune system may depend on the stage of development at the time of exposure. As key regulators of the innate immune system, cytokine expression is often used as an indication of the immunomodulatory potential of chemicals. The present study compared the effects of a known immunotoxicant, benzo[a]pyrene (B[a]P), on morphological, immune and biotransformation responses across early life-stages of *Xenopus laevis*. Short-term waterborne exposures to B[a]P (water control, DMSO vehicle control, 10, 175 or 350 µg/L B[a]P) were conducted at three distinct life-stages (embryo-larval, pre- and pro-metamorphosis) during tadpole development. Mortality, body weight and total length and any abnormalities were recorded, and expression of pro-inflammatory cytokines (tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), colony-stimulating factor-1 (CSF-1)), as well as cytochrome P450 1A7 (CYP1A7) and aryl hydrocarbon receptor (AhR1B), were measured by qPCR. Exposure to B[a]P decreased total length in individuals exposed during the embryo-larval life-stage and pre-metamorphosis but had no effect on length in the pro-metamorphic tadpoles. Exposure to B[a]P at all life-stages resulted in CYP1A7 mRNA induction, indicating that biotransformation, and subsequent production of reactive metabolites, can occur across larval development. Pre-metamorphic tadpoles exposed to B[a]P had reduced TNFα and CSF-1 and no change in IL-1β transcript levels, while the opposite was true for pro-metamorphic tadpoles. Together, these results demonstrate that B[a]P exposure differentially affects *X. laevis* tadpoles across developmental stages. This study offers new insights into the biotransformation capacity across tadpole life-stages and the stage-specific impacts of B[a]P exposure on the innate immune system.

3.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of contaminants released into the environment due to the incomplete combustion of organic matter. They are released through both natural and anthropogenic sources, with the latter being primarily responsible for their widespread occurrence and environmental contamination (Lawal, 2017; Samanta et al., 2002). Complex mixtures of PAHs are ubiquitously distributed in the environment and can be detected in samples of air, soil, sediment and water (Lawal, 2017). Benzo[a]pyrene (B[a]P) is often a component of PAH mixtures in the environment and is extensively studied as a representative PAH (Hylland, 2006).

Exposure to B[a]P causes adverse effects, such as impaired growth and development, cancer, mutations, teratogenicity and immunotoxicity in a variety of organisms ranging from fish to mammals (reviewed in Reynaud and Deschaux, 2006; Samanta et al., 2002). Decreased body weight and total length, as well as increased incidence of deformities, are commonly observed in fish and amphibians when exposure occurs during early life-stages (Corrales et al., 2014; Currie, 2018; Gravato and Guilhermino, 2009; Kim et al., 2008). The adverse effects of B[a]P are thought to be mediated, at least in part, by reactive metabolites produced during biotransformation (Miller and Ramos, 2001). B[a]P binds to the aryl hydrocarbon receptor (AhR), and this receptor undergoes a conformational change that exposes a nuclear localization sequence (Denison et al., 2011). Upon translocation to the nucleus, the AhR forms a heterodimeric complex with the aryl hydrocarbon nuclear translocator (ARNT), which can bind the promoter region of AhR responsive genes called the xenobiotic response element (XRE) (Beischlag et al., 2008; Hankinson, 1995). The most commonly documented target gene with an XRE is cytochrome P450 1A (CYP1A), a phase I enzyme that aids in the detoxification and elimination of many planar aromatic hydrocarbon compounds, including B[a]P (Schrenk, 1998). However, CYP1A also promotes the production of reactive B[a]P metabolites and reactive oxygen species (ROS) responsible for the carcinogenic, mutagenic and immunotoxic effects of B[a]P (Miller and Ramos, 2001). Although characterization of the metabolic activation of B[a]P is most extensive in mammals, the mechanism is hypothesized to be conserved across vertebrate species and similar pathways exist in numerous aquatic vertebrates (Lavine et al., 2005; Miller and Ramos, 2001; Reynaud and Deschaux, 2006). In *Xenopus laevis* specifically, CYP1A6 and

CYP1A7 are expressed as paralogs of the mammalian CYP1A1, and respond to similar AhR ligands (Lavine et al., 2005).

Amphibians can inhabit PAH-contaminated aquatic systems and live in close proximity to contaminated sediments. For example, many amphibian species inhabit the Athabasca oil sands region (Hersikorn and Smits, 2011; Melvin and Trudeau, 2012), where high concentrations of PAHs have been quantified in the sediment of wetlands surrounding oil sands extraction facilities (Bilodeau, 2017). Amphibians can accumulate PAHs in contaminated environments from the water via dermal absorption and especially from sediment while feeding on detritus (Bilodeau et al., 2019). Amphibians are particularly susceptible to PAH uptake and toxicity during early life stages when they are confined to the aquatic environment and interact with contaminated sediments during critical periods of development (Linder et al., 2010). However, this vulnerability may not be uniform across tadpole development. Many studies report stage-specific differences in the sensitivity to chemicals across tadpole development. For example, the common South American toad (*Rhinella arenarum*) exhibited a 4-fold difference in 96 hr 50% lethal concentrations (LC_{50}) values between the embryo and larval life-stages for a commonly used fungicide formulation (Svartz et al., 2018). In another study, western spadefoot toad (*Pelobates cultripes*) hatchlings exposed to ammonium nitrate for 96 hr had 100% mortality, whereas larvae exhibited 40% mortality when exposed to the same concentration and duration (Ortiz-Santaliestra et al., 2006). Studies such as these highlights the importance of assessing differential sensitivity of developmental stages of amphibians to chemicals although many primarily focus on lethality as an endpoint rather than sub-lethal effects that could have negative consequences for amphibian health.

The dynamic nature of amphibian development includes significant morphological and functional changes in various physiological systems, such as the immune system. Early life-stage tadpoles rely primarily on the innate immune system for protection against pathogens in their environment, as components of the adaptive immune system have limited function until pro-metamorphosis and metamorphic climax (Flajnik et al., 1987; Rollins-Smith, 1998). Cytokines are chemical messengers secreted by a wide range of cell types (both immune and non-immune), and are integral to the regulation and function of both the innate and adaptive immune systems (Segner et al., 2012b). In amphibians, cytokines are important even during tadpole life-stages – increased susceptibility to the amphibian pathogen ranavirus (FV3) was at least in part

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attributable to the diminished and delayed pro-inflammatory immune response in tadpoles compared to adults (De Jesús Andino et al., 2012). Cytokine expression is often used as a tool for assessing immune status in both fish and amphibians, and there is evidence that exposure to chemicals can modulate cytokine expression and production with postulated consequences for pathogen defence (Fang et al., 2013; Hur et al., 2013; Martini et al., 2012). A study by Hur et al. (2013) showed that short-term exposure of olive flounder (*Paralichthys olivaceus*) to B[a]P induced expression of the pro-inflammatory cytokine tumor necrosis factor-α (TNFα) through the AhR pathway in the head kidney. Based on this response, along with that of other cytokines such as interleukins (ILs), the authors suggested that exposure to B[a]P triggers an inflammatory cascade in this immune organ that likely contributes to immunotoxicity of B[a]P (Hur et al., 2013). We have shown that these cytokines are differentially expressed throughout tadpole development (Gallant and Hogan, 2018), but have not yet established whether they are differentially modulated by chemical exposure depending on the stage of development at the time of exposure. Therefore, the aim of this study was to identify the sensitive developmental phases for sub-lethal effects of B[a]P in larval amphibians using the standard amphibian test species, *Xenopus laevis*. The study evaluated the impacts of short-term exposure at specific developmental stages (embryo-larval, pre-metamorphosis, pro-metamorphosis) on morphometrics, developmental stage, and incidence of abnormalities. The expression of select genes related to the inflammatory response and immune function, specifically TNFα, interleukin-1β (IL-1β), and colony stimulating factor-1 (CSF-1) was assessed in the liver along with genes involved in the biotransformation pathway for B[a]P (AhR1B and CYP1A7).

3.2. Material and Methods

3.2.1. Breeding and rearing of *Xenopus laevis*

    Xenopus laevis (X. laevis) breeding and egg collection were performed as described in Gallant and Hogan (2018). Briefly, collected embryos and tadpoles were maintained in the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan in static conditions at 22°C and a 16 h light: 8 h dark cycle, where light hours began at 06h00. From five days post-fertilization onwards, tadpoles were fed Sera Micron® (Sera, Heinsberg, Germany) daily, *ad libitum*. A 75% water change and treatment renewal was performed daily after feeding. Water parameters were monitored and were maintained throughout the exposure periods (pH 7-

7.5, dissolved oxygen > 90%, temperature = 20 ± 0.5°C). The care and treatment of animals used in this study were approved by the University of Saskatchewan's Animal Research Ethics Board (protocol no. 20120062) and adhered to the Canadian Council on Animal Care guidelines for humane animal use (CCAC, 1993).

3.2.2. Benzo[a]pyrene exposures and sampling

Waterborne exposures to B[a]P (Cat: B1760; Sigma-Aldrich, Oakville, ON, Canada) were conducted at three developmental stages: embryo-larval, pre-metamorphosis and pro-metamorphosis. For each set of exposures, treatment groups included control, solvent control (dimethyl sulfoxide; DMSO; Thermo Fisher Scientific, Waltham, MA, USA), and three nominal concentrations of B[a]P at 10, 175 and 350 μg/L. B[a]P was dissolved in DMSO, and the final solvent concentration was 0.007% v/v in all treatments. Daily water changes were performed during the exposures to ensure consistent water quality (pH 7-7.5, dissolved oxygen > 90%, temperature = 20 ± 0.5°C), and tanks were subsequently re-dosed to maintain the target nominal concentrations.

For the 96 hr embryo-larval exposure, viable embryos (Nieuwkoop and Faber (NF) stage 8-9; Nieuwkoop and Faber, 1956) were obtained from bred females and immediately placed in glass petri dishes with 50 mL of dechlorinated facility water. They were maintained under 100% static renewal conditions with water changes and dosing occurring daily. Each of the five treatments was replicated four times, with ten embryos per dish. Individuals were monitored twice daily for mortality throughout the exposure. Following the 96 hr exposure, all individuals were euthanized and photographed for length measurements using the Olympus model SZ-CTV dissecting microscope (Olympus, Melville, NY, USA) before being pooled in triplicate and flash-frozen for later gene expression analysis.

Exposures were also conducted with tadpoles at pre-metamorphosis (NF stage 51) and pro-metamorphosis (NF stage 56) and conditions for these exposures were identical apart from the developmental stage of tadpoles at which the experiment began. Tadpoles were housed and reared under the conditions described above until there were enough tadpoles at the appropriate stage of development to commence the exposures. Tadpoles (24 individuals/tank) were exposed to B[a]P in glass aquarium tanks with 7 L of dechlorinated facility water, and each treatment was conducted in triplicate. Exposures were conducted for seven days with sampling after 2 d of

exposure and again at the end of exposure (12 tadpoles/tank sampled at two time points). Tadpoles were euthanized and body weight (to the nearest 0.01 g), total length (to the nearest 0.1 cm), and NF developmental stage were recorded. Tadpoles were segmented into head, trunk and tail and tissues were flash frozen for later gene expression analysis, as described in Gallant and Hogan (2018).

3.2.3. Gene expression analysis

Embryo-larval individuals (whole-body pooled samples) and pre- and pro-metamorphosis tadpoles (trunk samples) after 2 d B[a]P exposure were analyzed for changes in mRNA expression of target genes related to biotransformation (CYP1A7, AhR1B) and cytokines (IL-1β, TNFα and CSF-1). Expression of two housekeeping genes (glyceraldehyde 3-phosphate dehydrogenase, GAPDH, and elongation factor 1α, EF1α) was also analyzed and used for sample normalization. Samples were homogenized using the Bead Ruptor (Omni International, Kennesaw, GA, USA) at 40 Hz for 2 cycles of 45 s. Total mRNA was extracted using the Qiagen RNeasy Mini Kit as described by the manufacturer (Qiagen, Mississauga, ON, Canada) and re-suspended in RNAse-free water. DNAse treatment was performed using the Turbo DNA Free Kit (Ambion, Burlington, ON, Canada) as described by the manufacturer. Quantity and quality of the RNA were verified using the NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific) and 1% agarose gel, respectively. Using the iScript cDNA synthesis kit as directed by the manufacturer (BioRad Laboratories, Hercules, CA, United States), 1 μg of total RNA was reverse transcribed and resulting cDNA samples were diluted 10-fold prior to gene expression analysis.

Gene expression analysis was performed by real-time polymerase chain reaction (qPCR) using the CFX96 Real-time C1000 Thermal Cycler (BioRad Laboratories). Gene-specific primers (Invitrogen, Carlsbad, CA, United States) for target genes AhR1B, CYP1A7, IL-1β, TNFα, and CSF-1, and housekeeping genes GAPDH and EF1α were designed in-house or sourced from the literature (Table 3.1). Sequences of the single amplicon for each target gene were obtained from Plant Biotechnology Institute of Canada (National Research Council, Saskatoon, SK, Canada) to confirm the specificity of primers. Each primer set was optimized for annealing temperature (Table 3.1), cDNA input resulting in minimal C_t and a single sequence-specific peak in the melt curve using a SYBR Green-based detection systems. Each qPCR

reaction contained 1x SsoFast EvaGreen Supermix (BioRad Laboratories), 2 µL of diluted cDNA template and optimized concentrations of each primer set (4 nM, Invitrogen), for a total volume of 20 µL. The thermal cycle program included an enzyme activation step at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C and 5 s at the gene-specific annealing temperature (Table 3.1); after the amplification phase, there was a denaturation at 95°C for 1 min, and then a melt curve analysis from 55°C to 95°C, increasing in 1°C increments every 30 s.

In every qPCR assay, samples and standards were run in duplicate along with a no template control (rNase-free water instead of cDNA template) and a no reverse transcriptase control (cDNA template for which water was added instead of reverse transcriptase enzyme). The standard curve was made from a representative pool of cDNA that was serially diluted to encompass a wide range of gene expression. Standard curves for all primer pairs were optimized to have an efficiency of 90-110% and $R^2 > 0.98$. The relative standard curve method was used to interpolate relative mRNA abundance of target and reference genes. Duplicate data for each sample were averaged, and target genes were normalized to the geometric mean of the reference genes. Data are expressed as fold-change relative to the pooled control sample (combined water and solvent control sample) since the dilution water and solvent control groups were not statistically significantly different (Green and Wheeler, 2013).

Table 3.1. Real-time qPCR primers and conditions for gene expression analysis in *Xenopus laevis*.

Target	Primer	Sequence (5' to 3')	Accession number	Amplicon Length (bp)	Annealing Temp (°C)	References
IL-1β	Fwd	GGCCTCAATGAAACCTCCAC	NM_001085605.1	232	60	Gallant and Hogan, 2018
	Rev	AGGCAGATATCTCCCAGCAC				
CSF-1	Fwd	ATCGAACTCTGTCCAAGCTGGATG	NM_001280600.1	123	60	Sifkarovski et al., 2014
	Rev	GGACGAAGCAAGCATCTGCCTTAT				
TNFα	Fwd	TGTCAGGCAGGAAAGAAGCA	AB298595.1	203	62	Sifkarovski et al., 2014
	Rev	CAGCAGAGAGCAAAGAGGATGGT				
AhR1B	Fwd	TGGAGCGAAGGGGTAGTCGC	AY635783.1	138	62	This chapter
	Rev	AAGCGCGAGAGGGATGACGG				
CYP1A7	Fwd	CCTTTGCTACTTCCCCTTCACCC	NM_001097072.1	155	62	This chapter
	Rev	GGCAACAGAGACCACCACGTACC				
GAPDH	Fwd	GACATCAAGGCCGCCATTAAGACT	AF549496.1	130	58.4	Sifkarovski et al., 2014
	Rev	AGATGGAGGAGTGAGTGTCACCAT				
EF1α	Fwd	GAGGGTAGTCTGAGAAGCTCTCCACG	NM_001086133.1	221	60	Gallant and Hogan, 2018
	Rev	CCTGAATCACCCAGGCCAGATTGGTG				

Fwd, forward; Rev, reverse

3.2.4. Statistical analysis

All data were tested for normality using the Kolmogorov-Smirnov test on residuals and for homogeneity of variance using Levene's test. Data not meeting the assumptions of parametric statistics were log-transformed or transformed using Taylor's power law. Gene expression data for each exposure was analyzed using a one-way ANOVA, followed by a Tukey's post-hoc test to evaluate significant differences between groups. Data for tadpole body weight and total length were analyzed using a nested ANOVA, with tank nested within treatment to ensure that there were no tank effects on development. Tukey's post-hoc test was used to evaluate differences between treatment groups. Stage of development data are categorical and therefore differences in median NF stage were analyzed using the Jonckheere-Terpstra test. All statistical analyses were conducted using SPSS, version 22 (SPSS Inc, IBM, North Castle, NY, United States).

3.3. Results

3.3.1. Morphology

Effect of B[a]P exposure on morphometrics and developmental stage for embryo-larval, pre-metamorphic and pro-metamorphic tadpoles are presented in Table 3.2. There was no effect of B[a]P on mortality or incidence of deformities for any of the life-stages tested ($< 5\%$ in all treatments). Embryo-larval tadpoles exposed to 350 µg/L of B[a]P for 96 hr had reduced total length (mean $\pm$ SEM; 0.894 ± 0.007 cm) relative to the solvent control treatment (0.938 ± 0.005 cm; $F_{(4, 35)} = 4.634$, $p = 0.004$). Pre-metamorphic tadpoles exposed to 175 and 350 µg/L of B[a]P (7 d exposure) had reduced body weight relative to the pooled control group ($F_{(4, 33)} = 12.97$, $p < 0.0001$) and an earlier median NF stage of development ($p = 0.004$). Pre-metamorphic tadpoles exposed to 175 and 350 µg/L of B[a]P (7 d exposure) had reduced total length relative to the solvent control treatment ($F_{(4, 33)} = 9.41$, $p < 0.0001$). Pro-metamorphic tadpoles 350 µg/L of B[a]P (7 d exposure) had reduced body weight relative to the pooled control group (0.80 ± 0.04 g vs 0.97 ± 0.03 g; $F_{(4, 35)} = 4.15$, $p = 0.008$), but there was no change in the total length ($F_{(4, 35)} = 2.588$, $p = 0.058$).

Table 3.2. Morphometric measurements[1] and developmental stage[2] of *Xenopus laevis* exposed benzo[a]pyrene (B[a]P) at different stages of development.

Life stage at exposure	Endpoint	Control	B[a]P		
			10 μg/L	175 μg/L	350 μg/L
Embryo-larval (96 hr exposure)	Total length (cm)	0.938 ± 0.005^a	0.917 ± 0.006^{ab}	0.905 ± 0.006^{ab}	0.894 ± 0.007^b
	NF Stage	46 ± 0	46 ± 0.25	46 ± 1	46 ± 0
Pre-metamorphosis (7 d exposure)	Body weight (g)	0.50 ± 0.02^a	0.49 ± 0.02^a	0.37 ± 0.02^b	0.38 ± 0.02^b
	Total length (cm)[3]	4.10 ± 0.09^a	4.01 ± 0.08^a	3.63 ± 0.06^b	3.69 ± 0.08^b
	NF Stage	55 ± 1^a	55 ± 1^a	54 ± 1^a	53 ± 1^b
Pro-metamorphosis (7 d exposure)	Body weight (g)	0.97 ± 0.03^a	0.92 ± 0.05^{ab}	0.85 ± 0.03^{ab}	0.80 ± 0.04^b
	Total length (cm)[3]	5.53 ± 0.09	5.30 ± 0.11	5.36 ± 0.07	5.06 ± 0.17
	NF Stage	58 ± 1	58.5 ± 1	58.5 ± 1.75	59 ± 1

[1]Total length (cm), and body weight (g), are presented as mean ± SEM and analyzed with one-way ANOVA followed by Tukey's post hoc test.
[2]Developmental stage based on Nieuwkoop and Faber (NF) and presented as median ± interquartile range and analyzed with Jonckheere-Terpstra test.
[3]Analyzed against the solvent control group rather than the pooled control group, because control groups were significantly different from each other.
[a-c]Different letters indicate significant differences between treatment groups within a stage.

3.3.2. Expression of biotransformation-related genes

The effects of B[a]P exposure on expression of biotransformation-related genes across developmental stages of *X. laevis* are presented in Figure 3.1. There was no change in AhR1B mRNA expression with B[a]P exposure at the embryo-larval life stage (Figure 3.1A; $F (4, 35) = 2.506$, $p = 0.052$) nor in pro-metamorphic tadpoles (Figure 3.1C; $F (4, 35) = 0.533$, $p = 0.663$). In contrast, pre-metamorphic tadpoles exposed to B[a]P had decreased AhR1B mRNA levels, with the 350 µg/L being significantly lower than control treatment (Figure 3.1B; $F (4, 33) = 15.776$, $p < 0.0001$). In all three life-stages examined, CYP1A7 mRNA expression was increased in individuals exposed to B[a]P (Figure 3.1D-F). For embryo-larval individuals, CYP1A7 was upregulated in whole body samples at 10 µg/L and then further increased at 175 and 350 µg/L compared to control treatment (Figure 3.1D; $F (4, 35) = 90.489$, $p < 0.0001$), For pre-metamorphic and pro-metamorphic tadpoles, CYP1A7 in liver was similar to control treatment at 10 µg/L B[a]P but was increased at 175 and 350 µg/L (Figure 3.1E-F; pre-metamorphic: $F (4, 33) = 45.581$, $p < 0.0001$; pro-metamorphic: $F (4, 35) = 57.998$, $p < 0.0001$).
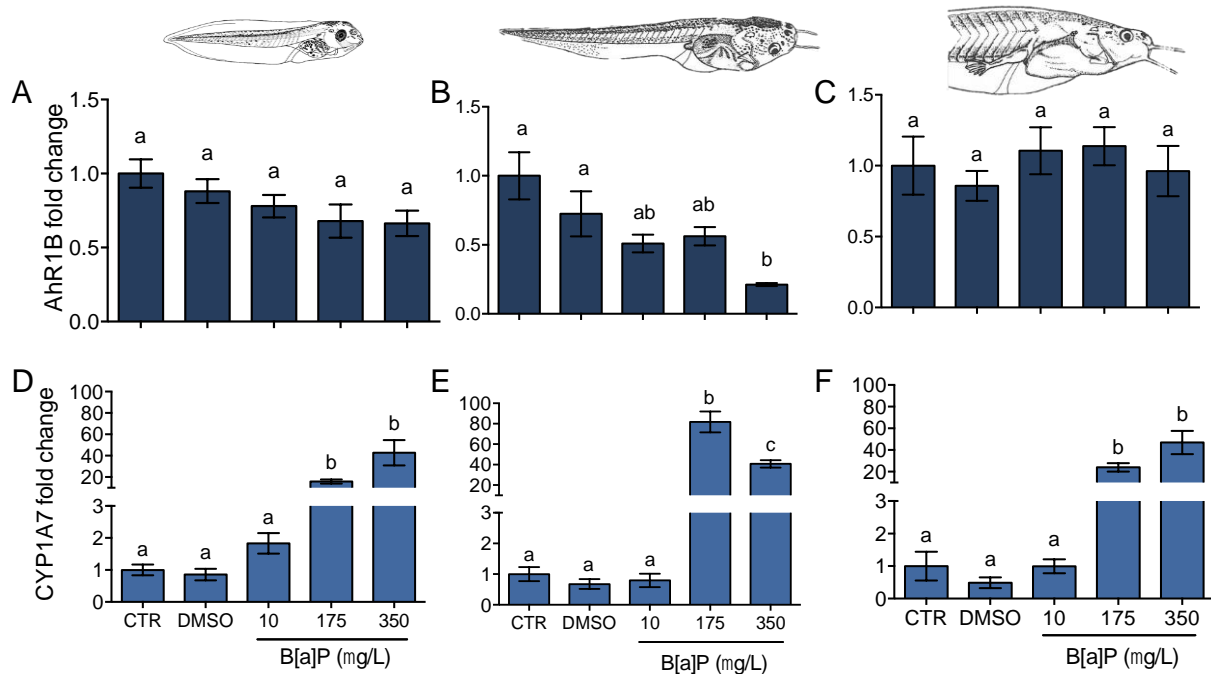
Figure 3.1. Expression of AhR1B (A – C) and CYP1A7 (D – F) in *Xenopus laevis* following exposure to 10, 175 or 350 µg/L B[a]P during the embryo-larval (96 hrs), pre-metamorphic (2 days) or pro-metamorphic (2 days) life-stage. Within each life-stage and target gene, data were normalized to the geometric mean of GAPDH and EF1α, and expressed relative to the pooled control group (combined water and solvent control group, CTL). Bars represent the mean ± SEM (n = 7-8 pooled samples for embryo-larval and individuals for pre- and pro-metamorphic tadpoles). Data were analyzed using one-way ANOVA followed by Tukey's post hoc test; alpha was set at 0.05 and different letters indicate significant differences among treatments. Tadpole images from Nieuwkoop and Faber (1956).

3.3.3. Cytokine expression

The effects of B[a]P exposure on cytokine mRNA expression across developmental stages of *X. laevis* are presented in Figure 3.2. The expression of TNFα was decreased in pre-metamorphic tadpoles exposed to 350 μg/L B[a]P relative to control treatment (Figure 3.2B; $F_{(4, 33)} = 4.256$, $p = 0.012$) but exposure at the other two life-stages had no effect on TNFα levels (Figure 3.2A embryo-larval: $F_{(4, 35)} = 1.198$, $p = 0.324$; Figure 3.2C pro-metamorphic: $F_{(4, 35)} = 0.661$, $p = 0.582$). With CSF-1, expression of was decreased in pre-metamorphic tadpoles exposed to 350 μg/L B[a]P relative to control treatment (Figure 3.2H; $F_{(4, 33)} = 3.094$, $p = 0.04$) and no changed in expression with B[a]P exposure at the embryo-larval (Figure 3.2G; $F_{(4, 35)} = 2.469$, $p = 0.079$) or pro-metamorphic life-stage (Figure 3.2I; $F_{(4, 35)} = 0.157$, $p = 0.924$). Embryo-larval tadpoles exposed to 350 μg/L B[a]P for 96 hr had IL-1β mRNA expression that were 2-fold higher relative to the control treatment (Figure 3.2D; $F_{(4, 35)} = 5.535$, $p = 0.004$) while pro-metamorphic tadpoles exposed to 350 μg/L B[a]P displayed a 2.6-fold decrease in IL-1β expression relative to control treatment (Figure 3.2F; $F_{(4, 35)} = 6.349$, $p = 0.002$). Conversely, there were no differences in the expression of IL-1β in the trunk of pre-metamorphic tadpoles exposed to B[a]P (Figure 3.2E; $F_{(4, 33)} = 1.048$, $p = 0.385$).
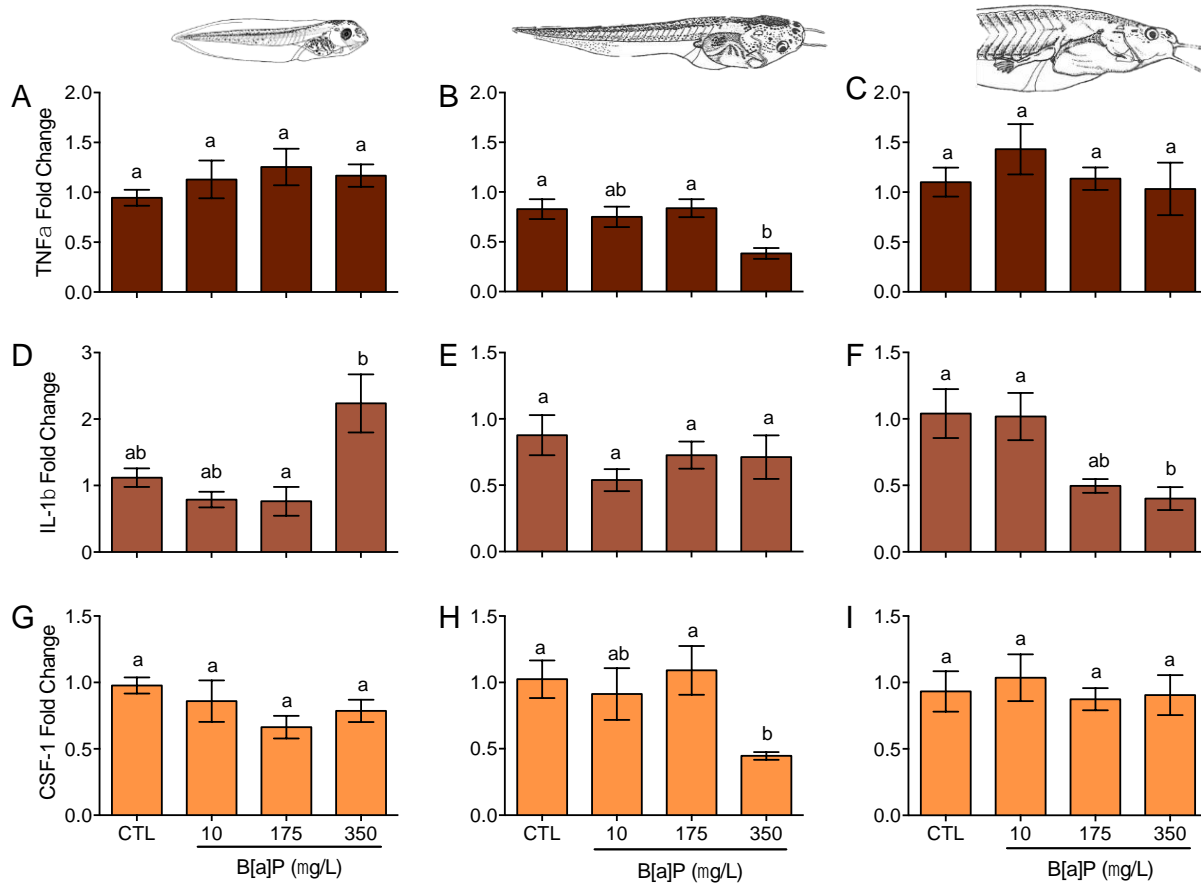
Figure 3.2. Expression of TNFα (A – C), IL-1β (D – F), and CSF-1 (G – I) in *Xenopus laevis* following exposure to 10, 175 or 350 µg/L B[a]P during the embryo-larval (96 hrs), pre-metamorphic (2 days) or pro-metamorphic (2 days) life-stage. Within each life-stage and target gene, data were normalized to the geometric mean of GAPDH and EF1α, and expressed relative to the pooled control (combined water and solvent control, CTL). Bars represent the mean ± SEM (n = 7-8 pooled samples for embryo-larval and individuals for pre- and pro-metamorphic tadpoles). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test; alpha was set at 0.05 and different letters indicate significant differences among treatments. Tadpole images from Nieuwkoop and Faber (1956).

3.4. Discussion

The present study used specific timing of B[a]P exposures during larval development of *X. laevis* to determine if developmental windows respond differently in terms of morphometrics, rate of development, and expression of genes related to biotransformation and immunity. While some studies have examined how B[a]P exposure affects tadpoles and post-metamorphic frogs (Martini et al., 2012; Sadinski et al., 1995), this is the first investigation of the effects of differential timing of B[a]P exposure throughout larval development. The tadpole life-stages represent a dynamic period of amphibian development, when drastic changes in organ/tissue organization and molecular signalling occur across many systems, including those related to metabolic processes and the immune system (Shi, 2000). The immunotoxicity of B[a]P in fish and mammals is dependent on biotransformation of B[a]P into reactive metabolites, and subsequent interaction of the metabolites with cellular and/or humoral components of the immune system (reviewed in Miller and Ramos, 2001; Reynaud and Deschaux, 2006). Due to the dynamic nature of amphibian development, the biotransformation capacity and components of the immune system present at any given time during development are continually changing (Rollins-Smith, 1998; Shi, 2000). Thus, early amphibian life-stages may respond differently to B[a]P depending on the timing of exposure, and this may have implications for the immunocompetence of tadpoles when challenged with a pathogen exposure. Results of the present study show increased mRNA expression of the phase I enzyme CYP1A7, suggesting metabolic capacity for B[a]P at all life-stages examined. Conversely, we found that the impact of B[a]P on the immune-related gene expression varied based on the stage of development at the time of exposure, which may be due to the progressive maturation of the immune system. Although concentrations of B[a]P used in this study exceed the limit of solubility and above environmentally relevant concentrations for B[a]P in aquatic systems, previous studies with *Xenopus* using similar concentrations have confirmed that these concentrations are stable in water for a 24 hr period, are sublethal for larval life-stages, and can alter expression of immune-related genes (Martini et al., 2012; Saka, 2004).

Pre-metamorphosis was identified as the most sensitive life-stage for morphological impacts of B[a]P. Pre-metamorphic tadpoles exposed to B[a]P for seven days had significantly decreased body weight, total length, and stage of development compared to control group, whereas embryo-larval and pro-metamorphic tadpoles exposed to B[a]P had decreased total

length or body weight, respectively. By examining responses across life-stages, this study incorporates basic biological differences between the life-stages to determine how they may influence the response to B[a]P. For example, *X. laevis* tadpoles begin external feeding at NF stage 45 and therefore, in the present study, dietary ingestion of B[a]P sorbed to food particles would be a relevant route of exposure only for pre- and pro-metamorphic tadpoles. Reduced body weight, total length and delayed development have been reported in larval amphibians with exposure to known AhR ligands, such as TCDD (Walker et al., 1991) as well as coal-tar-based pavement sealer (Bryer et al., 2006). Furthermore, morphological effects such as decreased total length, decreased swim bladder area, and increased pericardial edema, have also been reported in larval zebrafish exposed to B[a]P (Alharthy et al., 2017; Corrales et al., 2014). The B[a]P-induced stage-specific effects observed in our study may be attributable to differences in the growth rates between the stages combined with the energetic expense associated with up-regulation of detoxification pathways (Segner et al., 2012b). Even with short-term exposure, B[a]P disrupts pathways associated with liver function and metabolism in *X. tropicalis* (Regnault et al., 2016; 2014). Furthermore, demersal fish (*Solea ovata*) and rainbow trout (*Oncorhynchus mykiss*) exposure to B[a]P exhibited glycogen depletion and increased ethoxy-resorufin O-deethylase (EROD) activity in liver, suggesting increased energy expenditure to cope with detoxification of B[a]P (Au et al., 1999; Tintos et al., 2008). Pre-metamorphic tadpoles may be particularly vulnerable to energy deficits, resulting in the B[a]P-induced decreases in body weight, total length and stage of development.

Regardless of the stage of tadpole development, short-term exposure to B[a]P (≥ 175 μg/L) effectively induced expression of CYP1A7 in whole-body of embryo-larval stage individuals or in the trunk of tadpoles at pre- and pro-metamorphosis. Although previous studies have focused on a single, specific life-stage in examining effects of AhR ligands on amphibians, (Collier et al., 2008; Jönsson et al., 2011; Martini et al., 2012), this is the first study to compare responses between distinct developmental stages. In this study, all tadpole life-stages exposed to either 175 or 350 μg/L B[a]P had a greater than 15-fold induction of CYP1A7 mRNA. During metabolism, B[a]P can be detoxified or bioactivated by CYP1A. A substantial amount of evidence supports the connection between increased CYP1A mRNA expression and the subsequent production of ROS (Valavanidis et al., 2006). Increased CYP enzyme activity, as assessed through EROD induction has been reported in *X. tropicalis* exposed to AhR ligands

(Jönsson et al., 2011), and likely contributes to ROS production (Miller and Ramos, 2001). The tadpole liver serves as both a major organ for biotransformation and a secondary immune organ (Colombo et al., 2015; Manning and Horton, 1969); therefore, ROS produced by CYP1A7 in the tadpole liver are in proximity to the immune cells and has the potential to cause cellular and molecular damage (N'Diaye et al., 2006; Platzer et al., 2009) in a stage-specific manner.

In terms of AhR, effects of B[a]P exposure appeared to be stage-specific where B[a]P reduced AhR1B mRNA levels in liver of pre-metamorphic tadpoles but had no effect on AhR1B expression in either the pro-metamorphic or embryo-larval tadpoles. As an AhR ligand, B[a]P binds to the AhR and causes the transcriptional up-regulation of the AhR gene battery (reviewed in Miller and Ramos, 2001), a set of genes with the xenobiotic response element (XRE) promoter sequence that aid in the metabolism of AhR ligands (Beischlag et al., 2008). In a previous study, exposure of *X. tropicalis* to AhR ligands (3,3′,4,4′,5-pentachlorobiphenyl (PCB126), β-naphthoflavone and indigo) also induced expression of CYP isoforms without affecting AhR expression (Jönsson et al., 2011). In fish and mammals, instances of decreased AhR mRNA, as observed in the pre-metamorphic life-stage, following exposure to a ligand is attributed to the degradation of the transcription factor after binding to the XRE of AhR responsive genes (Pollenz, 2002). Taken together, these findings suggest that metabolism of B[a]P by CYP1A7 can occur across all tadpole life-stages examined and that AhR1B may not be an effective marker of exposure to B[a]P in *X. laevis* tadpoles.

Cytokines are released from both immune and non-immune cells, with each cell type capable of releasing a specific profile of cytokines. The ROS produced by CYP1A enzymes are hypothesized to be in part responsible for the immunotoxicity of PAHs, including B[a]P, in fish and mammals (reviewed in Reynaud and Deschaux, 2006; White et al., 1994), and the damage inflicted on the immune cells may ultimately affect the number and capacity of cells to produce cytokines (N'Diaye et al., 2006; van Grevenynghe et al., 2003). In the present study, B[a]P exposure decreased TNFα and CSF-1 expression in pre-metamorphic tadpoles and decreased IL-1β expression in pro-metamorphic tadpoles. Differences in transcript levels of cytokines across developmental stages in responses to B[a]P exposure could be attributed to specific immune cell types that are responsible for specific cytokine production and when these cell types are most abundant/active during amphibian development. During pre-metamorphosis for example, circulating and resident macrophage populations are first established, and these cells produce

both TNFα and CSF-1 (Grayfer et al., 2014b; Tracey and Cerami, 1993). These newly developed macrophage populations could be a target for B[a]P-induced ROS production, resulting in the impaired production of TNFα and CSF-1. In contrast, IL-1β is primarily produced by monocytes, macrophages and dendritic cells, which have all been shown to express high basal levels of AhR (Kreitinger et al., 2016). Once these populations are established in the pro-metamorphic tadpoles, the internal ROS production following B[a]P exposure in all three cell types may alter global IL-1β mRNA production during pro-metamorphosis. Although this study did not assess levels of cytokines released, B[a]P-induced changes in cytokine expression across developmental stages in *X. laevis* could be attributed to differences in the maturation state of the immune system at the time of exposure. Future co-exposure studies with B[a]P and AhR antagonists, such as α-naphthoflavone or CH223191, could provide additional insight into the role of AhR activation and CYP1A induction on affecting cytokine levels across developmental stages.

Given their integral role in immune defences against pathogens, impairment of cytokine regulation by B[a]P has the potential to impact disease susceptibility in amphibians. Several studies have demonstrated that exposure of *X. laevis* to the *Ranavirus* Frog virus 3 (FV3) or the simulated bacterial pathogen lipopolysaccharide (LPS) induces expression of the pro-inflammatory cytokines IL-1β, TNFα, CSF-1, IFN-γ (De Jesús Andino et al., 2012; Grayfer and Robert, 2013; Morales et al., 2010; Zou et al., 2000). Up-regulation of these cytokines in response to pathogens is important for the organism to mount a coordinated immune response across the diverse immune cells that are dispersed the body (Segner et al., 2012b). Furthermore, increased susceptibility to FV3 in the tadpole life-stage is at least in part attributable to the diminished and delayed pro-inflammatory cytokine immune responses to the pathogen in tadpoles compared to adults (De Jesús Andino et al., 2012). Therefore, B[a]P-induced impairment of pro-inflammatory cytokines in tadpoles may further contribute to their susceptibility to this environmentally relevant pathogen.

In conclusion, we demonstrated that the adverse outcomes of B[a]P exposure varies among life stages during which exposure occurs, with the pre-metamorphic tadpoles being the most affected in terms of morphological effects. In addition to the growth and developmental effects of B[a]P, our results suggest that activation of detoxification and inflammatory pathways are a consequence of B[a]P exposure in tadpoles that should also be considered and further

explored. Stage-specific effects that we observed at the gene expression level are likely attributable to the maturation state of the immune system at the time of exposure combined with the dual role of the liver in biotransformation of toxicants and as a secondary immune organ. However, the underlying mechanisms of the stage-specific effects remain unclear and warrant continued investigation of contaminant-induced immunotoxicity is amphibians – especially given potential consequences for amphibian disease susceptibility during larval life-stages and long-term population health.

CHAPTER 4:

BENZO[A]PYRENE-INDUCED IMPAIRMENT OF IMMUNE-ASSOCIATED RESPONSES IN THE AMPHIBIAN *XENOPUS LAEVIS*

Preface

The goal of the research presented in Chapter 4 of this thesis was to determine if the ability to respond to immune stimulation in *Xenopus laevis* is compromised by a short-term waterborne exposure to B[a]P. In order to accomplish this objective, a number of tools for *Xenopus laevis* immunotoxicity studies were developed, including a model for an immune challenge assays and flow cytometric analysis of white blood cell populations. Currently, the information on contaminant-induced immunotoxicity in amphibians is relatively scarce. This research not only explores the immunotoxic consequences of exposure to a widespread contaminant of concern but also provides an approach for evaluating other chemicals for their potential to impact the immune system of amphibians. The content of Chapter 4 is being prepared as a manuscript for submission to *Aquatic Toxicology* under the joint authorship of Melanie Gallant, Nicole Baldwin and Natacha Hogan.

Author contributions:

Melanie Gallant (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Nicole Baldwin (University of Saskatchewan) assisted in the design and management of the experiment, commented on and edited the manuscript.

Natacha Hogan (University of Saskatchewan) provided inspiration, scientific input and guidance on the design of the experiment, supervised Melanie Gallant commented on and edited the manuscript, and obtained funding for the research.

Abstract

Numerous contaminants present in the aquatic environment have the potential to disrupt immune defences and increase disease susceptibility. Amphibians are known to inhabit contaminated environments, and there is growing concern that immunotoxic contaminants, such as the polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene (B[a]P), may magnify the impacts of global emerging infectious diseases. The goal of this study was to determine if a short-term waterborne exposure to B[a]P affects the ability of juvenile *Xenopus laevis* to respond to an immune challenge. Lipopolysaccharide (LPS) was used as a simulated pathogen as it is a highly-conserved pathogen-associated molecular pattern that effectively induces immune responses. In a preliminary exposure to determine LPS dose for immune challenge, juvenile *X. laevis* were exposed to control or sham-injected (PBS) control treatments, 0.3, 3 or 30 µg LPS/g body weight via intraperitoneal (i.p.) injection and sampled 1- and 3-days post injection (dpi). *X. laevis* responded to LPS exposure by mounting a typical and robust leukocyte response (increased lymphocytes, decreased granulocyte:lymphocyte (G:L) ratio, and increased monocytes) and induction of cytokines (interleukin-1β, IL-1β; tumour necrosis factor-α, TNFα; colony-stimulating factor-1, CSF-1) following exposure to LPS at 1 dpi, with the response subsiding by 3 dpi. Using this preliminary information, an immune challenge experiment was conducted by pre-exposing juveniles to B[a]P (70 or 350 µg/L or a DMSO vehicle control treatment) for seven days and examining immune parameters 1 dpi in both an immune-resting (PBS injected) and immune-stimulated (10 µg/g LPS) states. The inflammatory response to LPS was impaired with pre-exposure to the highest concentration of B[a]P as indicated by an inability to induce G:L ratio or IL-1β mRNA expression. This study demonstrates that B[a]P can impair the inflammatory immune response to a simulated pathogen, and thus the immunotoxic potential of this compound in amphibians. Given the fundamental role of inflammation in immune responses, these findings may have implications for the susceptibility of amphibians to pathogen infection and associated diseases in contaminated environments.

4.1. Introduction

Aquatic systems are sinks for diverse organic and inorganic contaminants, which can be discharged via point and non-point sources (Davis et al., 2008). These inputs pose a significant risk to the health of aquatic organisms, with reported adverse effects ranging from altered behaviour to impaired development, reproduction or immune defences. In particular, the immune system is recognized as exceedingly sensitive to contaminant exposure, with impairment of immune defences often occurring at lower concentrations than other adverse effects (Bols et al., 2001; Rehberger et al., 2017; Van Loveren et al., 2003). Contaminants such as pesticides (Forson and Storfer, 2006b; Gilbertson et al., 2003; Rohr et al., 2008), polybrominated diphenyl ethers (Cary et al., 2014), endocrine disrupting compounds (reviewed in Casanova-Nakayama et al., 2011), metals (reviewed in Zelikoff, 1993), and polycyclic aromatic hydrocarbons (Carlson et al., 2004a; Martini et al., 2012; McNeill et al., 2012) can suppress immune responses in aquatic vertebrates. Disruption of immune defences can often lead to decreased host resistance against pathogens and increased incidence of disease and mortality in aquatic organisms (Pannetier et al., 2019; Rohr et al., 2008; Wenger et al., 2011).

As a chemical class, PAHs are widely distributed in the aquatic environment due to releases from both natural and anthropogenic sources (Abdel-Shafy and Mansour, 2016; Lawal, 2017). While some PAHs have relatively low toxicity, others, such as benzo[a]pyrene (B[a]P), are considered carcinogenic, teratogenic, mutagenic and immunotoxic (Lawal, 2017; Reynaud and Deschaux, 2006). Benzo[a]pyrene is often measured as a component of PAH mixtures and is one of the most extensively studied PAHs (Hylland, 2006). The immunotoxicity associated with B[a]P exposure is attributed to *in situ* metabolism of B[a]P through activation of the aryl hydrocarbon receptor (AhR) and subsequent production of reactive metabolites by cytochrome P450 1A (CYP1A) enzymes (Möller et al., 2013; Reynaud and Deschaux, 2006). Fish exposed to B[a]P exhibit decreased immune-related endpoints such as lymphocyte proliferation, superoxide production and immune cell numbers, as well as reduced host resistance as displayed by a suppressed immune response to a pathogen challenge (Carlson et al., 2004a; 2002; Phalen et al., 2014). Such exposure studies in aquatic species help identify parameters indicative of immune disruption by contaminants such as B[a]P and provide insight into potential immunological consequences such as disease susceptibility in exposed populations.

Amphibians inhabit PAH-contaminated environments where they can uptake and metabolize PAHs. In the Athabasca oil sands region, sediment from wetlands closest to oil sands extraction facilities have the highest concentrations of PAHs (Mundy et al., 2019). In laboratory exposure experiments, wood frog (*Lithobates sylvaticus*) tadpoles exposed to this sediment were shown to rapidly accumulate these compounds within 12 h of exposure (Bilodeau, 2017). Reduced growth and delayed development were observed in tadpoles raised in wetlands containing oil sands-impacted sediment (Hersikorn and Smits, 2011). Given the abundance of evidence for immunotoxic effects of PAHs, and especially B[a]P, in fish (reviewed in Reynaud and Deschaux, 2006), it is quite surprising the lack of similar studies for amphibians. Impairment of amphibian immune defences by PAHs could result in weakened defences against pathogen infection. Emerging infectious diseases are a major factor in the dramatic worldwide decline of amphibian populations (Carey et al., 1999; Daszak et al., 2003; Gray et al., 2009) and PAH exposure may compromise immune function and increase disease susceptibility of amphibians inhabiting contaminated environments (Blaustein et al., 2012; Hof et al., 2011).

The study of immunotoxicity in the context of ecotoxicology suffers from a lack of established assays and validated parameters (Rehberger et al., 2017). This is even more limited for amphibians as compared to fish. Endpoints and assays previously used to evaluate immune function in amphibians include the phytohaemagglutinin skin-swelling test (Brown et al., 2011; Venesky et al., 2012), lymphocyte proliferation (Christin et al., 2003), phagocytosis assay (Christin et al., 2004; 2003), and cytokine expression (De Jesús Andino et al., 2012; Gantress et al., 2003b; Sifkarovski et al., 2014). Cytokines are immune mediators that allow communication between many cell types and immune organs distributed around the body (Segner et al., 2012a) and as such, normal function and regulation of cytokines are a key component in mounting an effective immune response. Contaminants such as perfluorooctane sulfonate (Fang et al., 2013), B[a]P (Hur et al., 2013; Martini et al., 2012) and cypermethrin (Jin et al., 2011) can alter cytokine expression in aquatic vertebrates and thus have been used as indicators of immune dysfunction. At the cellular level, leukocyte profiling or differential white blood cell (WBC) counts are a hematological endpoint that can provide information about the health of fish and amphibians. Upon pathogen infection, leukocytes respond as the first line of defence (Davis et al., 2008; Köllner et al., 2002); however, if contaminant exposure alters the ability of specific cell types to respond, this could reduce host immunocompetence. For example,

immunocompetence of juvenile rainbow trout (*Oncorhynchus mykiss*) was compromised following exposure to ultraviolet B (UVB) irradiation where increased bacterial and trematode load in UVB-exposed fish was, in part, attributed to changes in circulating leukocyte profiles (Markkula et al., 2007). Traditional methods of manual leukocyte identification and counts are labour intensive, time-consuming and require training to differentiate cell types accurately. Recently, a flow cytometric method using the lipophilic dye 3,3′-dipentyloxacarbocyanine iodide ($DiOC_5(3)$) was developed to evaluate changes in leukocyte profiles due to physiological stress in western spadefoot toad (*Pelobates cultripes*; Burraco et al., 2017). It was used to identify a reduction in circulating and tissue-specific leukocyte populations upon B[a]P exposure in rainbow trout (Phalen et al., 2014). Therefore, this method may also be valuable for evaluating how exposure to B[a]P and other contaminants affect the immune system in amphibians at the cellular level.

It has been emphasized that assessment of immunotoxicity should be based on measuring chemical effects on the activated rather than on the resting immune system (Köllner et al., 2002; Segner et al., 2012b). For example, host resistance assays are performed as challenge assays in which organisms are exposed to the contaminant of concern and simultaneously or subsequently exposed to a defined concentration of a pathogen (Segner et al., 2012). While the effect parameter typically used in these challenge tests is the pathogen-induced mortality, molecular or cellular immune marker parameters can be used in conjunction with a sub-lethal immune challenge to elucidate underlying mechanisms by which a chemical impairs the ability of the host to mount an effective immune response. Previous studies looking at immunocompetence of amphibians with pesticide exposure have mainly focused on host mortality and infection rates (Davidson et al., 2007; Forson and Storfer, 2006a; Rohr et al., 2008), but did not evaluate specific immune parameters associated with an immune response. With this in mind, the overall goal of this study was to determine whether short-term waterborne exposure to B[a]P affects the ability of *Xenopus laevis* (*X. laevis*) to respond to a subsequent immune stimulation, via administration of lipopolysaccharide (LPS). As the main component of the external membrane of gram-negative bacteria, LPS is recognized by the innate immune system as a highly-conserved pathogen coded molecular structure and effectively induces leukocyte responses and mRNA expression of molecules involved in inflammation (such as cytokines) in both mammals and fish (Holen et al., 2012). The first specific objective was to validate a flow cytometric method with

the stain DiOC$_5$(3) for leukocyte profiling in blood of *X. laevis*. We then conducted an LPS immune challenge study to determine the sublethal effects of B[a]P on immune parameters in both an immune-resting and immune-stimulated state, evaluating blood leukocyte profiles and expression of specific immune mediators (cytokines). Benzo[a]pyrene induces the expression of CYP1A as part of phase I metabolism, and thus CYP1A7 expression was used as an indicator of B[a]P exposure in the immune challenge study.

4.2. Material and Methods

4.2.1. Animal care

Xenopus laevis used in this study were bred and reared in the Aquatic Toxicology Research Facility (ATRF) as described in Gallant and Hogan et al. (2018). Briefly, tadpoles were housed in a flow-through system maintained at 22°C with a 16 h light: 8 h dark cycle. Tadpoles were fed daily, *ad libitum*, commercially available Sera Micron (Sera, Heinsberg, Germany) and transitioned to frog brittle (Nasco, Fort Atkinson, WI, USA) after metamorphosis. Water quality was monitored and recorded daily during rearing and exposures (pH 7-7.5, dissolved oxygen > 90%, temperature = 22 ± 0.5°C). The care and treatment of animals used in this study were approved by the University of Saskatchewan's Animal Research Ethics Board (protocol no. 20130045), and adhered to the Canadian Council on Animal Care guidelines for humane animal use (CCAC, 1993).

4.2.2. Preliminary lipopolysaccharide challenge

An LPS challenge was conducted to determine the appropriate concentration and time for LPS to induce an immune response in juvenile *X. laevis*. A total of 88 animals were reared to three months post-metamorphosis and transferred to an environmental chamber to acclimate for two weeks before the exposure. Three days prior to injection, individuals were weighed and sorted to ensure an even distribution of sizes across treatments. LPS (E. coli 055:B5; Sigma-Aldrich, St. Louis, MN, USA) was solubilized in sterile PBS on the day of injections at stock concentrations of 7.8, 78, and 780 µg/mL. Animals were lightly anesthetized by immersion in buffered 0.2 g/L tricaine methanesulfate (MS-222) prior to injection. Individuals were injected intraperitoneally (i.p.) on the lower left portion of the abdomen with 50 µl using a 29-gauge needle to deliver 0.3, 3 or 30 µg LPS/g body weight or sham-injected (PBS) control group. These

LPS concentrations were chosen based on previous studies with *X. laevis* (Zou et al., 2000) and zebrafish (Novoa et al., 2009) that used similar concentrations and volumes for an LPS challenge. After injections, individuals were placed in clean water to recover and were monitored for survivorship.

Animals (n = 8/treatment) were euthanised by immersion in buffered MS-222 (1 g/L) at 1 day and 3 days post-injection (dpi). There were also eight individuals euthanized and sampled prior to LPS injections (0 dpi) as a "no handling" control group. For all sampling, individuals were measured for total wet weight (to the nearest 0.01 g) and snout-vent length (to the nearest 0.1 cm). The delivered dose of LPS was calculated using individual weights, and the known concentration of LPS injected, then averaged by treatment. There was no difference between the calculated and nominal delivered LPS dose for any treatment at either 1 or 3 dpi (Appendix A). Whole blood (10 µl) was collected by cardiac puncture and was divided for blood smears and flow cytometry (see section 4.2.4.). Livers were dissected, weighed (to the nearest 0.01 g) and immediately flash frozen for later gene expression analysis (see section 4.2.5.).

4.2.3. Benzo[a]pyrene exposure with lipopolysaccharide challenge

As in the preliminary LPS exposure, 72 juvenile *X. laevis* were allowed to acclimate in the environmental chamber for two weeks, and individuals were sorted by weight three days before the exposure. The B[a]P (cat no. B1760, Sigma-Aldrich) was dissolved in DMSO and added to exposure tanks for a final solvent concentration of 0.007% v/v. Treatments consisted of B[a]P at nominal concentrations of 70 or 350 µg/L and a DMSO vehicle control treatment. Individuals were exposed to B[a]P for seven days under static renewal conditions with triplicate tanks per treatment and eight tadpoles per tank. Water parameters were recorded daily (pH 7-7.5, dissolved oxygen > 90%, temperature = 20 ± 0.5°C) and a 75% water change was conducted where tanks were re-dosed with B[a]P to maintain the nominal target concentrations. After seven days of B[a]P exposure, each tank was divided so that four individuals per tank received PBS injection and four received the LPS injection. This experimental design resulted in a 2 x 3 factorial design, with six unique treatments and 12 individuals per treatment. A nominal LPS dose of 10 µg/g was chosen based on the preliminary LPS challenge in order to stimulate the immune system as measured by an induction in cytokine expression and changes in blood leukocyte profiles. Using the individual weights taken before the exposure began, a stock

concentration of 142 µg/mL was prepared on the day of injections. Anesthesia, injections and recovery were conducted as previously described, with individuals receiving 50 µl of the appropriate solution (PBS or LPS), returned to clean water and sampled 1 dpi (chosen based on data from the preliminary LPS challenge showing maximal immune stimulatory response). Euthanasia, sampling, and average delivered dose calculations were were performed as described for the preliminary LPS exposure. There was no difference in the average delivered LPS dose across B[a]P exposure groups (Appendix B).

4.2.4. Leukocyte profiles and 3,3′-dipentyloxacarbocyanine iodide (DiOC$_5$(3)) validation in *Xenopus laevis*

Whole blood was collected by puncturing the ventricle of the heart and placing a heparinized micro-hematocrit capillary tube (Fisher Scientific) in the incision. Whole blood was divided at the time of sampling for leukocyte profiling using flow cytometric and blood smear analyses. For flow cytometry, 1 µl of blood was placed in heparinized vacutainers (32 IU sodium heparin; BD Bioscience, Franklin Lakes, NJ, USA) containing 1 mL of cold PBS in duplicate per individual. Samples were stored on ice for a maximum of 1 h before staining and preparation for flow cytometry analysis as described in Burraco et al. (2017). Briefly, 1 µl of 2 mM DiOC$_5$(3) was added to the blood and PBS solution, for a final concentration of 2 µM DiOC$_5$(3). Samples were gently vortexed and incubated at room temperature for 5 min. Samples were then centrifuged at 1,000 rpm for 5 min at 4°C, supernatant was removed, and cells were re-suspended in PBS. Stained samples were analyzed by flow cytometry on the Accuri C6 (BD Bioscience), where 100,000 events were recorded per sample.

Gates in flow cytometry are electronic windows that delineate particular cell populations and allow the user to quantify the number of cells belonging to each population; therefore, accurate gating is required for reliable flow cytometry data. The DiOC$_5$(3) binds to lipid bilayers, including the plasma membrane and the membrane of organelles. This affinity for all lipid bilayers allows DiOC$_5$(3) to identify an increasing gradient of organelle content within cells. Erythrocytes, lymphocytes, granulocytes and monocytes were identified and gated based on their distinct properties. For each cell, three measurements were taken: forward scatter (FSC) indicative of the size of the cell, side scatter (SSC) measuring the granularity or complexity of the cell, and the fluorescent intensity of the cell in the FL1 channel. Given SSC and fluorescence

of the cells along with known characteristics for each leukocyte type, gates were set to encompass homogenous cell populations (Figure 4.1.). Gated populations were further validated by selecting the specific populations and plotting the FSC vs SSC to ensure homogenous cell size and granularity within each population.

To validate the flow cytometric analysis of leukocyte populations, manual leukocyte counts using blood smears were conducted under light microscopy. A total of 21 individuals were randomly selected from the 1 dpi preliminary LPS exposure for this validation. For these individuals, 4 µl of blood was smeared on each duplicate glass slides and allowed to air dry at room temperature. Slides were stained using the Hema 3 staining set (Thermo Fisher Scientific, Waltham, MA, USA) as described by the manufacturer, and a coverslip was affixed using Permount (Thermo Fisher Scientific). Blood smears were viewed on an Axiostar Plus microscope (Carl Zeiss, Oberkochen, Germany) at 100X magnification with oil immersion. One hundred leukocytes were counted per slide, for a total of 200 cells per individual. Lymphocytes, neutrophils, monocytes, basophils and eosinophils (Figure 4.2.) were identified with manual leukocyte counts; however, the three granulocyte types (neutrophils, basophils and eosinophils) were pooled for analysis to compare with flow cytometry data.
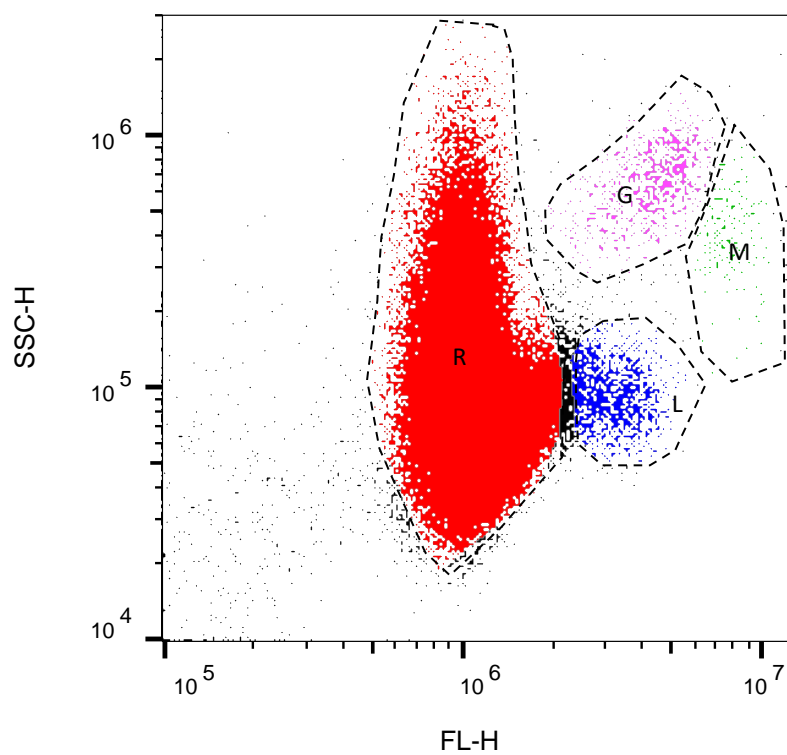
Figure 4.1. Dot plot of *Xenopus laevis* whole blood stained with $DiOC_5(3)$ and gated by cell type (red blood cells, lymphocytes, monocytes and granulocytes, designated by R, L, M, and G, respectively) based on side scatter (SSC) and fluorescent properties.
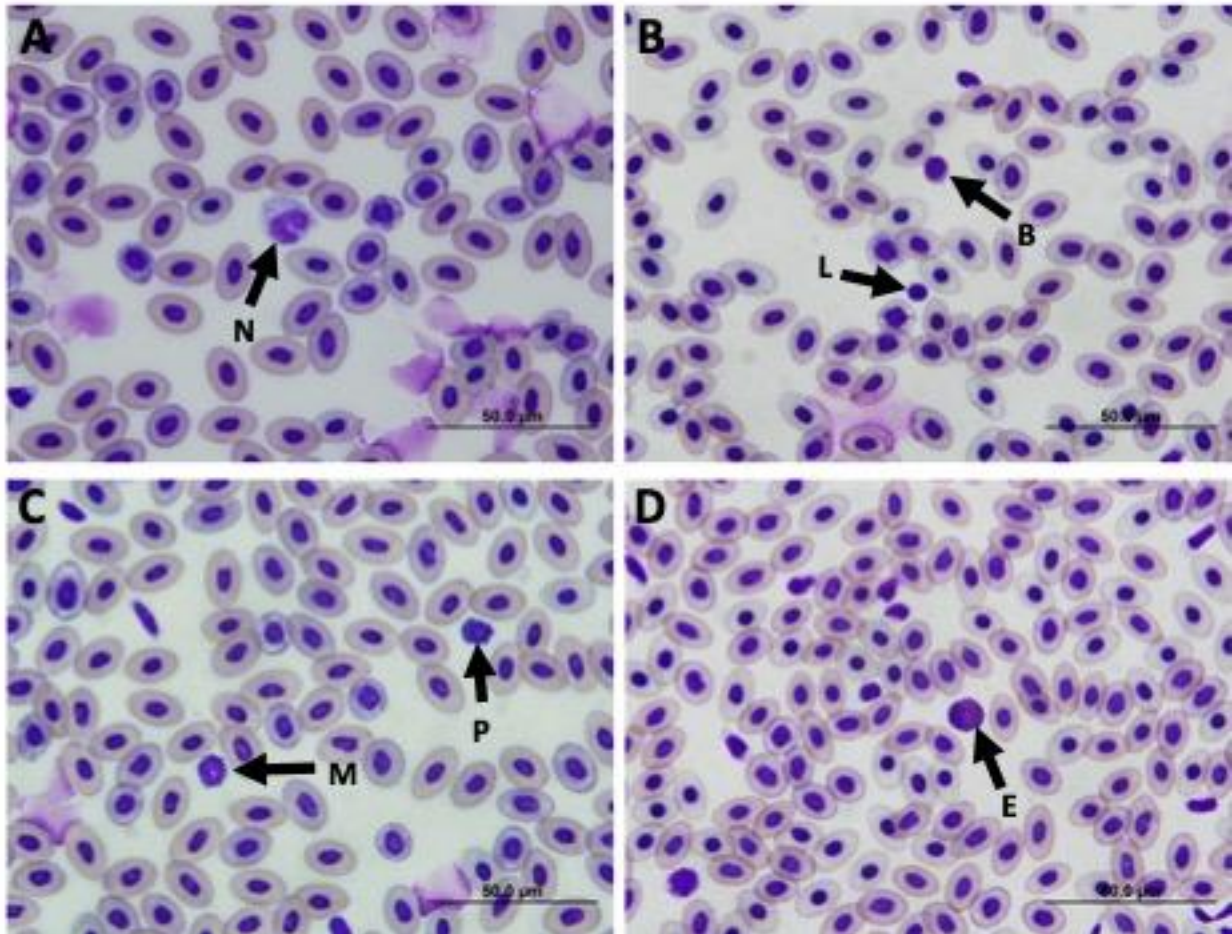
Figure 4.2. Cell types identified in blood smears prepared with whole blood from juvenile *Xenopus laevis*. Panels have specific cell types indicated with arrows: (A) neutrophil, (B) lymphocyte and basophil, (C) monocyte and polychromatic red blood cell (D) eosinophil. Images taken at 100x magnification, scale bar represents 50 µm.

4.2.5. Gene expression analysis

Liver tissues were homogenized using the Bead Ruptor (Omni International, Kennesaw, GA, USA) at 40 Hz for two cycles of 45 s. Total mRNA was extracted using TRIzol reagent as described by the manufacturer (Thermo Fisher Scientific) and re-suspended in rNase-free water. Quality and quantity of the extracted RNA were verified using a 1% agarose gel and the NanoDrop-1000 (Thermo Fisher Scientific), respectively. gDNA cleanup and cDNA synthesis of 1 µg total RNA was performed using the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada) as described by the manufacturer. Samples were diluted 10-fold prior to real-time polymerase chain reaction (qPCR). Gene expression analysis was performed by qPCR using the CFX96 Real-time C1000 Thermal Cycler (Bio-Rad Laboratories) and SsoFast EvaGreen Supermix (Bio-Rad Laboratories) using previously described methods (Gallant and Hogan, 2018). Gene-specific primer sets for targets CYP1A7, IL-1β, TNFα and CSF-1 as well as GAPDH and EF1α, which were used as reference genes, were sourced from the literature (Table 4.1.). Each qPCR assay was optimized for the primer concentration, cDNA input and annealing temperature that resulted in minimal C_t and a single sequence-specific peak (Table 4.1). The mRNA abundance of target and reference genes was interpolated using the relative standard curve method. Duplicate data for each sample were averaged, and target genes were normalized to the geometric mean of the reference genes. Data were expressed as fold-change relative to the respective control group for each experiment. Data for the preliminary LPS experiment were expressed relative to the 0 dpi no handling control group, and data for host resistance assay was expressed relative to the DMSO exposed + PBS-injected group.

Table 4.1. List of target genes and the specific forward and reverse primers used for transcript expression analysis by qPCR in *Xenopus laevis*.

Target	Primer	Sequence (5' to 3')	Accession number	Amplicon Length (bp)	Annealing Temp (°C)	References
IL-1β	Fwd Rev	GGCCTCAATGAAACCTCCAC AGGCAGATATCTCCCAGCAC	NM_001085605.1	232	60	Gallant and Hogan, 2018
CSF-1	Fwd Rev	ATCGAACTCTGTCCAAGCTGGATG GGACGAAGCAAGCATCTGCCTTAT	NM_001280600.1	123	60	Sifkarovski et al., 2014
TNFα	Fwd Rev	TGTCAGGCAGGAAAGAAGCA CAGCAGAGAGCAAAGAGGATGGT	AB298595.1	203	62	Sifkarovski et al., 2014
CYP1A7	Fwd Rev	CCTTTGCTACTTCCCCTTCACCC GGCAACAGAGACCACCACGTACC	NM_001097072.1	155	62	Chapter 3
GAPDH	Fwd Rev	GACATCAAGGCCGCCATTAAGACT AGATGGAGGAGTGAGTGTCACCAT	AF549496.1	130	58.4	Sifkarovski et al., 2014
EF-1α	Fwd Rev	GAGGGTAGTCTGAGAAGCTCTCCACG CCTGAATCACCCAGGCCAGATTGGTG	NM_001086133.1	221	60	Gallant and Hogan, 2018

Fwd, forward; Rev, reverse

4.2.6. Statistical analysis

Data were tested for normality using the Kolmogorov-Smirnov test on residuals and for homogeneity of variance using Levene's test. Data not meeting the assumptions of parametric statistics were log-transformed or transformed using Taylor's power law. The calculated delivered dose in the preliminary LPS challenge was tested for differences from the nominal concentrations using one-sample t-tests. For the preliminary LPS challenge experiment, leukocyte and gene expression data were analyzed for differences among treatments using a one-way ANOVA followed by Tukey's post-hoc test to evaluate differences between groups. Validation of flow cytometry methods was done using the subset of 21 individuals from the preliminary LPS exposure for which manual leukocyte counts were obtained from blood smears. Spearman correlations were used to evaluate the relationship between manual counts and flow cytometry data for each leukocyte type. The LPS-injected groups in the immune challenge assay were tested for differences in delivered LPS dose across B[a]P concentrations using a one-way ANOVA. For the immune challenge assay with B[a]P and LPS, a two-way ANOVA was used to determine significant differences due to interactions (exposure treatment × injection challenge) or main factors (exposure treatment and injection challenge). When significant main effects were detected, a Tukey's post-hoc test was used to distinguish between B[a]P exposure groups, whereas LPS main effects were examined with Sidak's multiple comparisons test. When a significant interaction was detected, a Tukey's post-hoc test was used to compare across all treatments. Hypothesis testing was two-tailed, and results were considered significantly different at an α-level of 5% ($p < 0.05$). All statistical analyses were conducted using SPSS version 22 (SPSS Inc, IBM, North Castle, NY, United States).

4.3. Results

4.3.1. Preliminary lipopolysaccharide challenge

4.3.1.1. Morphometrics

All individuals recovered from handling and injection, and there was no morbidity or mortality observed during the experiment. Morphometrics after 1 and 3 dpi of LPS are shown in Appendix C). Following LPS exposure, there were no differences in the total body weight (1 dpi: $F_{(4, 35)} = 0.175$; $p = 0.950$; 3 dpi: $F_{(4, 34)} = 0.151$; $p = 0.961$) or length (1 dpi: $F_{(4, 35)} = 0.290$; $p = 0.883$; 3 dpi: $F_{(4, 34)} = 0.168$; $p = 0.953$) across treatments. Liver somatic index

(LSI) was higher (1.4-fold) with 30 µg/g LPS at 1 dpi when compared to the PBS-injected control group. There was no difference in LSI at 3 dpi (F (4, 34) = 0.502; p = 0.734).

4.3.1.2. Effect of lipopolysaccharide on blood leukocyte counts and validation of 3,3'-dipentyloxacarbocyanine iodide (DiOC$_5$(3)) staining method with flow cytometry

The effects of LPS injection on the number of specific leukocytes (lymphocytes, granulocytes, monocytes) in whole blood at 1 and 3 dpi are presented in Figure 4.3. After 1 dpi with LPS, there was approximately a 2-fold decrease in the number of lymphocytes at the two highest doses of LPS compared to the no-injection control group but not the PBS-injected control group (Figure 4.3.A; F (4, 35) = 3.61, p = 0.015). There was an increase in the number of granulocytes with increasing LPS doses at 1 dpi (Figure 4.3.B; F (4, 35) = 3.653, p = 0.014). Individuals injected with 3, and 30 µg/g LPS had an approximately 9-fold increase in the number of monocytes relative to the PBS-injected control treatment at 1 dpi (Figure 4.3.C; F (4, 35) = 30.98, p < 0.001). There was a dose-dependent increase in the G:L ratio (Figure 4.3.D; F (4, 35) = 14.904, p < 0.001) with increasing LPS dose at 1 dpi. There was no effect of LPS injection on the number of any of the three leukocyte types at 3 dpi (Figure 4.3.A-D; lymphocytes: F (4, 34) = 0.925, p = 0.461; granulocytes: F (4, 34) = 0.996, p = 0.423; monocytes: F (4, 34) = 2.60, p = 0.053; G:L ratio: F (4, 34) = 1.655, p = 0.183).

From the samples analyzed by flow cytometry, a random subset (n = 21) were chosen and the proportion of leukocytes determined by analyzing blood smears to validate that the DiOC$_5$(3) staining method with flow cytometry was providing the same results as traditional manual cell counts. For the proportion of lymphocytes, the subset of individuals had an average (± standard error of the mean; SEM) intra-sample coefficient of variation (CV) of 15.0 ± 2.4% for manual counts and 13.3 ± 2.5% for flow cytometry. The proportion of granulocytes had an intra-sample CV of 31.1 ± 5.2% for manual counts and 11.7 ± 2.3% for flow cytometry. The intra-sample CV for the proportion of monocytes was 12.5 ± 2.4% for manual counts and 20.5 ± 3.0% for flow cytometry. Identified cell populations plotted as SSC vs FSC showed tight grouping, indicating an effective gating strategy. Lymphocytes and granulocytes showed good correlation between results obtained by blood smear and flow cytometry (Figure 4.4.A and B; lymphocytes: rho = 0.525 and P = 0.015; granulocytes: rho = 0.431 and P = 0.049). There was no correlation

between values obtained by flow cytometry and blood smears for monocytes (Figure 4.4.C; rho = -0.325 and P = 0.15).
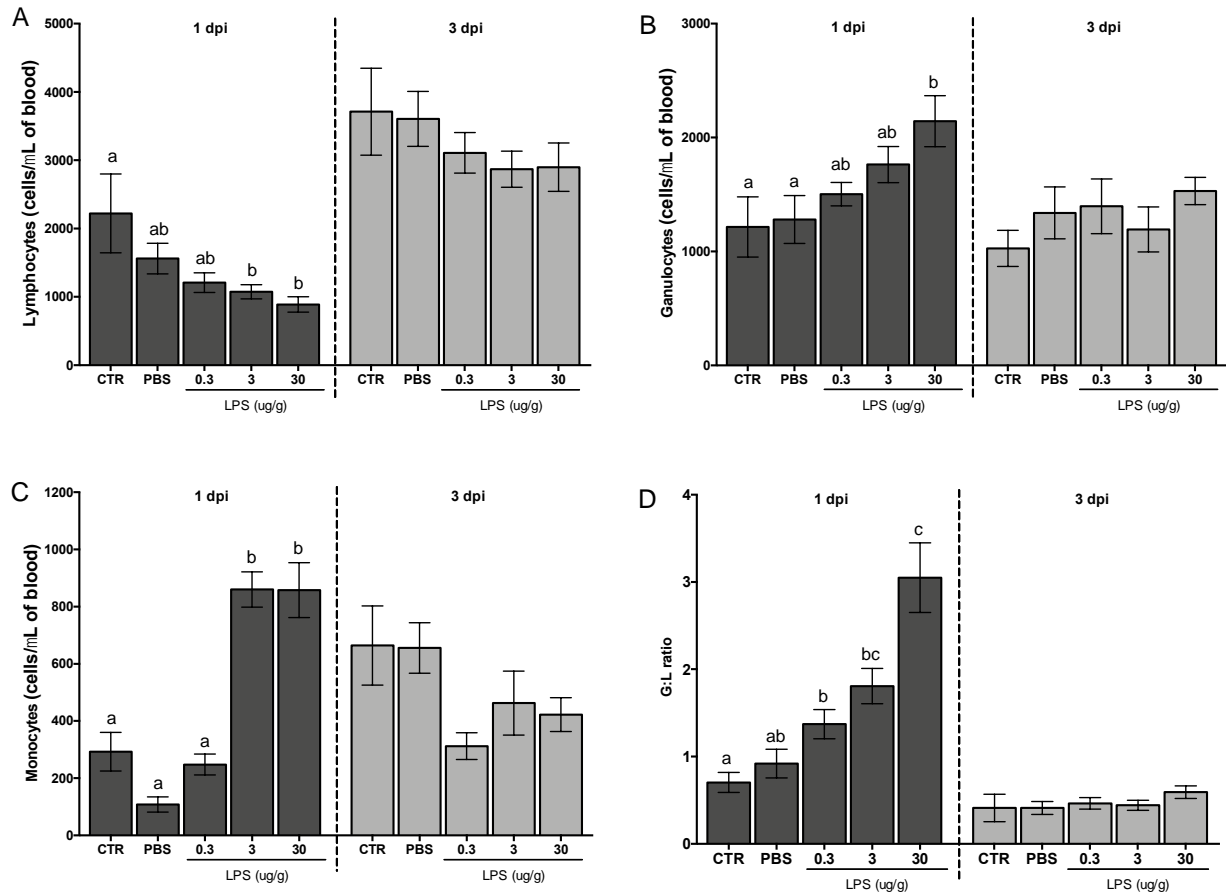
Figure 4.3. Effects LPS injection (PBS, 0.3, 3 or 30 µg/g) on leukocyte numbers (cells/µL whole blood) in juvenile *Xenopus laevis* at 1 dpi (dark grey) and 3 dpi (light grey) as measured by $DiOC_5(3)$ staining and flow cytometry. Panels represent number of (A) lymphocytes, (B) granulocytes, (C) monocytes, and (D) G:L ratio. Bars represent mean ± SEM (n = 7-8). One-way ANOVA was used to compare groups within 1 and 3 dpi followed by Tukey's post hoc test. Letters indicate significant differences at $p < 0.05$.
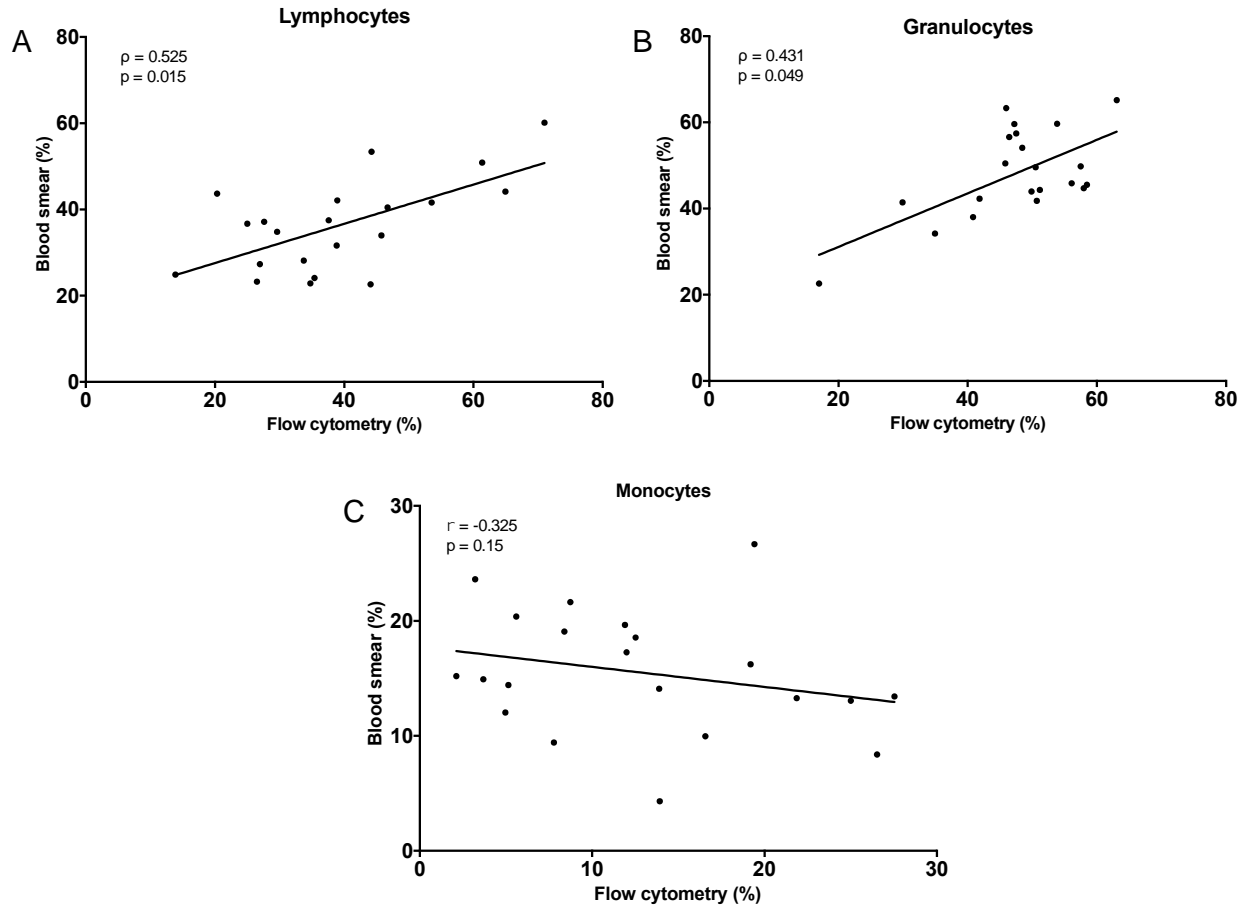
Figure 4.4. Correlations between proportion of (A) lymphocytes, (B) granulocytes and (C) monocytes in whole blood as determined by flow cytometry and blood smear counts. Individuals used in this comparison were randomly chosen from the preliminary LPS injection study (1 dpi, n = 21). For both methods, duplicate measurements were taken per individual and averaged; data were analyzed by Spearman correlation.

4.3.1.3. Effect of LPS on cytokine expression

Since there were no LPS-induced changes in the number of leukocytes at 3 dpi, gene expression was only analyzed for 1 dpi samples (Figure 4.5.). After 1 dpi, TNFα mRNA expression was 2.0, 3.2, and 3.6-fold higher with injection of 0.3, 3 and 30 µg/g of LPS, respectively, relative to the PBS-injected control treatment (Figure 4.5.A; $F_{(5, 41)} = 18.273$, $p < 0.001$). Expression of IL-1β mRNA increased 8.6-fold with 3 µg/g LPS and 14.2-fold with 30 µg/g LPS, relative to the PBS-injected control treatment (Figure 4.5.B; $F_{(5, 41)} = 26.896$, $p < 0.001$). Individuals injected with 30 µg/g LPS had 2.6-fold higher CSF-1 mRNA expression relative to PBS-injected control treatment at 1 dpi (Figure 4.5.C; $F_{(5, 41)} = 7.971$, $p < 0.001$)

Based on the effective dose and time-course response of blood leukocyte populations and cytokine expression to LPS, an injected dose of 10 µg/g LPS and sampling at 1 dpi were selected for the subsequent immune stimulation experiment with B[a]P.
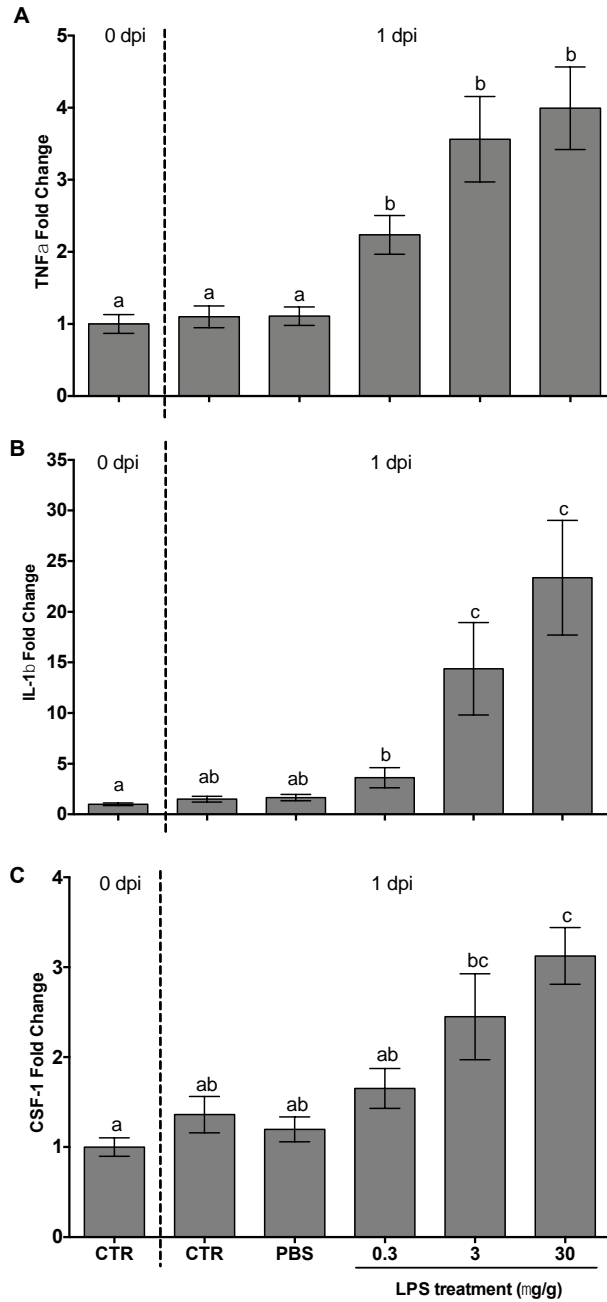
Figure 4.5. Effects of LPS injection on the expression of (A) TNFα, (B) IL-1β, and (C) CSF-1 in juvenile *Xenopus laevis* exposed to control, PBS, 0.3, 3, or 30 µg/g LPS and sampled 0 and 1 dpi. Data were normalized to the geometric mean of GAPDH and EF1α and expressed relative to the 0 dpi control group. Bars represent mean ± SEM (n = 7-8). One-way ANOVA was used to compare groups followed by Tukey's post-hoc test. Letters indicate significant differences at p < 0.05.

4.3.2. Benzo[a]pyrene exposure with lipopolysaccharide challenge

   4.3.2.1. Morphometrics

   All individuals recovered from handling and injection, and there was no morbidity or mortality observed during the experiment. There were no changes in body weight (B[a]P exposure: $F_{(2, 66)} = 0.012$, $p = 0.989$; LPS injection: $F_{(1, 66)} = 0.007$, $p = 0.932$; B[a]P $\times$ LPS interaction: $F_{(2, 66)} = 0.379$, $p = 0.686$), body length (B[a]P exposure: $F_{(2, 66)} = 0.396$, $p = 0.675$; LPS injection: $F_{(1, 66)} = 0.058$, $p = 0.810$; B[a]P $\times$ LPS interaction: $F_{(2, 66)} = 0.165$, $p = 0.848$) or LSI (B[a]P exposure: $F_{(2, 66)} = 0.309$, $p = 0.735$; LPS injection: $F_{(1, 66)} = 1.478$, $p = 0.228$; B[a]P $\times$ LPS interaction: $F_{(2, 66)} = 0.738$, $p = 0.482$) following B[a]P exposure with LPS challenge (Appendix D).

   4.3.2.2. Effect of benzo[a]pyrene on lipopolysaccharide-stimulated leukocyte numbers

   Number of leukocytes in blood taken from cardiac puncture was determined using $DiOC_5(3)$ cell staining together with flow cytometry (Figure 4.6.). For the number of lymphocytes in blood (Figure 4.6.A), there was a B[a]P $\times$ LPS interaction ($F_{(2, 66)} = 3.565$, $p = 0.034$). Multiple comparisons revealed that DMSO- exposed LPS-injected individuals had decreased blood lymphocytes compared DMSO-exposed PBS-injected group. However, individuals pre-exposed to 70 or 350 µg/L B[a]P and injected with PBS had leukocyte levels equal to those injected with LPS. There was no effect of B[a]P exposure ($F_{(2, 66)} = 1.001$, $p = 0.373$), LPS injection ($F_{(1, 66)} = 3.965$, $p = 0.051$), or B[a]P $\times$ LPS interaction ($F_{(2, 66)} = 0.823$, $p = 0.444$) on the number of granulocytes in blood (Figure 4.6.B). For monocytes (Figure 4.6.C), there was a B[a]P $\times$ LPS interaction ($F_{(2, 66)} = 4.478$, $p = 0.015$). Multiple comparisons revealed that all LPS treatments had increased monocytes numbers relative to the respective PBS treatments. Furthermore, there was decreased monocyte numbers in all B[a]P-exposed and LPS-injected treatments relative to the DMSO-exposed and LPS-injected treatment. There was a main effect of LPS injection on G:L ratio ($F_{(1, 66)} = 9,328$, $p = 0.003$), but no effect of B[a]P ($F_{(2, 66)} = 1.906$, $p = 0.157$) or B[a]P $\times$ LPS interaction ($F_{(2, 66)} = 2.059$, $p = 0.136$). Individuals exposed to DMSO and 70 µg/L B[a]P had increased G:L ratio with LPS exposure but individuals exposed to 350 µg/L B[a]P did not have a similar increase (Figure 4.6.D).
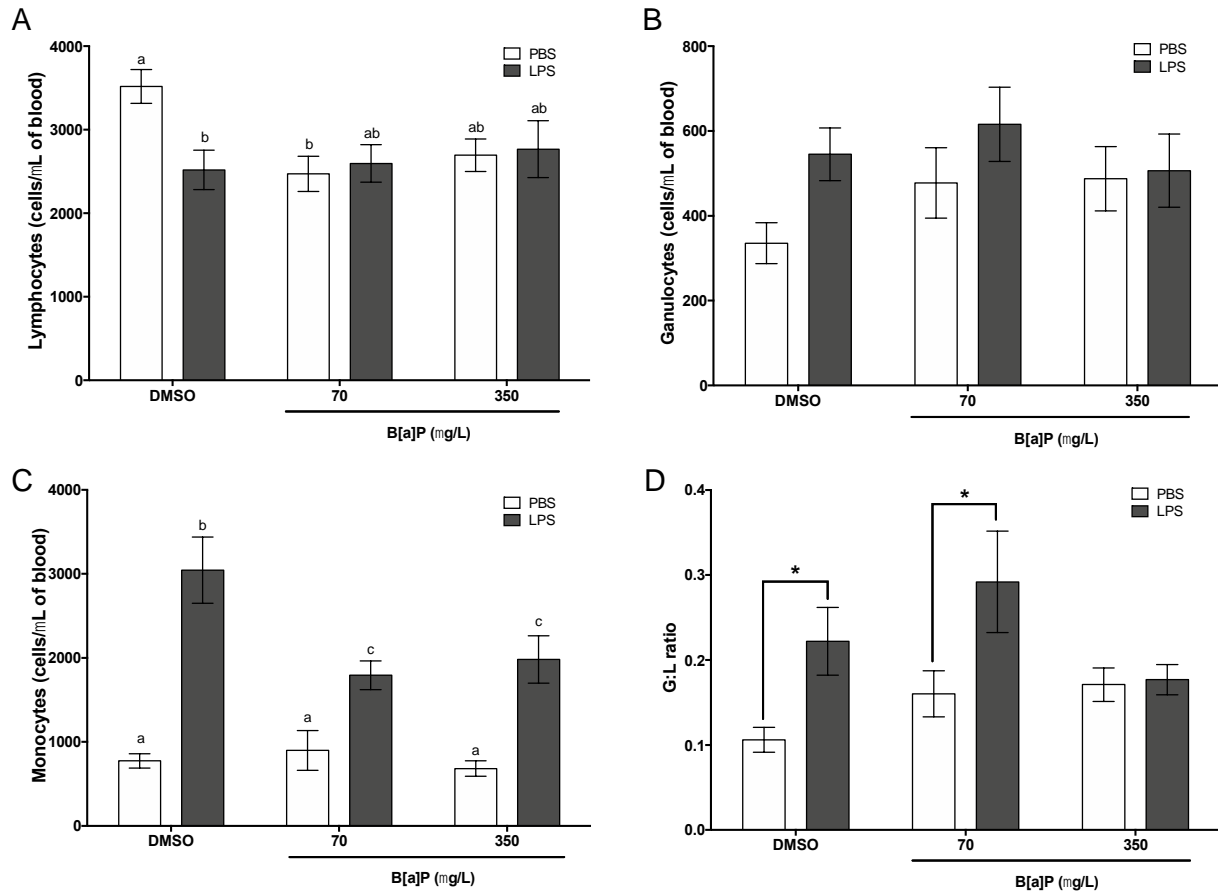
Figure 4.6. Effects B[a]P exposure and LPS injection on the number (cells/µL whole blood) of (A) lymphocytes, (B) granulocytes, (C) monocytes, and (D) G:L ratio in whole blood of *Xenopus laevis* as measured by $DiOC_5(3)$ staining and flow cytometry. Individuals were exposed to B[a]P (DMSO, 70 or 350 µg/L) for 7 d and then injected with PBS or LPS (10 µg/g) and sampled 1 dpi. Bars represent mean ± SEM (n = 12). Data were analyzed using two-way ANOVA with B[a]P and injections as factors. Letters indicate significant differences at $p < 0.05$ among B[a]P treatments (Tukey's post-hoc test), whereas asterisks indicate significant differences among injection treatments (Sidak's multiple comparisons).

4.3.2.2. Effect of benzo[a]pyrene on lipopolysaccharide-stimulated gene expression

Expression of CYP1A and specific cytokines were measured in liver tissue by qPCR (Figure 4.7.). Levels of CYP1A7 mRNA were affected by B[a]P exposure ($F_{(2, 54)} = 3.248$, $p = 0.046$) but there was no effect of LPS injection ($F_{(1, 54)} = 0.346$, $p = 0.559$) and no or B[a]P × LPS interaction ($F_{(2, 54)} = 0.009$, $p = 0.991$; Figure 4.7.A). Multiple comparison of treatment revealed an increase in expression of CYP1A7 in individuals exposed to 350 µg/L B[a]P relative to the DMSO-exposed group. For TNFα, there was a main effect of LPS injection ($F_{(1, 54)} = 38.564$, $p < 0.001$), with no effect of the B[a]P exposure ($F_{(2, 54)} = 0.734$, $p = 0.485$) or B[a]P × LPS interaction ($F_{(2, 54)} = 0.193$, $p = 0.824$; Figure 4.7.B). Multiple comparisons showed induction of TNFα expression with LPS-injection, regardless of B[a]P exposure level. There was a main effect of LPS on CSF-1 expression ($F_{(1, 54)} = 6.537$, $p = 0.014$), but the post-hoc could not distinguish specific treatments exhibiting the response (Figure 4.7.C). There was no effect of B[a]P ($F_{(2, 54)} = 0.760$, $p = 0.473$) or B[a]P × LPS interaction ($F_{(2, 54)} = 0.303$, $p = 0.742$) on CSF-1 expression. There was a main effect of LPS on expression IL-1β mRNA ($F_{(1, 54)} = 47.848$, $p < 0.001$) and multiple comparisons revealed an increased in IL-1β mRNA with LPS-injection in individuals pre-exposed to DMSO and 70 µg/L B[a]P (compared to their PBS-injected control treatment), but this was not the case at 350 µg/L B[a]P (Figure 4.7.D). There was no effect of the B[a]P exposure ($F_{(2, 54)} = 0.734$, $p = 0.485$) or B[a]P × LPS interaction ($F_{(2, 54)} = 2.482$, $p = 0.094$) on IL-1β expression in liver.
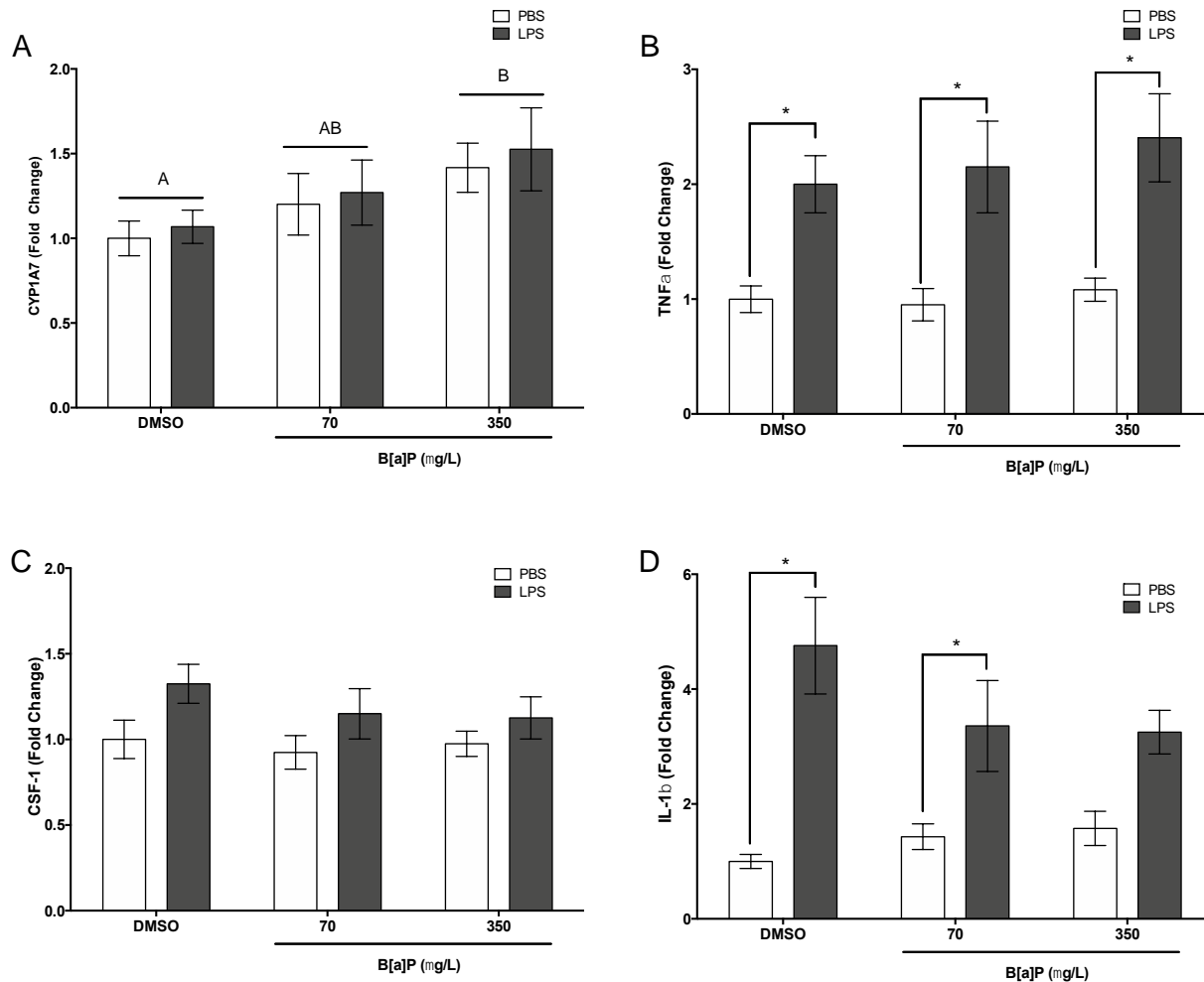
Figure 4.7. Effects B[a]P exposure and LPS injection on mRNA expression of (A) CYP1A7 and immune response genes, specifically (B) TNFα, (C) IL-1β and (D) CSF-1 in liver of *Xenopus laevis* as measured by qPCR. Individuals were exposed to B[a]P (DMSO, 70 or 350 µg/L) for 7 d and then injected with PBS or LPS (10 µg/g) and sampled 1 dpi. Data were normalized to the geometric mean of GAPDH and EF1α and expressed relative to the DMSO-exposed and PBS-injected group. Bars represent the mean ± SEM (n = 10). Data were analyzed using two-way ANOVA with B[a]P and LPS injection as factors. Letters indicate significant differences at p < 0.05 among B[a]P treatments (Tukey's post-hoc test), whereas asterisks indicate significant differences among injection treatments (Sidak's multiple comparisons).

4.4. Discussion

Immunotoxicity in amphibians as a result of contaminant exposure has received increased attention due to increasing reports of disease outbreaks around the world (Carey et al., 1999; Daszak et al., 2003; 2000; Engering et al., 2013) and studies that show potential for contaminants to compromise immune defences in various amphibian species (Christin et al., 2003; Forson and Storfer, 2006b; Gilbertson et al., 2003; Rohr et al., 2008). Nevertheless, the effects of exposure to contaminants on immune responses and associated molecular mechanisms remain unclear in amphibians. For the first time, our data suggest that short-term B[a]P exposure can alter number of circulating immune cells in amphibians that are critical for mounting an effective immune response to pathogens. Exposure to B[a]P also modulates the LPS-induction of mRNA levels of genes encoding key mediators of the innate immune response. The cellular and molecular responses to B[a]P reported herein support the well-documented effects reported in studies with fish and may consequently increase the susceptibility of amphibians to pathogen-associated disease.

4.4.1. 3,3′-Dipentyloxacarbocyanine iodide ($DiOC_5(3)$) staining for profiling leukocytes in amphibian blood

We evaluated leukocyte populations in whole blood taken from juvenile *X. laevis* and found that $DiOC_5(3)$ staining along with flow cytometric analysis is a suitable alternative to traditional manual cell counts from blood smears. Lymphocytes and granulocytes, which make up a large proportion of blood leukocytes, showed the strongest correlations between the two quantification methods. In contrast, there was no correlation between the two methods for monocytes, and this is likely attributed to a number of factors, such as the visual similarity of monocytes with polychromatic red blood cells, the small sample size obtained in blood smears (200 total cells/individual), and the infrequency of monocytes (0.6–2.8% of total leukocytes in amphibians; reviewed in Davis et al., 2008). Together, these factors contribute to the high variability in monocyte numbers when measured using direct cell counts. In contrast, monocytes are easily distinguishable from polychromatic red blood cells in $DiOC_5(3)$-stained cells due to the vast difference in the number of organelles between the two cell types. Both cell types are widely variable in their complexity (SSC), but monocytes have higher organelle contents

resulting in an order of magnitude higher fluorescence, giving flow cytometry the advantage of easy gating and identification of this cell type.

Flow cytometry gains another advantage for identification and quantification of blood cells as a smaller volume of blood can be used to count a larger number of cells in a fraction of the time. The power and accuracy of flow cytometry are much greater given that 100,000 events are recorded per replicate compared to 100 leukocytes per replicate in blood smears. However, direct blood cell counts offer the advantage of discerning neutrophil, basophil and eosinophil cell types, whereas the non-specific $DiOC_5(3)$ dye does not distinguish between these different granulocytes (Burraco et al., 2017). The benefit of identifying types of granulocytes using blood smears types may not be important for amphibians given that the majority of granulocytes are neutrophils (Davis et al., 2008) and the role of basophils and eosinophils in the immune response of amphibians remains unclear. Regardless of some of the limitations, we confirmed that flow cytometry with $DiOC_5(3)$ staining of cells can be used to obtain differential leukocyte counts in *X. laevis* with greater efficiency and power when compared to manual counts. Perhaps most importantly, this method has the potential to be used for comparative immunology and immunotoxicity studies with other amphibian species and contribute to the immunological assays available for non-standard amphibian species.

4.4.2. *Xenopus laevis* exhibits a robust immune response to lipopolysaccharide

We evaluated cellular and molecular responses to a single LPS injection to establish an immunostimulatory dose of LPS that could be used in subsequent immune challenge assays. Injection of LPS resulted in decreased lymphocytes, increased monocytes and increased G:L ratio in whole blood 1 dpi. Lymphocytes and neutrophils are the most abundant leukocytes in circulation and made up a combined proportion of ~80% of leukocytes in juvenile *X. laevis* in this study, which is similar to what is reported for other amphibian species (Davis et al., 2008). Reduction in the number of circulating lymphocytes in response to immune stimulation by LPS injections is likely due to an exodus of lymphocytes from the blood to the tissues (Davis et al., 2008; Dhabhar, 2009), rather than widespread destruction of the cells. In contrast, injection of LPS did not affect the composite number of granulocytes, which includes neutrophils, basophils and eosinophils. Neutrophils dominate the granulocyte population, and as the primary phagocytic leukocytes in circulation, neutrophils are expected to increase in response to immune stimulation

(reviewed in Davis et al., 2008). There are a few possible explanations for the lack of changes in the number of granulocytes with LPS injection. Firstly, the duration or magnitude of the immune challenge may have been insufficient to elicit measurable proliferation of the neutrophils in circulation. For example, wood frogs (*Lithobates sylvatica*) exposed to ranavirus (Frog Virus 3; FV3) had increased neutrophils in circulation four days following FV3 injection, and levels continued up to two weeks post injection (Forzán et al., 2016). Alternatively, previous studies in amphibians report decreases in eosinophils in response to pathogens such as *Batrachochytrium dendrobatidis* (Davis et al., 2010; Woodhams et al., 2007). Given that the $DiOC_5(3)$ assay cannot distinguish types of granulocytes (neutrophil, eosinophil, or basophils), it is possible that contrasting responses in neutrophil and eosinophil populations resulted in no measurable granulocyte response to LPS. The number of monocytes increased with LPS injection; this is a characteristic response of these long-lived phagocytic cells, which are often reported to increase in circulating blood in response to bacterial infections (Allender and Fry, 2008; Davis et al., 2008). Overall, leukocytes are an integral part of the initial response against an immune stimulus in amphibians and the dramatic changes in blood leukocytes in response to LPS measured herein clearly demonstrate that LPS is capable of inducing an immune response in *X. laevis*.

The response to LPS injection in juvenile *X. laevis* was time-dependent in that there was a robust effect on blood leukocyte populations at 1 dpi, which disappated after 3 dpi. A temporal response to LPS has never been characterized in *X. laevis*, so we tested two time-points to capture the optimal response to LPS in terms of blood leukocytes and cytokine expression. The lack of changes in the leukocyte populations after 3 dpi is not entirely surprising. Lipopolysaccharide is a component of bacterial outer membranes and not intact proliferating bacteria; therefore, once the injected LPS particles are recognized by the toll-like receptors and neutralized by the immune system, the pro-inflammatory status of the animal will resolve and homeostatic conditions would return (O'Carroll et al., 2013). In zebrafish, LPS-induced mortality reached maximal levels one day after waterborne exposure (Novoa et al., 2009), demonstrating that the impacts of LPS in aquatic vertebrates occur over a relatively short time-frame. Overall, we observed a characteristic LPS-induced change in leukocyte numbers at 1 dpi, indicating activation of the immune response. Given that the immune response had largely subsided by 3 dpi, subsequent investigation of the immune response at the molecular level was only conducted at the earlier time point.

We examined the cytokine signalling response in liver tissues of *X. laevis* to further characterize their response to LPS stimulation. Injection of LPS induced the expression of pro-inflammatory cytokines in the liver, specifically IL-1β, TNFα and CSF-1. These cytokines play an integral role in the initial pro-inflammatory immune responses and therefore are a strong indication of immune activation. Cytokines are soluble proteins that activate cellular components of both the innate and adaptive immune systems in response to pathogens and can be released by both immune and non-immune cells (Bartee et al., 2008). In a study by Zou et al. (2000), adult *X. laevis* exposed to approximately 1 µg/g body weight LPS for 24 h had increased IL-1β mRNA expression in kidney, liver and spleen. Cytokine expression is also induced in amphibians with pathogen exposure. Both tadpoles and adult *X. laevis* up-regulate IL-1β, TNFα and CSF-1 mRNA with ranavirus (FV3) injection, as early as 1 dpi (De Jesús Andino et al., 2012; Gantress et al., 2003b; Sifkarovski et al., 2014). In particular, TNFα and CSF-1 play an essential role in the immune response against pathogen invasion by propagating the inflammatory response and supporting the differentiation of macrophages from monocytes in the liver of amphibians (Grayfer et al., 2014a; Grayfer and Robert, 2013; Hadji-Azimi et al., 1990; MacKenzie et al., 2003). In fish, LPS is a potent stimulator of both monocytes/macrophages (Swain et al., 2008) and although we did not measure macrophages in liver, the concomitant induction of TNFα and CSF-1 indicates activation of signal transduction pathways involved in apoptosis, lipid metabolism, macrophage development and differentiation, and immunological regulation (Grayfer et al., 2014b; Grayfer and Robert, 2013; MacKenzie et al., 2006). Collectively, the clear leukocyte and cytokine responses to LPS observed in the present study confirm that *X. laevis* is mounting an immune response to this simulated pathogen. This allowed for identification of an appropriate dose of LPS for an immune challenge assay to evaluate the effects of B[a]P exposure on a defined immune response.

4.4.3. Benzo[a]pyrene exposure modulates the immune response to lipopolysacchride

Exposure to B[a]P appeared to impair the normal inflammatory response to LPS in that individuals exposed to the highest concentration of B[a]P did not exhibit an LPS-stimulated increase in G:L ratio. The G:L ratio is a subclinical marker of inflammatory status that is calculated by dividing the number of granulocytes by the number of lymphocyte in an individual (Forget et al., 2017; Socorro Faria et al., 2016). Increased G:L ratio is indicative of an

inflammatory response and would be expected during an immune response to a pathogen due to a combination of granulocyte proliferation and re-distribution of lymphocytes (and therefore a decrease) from circulation to the tissues (Davis et al., 2008) – exposure to 350 µg/L B[a]P impaired this response. Similarly, while LPS is a strong inducer of IL-1β, individuals exposed to 350 µg/L B[a]P did not exhibit this response. The cytokine IL-1β has numerous roles during an immune response, including promotion of pro-inflammatory pathways involved in cell proliferation, differentiation and apoptosis (Dinarello, 2017). Effective inflammatory responses in amphibians are required to combat infection from a variety of amphibian pathogens. For example, an amphibian study demonstrated that increased susceptibility to ranavirus (FV3) in the tadpole life-stage is at least in part attributable to the diminished and delayed inflammatory immune responses in tadpoles compared to adults (De Jesús Andino et al., 2012). The ability of B[a]P to impair inflammatory responses against the simulated pathogen LPS suggests that exposure could also inhibit similar responses to a pathogen such as ranavirus, possibly increasing susceptibility to disease.

Exposure to B[a]P resulted in decreased lymphocyte populations in the resting (70 µg/L B[a]P and non LPS-stimulated) immune system and impaired G:L ratio and IL-1β responses to LPS injection. These results are consistent with previous findings in various fish species where B[a]P demonstrated selective toxicity towards lymphocyte populations (Carlson et al., 2002; Nakayama et al., 2008; Phalen et al., 2014). For example, Phalen et al. (2014) reported reduced B-cell populations in circulating blood, spleen and head kidney in rainbow trout (*Oncorhynchus mykiss*) three weeks after i.p. injection of B[a]P. Splenic lymphocyte proliferation decreased 48 h after i.p. injection of B[a]P in Japanese medaka (*Oryzias latipes*; Carlson et al., 2002). Several mechanisms have been proposed for B[a]P-induced toxicity in lymphocytes in fish and mammals, including DNA damage, altered calcium homeostasis, CYP1A-dependent effects, or alternative hypotheses such as migration of lymphocytes to different compartments or impaired proliferation (Carlson et al., 2004a; Curtis et al., 2011; Gao et al., 2008; Reynaud et al., 2001). Fish lymphocytes are known to express inducible CYP1A (Nakayama et al., 2008), and impairment of the lymphocyte proliferation response by B[a]P injection is ameliorated when Japanese medaka are co-exposed to the CYP1A inhibitor α-naphthoflavone (Carlson et al., 2004a). These findings suggest that CYP1A induction following B[a]P exposure is at least in part responsible for the impaired lymphocyte proliferation responses. Overall, findings from this

study are consistent with studies performed in fish, where lymphocyte populations appear to be the most severely impacted leukocyte cell type, and reduction of lymphocytes may be mediated through induction of CYP1A7. Metabolism of B[a]P by CYP1A7 can result in the formation of reactive metabolites that can ultimately impair proliferation or cause apoptosis in lymphocyte populations (Reynaud and Deschaux, 2006). However, the mechanism by which B[a]P affects lymphocyte population in amphibian blood cannot be differentiated based on the results from this study.

Evaluating the effects of contaminants on the activated immune system provides insight into contaminant-induced deficits in the ability of the organism to respond to an immune stimulus. In this study, the impact of B[a]P on the G:L ratio and IL-1β expression is only discernable when then immune system has been activated with LPS, while these endpoints remain unchanged in the resting immune system. These findings emphasize the value of evaluating contaminant-induced immunotoxicity on the activated immune system (Köllner et al., 2002; Segner et al., 2012b), and highlight that these adverse effects may be overlooked when assessing contaminant impact only on the resting immune system. Ranavirus is a viral pathogen that has impacted amphibian populations around the word (Gray et al., 2009; Miller et al., 2011) and inflammatory responses are critical for effective immune defences against this pathogen. Increased susceptibility to ranavirus (FV3) in tadpoles compared to adults is attributed to reduced and delayed pro-inflammatory cytokine responses (including IL-1β; De Jesús Andino et al., 2012). Exposure to carbaryl during tadpole development resulted in persistent effects on immune responses of juvenile *X. laevis* to ranavirus (FV3), including suppressed cytokine expression (IL-1β and IFN-I) and increased viral load (De Jesús Andino et al., 2017). Therefore, impairment of inflammatory immune response by B[a]P exposure as observed in this study may have implications for susceptibility to environmentally relevant amphibian pathogens, such as ranavirus. Overall, the approach taken in this study has the potential to be extended to other contaminants and pathogens of concern.

In conclusion, we have validated a new method for leukocytes counts using $DiOC_5(3)$ in conjunction with flow cytometry, adding to the toolbox for investigating sublethal impairment of the immune system in amphibians. The results from this study demonstrated the ability of *X. laevis* to recognize LPS and respond through the activation of the innate immune system. The PAH exposure impaired the ability of *X. laevis* to induce inflammatory responses (G:L ratio and

IL-1β) against the simulated pathogen LPS, and the overall number of lymphocytes is lowered in the blood of B[a]P-exposed frogs. Induction of CYP1A7 indicates activation of metabolic pathways in the liver that can produce toxic metabolites whose activity has been previously linked to immune cell toxicity and immunosuppressive effects of this PAH. Gaining a better understanding of the impacts of contaminants on the amphibian immune system may provide some insight into the factors contributing to the disease-driven amphibian population decline.

CHAPTER 5:

CHARACTERIZING THE *IN VITRO* RESPONSE OF *XENOPUS LAEVIS* A6 KIDNEY EPITHELIAL CELLS TO BENZO[A]PYRENE EXPOSURE AND LIPOPOLYSACCHARIDE CHALLENGE

Preface

Despite the fact that B[a]P-induced immunotoxicity has been described in fish and mammals, the potential mechanism of immunomodulation remains largely unknown in amphibians. The goal of the research presented in Chapter 5 of this thesis was to establish the mechanism of action of B[a]P in *Xenopus laevis* A6 kidney epithelial cells and to determine if pre-exposure to B[a]P compromised the response to an immunostimulatory agent. The content of Chapter 5 is being prepared as a manuscript for submission to *Toxicology In Vitro* under the joint authorship of Melanie Gallant and Natacha Hogan.

Author contributions:

Melanie Gallant (University of Saskatchewan) conceived, designed, and managed the experiment, generated and analyzed the data, prepared all figures, and drafted the manuscript.

Natacha Hogan (University of Saskatchewan) provided inspiration, scientific input and guidance on the design of the experiment, supervised Melanie Gallant commented on and edited the manuscript, and obtained funding for the research.

Abstract

Amphibians are exceptionally sensitive to contaminant exposure and are currently experiencing disease-driven population declines, which makes it increasingly important to identify and understand the mechanisms of contaminant-induced immunotoxicity in this taxon. Of particular interest is the potential immunotoxicity of polycyclic aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (B[a]P), which are known to contaminate amphibian habitats and cause immunotoxicity in other vertebrates. Despite our relatively complete understanding in fish and mammals, the mechanism of action for B[a]P-induced immunotoxicity in amphibians remains under-studied. The *Xenopus laevis* kidney epithelial cell line (A6 cells) is a promising model for amphibian immunotoxicity studies given that in amphibians, the kidney plays a role in the immue system and an infection target for some pathogens such as Ranavirus. As such, this study aimed to identify the response of A6 cells to B[a]P and determine the impact of this contaminant on the ability of the cells to respond to immune stimulation. Here we show that *X. laevis* A6 kidney epithelial cells respond to the simulated pathogen lipopolysaccharide (LPS; 1 or 10 µg/mL LPS for 3 or 6 h), and optimized the time and concentrations required to mount a robust pro-inflammatory cytokine response. Furthermore, a time-course experiment (50 or 500 ng/mL B[a]P for 3, 6, 12 or 24 h) was performed to examine the mechanism of action of B[a]P in A6 cells and the time-frame over which they occur. There were significant increases of the Phase I biotransformation enzyme CYP1A7 mRNA expression and reactive oxygen species production at all time-points with B[a]P exposure. However, the Phase II detoxification enzyme glutathione-S-transferase mu 1 (GSTM1) mRNA expression was only induced after 24 h of exposure to 500 ng/mL B[a]P. Finally, there were decreases in the metabolic capacity of the cells (MTT assay) with exposure to 500 ng/mL B[a]P for 12 and 24 h, with no effect on cell viability. However, pre-exposure to B[a]P (50 or 200 ng/mL for 12 h) did not appear to impair the ability of the A6 cells to mount an inflammatory response to LPS (2 µg/mL for 3 h). Taken together, findings from this study support the mechanism of AhR activation and CYP-induced damage through ROS production; however, this mechanism does not appear to impair immune responses, as evidenced in other vertebrates.

5.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of priority pollutants that mainly originate from combustion of fossil fuel as well as release of petroleum and associated products (Abdel-Shafy and Mansour, 2016; Lawal, 2017). Within aquatic environments, PAHs can be detected in the water column and the sediment (Abdel-Shafy and Mansour, 2016) and the adverse effects of PAH contamination in aquatic species, particularly fish, can include immunotoxicity, hepatic neoplasia, endocrine disruption, impaired growth and development, and epigenetic changes (reviewed in Collier et al., 2013). Amphibians also reside in PAH-contaminated habitats, making them a relevant, yet under-studied, taxa at risk for adverse effects from exposure to these environmental contaminants. High concentrations of PAHs exist in the sediment of wetlands surrounding oil sands extraction facilities (Bilodeau, 2017) and these areas also serve as habitats for wood frogs (*Lithobates sylvaticus*) and Northern Leopard frogs (*Lithobates pipiens*) (Hersikorn and Smits, 2011; Melvin and Trudeau, 2012). Additionally, high body burdens of PAHs have been measured in wild amphibians relative to concentrations in their surrounding environment (Garrigues et al., 2004; Grinfield et al., 1986; Sparling, 2010a).

Benzo[a]pyrene (B[a]P) is one of the most potent PAHs in terms of carcinogenicity, mutagenicity, and immunotoxicity (Ball and Truskewycz, 2013; Miller and Ramos, 2001), and the effects of B[a]P exposure in fish are at least in part mediated through activation of the aryl hydrocarbon receptor (AhR) and the subsequent induction of cytochrome P450 (CYP) enzymes and oxidative damage (reviewed in Reynaud and Deschaux, 2006). Furthermore, a recent meta-analysis of the literature focusing on PAH effects in fish found that oxidative stress indicators such as ethoxyresorufin-O-deethylase (EROD), glutathione S-transferase mu 1(GSTM1), superoxide dismutase, glutathione peroxidase are significantly affected by PAH exposure (Santana et al., 2018). Given that similar molecular components exist across vertebrates, it has been hypothesized that this pathway is phylogenetically conserved from fish to mammals (reviewed in Reynaud and Deschaux, 2006). However, it is important to note that the effect threshold of some PAHs, such as B[a]P, may not be comparable between taxa; for example, amphibians are reportedly insensitive to the lethality induced by AhR ligands (*e.g.* TCDD; Beatty et al., 1976; Jung and Walker, 1997; Mima, 1997). In fact, amphibians are estimated to be 100 to 1,000 fold less sensitive to TCDD-induced lethality than early life-stages of fish (Jung and Walker, 1997).

As evidenced by the vast differences in the lethality of AhR ligands between fish and amphibians, extrapolating the sub-lethal impacts of PAHs exposure in fish to amphibians may be problematic. A particular sub-lethal response to PAH exposure often evaluated in fish and that is of special relevance to amphibians is immunotoxicity (Bado-Nilles et al., 2014; Carlson et al., 2004b; Phalen et al., 2014; Reynaud and Deschaux, 2005). Many amphibian species have experienced disease-driven population declines in recent years (Skerratt et al., 2007) and anthropogenic chemicals, including PAHs, are thought to contribute to reduced immunocompetence and increased disease susceptibility in fish (Arkoosh et al., 1998) and amphibians (Blaustein and Kiesecker, 2002; Sparling, 2010b). In amphibians, exposure to B[a]P has effects on the immune system, including altered cytokine expression (Martini et al., 2012), inflammatory cell numbers in the liver (Fanali et al., 2018), phagocytic capacity of splenic cells (Regnault et al., 2016), and circulating blood leukocytes (Chapter 4). Although the mechanisms underlying these responses have not been studied in amphibians, some proposed mechanisms for B[a]P-induced immunotoxicity in other vertebrates include DNA damage, altered calcium homeostasis, CYP1A-dependent effects, or alternative hypotheses such as migration of lymphocytes to different compartments or impaired proliferation (Carlson et al., 2004a; Curtis et al., 2011; Gao et al., 2008; Reynaud et al., 2001).

In vitro approaches have long been valued for mechanistic studies in the field of toxicology and are used in toxicity screening of chemicals for potential adverse effects in humans and fish (Knauer et al., 2007; Roggen, 2011; Stadnicka-Michalak et al., 2015). *Xenopus laevis* A6 cells were produced from kidney epithelial cells and have been regularly used in biomedical research (Ali et al., 1996; Greenlee et al., 2015; Ichigi and Asashima, 2001; Ohan et al., 1998; Puoti et al., 1995) with a few references in toxicological studies (Gorrochategui et al., 2016; Lavine et al., 2005). This amphibian kidney epithelial cell line is a promising model for amphibian immunotoxicity studies since in amphibians, the kidney is a key immune organ (Chen and Robert, 2011) and an infection target for some pathogens such as Ranavirus, a prevalent viral agent plaguing wild amphibian populations (De Jesús Andino et al., 2012). In addition, *in vitro* systems provide simple, inexpensive and rapid methods for initial toxicity screening while limiting the number of animals and space required for assessment.

This study aimed to evaluate the *X. laevis* kidney epithelial cell line (A6 cells) in terms of its relative sensitivity to different B[a]P concentrations and exposure time and to identify specific

mechanisms that may be involved in adverse effects of B[a]P on amphibians, particularly those relevant to immunotoxicity. To do this, we first exposed A6 cells to graded concentrations of B[a]P for 3, 6, 12 and 24 h and measured their response in terms of CYP1A7 mRNA expression, ROS production, expression of the Phase II detoxification (conjugation) enzyme GSTM1, and cytotoxicity. We then used this data to identify the concentrations and time of B[a]P exposure used in the LPS-challenge assay to determine if B[a]P exposure impairs the ability of the A6 cells to respond to an immune stimulation.

5.2. Materials and Methods

5.2.1. Cell culture and treatments

$X.\ laevis$ kidney epithelial A6 cells were obtained from the American Type Culture Collection (ATCC; Cat # CCL-102, Rockville, MD, USA). Cells were cultured in complete media, consisting of Leibovitz (L)-15 media (Sigma-Aldrich, Oakville, ON, Canada) and supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin to a final concentration of 100 units/mL at 26°C in T125 cm^2 flasks (Thermo Fisher Scientific, Waltham, MA, USA). Cells were maintained as recommended by ATCC by passaging cells twice per week when cells reached 80-90% confluency. For all exposures, cells were seeded in 6-well (2 mL total volume) or 96-well (100 μl) plates at a density of approximately 2.5 x 10^4 cells/cm^2 and left to adhere for 24 h at 26°C. After 24 h, the media was replaced with serum-free (SF) media (L-15 media supplemented with 100 units/mL penicillin/streptomycin) and left for 18 h at 26°C before exposures. All exposures were conducted in SF media to avoid the presence of endogenous AhR ligands in serum which could activate the AhR and confound the results (Adachi et al., 2004; 2001; Procházková et al., 2011).

5.2.2. Experimental design

To characterize the response of $X.\ laevis$ A6 kidney epithelial cells to B[a]P, a preliminary dose-response experiment was performed where cells were exposed to B[a]P concentrations ranging from 50 to 3200 ng/L for 3 h. Results indicated that 3 h exposure to 50 or 500 ng/mL B[a]P did not negatively impact viability or metabolic capacity, yet induced CYP1A7 mRNA expression (see Appendix E). Therefore, in the time-course experiment, cells were exposed to media control or solvent control (dimethyl sulfoxide, DMSO; Thermo Fisher

Scientific) treatments, 50 or 500 ng/mL B[a]P (B1760, Sigma-Aldrich) for 3, 6, 12 or 24 h. Except for the media control group, all treatments had a final concentration of 0.1% v/v DMSO. Each test concentration was represented on every plate, and one plate represented one time-point; thus, statistical comparisons can only be made within a time-point.

Based on the results of the B[a]P time-course experiment, an LPS-challenge experiment was conducted to determine the impact of B[a]P on the ability of the A6 cells to mount an immune response to LPS. Cells were first exposed to B[a]P (DMSO, 50 or 200 ng/mL) for 12 h, at which time cells were exposed to 2 µg/mL LPS (E. coli 055:B5; Sigma-Aldrich, St. Louis, MN, USA) for 3 h. The concentration of LPS was chosen based on a preliminary test to determine immune stimulatory concentrations of LPS in A6 cells and confirmed that exposure to 2 µg/mL LPS for 3 h did not negatively impact cell viability or metabolic capacity (Appendix F), yet significantly induced cytokine mRNA expression (TNFα, IL-1β, CSF-1; Figure 5.1). Two plates were seeded for each biological replicate, each plate contained every concentration of B[a]P for the pre-exposure, then one plate was challenged with LPS (2 µg/mL), and the other was exposed to SF media. All treatments had a final concentration of 0.1% v/v DMSO. This experimental design resulted in a 2 x 3 factorial design, with six unique treatments.

For both the B[a]P time-course exposure and the LPS-challenge exposure, samples were collected to measure cell viability using the trypan blue exclusion assay (see section 5.2.3.), metabolic capacity using the MTT assay (see section 5.2.3.), ROS production using 2',7'dichlorodihydrofluorescein diacetate (H_2DCFDA) assay (see section 5.2.4.), and gene expression analysis by real-time PCR (see section 5.2.5.).

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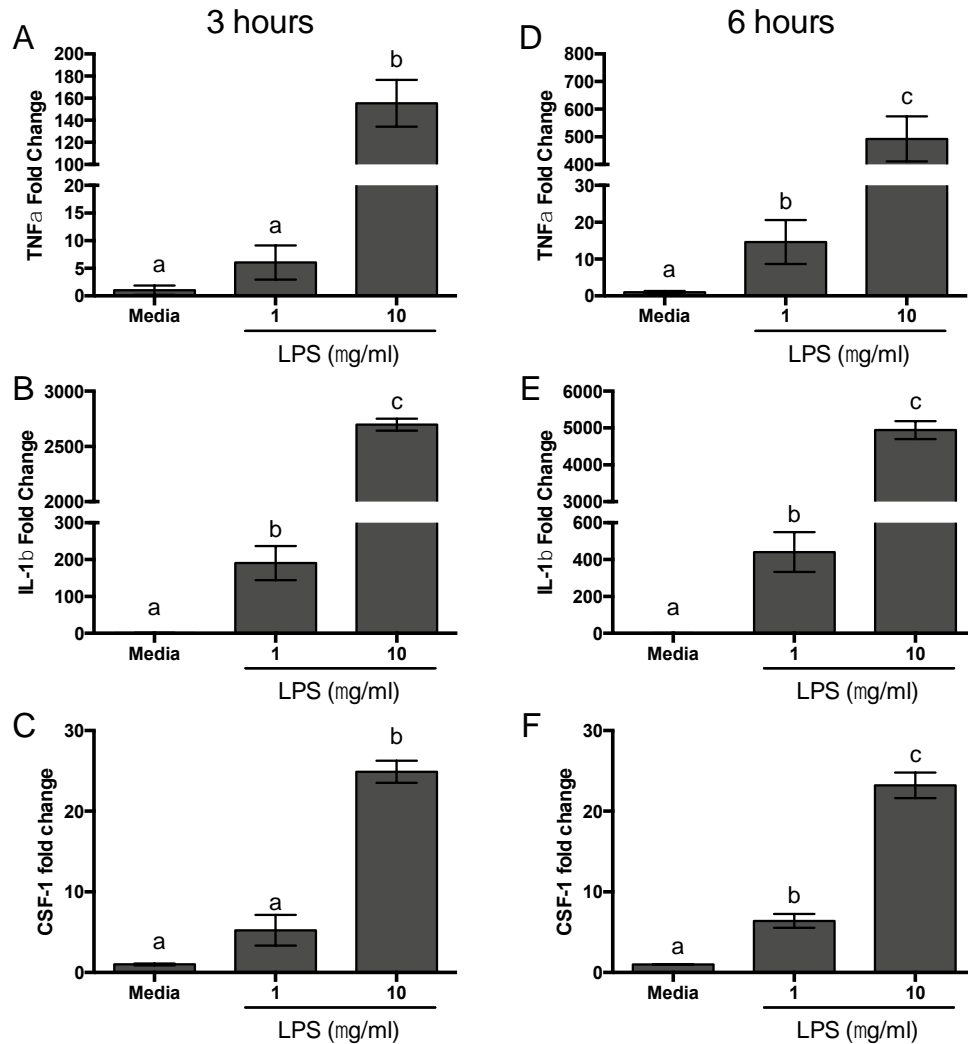
Figure 5.1. The effect of LPS on the mRNA expression of (A and D) TNFα, (B and E) IL-1β, (C and F) CSF-1 (D) in *Xenopus laevis* A6 kidney epithelial cells. Cells were exposed to media control, 1 or 10 μg/mL LPS for 3 or 6 h. For each biological replicate of the assay (n = 4), duplicate technical replicates were averaged, and data were normalized to the geometric mean of GAPDH and EF1α. Normalized data were expressed as fold-change relative to the media control treatment. Data are presented as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey's post hoc test within each time point. Different letters above columns indicate significant differences between treatments at the level of $p < 0.05$.

5.2.3. Assessment of cell viability and metabolic capacity

For the trypan blue exclusion assay, all cells and overlaying media were collected from 6-well plates, pelleted and re-suspended in 500 µl of media. Triplicate 10 µl sub-samples were stained with a final concentration 0.2 % Trypan blue solution and live/dead determination was done by the Countess II FL Automated Cell Counter as described by the manufacturer (Thermo Fisher Scientific).

Metabolic capacity was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay using the Cell Proliferation Kit I (Roche, Risch-Rotkreuz, Switzerland). Exposures were conducted in 96-well plates and following exposures the MTT labelling reagent was added to each well to a final concentration of 5 mg/mL and incubated for 4 h at 26°C. After incubation with the labelling reagent, 100 µl of solubilization solution was added to each well, and the plate was left to incubate for 18 h. Absorbance was read at 550 and 690 nm using an EPOCH 2 microplate reader (BioTek, Winooski, VT, United States).

5.2.4. Detection of reactive oxygen species by 2',7'dichlorodihydrofluorescein diacetate (H_2DCFDA) assay

Intracellular ROS levels were measured by flow cytometry using H_2DCFDA (Thermo Fisher Scientific). When applied to cells, H_2DCFDA easily diffuses into cells where acetate groups are cleaved by intracellular esterases to form 2',7'-dichlorodihydrofluorescein (H_2DCF), which cannot diffuse out of the cell. This product is then oxidized to the highly fluorescent 2',7'-dichlorofluorescein (DCF) by intracellular ROS and the redox state of the sample can be monitored by detecting the increase in fluorescence (Eruslanov and Kusmartsev, 2009). Cells were cultured in 6-well plates and exposures conducted as described in section 5.2.2. Exposure media was removed, cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS) and then incubated with 2 mL of the loading buffer (DPBS containing 1 µM of H_2DCFDA) for 1 h at 26°C. Loading buffer was then replaced with SF media to allow the cells to recover for 30 min before cells were collected and analyzed by flow cytometry, as described in Eruslanov and Kusmartsev (2009). For each biological replicate, 10,000 events were analyzed for DCF fluorescence using the Accuri C6 flow cytometer (BD Bioscience, Franklin Lakes, NJ, USA) at excitation and emission wavelengths of 485 and 535 nm, respectively. An additional unexposed and unstained control group was included in this assay to correct for potential auto-fluorescence

within the cells. Data were corrected with the unexposed and unstained control group before being expressed relative to the respective media control groups.

5.2.5. Measuring mRNA expression

For total RNA isolation and gene expression analysis, A6 cells were cultured in 6-well plates and then underwent B[a]P or B[a]P+LPS exposures as described in section 5.2.2. At the end of exposures, media was removed, and cells were rinsed with 1 mL of DPBS. Cells were lysed by adding 1 mL of trizol reagent (Thermo Fisher Scientific) per well and stored at -80°C until RNA extraction. RNA extraction was performed according to the Trizol reagent manufacturer protocol (Thermo Fisher Scientific), and RNA was re-suspended in 20 µl of rNase-free water. Quantity and purity of RNA were verified using NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific), while quality was verified with a 1% agarose gel. Using the Quantitect cDNA synthesis kit (Qiagen, Hilden, Germany), 500 ng of total RNA was reverse transcribed and resulting cDNA was diluted 10-fold before use in real-time polymerase chain reaction (qPCR).

Gene expression analysis was performed by qPCR using the CFX96 Real-time C100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) and methods previously described (Gallant and Hogan, 2018). Briefly, gene-specific primers (Invitrogen, Carlsbad, CA, USA) for TNFα, IL-1β, CSF-1, AhR, CYP1A7, GSTM1, GAPDH and EF1α were used according to previously optimized reaction conditions (Table 5.1). Each qPCR reaction contained 1x SsoFast EvaGreen Supermix (Bio-Rad Laboratories), optimized concentrations of the appropriate primer set (Table 5.1) and 2 µL of diluted cDNA template. The thermal cycle program was performed according to manufacturer protocol (Bio-Rad Laboratories), where an enzyme activation step at 95°C for 30 s was followed by 40 cycles of 5 s at 95°C and 5 s at the gene-specific annealing temperature (Table 5.1). After the amplification phase, there was a denaturation at 95°C for 1 min, and then a melt curve analysis from 55°C to 95°C, increasing in 1°C increments every 30 s.

Every qPCR assay contained samples and standards in duplicate, in addition to no template control samples (NTC) and no reverse transcriptase control samples (NoRT). A representative pool of cDNA was used to construct the standard curve, which was serially diluted to encompass a wide range of gene expression. Standard curves for all primer pairs were optimized to an efficiency of 90-110% and $R^2 > 0.98$. The relative standard curve method was

used to interpolate relative mRNA abundance of target and reference genes. Duplicate data for each sample were averaged, and target genes were normalized to the geometric mean of the reference genes (GAPDH and EF1α). Data were expressed as fold-change relative to the respective media control groups.

Table 5.1. List of target genes and the specific forward and reverse primers used for transcript expression analysis by qPCR in *Xenopus laevis*.

Target	Primer	Sequence (5' to 3')	Accession numbers	Amplicon Length (bp)	Annealing Temp (°C)	References
IL-1β	Fwd Rev	GGCCTCAATGAAACCTCCAC AGGCAGATATCTCCCAGCAC	NM_001085605.1	232	60	Gallant and Hogan, 2018
CSF-1	Fwd Rev	ATCGAACTCTGTCCAAGCTGGATG GGACGAAGCAAGCATCTGCCTTAT	NM_001280600.1	123	60	Sifkarovski et al., 2014
TNFα	Fwd Rev	TGTCAGGCAGGAAAGAAGCA CAGCAGAGAGCAAAGAGGATGGT	AB298595.1	203	62	Sifkarovski et al., 2014
GSTM1	Fwd Rev	GTTGGCTCATTCTATCCGTCTCC ATCCCAGCTTCTCTTTTTCATTCA	NM_001087371.2	128	60	Ziková et al., 2013
CYP1A7	Fwd Rev	CCTTTGCTACTTCCCCTTCACCC GGCAACAGAGACCACCACGTACC	NM_001097072	155	60	Chapter 3
GAPDH	Fwd Rev	GACATCAAGGCCGCCATTAAGACT AGATGGAGGAGTGAGTGTCACCAT	AF549496.1	130	58.4	Sifkarovski et al., 2014
EF-1α	Fwd Rev	GAGGGTAGTCTGAGAAGCTCTCCACG CCTGAATCACCCAGGCCAGATTGGTG	NM_001086133.1	221	60	Gallant and Hogan, 2018

Fwd, forward; Rev, reverse

5.2.6. Statistical analyses

All data were tested for normality using the Kolmogorov-Smirnov test on residuals and for homogeneity of variance using Levene's test. Data not meeting the assumptions of parametric statistics were log transformed, or the equivalent non-parametric test was selected. For the B[a]P time-course experiment, data within each time-point were analyzed using one-way ANOVAs to assess effects across treatments. One-way ANOVAs were followed by a Tukey's post-hoc test to evaluate significant differences between groups. To determine the effect of B[a]P on the ability of the cells to mount an immune response in the LPS-challenge trial, two-way ANOVAs were performed (B[a]P concentration and LPS treatment as fixed factors). If a main effect of B[a]P was detected, a Tukey's post-hoc test was used to evaluate differences between groups, whereas an LPS main effect was discerned by a Sidak's multiple comparisons test. Alternatively, if a significant interaction was detected Tukey's post-hoc test was used to compare across treatments. All statistical analyses were conducted using SPSS, version 22 (SPSS Inc, IBM, North Castle, NY, United States) and all graphs were created in Prism, version 6 (GraphPad, La Jolla, California, United States).

5.3. Results

5.3.1. Characterizing the response of A6 cells to benzo[a]pyrene

Results of the B[a]P time-course experiment are presented in Figures 5.2–5.4. No effects on cell viability were observed at any time or concentration tested (Figure 5.2A; 3 h: $F_{(2, 6)}$ = 3.215, p = 0.112; 6 h: $F_{(2, 6)}$ = 0.504, p = 0.617; 12 h: $F_{(2, 6)}$ = 0.426, p = 0.666; 24 h: $F_{(2, 6)}$ = 1.803, p = 0.244). However, relative to the solvent control treatment, the metabolic capacity of cells decreased 24% and 45% with exposure to 500 ng/mL B[a]P at 12 and 24 h, respectively (Figure 5.2B; 3 h: $F_{(2, 6)}$ = 0.061, p = 0.942; 6 h: $F_{(2, 6)}$ = 0.344, p = 0.716; 12 h: $F_{(2, 6)}$ = 17.619, p = 0.001; 24 h: $F_{(2, 6)}$ = 22.295, p = 0.002). At 3, 6 and 12 h of exposure, there was an increase in ROS production with each concentration of B[a]P when compared to the solvent control treatment (Figure 5.3; 3 h: $F_{(2, 9)}$ = 23.033, p < 0.0001; 6 h: $F_{(2, 9)}$ = 54.806, p > 0.0001; 12 h: $F_{(2, 9)}$ = 191.155, p < 0.0001). At 24 h, there was no effect of 50 ng/mL B[a]P on ROS production but an increase at 500 ng/mL (Figure 5.3; 24 h: $F_{(2, 9)}$ = 39.582, p < 0.0001). Expression of CYP1A7 mRNA increased at all time-points and B[a]P exposure concentrations

(Figure 5.4A). After 3 h, there was an equal induction of CYP1A7 mRNA at 50 and 500 ng/mL relative to control treatment (Figure 5.4A; 3 h: $F_{(2, 9)} = 327.365$, $p < 0.0001$) while after 6, 12 and 24 h, there was a dose-dependent increase in CYP1A7 mRNA expression with B[a]P exposure (Figure 5.4A; 6 h: $F_{(2, 9)} = 149.370$, $p < 0.0001$; 12 h: $F_{(2, 9)} = 9582.298$, $p < 0.0001$; 24 h: $F_{(2, 9)} = 1660.920$, $p < 0.0001$). There was no effect of B[a]P on GSTM1 expression after 3, 6, or 12 h of exposure but GSTM1 mRNA increased with exposure to 500 ng/mL B[a]P after 24 h (Figure 5.4B; 3 h: $F_{(2, 9)} = 0.719$, $p = 0.560$; 6 h: $F_{(2, 9)} = 1.641$, $p = 0.232$; 12 h: $F_{(2, 9)} = 0.993$, $p = 0.429$; 24 h: $F_{(2, 9)} = 6.189$, $p = 0.009$).
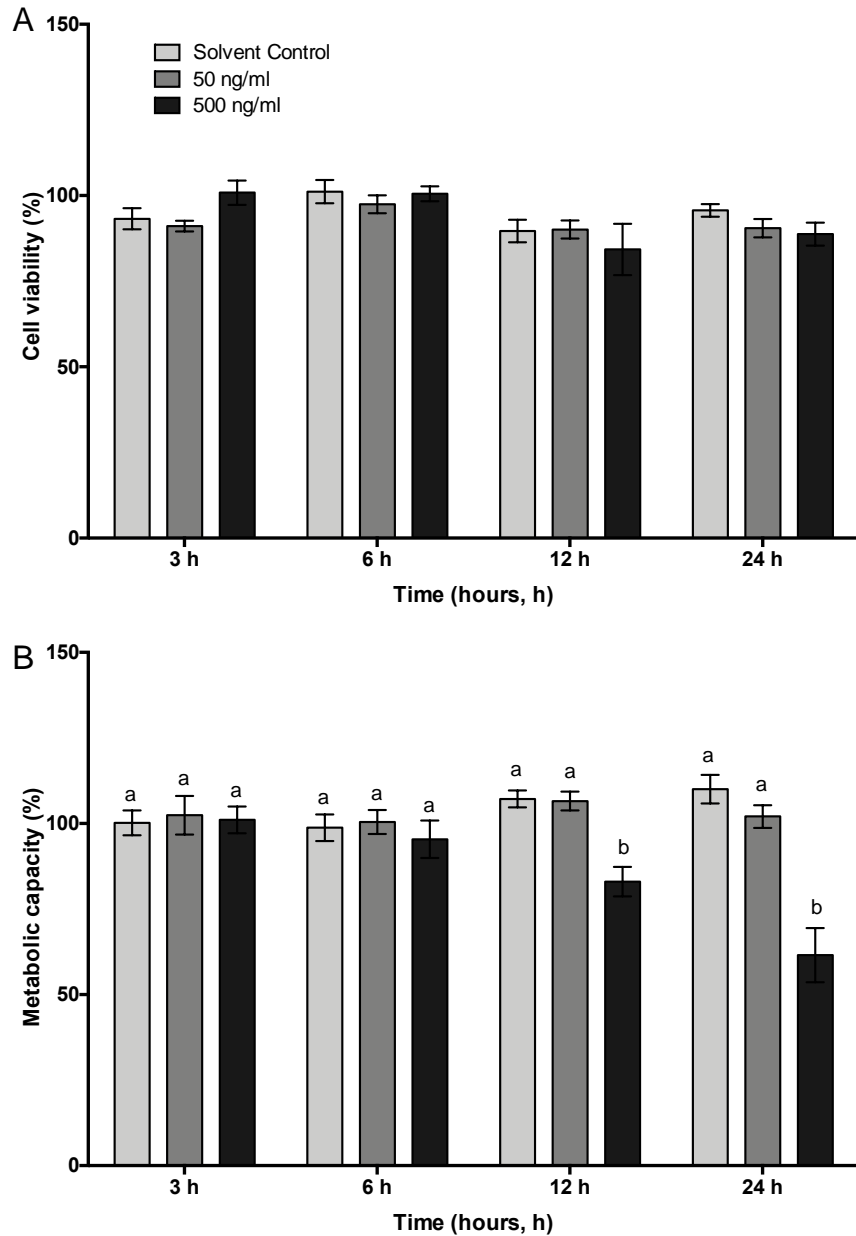
Figure 5.2. The effect of B[a]P on measures of (A) cell viability (trypan blue exclusion assay) and (B) metabolic capacity (MTT assay) in *Xenopus laevis* A6 kidney epithelial cells exposed to media control, solvent control 50 or 500 ng/mL B[a]P for 3, 6, 12 or 24 h. For each biological replicate of the assays (n = 3), triplicate technical replicates were averaged, and data were expressed relative to the media control treatment of the respective time-point. Data are presented as mean $\pm$ SEM and were analyzed by one-way ANOVA followed by Tukey's post hoc test within each time point. Different letters above columns indicate significant differences between treatments at the level of $p <$ 0.05.
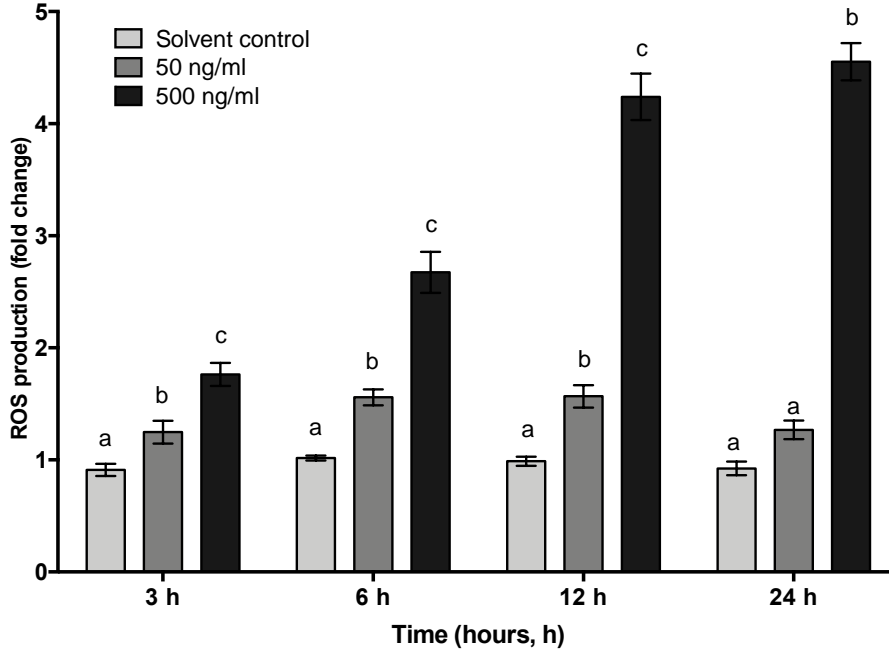
Figure 5.3. The effect of B[a]P on the production of reactive oxygen species (ROS) in *Xenopus laevis* A6 kidney epithelial cells as measured by the H_2DCFDA assay. Cells were exposed to B[a]P (media control, solvent control, 50 or 500 ng/mL) for 3, 6, 12 or 24 h. For each biological replicate of the assay (n = 4), triplicate technical replicates were averaged, and data were expressed relative to the media control treatment of the respective time-point. Data are presented as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey's post hoc test within each time-point. Different letters above columns indicate significant differences between treatments at the level of $p < 0.05$.
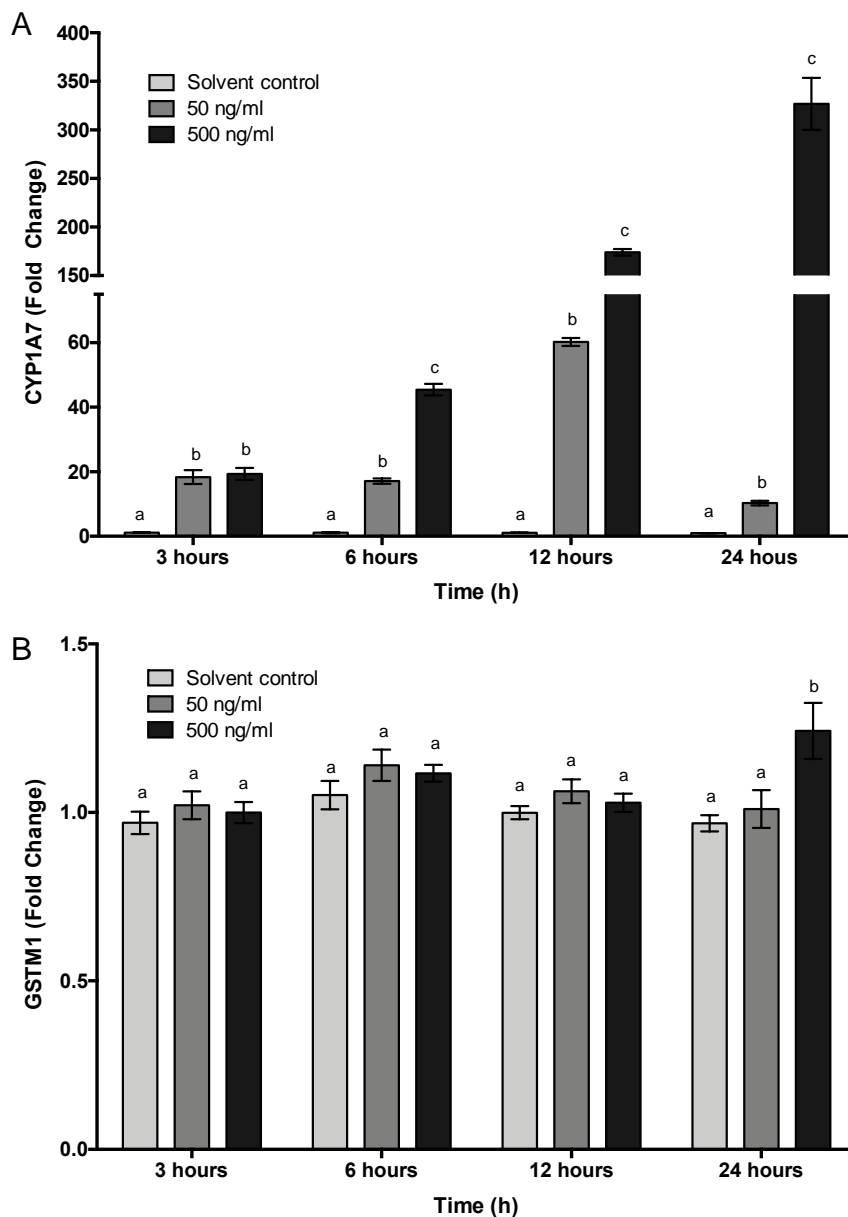
Figure 5.4. The effect of B[a]P on the expression of (A) CYP1A7 and (B) GSTM1 in *Xenopus laevis* A6 kidney epithelial cells exposed to media control, solvent control 50 or 500 ng/mL B[a]P for 3, 6, 12 or 24 h. For each biological replicate of the assay (n = 4), duplicate technical replicates were averaged, and data were normalized to the geometric mean of GAPDH and EF1α. Normalized data were expressed as fold-change relative to the media control treatment of the respective time-point. Data are presented as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey's post hoc test within each time-point. Different letters above columns indicate significant differences between treatments at the level of $p < 0.05$.

5.3.2. Effect of benzo[a]pyrene exposure on the response of A6 cells to lipopolysaccharide

In a preliminary study, the response of *X. laevis* A6 kidney epithelial cells to 1 or 10 µg/mL LPS for 3 or 6 h was evaluated for effects on cell viability, metabolic capacity and cytokine expression. LPS treatments did not affect cell viability (Appendix F; 3 h: $F_{(2, 9)}$ = 0.128, p = 0.882; 6 h: $F_{(2, 9)}$ = 0.925, p = 0.446) or metabolic capacity (Appendix F; 3 h: $F_{(2, 9)}$ = 0.299, p = 0.752; 6 h: $F_{(2, 9)}$ = 0.964 p = 0.439). After 3 h, expression of all cytokines increased with 10 µg/mL LPS treatment (Figure 5.1 A-C; TNFα 3 h: $F_{(2, 9)}$ = 21.981, p < 0.0001; IL-1β 3 h: $F_{(2, 9)}$ = 38.400, p < 0.0001; CSF-1 3 h: $F_{(2, 9)}$ = 114.432, p < 0.0001) while only IL-1β expression was increased with exposure to 1 µg/mL LPS. There was a dose-dependent increase in expression of all cytokines after 6 h of exposure to LPS (Figure 5.1 D-F; TNFα 6 h: $F_{(2, 9)}$ = 51.622, p < 0.0001; IL-1β 6 h: $F_{(2, 9)}$ = 38.400, p < 0.0001; CSF-1 6 h: $F_{(2, 9)}$ = 90.967, p < 0.0001). Based on these results, an LPS concentration of 2 µg/mL and exposure time of 3 h were carried forward for the LPS-challenge with B[a]P exposure.

Results for the B[a]P exposure with LPS-challenge are presented in Figures 5.5-5.7. There was no effect of this combinatorial exposure on cell viability (Figure 5.5A; main effect of B[a]P: $F_{(2, 12)}$ = 0.104, p = 0.902; main effect of LPS: $F_{(1, 12)}$ = 0.183, p = 0.676; B[a]P × LPS interaction: $F_{(2, 12)}$ = 0.057, p = 0.945). There was a 13% decrease in the metabolic capacity of the cells exposed to 200 ng/mL B[a]P relative to the solvent control groups (Figure 5.5B; main effect of B[a]P: $F_{(2, 12)}$ = 4.650, p = 0.032; main effect of LPS: $F_{(1, 12)}$ = 0.070, p = 0.796; B[a]P × LPS interaction: $F_{(2, 12)}$ = 1.432, p = 0.277). There was a main effect of B[a]P on ROS production, where cells exposed to 200 ng/mL B[a]P had higher ROS production than both 50 ng/mL B[a]P and solvent control treatments (Figure 5.6; main effect of B[a]P: $F_{(2, 12)}$ = 27.882, p < 0.0001; main effect of LPS: $F_{(2, 12)}$ = 6.109, p = 0.029; B[a]P × LPS interaction: $F_{(2, 12)}$ = 1.161, p = 0.346). There was also a main effect of LPS on ROS production, but the post-hoc could not distinguish specific treatments exhibiting the response (Figure 5.6). There was a main effect of B[a]P exposure on CYP1A7 mRNA expression, independent of LPS treatment (Figure 5.7A; main effect of B[a]P: $F_{(2, 30)}$ = 138.610, p < 0.0001; main effect of LPS: $F_{(1, 30)}$ = 2.576, p = 0.119; B[a]P × LPS interaction: $F_{(2, 30)}$ = 0.918, p = 0.410). There was no effect of B[a]P exposure on cytokine induction by LPS treatment for TNFα (Figure 5.7B; main effect of B[a]P: $F_{(2, 30)}$ = 0.747, p = 0.483; main effect of LPS: $F_{(1, 30)}$ = 95.711, p < 0.0001; B[a]P × LPS interaction: $F_{(2, 30)}$ = 0.156, p = 0.856),

IL-1β (Figure 5.7C IL-1β; main effect of B[a]P: $F_{(2, 30)} = 1.367$, $p = 0.270$; main effect of LPS: $F_{(1, 30)} = 101.998$, $p < 0.0001$; B[a]P $\times$ LPS interaction: $F_{(2, 30)} = 0.580$, $p = 0.566$), or CSF-1 (Figure 5.7D; main effect of B[a]P: $F_{(2, 30)} = 0.846$, $p = 0.439$; main effect of LPS: $F_{(1, 30)} = 729.760$, $p < 0.0001$; B[a]P $\times$ LPS interaction: $F_{(2, 30)} = 0.238$, $p < 0.790$).
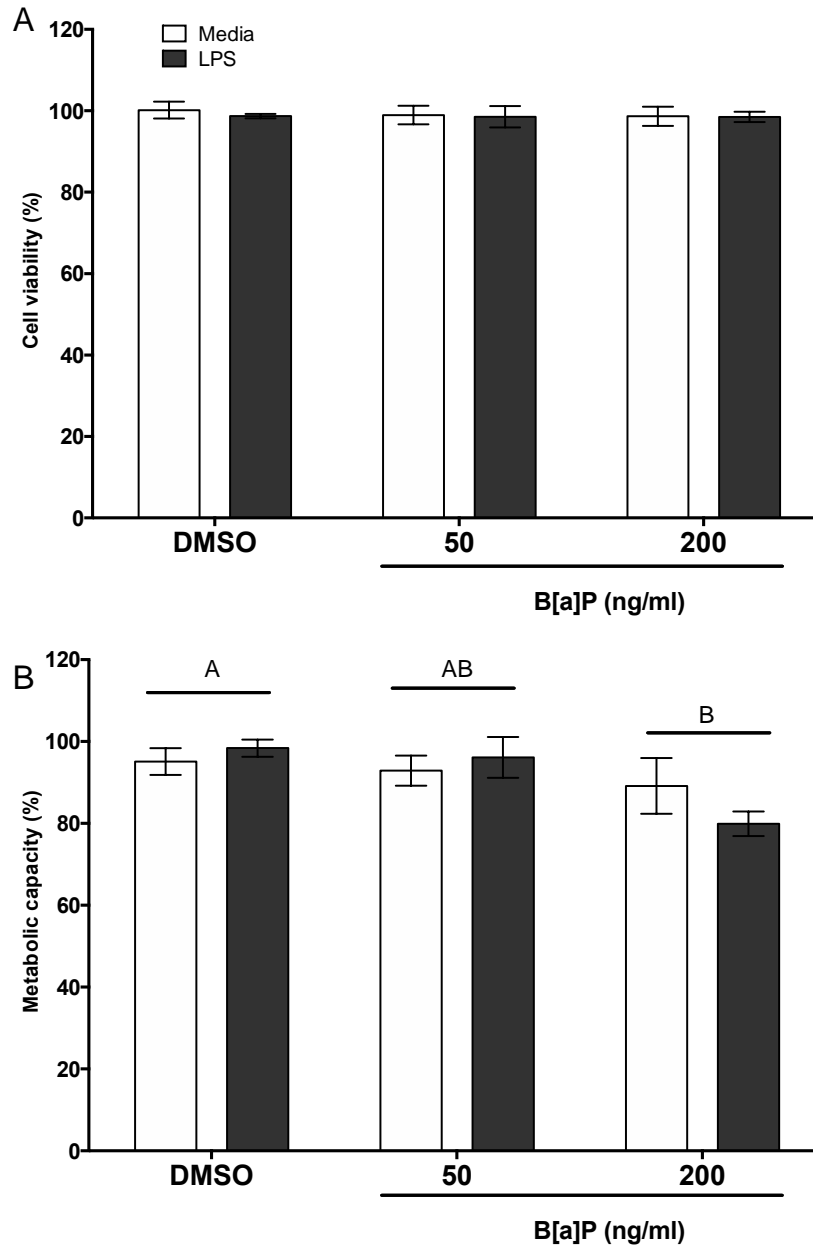
Figure 5.5. Effects of B[a]P and LPS on (A) cell viability (trypan blue exclusion assay) and (B) metabolic capacity (MTT assay) in *Xenopus laevis* A6 kidney epithelial cells. Cells were exposed to media control, solvent control 50 or 200 ng/mL B[a]P for 12 h followed by a 3 h exposure to either SF media or 2 µg/mL LPS. For each biological replicate of the assays (n = 3), triplicate technical replicates were averaged, and data were expressed relative to the media control. Data are presented as mean ± SEM and were analyzed using two-way ANOVA with B[a]P and LPS as factors. Letters indicate significant differences at $p < 0.05$ among B[a]P treatments (Tukey's post-hoc test).
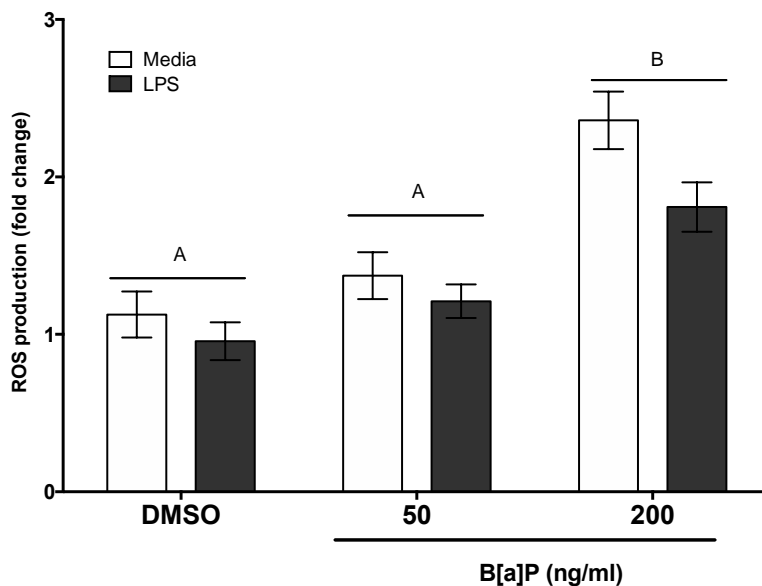
Figure 5.6. Effects of B[a]P and LPS on ROS production in *Xenopus laevis* A6 kidney epithelial cells. Cells were exposed to media or solvent control, 50 or 200 ng/mL B[a]P for 12 h followed by a 3 h exposure to either SF media or 2 µg/mL LPS. For each biological replicate of the assay (n = 3), triplicate technical replicates were averaged, and data were expressed relative to the media control treatment. Data are presented as mean ± SEM and were analyzed using two-way ANOVA with B[a]P and LPS as factors. Letters indicate significant differences at $p < 0.05$ among B[a]P treatments (Tukey's post-hoc test).
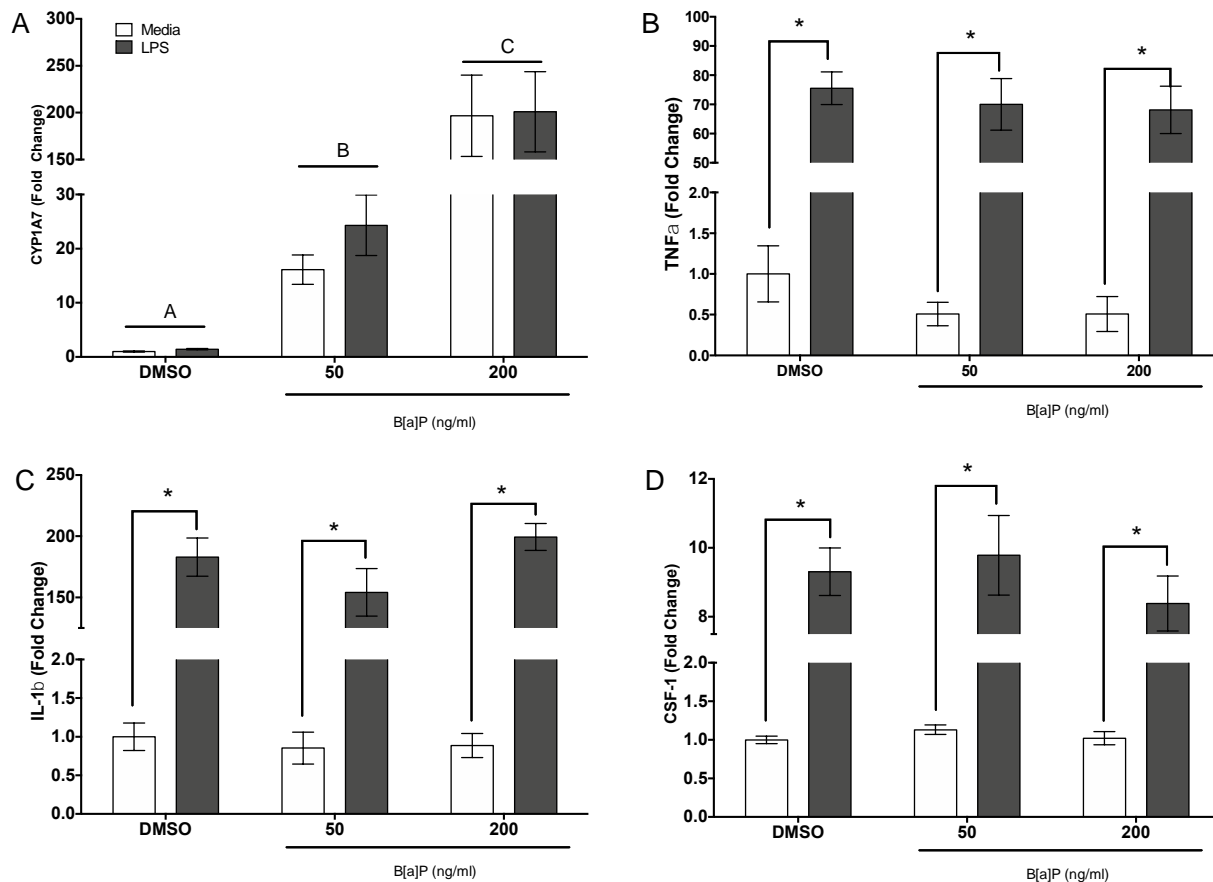
Figure 5.7. The effect of B[a]P on the expression of (A) CYP1A7, (B) TNFα, (C) IL-1β, (D) CSF-1 in *Xenopus laevis* A6 kidney epithelial cells. Cells were exposed to media or solvent control treatments 50 or 200 ng/mL B[a]P for 12 h followed by a 3 h exposure to either SF media or 2 µg/mL LPS. For each biological replicate of the assay (n = 6), duplicate technical replicates were averaged, and data were normalized to the geometric mean of GAPDH and EF1α. Normalized data were expressed as fold-change relative to the media control treatment. Data are presented as mean ± SEM and were analyzed using two-way ANOVA with B[a]P and LPS as factors. Letters indicate significant differences at p < 0.05 among B[a]P treatments (Tukey's post-hoc test), whereas asterisks indicate significant differences among injection treatments (Sidak's multiple comparisons).

5.4. Discussion

Although the immunotoxicity of B[a]P is well studied in many vertebrates, amphibians remain an understudied in this capacity. This gap in the literature is surprising given that amphibians are facing widespread disease driven population declines, which may be exacerbated by exposure to immunotoxic chemicals, such as B[a]P. *In vitro* exposures with cell lines represent rapid and robust test systems for mechanistic toxicity studies and provide data to complement whole animal studies. In the present study, cytotoxicity of B[a]P on *Xenopus laevis* A6 kidney epithelial cell line, as well as changes in ROS production and the regulation of genes related biotransformation pathways and inflammatory response were analyzed in order to increase our knowledge of B[a]P toxicity in amphibians. We also optimized methods for an *in vitro* LPS-challenge exposure model with this amphibian cell line, which provides a novel approach to test the potential mechanisms by which B[a]P, and other compounds, may have immunotoxic effects in amphibians.

A step-wise induction in the expression of CYP1A7 mRNA was observed at all concentrations of B[a]P and time points examined, with the notable exception of 50 ng/mL after 24 hours of exposure. Given that the mechanism of PAH-induced toxicity is hypothesized to be phylogenetically conserved (Reynaud and Deschaux, 2006), activation of the classical AhR gene battery is not surprising. A previous study using the amphibian A6 cell line demonstrated that potent AhR ligands bind to the AhR, albeit with lower affinity when compared to cells from mice (Lavine et al., 2005). Once a ligand binds to the AhR, the complex moves to the nucleus where it can bind to ARNT to form the heterodimeric ligand:AhR:ARNT complex (Denison et al., 2011; Hankinson, 1995). This heterodimeric complex binds to the XRE present in genes within the AhR gene battery, inducing the transcription and translation of several of these genes, including CYP1A7 (Denison et al., 2011). This sequence of events is consistent with the induction of CYP1A7 mRNA observed in the present study. Furthermore, our results support the findings of Lavine et al. (2005), where ligand binding to the AhR led to the induction of members of the CYP family (including CYP1A7) in *X. laevis* A6 cell line.

In the present study, both CYP1A7 mRNA expression and ROS production increased with B[a]P exposure while metabolic capacity of the cells decreased at the highest concentration (500 ng/mL) after 12 and 24 h of exposure. Induction of CYP1A7 aids in the detoxification of B[a]P and can produce ROS as a by-product of this detoxification process (Miller and Ramos,

2001). Excessive ROS production can lead to oxidative stress, with numerous cellular consequences (Valavanidis et al., 2006). The first line of defence for cells against ROS and reactive metabolites is the induction of detoxification enzymes (Kohen and Nyska, 2002; Valavanidis et al., 2006). However, in the present study, the conjugation enzyme GSTM1 was induced only after 24 h exposure to the highest concentration of B[a]P despite the fact that CYP1A7 mRNA expression and ROS production were induced at all time points and B[a]P concentrations. If ROS production remains un-opposed by the detoxification system, it is likely to cause impaired energy production and membrane integrity. Decreased metabolic capacity and viability of the cells observed in this study may be the result of cell damage from increased production of ROS and reactive metabolites. The hypothesized mechanism of B[a]P-induced toxicity in *X. laevis* A6 kidney epithelial cells based on the results of this study is indicated in Figure 5.8.
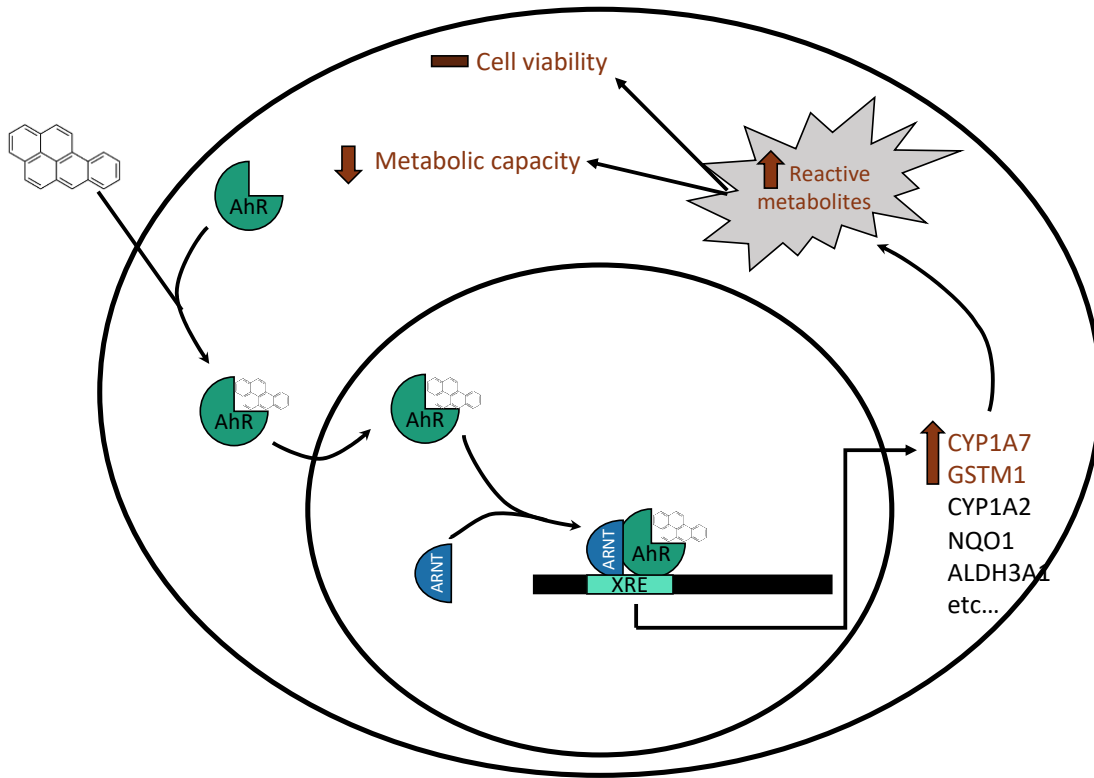
Figure 5.8. The hypothesized mechanism of benzo[a]pyrene (B[a]P)-induced toxicity in *Xenopus laevis* A6 kidney epithelial cells based on the results of this study (indicated in red).

Interestingly, at the lower concentration of B[a]P (50 ng/mL) we observed a time-dependent response where A6 cells exposed for 24 h had markedly lower magnitude of CYP1A7 mRNA induction compared to 12 h of exposure (60-fold induction at 12 h compared 10-fold induction at 24 h). Furthermore, there was no detectable ROS production in cells exposed to 50 ng/mL for 24 h relative to the solvent control treatment. These data suggest that the cells may have metabolized the B[a]P, and thus, CYP1A7 mRNA induction was no longer required, and the cells were no longer producing ROS. However, further studies characterizing the presence of the parent compound and metabolites would be required to confirm this hypothesis. The early induction CYP1A7 mRNA expression and ROS production relative to the induction of GSTM1 suggests that the ROS remains un-opposed in the system until 24 h of exposure and that 12 h of exposure to B[a]P represents an interesting time-point in terms of potential oxidative damage incurred by the cells. Although other conjugation enzymes besides GSTM1 are present in amphibians (Jones et al., 2010), excessive ROS production and subsequent damage are supported by the reduced metabolic capacity of the cells prior to GSTM1 induction. Taken together, the impaired metabolic capacity without GSTM1 induction indicates that the cells are compromised and provided an interesting time-point in the subsequent LPS-challenge study.

Various mechanisms of B[a]P-induced toxicity have been studied in fishes (reviewed in Reynaud and Deschaux, 2006) and may also occur in amphibians. For example, PAH-induced alterations in calcium homeostasis are proposed to contribute to the immunotoxicity observed in common carp (Reynaud et al., 2003; 2001). Thus, although the data of this study support the pathway of AhR activation and CYP-induced damage by B[a]P, it is possible that other mechanisms are also at play. Given the importance of calcium homeostasis in signal transduction, prolonged increases in intracellular calcium concentrations generally precede apoptosis and therefore has the potential to result in immunosuppression when specific immune cells are affected (Davila et al., 1995; Reynaud and Deschaux, 2006). Thus, future studies should consider measuring intra-cellular calcium concentrations to elucidate the potential role of this mechanism in B[a]P-induced toxicity in amphibians.

Exposure to B[a]P did not alter the capacity of A6 cells to respond to an LPS challenge as measured by cytokine expression. Exposure to LPS alone induced TNFα, IL-1β, CSF-1 mRNA expression, and this induction was not affected by the cellular/molecular responses (ROS production, CYP1A7 induction) simultaneously occurring with B[a]P exposure. This was

surprising given the evidence that B[a]P and other PAHs alter immune parameters both in vivo and *in vitro* in a variety of organisms ranging from fish to mammals. For amphibians specifically, we previously demonstrated that a short-term B[a]P exposure alters the response of circulating white blood cells (lymphocytes and granulocyte:lymphocyte ratio) and cytokine expression (IL-1β) upon LPS stimulation in post-metamorphic *X. laevis* (Chapter 4). Following PAH exposure, immune impairment can also be observed in numerous fish species, including rainbow trout (*Oncorhyncus mykiss*), Japanese medaka (*Oryzias latipes*), sea bass (*Dicentrarchus labrax*) and common carp (*Cyprinus carpio*) (Carlson et al., 2002; Danion et al., 2011; Phalen et al., 2014; Reynaud et al., 2003; 2001). The immunotoxicity observed in these fish species is, in some cases, attributed to the metabolism of the PAH by similar mechanisms as those observed in the present study. For example, co-exposure with B[a]P or another PAH 3-methylcholanthrene (3-MC) and the CYP1A inhibitor α-naphthoflavone resulted in the reversal of the immunotoxicity in Japanese medaka and common carp (Carlson et al., 2004a; 2002; Reynaud et al., 2003; 2001), indicating that metabolism of B[a]P by CYP1A is required for the adverse effects.

As demonstrated in previous *in vivo* studies, exposure to B[a]P can modulate cytokine expression and impact the immune system in fish and amphibians (Carlson et al., 2002; Martini et al., 2012); however, we did not observe any impairment of the cytokine response to LPS in amphibian kidney cells pre-exposed to B[a]P. There are a few possible explanations for this apparent discrepancy between *in vivo* and *in vitro* responses. A previous study in mice revealed contrasting detoxification and bioactivation roles of CYP1A between *in vitro* and *in vivo* scenarios – hepatocytes *in vivo* were more likely to detoxify B[a]P, whereas their *in vitro* counterparts preferentially bioactivated B[a]P (Arlt et al., 2008). If detoxification and bioactivation pathways differ across amphibian model systems, it may account for the B[a]P-induced immune-modulation observed *in vivo* (Chapter 4), but not *in vitro* (Chapter 5). While there are many advantages to using *in vitro* models for investigating the mechanistic underpinning of adverse effects, the complexity of *in vivo* is not captured in *in vitro* models. However, the lack of cytokine impairment *in vitro* may also be a consequence of the cell type used in this study. Kidney cells are targets for the pathogenesis of Ranavirus and release cytokines in response to infection (De Jesús Andino et al., 2015; Gantress et al., 2003a). However, classical immune cells, such as macrophages, lymphocytes or neutrophils, may be

more susceptible to contaminant-induced immunotoxicity than kidney epithelial cells, which are not strictly immune cells. Future studies may extend the methods employed in this study to primary immune cells to further determine whether an amphibian *in vitro* LPS-challenge assay can be used to investigate the mechanistic basis of contaminant-induced immunotoxicity.

CHAPTER 6:
GENERAL DISCUSSION

6.1. Project overview and summary of findings

Amphibian populations are declining at an unprecedented rate and this is attributed to numerous factors including climate change, habitat destruction, invasive species, UV radiation, emerging infectious diseases and environmental contaminants (Blaustein et al., 2012; Rollins-Smith and Smits, 2005). Amphibians are sensitive to environmental disruptions (Blaustein and Bancroft, 2007), and the causes for the observed declines likely differ by populations or species and in fact could be attributed to a combination of the above mentioned stressors (Hof et al., 2011). The interaction between pathogens and contaminants in amphibian habitats is of particular concern for two main reasons: (1) contaminants present in amphibian habitats, such as polycyclic aromatic hydrocarbons (PAHs), have the potential to suppress the immune system and increase the severity of disease outbreaks (Bilodeau, 2017; Hersikorn and Smits, 2011; Marquis et al., 2006; Melvin and Trudeau, 2012; Reynaud and Deschaux, 2006) and (2) immunotoxicity is increasingly recognized as a distinct mechanism of action for environmental contaminants (Rehberger et al., 2017; Segner et al., 2012b). Thus, the overall goal of this thesis was to gain a better understanding of developmental and contaminant-induced changes in the immune system of amphibians, and ultimately how exposure to contaminants modulates the response of the immune system to a stimulus.

Throughout this thesis, four studies were conducted to address specific questions within the broader objective. Findings presented herein support that the tadpole immune system of *Xenopus laevis* is transient over development and that the impacts of the prototypical PAH, benzo[a]pyrene (B[a]P), are dependent on the stage of development at the time of exposure. Furthermore, an immune challenge model was developed for juvenile *X. laevis*, which identified immunostimulatory concentrations of the bacterial mimic lipopolysaccharide (LPS) and confirmed that B[a]P can disrupt the ability of the host to mount an immune response against LPS. Finally, this research began to uncover potential mechanisms underlying these immunotoxic consequences of B[a]P in amphibians through both *in vivo* and *in vitro* observations. The final chapter of this thesis will place these findings in the broader context of the literature, explore the implications, and present avenues for future research.

6.2. Furthering our understanding of amphibian immune system development

Amphibian metamorphosis is one of the most dramatic examples of morphological, physiological and biochemical re-organization occurring during post-embryonic development (Tata, 2006). Research presented in this thesis further characterizes the dynamic nature of immune system development and underscores the importance of considering the stage of development when evaluating the effects of chemical exposure on tadpole development, particularly immune system development. Previous studies demonstrate the extensive re-modelling of tadpole tissues and organs into the functionally mature adult counterparts, including the gastrointestinal tract, skin, respiratory organs, hematopoietic system, brain and spinal cord, eyes, liver, and immune organs (reviewed in Brown and Cai, 2007). In particular, the components and functions of the amphibian immune system continually develop as the organism progresses from the tadpole to adult life-stage (Flajnik et al., 1987; Rollins-Smith, 1998). The research conducted in Chapters 2 and 3 examined the development of the tadpole immune system at the molecular level and showed that contaminant-induced changes in immune parameters occur in a stage-dependent manner. Changes in the basal expression of cytokines over development (Chapter 2) provides additional support for the stage-dependent maturation state of the amphibian immune system, as previously seen in other components of the immune system such as immune organs (*e.g.* spleen, thymus, bone marrow), lymphocyte populations, anti-microbial peptides, natural killer cells (Flajnik et al., 1987; Pollet, 2010; Rollins-Smith, 1998). Differences in basal cytokine expression at different developmental stages may also influence how these cytokines respond to B[a]P exposure and contribute to the developmental sensitivity to the immunotoxic effects of this chemical (Chapter 3).

There is a long-standing recognition that the tadpole immune system develops in distinct phases (Rollins-Smith, 1998); therefore, life-stage should be taken into account when designing studies and interpreting responses to contaminant exposure. However, previous immunotoxicity studies examining the impacts of contaminant exposure on amphibians have seldom justified the developmental stage in their study design. For example, Martini et al. (2012) concluded that IL-1β expression, but not TNFα, was a good indicator B[a]P-induced changes in the immune system of amphibians; however, this study only conducted exposures using a single developmental stage. I demonstrated that B[a]P-induced changes in the expression of several cytokines is dependent on the stage of tadpole development at the time of exposure (Chapter 3). For instance, TNFα responded in embryo-larval and pre-metamorphic tadpoles (NF stage 51),

while IL-1β responded in embryo-larval and pro-metamorphic tadpoles (NF stage 56) exposed to B[a]P for 2 days (Chapter 3). Furthermore, I found that neither cytokine responded when B[a]P was administered to juvenile (post-metamorphic) frogs (Chapter 4). Overall, our studies show that the stage of development at the time of contaminant exposure is an important consideration in amphibian immunotoxicity studies.

6.3. Adding to the toolbox for amphibian immunotoxicology research

One of the major outcomes of this thesis is the development of novel methods and assays for immunotoxicology studies in the standard amphibian test species, *X. laevis.* Although the repertoire of immune-related endpoints for amphibians remains limited relative to other vertebrate taxa (*e.g.* fish, birds and mammals), this thesis has contributed to the toolbox for amphibian immunotoxicology research. Through this research, I validated primers for cytokine expression to detect dysregulation of the innate immune system across amphibian life-stages as well as within *in vitro* studies using the *X. laevis* A6 kidney epithelial cell line (Chapters 2, 3 and 5). In Chapter 4, a flow cytometric method for leukocyte profiling was optimized for *X. laevis* and this allowed for faster, more robust and more cost-effective analyses with smaller volumes of blood when compared to conventional differential counts using blood smears. Finally, I optimized an exposure approach for *X. laevis* that combines chemical exposure and an immune challenge in order to assess the potential for contaminant-induced immunotoxicity using the activated amphibian immune system (Chapter 4).

The information presented in Table 6.1. outlines the assays described in this thesis and related scientific literature for measuring immune parameters and evaluating immune modulation in amphibians. Overall, there is a bias towards assays that inform on innate immune responses with less focus on assessing adaptive immunity. This agrees with the conclusions drawn from a review of studies in fish (Rehberger et al., 2017). Given that juvenile and adult amphibians rely on both branches of the immune system for defence against pathogens (Rollins-Smith, 1998), immunotoxicity studies should ideally consider impacts of chemicals on the function of the adaptive immune system as well as the innate immune system in post-metamorphic individuals. The host resistance assay and the leukocyte counts developed in this thesis (Chapter 4) are integrative rather than purely innate-related endpoints, thus contributing to the diversity of tools available for amphibian immunotoxicity assessments.

Table 6.1. Most common assays used in evaluating contaminant-induced immune modulation in amphibians.

Assay	Sample type	Parameter/Function	Species (life-stage) investigated	Key References
Cytokines	Whole organ or whole body	TNF-α, IL-1β, IFN-γ, Mx1, CSF-1	*Xenopus laevis* (embryo-larval, tadpoles, juveniles)	Chapters 2, 3, and 4; De Jesús Andino et al., 2012; Martini et al., 2012; Morales et al., 2010
Leukocyte counts	Whole blood	Lymphocytes, neutrophils, eosinophils, monocytes, basophils, G:L ratio	*Xenopus laevis* (juveniles), *Pelobates cultripes* (tadpoles), *Lithobates catesbeiana* (tadpoles, juveniles), *Lithobates sylvatica* (adults), *Osteopilus septentrionalis* (tadpoles)	Chapter 4; Burraco et al., 2017; Davis, 2009; Forzán et al., 2016; Sears et al., 2013
Bacterial killing assay	Whole blood	*Escherichia coli* killing ability	*Lithobates sphenocephala* (tadpole)	Venesky et al., 2012
Phytohaemagglutinin challenge	Measurable appendage	Inflammation	*Lithobates pipiens* (juvenile), *Lithobates sphenocephala* (tadpole)	Gilbertson et al., 2003; Venesky et al., 2012
Lymphocyte proliferation assay	Splenocytes	Lymphocyte proliferation	*Lithobates pipiens* (juvenile)	Christin et al., 2003
Key-hole limpet hemocyanin assay	Whole blood	Specific immunoglobulin production	*Lithobates pipiens* (juvenile)	Cary et al., 2014; Gilbertson et al., 2003
Phagocytosis assay	Peritoneal leukocytes or splenocytes	Phagocytic activity of neutrophils or splenocytes	*Lithobates pipiens* (juvenile), *Xenopus laevis* (juveniles)	Cary et al., 2014; Christin et al., 2003; 2004
Zymozan-induced chemiluminescence	Whole blood	Oxidative burst	*Lithobates pipiens* (juvenile)	Gilbertson et al., 2003
Fungal growth inhibition assay	Skin peptides	Skin peptide antimicrobial activity	*Lithobates pipiens* (juvenile), *Lithobates boylii* (juvenile), *Xenopus laevis* (juveniles)	Cary et al., 2014; Davidson et al., 2007; Ramsey et al., 2010
Immune challenge assay	Whole organism	Morbidity/mortality, pathogen load, up-regulation of immune responses	*Xenopus laevis* (juvenile), *Lithobates pipiens* (juvenile)	Chapter 4; Christin et al., 2003; Rohr et al., 2008

Often, the choice of specific immune-related endpoints measured in developing amphibians is constrained by the small size of tadpoles and their maturation state at the time of sampling. However, the small body size of tadpoles was less of a barrier for measurement of immune parameters as part of this thesis research since enumeration of blood cells by flow cytometry and evaluation of gene expression by real-time PCR only require small amounts of tissue. The majority of assays and approaches for evaluating immune function in amphibians (as detailed in Table 6.1) were developed and optimized for metamorphic or post-metamorphic individuals, rather than larval life-stage. However, tadpoles are considered the most vulnerable life-stage as they are confined to the aquatic environment and interact with contaminated water and sediments as well as pathogens during critical periods of development (Linder et al., 2010). Therefore, there is a need to expand the repertoire of endpoints that can be used to assess contaminant-induced immunotoxicity in early-life-stages of amphibians. Methods developed for leukocyte counts in juveniles could be easily adapted to different amphibian life-stages and non-model species, as it requires small volumes and does not rely on sequence- or antibody-specific markers. Overall, this thesis has contributed to the development of tools for evaluating the amphibian immune system, particularly for small-bodied species and during the vulnerable tadpole life-stages.

6.4. Establishing an immune challenge model with *Xenopus laevis*

The potential for immunotoxic effects of contaminants is generally overlooked in risk assessments involving aquatic species for two main reasons: (1) the complexity of the immune system and/or (2) the lack for information linking changes in immune parameters to altered immunocompetence in these organisms (Segner et al., 2012). The consequence of contaminant exposure on immunity is typically assessed on the resting or non-stimulated immune system, which does not provide information on whether the exposure has compromised the ability of the individual to respond in the face of a pathogen or immune challenge (Segner et al., 2012; Van Loveren et al., 2003). I found there were minimal impacts of B[a]P on the resting juvenile immune system of *X. laevis*, whereas activation of the immune system with a simulated pathogen revealed B[a]P-induced deficiencies in the response (Chapter 4). Specifically, the inhibitory effect of B[a]P on the G:L ratio and IL-1β expression were only discernable when the immune system had been activated with LPS as these endpoints remain unchanged with B[a]P exposure

in the resting immune system (Chapter 4). Similarly, Wenger et al. (2011) showed that immunosuppression by 17β-estradiol exposure was only observed in fish challenged with a bacterial pathogen and this response was associated with increased mortality in fish in the combined chemical/pathogen exposure. A limitation of our work was that I could not assess whether B[a]P exposure truly resulted in immunocompromised individuals as LPS is not actually a pathogen. Agents that trigger immune or inflammatory responses without infecting individuals do not allow us to evaluate whether a chemical can affect pathogen load, the manifestation of disease symptoms or result in mortality. However, the ability of B[a]P to impair critical inflammatory responses to LPS suggests that there may be implications for amphibian health in the context of environmentally relevant pathogens. By using agents such as ranavirus or chytrid fungus, the immune challenge assay could be refined and be more reflective of the situation where an amphibian in a B[a]P-contaminated wetland encounters a pathogen. Results from such a study would provide more compelling evidence for whether contaminant exposure increases disease susceptibility in amphibians.

There are certainly some limitations and cautions for applying the findings of this thesis research to other amphibian species. Throughout this thesis, *X. laevis* was used to expand our knowledge of contaminant-induced immunotoxicity in amphibians. However, findings from *X. laevis* may or may not be applicable to the more environmentally relevant species in Canada due to differences in immune system characteristics. For example, ranid and bufonid species, many of which are found in Canada, possess lymphomyeloid nodes that act as blood-filtering organs, and these are absent in *X. laevis* (Robert, 2016). There is also evidence of species-specific differences in immune components influencing susceptibility to pathogens (Woodhams et al., 2007), in the absence of contaminant exposure. There is also the potential for species-specific differences in the metabolism of contaminants. Studies comparing the metabolism of PAHs across amphibian species have demonstrated that there are differences in the sequences, activity and metabolite formation from phase I and phase II enzymes (Nakayama et al., 2009; Ueda et al., 2011). Metabolic pathways across amphibian species may underlie some of the observed differences in toxicity across species. For example, the 96 h LC_{50} values for fluoranthene and UV light are 3-fold higher for northern leopard frogs compared to *X. laevis* (Hatch and Burton, 1998). These differences in the metabolism and toxicity of compounds between amphibian species is applicable across numerous compounds including PAHs, pesticides, metals, road salts

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(Collins and Russell, 2008; Goulet and Hontela, 2003; Hatch and Burton, 1998; Howe et al., 2004; Relyea and Jones, 2009). Therefore, understanding the functional implications of these differences in the immune systems and metabolic pathways between amphibian species will likely be a fruitful area of research and may aid in the interpretation and extrapolation of results from immune challenge studies to other environmentally relevant species.

Immune challenge assays can be performed with various agents, including pathogens or compounds that produce pathogen-associated molecular patterns (Köllner et al., 2002; Segner et al., 2012). Presently, the primary pathogens impacting amphibian populations and causing disease outbreaks are ranavirus and chytrid fungus (Chinchar, 2002; Daszak et al., 2003; 2000). In my research, LPS was employed as an immune stimulator in both the *in vivo* and *in vitro* immune challenge model. LPS is a major component of gram-negative bacterial outer-membranes that is recognized by the innate immune system, effectively induces inflammatory responses as demonstrated by increases pro-inflammatory cytokines and shifts in leukocyte profiles of fish, amphibians and mammals (Chapter 4; Holen et al., 2012). Without creating an infectious animal, LPS triggers an inflammatory response that is typical of a bacterial infection, including activation numerous immune cell types expressing toll-like receptors (*e.g.* macrophages, monocytes, Kupffer cells) and triggering signaling cascades that promote inflammatory and pyrogenic phenotypes (Novoa et al., 2009; West and Heagy, 2002). Administration of LPS, either *in vivo* through injection in juvenile animals (Chapter 4) or through *in vitro* exposure of amphibian cells (Chapter 5), produced a robust inflammatory response as measured by cytokine expression and this demonstrates that LPS is an effective agent for immune challenge and immunotoxicology studies. Although LPS induces a similar molecular pattern as a bacterial pathogen, an immune-challenge model with this simulated pathogen can provide useful information for contaminant-induced impairment of inflammatory responses required for defence against numerous pathogens. For example, a key component of the defence against ranaviral infection are inflammatory responses (De Jesús Andino et al., 2012). Thus, results from an immune challenge with LPS may have implications for environmentally relevant pathogens such as ranavirus. In contrast, antimicrobial peptides are the primary line of defence against chytrid fungus (Davidson et al., 2007), which is not directly tested in this immune challenge model. Therefore, future work would be required to establish an

immune challenge model that can be used to evaluate effects of contaminant exposure on the presence and function of antimicrobial peptides.

6.5. Elucidating the mechanism of benzo[a]pyrene-induced immunotoxicity in amphibians

The mechanism of B[a]P-induced immunosuppression is thought to be phylogenetically conserved from fish to mammals (Reynaud and Deschaux, 2006), yet few aspects of the mechanism had been confirmed in amphibians. Amphibians are known to express AhR as well as the AhR nuclear translocator (ARNT; Lavine et al., 2005) and CYP1A isoforms (Jönsson et al., 2011; Lavine et al., 2005). Exposure to B[a]P is reported to alter immune parameters in amphibians including increased mast cell number in the liver (Fanali et al., 2018), altered phagocytic activity and viability of spleen cells (Regnault et al., 2016), and altered interleukin-1β (IL-1β) expression (Martini et al., 2012). The research results presented in this thesis indicate that B[a]P activates the aryl hydrocarbon receptor (AhR), resulting in the induction of the cytochrome P450 1A enzyme and subsequent production of immunotoxic metabolites. The research presented in Chapters 5 is the first characterize the time-course response of amphibian cells to B[a]P and demonstrates that the CYP-induced ROS production and subsequent oxidative damage may be, at least in part, responsible for changes immune parameters and impaired response to an immune challenge. Furthermore, Chapter 3 of this thesis was the first study to demonstrate that B[a]P exposure can induce CYP1A7 expression throughout the larval life-stages, and therefore, this mechanism may apply throughout tadpole development. Additionally, B[a]P-induced cytokine dysregulation also occurs across amphibian life-stages, providing further support for this mechanism of action in tadpoles. Taken together, our studies begin to confirm the mechanism of B[a]P-induced immunotoxicity in amphibians across developmental stages and indicates that amphibians in contaminated environments may have compromised immune function.

6.6. Future directions

Currently, there is limited evidence in amphibians in terms of whether contaminant-induced changes to immune parameters translate into reduced immunocompetence and increased disease susceptibility in the whole animal. Such adverse outcomes at the individual level are the most relevant when evaluating the risk of chemicals to amphibian population health.

Contaminant impacts on the immune system are most often assessed on the resting immune system (*i.e.* no immune challenge), and changes in basal immune parameters are inferred to mean altered immunocompetence without evidence that immune function was truly altered. This information is crucial if we are to determine causal contaminants that alter the immune system and direct efforts to mitigate or manage chemicals that impact disease susceptibility. In addition, given the complexity and multifaceted nature of the immune system, a single assay or parameter is rarely sufficient to assess immunotoxic effects. Selection of appropriate endpoints can be particularly complex given the redundancies, compensatory mechanisms, and the diversity of potential targets of toxicity within the immune system (Rehberger et al., 2017). As the variety of endpoints available for amphibians continues to expand, it will become increasingly important to justify the choice of immune parameters selected to evaluate the function of the immune system following contaminant exposure.

There are tiered testing strategies to screen for potential immunotoxicity of chemicals in mammals (Hinton, 2000; Luster, 2014) and recent discussion regarding the feasibility of such a framework for fish (Rehberger et al., 2017); however, no such standardized testing has been proposed for amphibians. Although results from fish are often extrapolated to amphibians, meta-analyses remain inconclusive as to whether fish are appropriate surrogates for amphibians (Birge et al., 2010; Weltje et al., 2013) Therefore, establishing a standardized immunotoxicity testing strategy in amphibians would decrease the uncertainty associated with extrapolations across taxa and may allow immunotoxicity to be included in the hazard assessment for ecotoxicological risk assessments.

In order to have a predictive tool for chemicals that compromise immune function in amphibians, parameters and endpoints that most closely associate with and predict immunocompetence must first be identified. The immune system is delicately balanced and can compensate for some perturbances without altering the immunocompetence of the organism. This ability to compensate makes it difficult, if not impossible, to identify a single endpoint that will be predictive of immune function in the whole organism. Choosing the most appropriate predictive endpoints could be achieved through the use of a principal component analysis to determine which endpoints best correlate with the immune challenge assay and account for the most variability in the data. This approach was used in a mouse model to determine which of the 34 variables tested most closely associate with host resistance to tumor cell formation after

exposure to dexamethasone (Keil et al. 2001). The targeted endpoints could then make up the battery of tests to be used for screening compounds for their potential impacts on immunocompetence.

Another avenue of future research would be to confirm the mechanism of B[a]P-induced immunotoxicity in amphibians. Taken together, the results of this thesis research suggest that CYP-induced ROS production and subsequent oxidative damage is at least in part responsible for the observed immune modulation of B[a]P in amphibians. However, knock-down and/or inhibition experiments could be performed both *in vivo* and *in vitro* to functionally confirm the mechanism. If the mechanism of B[a]P-induced immunotoxicity is in fact mediated by CYP, we would expect genetically modified (*i.e.* knock-down) or chemically inhibited (*e.g.* α-naphthoflavone or CH223191) animals not to exhibit immune modulations. Additional studies could be performed with antioxidant treatments to determine if preventing oxidative damage ameliorates the immune impairment. Such 'loss of function' experiments would also contribute to our understanding of whether taxa-specific differences (*i.e.* amphibians compared to fish) in the responses to AhR ligands extend to B[a]P-induced effects on immune responses.

6.7. Conclusion

The impact of pathogens and contaminants to amphibian population health individually and together emphasizes the critical need to better understand how chemicals modulate immune function and influence disease from both a whole organism and mechanistic perspective. My research contributes to our overall understanding of the developmental and contaminant-induced changes in the immune system of amphibians, and ultimately how exposure modulates their response to immune stimulation. While my work provides a foundation for further study into the mechanism of B[a]P-induced immunotoxicity in amphibians specifically, it also provides tools and approaches that can be used in screening chemicals for their potential to alter immunocompetence and disease susceptibility. The tools and approaches developed as part of this research could be used to widely screen chemicals for their potential to cause immunotoxicity in amphibians and also be expanded to evaluate immune responses of ecologically relevant amphibian species who inhabit contaminated environments and may be at real risk of disease-driven population declines.

APPENDIX

Appendix A: Calculated delivered dose of LPS based on individual body weights for the preliminary LPS challenge.

Day	Nominal LPS (μg/g)	Body weight (g)[1]	Quantity of LPS (μg) injected in 50 μl	Delivered dose (μg/g)[1]	p value[2]
0	Control	1.37 ± 0.09	N/A	N/A	N/A
1	Control	1.36 ± 0.12	N/A	N/A	N/A
	PBS	1.44 ± 0.08	0	0 ± 0	N/A
	0.3	1.35 ± 0.08	0.39	0.3 ± 0.02	0.899
	3	1.35 ± 0.09	3.9	2.97 ± 0.18	0.854
	30	1.41 ± 0.10	39	28.89 ± 2.49	0.669

[1]Data presented as mean $\pm$ SEM.
[2]One-sample t-test comparing delivered and nominal LPS doses.

Appendix B: Calculated delivered dose of LPS based on individual body weights for B[a]P exposure with LPS challenge.

B[a]P treatment	Nominal LPS (μg/g)	Body weight (g)[1,2]	Quantity of LPS (μg) injected in 50 μl	Delivered dose (μg/g)[1, 3]
DMSO	PBS	1.0 ± 0.06	0	N/A
	10	0.96 ± 0.08	7.1	7.89 ± 0.59
70 μg/L	PBS	0.94 ± 0.08	0	N/A
	10	1.02 ± 0.11	7.1	7.77 ± 0.69
350 μg/L	PBS	1.02 ± 0.07	0	N/A
	10	0.96 ± 0.1	7.1	8.14 ± 0.73

[1]Data presented as mean $\pm$ SEM.
[2]Data were analysed using two-way ANOVA with B[a]P and LPS injection as factors.
[3]One-way ANOVA comparing delivered doses across B[a]P treatments (F = 0.081, p = 0.923).

Appendix C: Morphometric values for juvenile *X. laevis* exposed to LPS in the preliminary challenge.

Day	Nominal LPS (μg/g)	Body weight (g)[1,2]	Body length (mm)[1,2]	Liver somatic index[1,2]
0	Control	1.37 ± 0.09	23.31 ± 0.49	2.20 ± 0.13
1	Control	1.36 ± 0.12	23.50 ± 0.54	2.08 ± 0.23[a]
	PBS	1.44 ± 0.08	23.96 ± 0.39	2.58 ± 0.16[ab]
	0.3	1.35 ± 0.08	23.57 ± 0.59	2.42 ± 0.1[ab]
	3	1.35 ± 0.09	23.29 ± 0.54	2.51 ± 0.16[ab]
	30	1.41 ± 0.10	23.88 ± 0.50	2.93 ± 0.21[b]
3	Control	1.35 ± 0.12	23.11 ± 0.62	1.86 ± 0.32
	PBS	1.29 ± 0.09	22.71 ± 0.49	2.26 ± 0.28
	0.3	1.29 ± 0.08	23.04 ± 0.60	2.24 ± 0.18
	3	1.35 ± 0.11	23.15 ± 0.61	2.27 ± 0.22
	30	1.28 ± 0.07	23.33 ± 0.43	2.14 ± 0.18

[1]Data presented as mean ± SEM.
[2]Data were analysed using one-way ANOVA comparing endpoints within 1 or 3 dpi. Letters indicate significant differences at $p < 0.05$ among groups (Tukey's post-hoc test).

Appendix D: Morphometric values following immune challenge with B[a]P exposure and 1dpi LPS challenge in juvenile *X. laevis*.

B[a]P treatment	Nominal LPS (μg/g)	Body weight (g)[1,2]	Body length (mm)[1,2]	Liver somatic index (LSI)[1,2]
DMSO	PBS	1.0 ± 0.06	21.68 ± 0.39	3.02 ± 0.13
	10	0.96 ± 0.08	21.51 ± 0.46	3.22 ± 0.19
70 μg/L	PBS	0.94 ± 0.08	21.13 ± 0.53	2.87 ± 0.19
	10	1.02 ± 0.11	21.37 ± 0.73	3.27 ± 0.28
350 μg/L	PBS	1.02 ± 0.07	21.30 ± 0.55	3.00 ± 0.14
	10	0.96 ± 0.10	20.89 ± 0.70	2.95 ± 0.14

[1]Data presented as mean ± SEM.
[2]Data were analysed using two-way ANOVA with B[a]P and LPS injection as factors.
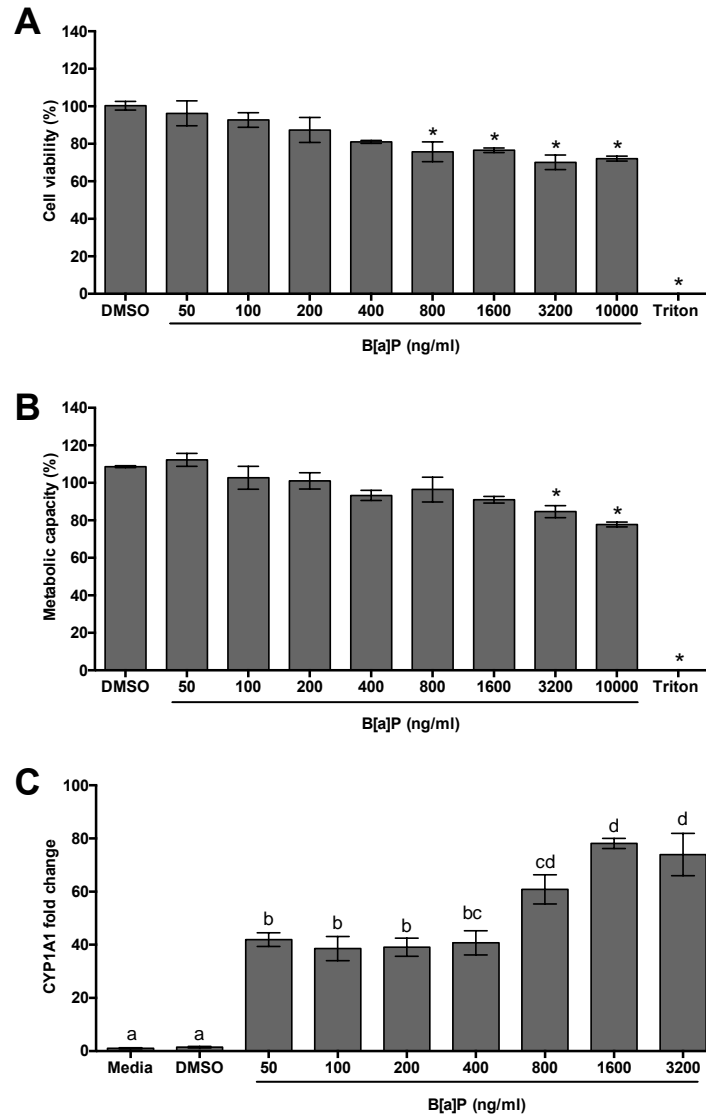
Appendix E: The effect of 3 h exposure B[a]P on measures of (A) cell viability (trypan blue exclusion assay), (B) metabolic capacity (MTT assay), and (C) CYP1A7 mRNA expression in *Xenopus laevis* A6 kidney epithelial cells. Data (mean ± SEM) are expressed relative to the media control treatment with n=3 cell viability and metabolic capacity and n=6 for gene expression. Different letters indicate $p < 0.05$ (one-way ANOVA and Tukey's post hoc test).
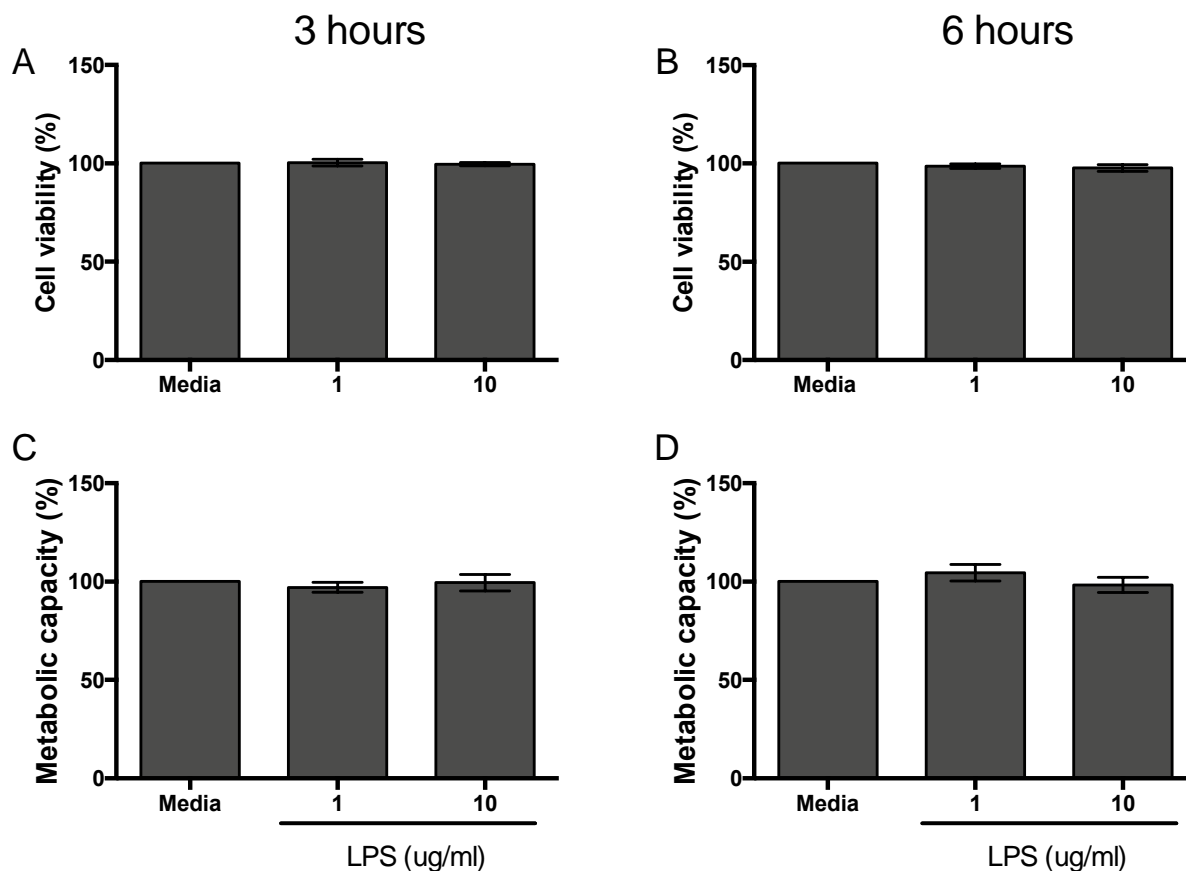
Appendix F: The effect of LPS on measures of (A and B) cell viability (trypan blue exclusion assay) and (C and D) metabolic capacity (MTT assay) in *Xenopus laevis* A6 kidney epithelial cells exposed to media control, 1 or 10 μg/mL LPS for 3 or 6 h. For each biological replicate of the assays (n = 3), triplicate technical replicates were averaged, and data were expressed relative to the media control treatment of the respective time-point. Data are presented as mean ± SEM and were analyzed by one-way ANOVA followed by Tukey's post hoc test within each time point. Different letters above columns indicate significant differences between treatments at the level of $p < 0.05$.

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