Immunotoxic Effects of Benzo[a]Pyrene on Rainbow Trout (*Oncorhynchus mykiss*)

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Thesis Abstract

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds with immunotoxic and carcinogenic potential that may pose a threat to fish populations. The mechanism underlying immune toxicity of PAHs in fishes remains unclear. Some evidence supports the requirement for metabolism to more toxic metabolites. This two-part study aims to utilize a newly developed fish immunotoxicology model to determine the immune tissue/cell population level effects of PAHs on rainbow trout, using benzo[a]pyrene (BaP) as a representative immunotoxic PAH. In the first part of the study, intraperitoneal injection of 25 or 100 mg/kg BaP resulted in sustained exposure as indicated by biliary fluorescence at BaP wavelengths for up to 42 d. A new flow cytometry method for absolute counts of differential leukocyte distributions in spleen, blood, and head kidney was developed by combining absolute quantitative counts of total leukocytes in the tissue (3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) dye) with relative differential counts using monoclonal antibodies for B cells, T cells, myeloid cells, and thrombocytes. Experiments indicated dose- and time-dependent decreases in the absolute number of B cells, myeloid cells, or T cells in blood, spleen, or head kidney after 7, 14 or 21 d of exposure. There was no change in the absolute numbers of erythrocytes or thrombocytes in any tissue. When rainbow trout were exposed to inactivated *Aeromonas salmonicida* after a 21 d exposure to 100 mg/kg BaP, circulating antibody concentrations were decreased by 56%. It was concluded that BaP has a cell lineage-specific toxic effect on some immune cells of rainbow trout, and causes a decrease in circulating antibody levels. The second part of the study examined whether intraperitoneal exposure of rainbow trout to BaP caused leukocyte mRNA expression changes in five cytochrome

P450 (CYP) enzymes; to their transcription factor, AhR; or to an extrinsic pathway apoptosis checkpoint, p53. mRNA expression was analyzed in immunomagnetically isolated B cells and thrombocytes from blood, spleen, or head kidney in an effort to clarify the tissue and cell specific toxicity of BaP. Significant inductions above control levels were observed in CYP1A1 in liver, blood B cells, and blood thrombocytes; CYP1B1 in blood B cells, and blood thrombocytes; CYP1A3 in liver, blood and spleen B cells; and AhR in spleen thrombocytes. No significant changes were found in CYP1C1, CYP1C2, or p53. Increased mRNA expression was observed 14 d after exposure, indicating a prolonged physiological effect of a single BaP injection. Although there were differences in expression, it was concluded that variations in the presence or induction of CYP enzymes is not enough to explain the difference in toxicity observed between B cells and thrombocytes. An induction of AhR in thrombocytes is interesting, but difficult to explain given current knowledge on endogenous AhR regulation and the limited toxicity in thrombocytes. Overall, our findings provide support for a complex mechanism of toxicity for chronic PAH exposure, and suggest that CYP expression profiles do not fully explain these effects.

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List of Abbreviations

3-MC – 3-methylcholanthrene
A.s. – *Aeromonas salmonicida*
AhR – Aryl hydrocarbon receptor
ANCOVA – Analysis of co-variance
ANOVA – Analysis of variance
ARNT – Aryl hydrocarbon receptor nucler translocator
BaP – Benzo[a]pyrene
CF – Condition factor
LSI – Liver somatic index
CYP – Cytomchrome P450
DiOC6 – 3,3'-Dihexyloxacarbocyanine iodide
DMBA – 7, 12-dimethylbenz[a]anthracene
DNA – Deoxyribonucleic acid
DRE – Dioxin response element
EDTA – Ethylenediaminetetraacetic acid
EF1α – Elongation factor 1α
ELISA – Enzyme-linked immunosorbent assay
FACS – Fluorescence assisted cell sorting
HCl – Hydrochloric acid
HPLC – High performance liquid chromatography
i.p. – Intraperitoneal
i*A.s.* – Formalin inactivated *Aeromonas salmonicida*
Ig - Immunoglobulin
mAbs – Monoclonal antibodies
mRNA – Messenger ribonucleic acid
MS-222 – Tricaine mesylate
p.e. – Post exposure
PAH – Polycyclic aromatic hydrocarbon
PAMP – Pathogen associated molecular pattern
PBS – Phosphate buffered saline
qPCR – Quatitative polymerase chain reaction
SSI – Spleen somatic index
VIE – Visual implant elastomer
XRE – Xenobiotic response element

Chapter 1

1.1 General Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds that consist of multiple fused aromatic rings, taking a planar shape (Fig. 1.1). They may also have any number of constituents on the ring structures, making this a diverse group of compounds. PAHs are formed by the incomplete combustion of organic materials including wood and petroleum. PAHs are found ubiquitously in the environment; they have been shown to disperse from their original source via atmospheric deposition and can be a component of various effluents and mining tailings (Brun *et al.*, 2004; Galarneau, *et al.*, 2014; Kelly *et al.*, 2009; Leppanen and Oikari, 2001; Schindler, 2014).

Once introduced to the environment, PAHs can attach to particles in air and water as well as accumulate in sediment and soils (Baek *et al.*, 1991). Because they are generally lipophilic, PAHs easily move into tissues of animals when inhaled, ingested, or taken up from water. However, they can be metabolised, meaning they do not necessarily build up over time. Lipophilicity, metabolism potential, and toxicity all vary depending on the unique structure of each compound.

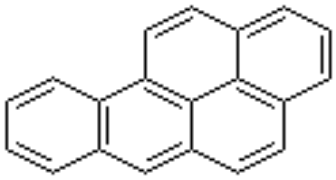

Fig. 1.1: The chemical structure of prototypical PAH, BaP.

Fish have a well conserved mechanism in place to breakdown and excrete these naturally occurring compounds. When planar aromatic compounds enter a cell, they bind to a receptor in the cytoplasm called the aryl hydrocarbon receptor (AhR). This activates

the receptor, signalling for chaperone molecules including Hsp90, XAP2, and p23 to attach, followed by translocation into the nucleus. Once in the nucleus, the complex is bound by another protein called aryl hydrocarbon receptor nuclear translocator (ARNT), yielding a functional transcription factor. This complex binds to what is known as a dioxin response element (DRE) or xenobiotic response element (XRE), transcribing the genes downstream (Hahn and Karchner, 2012; Zhou *et al.*, 2010). Known transcription products of this activation in fish include members of the cytochrome P450 1 (CYP1) family of enzymes such as CYP1A1, CYP1A3, CYP1B1, CYP1C1, CYP1C2, CYP1C3, and CYP1D (Gao *et al.*, 2011; Goldstone and Stegeman, 2008; Jonsson *et al.*, 2010). These enzymes are responsible for phase I biotransformation involving catalytic breakdown of PAHs and other planar aromatic compounds to facilitate excretion. Phase I reactions typically oxidize molecules by adding a functinal group, rendering them more reactive so they can be conjugated to water soluble compounds in phase II (eg. glucuorinidation).

Despite a physiological ability to remove these compounds, PAHs have been implicated in causing disease in animals. In the case of certain PAHs, including BaP, the more reactive phase I metabolites rather than the parent compound is thought to be responsible for some of the toxic effects of exposure. Therefore, exposure to the compound stimulates production of the enzyme that ultimately induces toxicity. It has been well documented that the BaP breakdown product benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide is capable of interaction with DNA, and is the agent responsible for carcinogenesis (Gelboin, 1980). Carcinogenic potential of PAHs is in part explained by

the presence of what is known as a bay region, which facilitates biological interaction (Fig. 1.2).

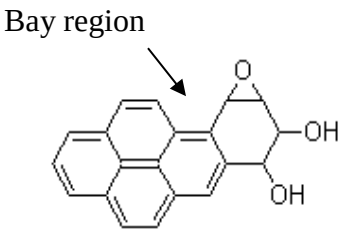

Fig. 1.2: The chemical structure of carcinogenic metabolite of BaP, benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide. Note the bay region, which facilitates interaction with biological molecules.

In addition to their carcinogenic potential, PAHs have also been implicated in causing immune modulation in mammals and fishes (reviewed by Reynaud *et al.*, 2008; Stockinger *et al.*, 2011). To study this phenomenon, knowledge of immune physiology in fishes is important. The head kidney in fishes is described as the equivalent to mammalian bone marrow, demonstrating hematopoietic activity and containing several immune cell types including granulocytes, macrophages, B cells, T cells, and containing reticular cells that aid in the function of lymphocytes (Uribe *et al.*, 2011; Zwollo *et al.*, 2005). The head kidney also serves a lymph node-like function, an important location for orchestration of immune-antigen interactions (Press and Evenson, 1999). Cells equivalent to B cells and accordingly named are also found in fishes (Uribe *et al.*, 2011), although they express different profiles of immunoglobulin (Ig). Most particularly, an IgM tetramer is the most prevalent Ig in plasma. IgD and the unique IgT can also be found in fishes (Hansen *et al.*, 2005; Uribe *et al.*, 2011). T cells have also been described in fishes, and shown to have CD4 and CD8 positive sub-populations and to interact with B cells (Uribe *et al.*, 2011). Fish also have a thymus, equivalent to that in humans, that is populated almost entirely by T cells. The spleen has also been shown to have both hematopoietic and lymphoid functions (Uribe *et al.*, 2011).

When isolating whole leukocyte populations from fish tissues - in addition to cells of myeloid lineage and lymphocytes - thrombocytes are included in the fraction. These cells are difficult to differentiate from lymphocytes when using staining and visual characterization analysis. Thrombocytes are responsible for clotting in fishes as they are in mammals (Hill and Rowley, 1996; Stosik *et al.*, 2002). Unlike in mammals, where thrombocytes are called platelets, thrombocytes in fishes are nucleated and there is some evidence that they participate in phagocytosis (Hill and Rowley, 1996; Stosik *et al.*, 2002), antigen presentation, and immune regulation (Köllner *et al.*, 2004).

Literature relating to immunotoxic potential of PAHs in fishes is limited and can often be conflicting. Many studies focus on functional end points of innate immunity including phagocytosis, lysozyme activity, and macrophage respiratory burst as reviewed by Reynaud and Deschaux (2006). Decreased phagocytosis after exposure to PAH polluted waters has been reported (Rice and Schlenk, 1995; Seeley and Weeks-Perkins, 1991; Weeks and Warinner, 1984; Weeks *et al.*, 1986), while one study showed an increase in phagocytic activity after exposure to high concentrations of PAHs (Kelly-Reay and Weeks-Perkins, 1994). Effect on macrophage respiratory burst activity is conflicting, with some studies showing increases in activity (Reynaud et al, 2002) and others showing impairment (Carlson *et al.*, 2004). Lysozyme, an enzyme that acts by splitting glycosydic linkages, has often been analyzed from fish blood serum after exposure to PAHs. Decreases in lysozyme activity were observed after exposure of rainbow trout to a low dose of diesel-oil drilling mud, but higher doses did not yield this effect (Tahir and Secombes, 1995). A study in dab (*Limanda limanda)* indicated decreased lysozyme activity after exposure to oil contaminated sediment, and after

exposure to an oil spill (Secombes *et al.*, 1997). Yet, when dab were exposed to PAHs in a laboratory setting, no effect was observed (Hutchison *et al.*, 2003). Although sometimes conflicting, these studies suggest the potential of environmental exposure to PAHs to be capable of modulating fish immune responses.

Other studies focus on the effect of PAHs on the acquired immune system. Plaque forming assays assess the ability of a leukocyte population to produce antibody in response to a mitogen *in vitro*. Smith *et al.* (1999) reported decreased plaque formation by tilapia (*Oreochromis niloticus*) head kidney leukocytes in response to a sheep red blood cell antigen after exposure to BaP. Decreases in plaque formation by chinook salmon (*Oncorhynchus tshawytscha*) head kidney and spleen leukocytes in response to another antigen (TNP-keyhole limpet hemocyanin) were reported after exposure to DMBA (Arkoosh *et al.*, 1994). Effects on the ability of lymphocytes to proliferate have also been reported: Reynaud *et al.* (2003) reported a decrease in common carp (*Cyprinus carpio*) peripheral blood leukocyte proliferation after *in vitro* exposure to a mitogen plus 3-methylchloranthrene (3-MC).

The above described studies examine scenarios in which fish leukocytes are isolated and then exposed to mitogen and toxicant *in vitro*. While these results are indicative of ability for PAHs to modulate fish immune cell function, perhaps more telling are alterations following *in vivo* exposures. Decreased peripheral blood leukocyte proliferation was also reported in a later study by Reynaud and Deschaux (2005) on common carp in which 3-MC was intraperitoneally (i.p.) injected and proliferation was assessed *in vitro*. Carlson *et al.* (2002a) report a decrease in Japanese medaka (*Oryzias latipes*) spleen leukocyte proliferation after i.p. injection of BaP, again, proliferation was

assessed *in vitro*. These studies indicate that *in vivo* exposures to PAHs have effects on the ability of lymphocytes to proliferate in response to an antigen.

An important scenario to consider in immune toxicology is one in which the animal is exposed to both a toxicant and an immune challenge, because many immune functions are not active without the presence of a stimulus. *In vivo* exposure to the antigen is ideal, given that immune cells are dependent upon communication between various cell types. Carlson *et al.* (2002a) exposed Japanese medaka i.p. to BaP and found that host resistance to bacterial pathogen *Yersinia ruckeri* was decreased. Arkoosh *et al.* (1998) report an increase in mortality from *Vibrio anguillarium* infection after exposure to DMBA. Decreased circulating antibody titres against inactivated *Aeromonas salmonicida* were reported in rainbow trout after waterborne exposure to BaP (Leclair *et al.*, 2013). These studies indicate that *in vivo* exposure to PAHs suppress the normal immune function of fishes.

Collectively, reports of impaired innate immune cell functions, decreased plaque formation, reductions in lymphocyte proliferation, decreased host immune resistance to disease, and decreased circulating antibody in various fish species after exposure to PAHs provide compelling evidence that these compounds are capable of modulating immune functions. However, such studies provide little insight into how PAHs cause this effect. The end points assessed in these studies are downstream effects of immune cell activity, but in no scenario are changes at the cellular level assessed *in vivo*.

A few studies provide insight into the cellular level effects of PAH exposure in fishes. Total head kidney and spleen leukocyte counts were depressed in tilapia after i.p. injection of DMBA as determined by flow cytometry. These findings were reflected by

histological analysis that showed hypocellularity in the lymphoid area of spleen (Hart *et al.*, 1998). Tilapia head kidney also had reduced leukocyte numbers after i.p. injection of BaP (Holladay *et al.*, 1998). A few very recent studies by our lab and that of colleagues have looked at effects of BaP on the rainbow trout immune system using monoclonal antibodies to distinguish leukocyte subpopulations from each other. LeClair *et al.* (2013) observed decreases in absolute numbers of B and T cells after 7 d of waterborne exposure to BaP, but also saw an induction of thrombocyte numbers after 7 d in blood. Möller *et al.* (2014) observed decreases in relative counts of blood thrombocytes after 24 h of exposure, followed by an increase at 96 h. They also report a stimulatory effect on liver IgM B cells and thrombocytes at 24 h. These studies indicate that exposure to PAHs can cause an alterationin immune cell number in fishes.

Cell population reductions may be explained by lack of proliferation, apoptosis, necrosis, or movement to other tissues. We have already seen studies in which leukocyte proliferation is reduced after *in vitro* exposure to antigen, but this phenomenon has not been investigated *in vivo*. Reynaud *et al.* (2004) report induction of apoptosis in common carp peripheral blood leukocytes and head kidney phagocytes after exposure to 3-MC *in vitro*. Holladay *et al.* (1998) report an increase in the number of apoptotic immune cells of the tilapia head kidney after i.p. injection to BaP. The specific mechanism by which PAHs can prevent immune cell proliferation or increase apoptosis in fishes has not been investigated.

Some studies indicate that BaP toxicity is dependent on metabolism (Carlson 2002b, 2004b). Yet 3-MC does not seem to depend on this pathway to induce toxicity despite being able to induce CYP expression, as determined by the use of a compound that

blocks interaction with AhR but does not activate it (Dorrington *et al.*, 2011; Reynaud *et al.*, 2005). BaP is capable of stimulating CYP enzymatic activity in immune tissues of rainbow trout (Möller *et al.*, 2013). CYP1A has been shown to be basally expressed in B cells and granulocytes of the head kidney (Nakayama *et al.*, 2008). Nakayama *et al.* (2008) also showed that CYP enzymatic activity was increased after BaP exposure. Such investigations indicate that it is important to consider whether PAH metabolism is necessary to induce toxicity. Also, interactions with the AhR-CYP-metabolism pathway provides a starting point to study the effects that PAHs may have on fish immune cells.

1.2 Thesis outline

In the following studies, we use existing knowledge and new tools to design novel studies in an attempt to further elucidate the effect of a PAH, BaP, on the rainbow trout immune system. A fundamental difference of the approach used here compared to the previous studies is the focus on cellular, rather than functional endpoints. We hypothesized that for a compound to be immunotoxic, selective toxicity to lymphatic tissue must occur, and this must in turn manifest in terms of tissue-specific impacts on those cell populations. The studies herein use monoclonal antibodies (mAbs) that allow for flow cytometry separation of the leukocyte population into 4 distinct sub categories including cells of myeloid lineage, thrombocytes, IgM^+ B cells, and T cells. Two of these mAbs are also suitable for use in immunomagnetic separation, allowing for separate analysis of the B cell and thrombocyte sub types. A secondary goal of this study is to contribute to the development of *in vivo* immunotoxicity bioassays for fishes. This is

examined with consideration for both toxicant dose, exposure duration, and has the additional consideration of a non-lethal immune challenge using bacterial antigen.

The hypotheses of the two distinct studies encompassed in this thesis are outlined below:

- Chapter 2: Intraperitoneal exposure to BaP will selectively reduce the number of certain leukocyte populations in a tissue-specific, dose dependent manner.
- Chapter 2: Pre-exposure to BaP will cause toxicity leading to a reduction in antibody production against a non-infectious antigen.
- Chapter 3: B cell mRNA expression profiles of AhR-CYP pathway relevant genes will differ from those in thrombocytes, potentially explaining the differential effect on cellular toxicity observed in Chapter 2.

Given the consistency with which investigations reveal the ability for PAHs to modulate fish immune parameters, we further hypothesize that there is a cellular mechanism that allows immune cell specific toxicity. These studies are our preliminary effort to begin homing in on the location of this effect. We aim to determine if the differential regulation of the traditional AhR-CYP pathway may help explain if and how immune cells become targeted.

1.3 Literature Cited

Arkoosh, M., Casillas, R., Clemons, R., Kagley, A.N., Olsen, R., Reno, P., Stein, J.E., 1998. Effect of pollution on fish disease: potential impacts on salmonid populations. J. Aquat. Anim. Health 10, 182–190.

Baek, S.O., Field, R.A., Goldstone, M.E., Kirk, P.W., Lester, J.N., Perry, R., 1991. A review of atmospheric polycyclic aromatic hydrocarbons: sources, fate, and behaviour. Water, Air, Soil Poll. 60, 279-300.

Boehm, T., Iwanami, N., Hess, I., 2012. Evolution of the immune system in the lower vertebrates. Ann. Rev. Genomics Hum Genet. 13, 127-149.

Boffetta, P., Jourenkova, N., Gustavsson, P., 1997. Cancer risk from occupational and environmental exposure to polycyclic aromatic hydrocarbons. Cancer Causes Control. 8, 444-472.

Brun, G.L., Vaidya, O.C., Leger, M.G., 2004. Atmospheric deposition of polycyclic aromatic hydrocarbons to Atlantic Canada: Geographic and temporal trends 1980-2001. Environ. Sci. Technol. 38, 1941-1948.

Carlson, E.A., Li, Y., and Zeilkoff, J.T., 2002a. Exposure of Japanese medaka (*Oryzias latipes*) to benzo[a]pyrene suppresses immune function and host resistance against bacterial challenge. Aquat. Toxicol. 56, 289-301.

Carlson, E.A., Li, Y., and Zeilkoff, J.T., 2002b. The Japanese medaka (*Oryzias latipes*) model: applicability for investigating the immunosuppressive effects of the aquatic pollutant benzo[a]pyrene (BaP). Mar. Environ. Res. 54, 565-568.

Carlson, E.A., Li, Y., Zelikoff, J.T., 2004. Suppressive effects of benzo[a]pyrene upon fish immune function: evolutionarily conserved cellular mechanisms of immunotoxicity. Mar. Environ. Res. 58, 731–734.

Dorrington, T., Zanette, J., Zacchi, F.L., Stegeman, J.J., Bainy, A.C.D., 2012. Basal and 3-MC induced expression of cytochrome P450 1A, 1B and 1C genes in the Brazilian guppy, *Poecilia vivipara*. Aquat. Toxicol. 124-125, 106-113.

Galarneau, E., Hollebone, B.P., Yang, Z., Schuster, J., 2014. Preliminary measurement-based estimates of PAH emissions from oil sands tailings ponds. Atmos Environ. 97, 332-335.

Gao, K., Brandt, I., Goldstone, J.V., Jönsson, M.E., 2011. Cytochrome P450 1A, 1B, and 1C mRNA induction patterns in three-spined stickleback exposed to a transient and a persistent inducer. Comp. Biochem. Phys. 154, 42-55.

Gelboine, H.V., 1980. Benzo[a]pyrene metabolism, activation, and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes.

Goldstone, J.V., Stegeman, J.J., 2008. Gene structure of the novel cytochrome P4501D genes in stickleback and medaka. Mar. Environ. Res. 66, 19-20. Physiol. Rev. 4, 1107-1154.

Hahn, M.E., Karchner, S.I., 2012. Structural and functional diversification of AhRs during metazoan evolution. The AH receptor in biology and toxicology. First ed., Wiley and Sons Inc. 389-403.

Hansen, J.D., Landis, E.D., Phillips, R.B., 2005. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. Proc. Natl. Acad. Sci. 102, 6919-6924.

Hart, L.J., Smith, S.A., Smith, B.J., Robertson, J., Besteman, E.G., Holladay, S.D., 1998. Aquat. Toxicol. 41, 17-29.

Hill, D.J., Rowley, A.F., 1996. The thromboxane mimetic, U-46619, induces the aggregation of fish thrombocytes. Brit. J. Haematol. 92, 200-211.

Hutchinson, T.H., Field, M.D., Manning, M.J., 2003. Evaluation of nonspecific immune functions in dab, *Limanda limanda* L., following shortterm exposure to sediments contaminated with polyaromatic hydrocarbons and/or polychlorinated biphenyls. Mar. Environ. Res. 55, 193–202.

Holladay, S.D., Smith, S.A., Besteman, E.G., Deyab, A.S.M.I., Gogal, R.M., Hrubec, T., Robertson, J.L., Ahmed, S.A., 1998. Vet. Immunol. Immunop. 64, 69-82.

Jönsson, M.E., Gao, K., Olsson, J.A., Goldstone, J.V., and Brandt, I., 2010. Induction patterns of new CYP1 genes in environmentally exposed rainbow trout. Aquat. Toxicol. 98, 311-321.

Kelly, E.N., Short, J.W., Schindler, D.W., Hodson, P.V., Ma, M., Kwan, A.K., Fortin, B., 2009. Oil sands development contributes polycyclic aromatic compounds to the Athabasca River and its Tributaries. Proc. Natl. Acad. Sci. 106, 22346-22351.

Kelly-Reay, K., Weeks-Perkins, B.A., 1994. Determination of the macrophage chemiluminescent response in Fundulus heteroclitus as a function of pollution stress. Fish Shellfish Immunol. 4, 95–105.

Köllner, B., Fischer, U., Rombout, J.H.W.M., Taverne-Thiele, Hansen, J.D., 2004. Potentil involvement of rainbow trout thrombocytes in immune functions: a study using a panel of monoclonal antibodies and RT-PCR. Dev. Comp. Immunol. 28, 1049-1062.

Leclair, L.A., MacDonald, G.Z., Phalen, L.J., Kollner, B., Hogan, N.S., van den Heuvel, M.R., 2013. The immunological effects of oil sands surface waters and naphthenic acids on rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 142-413, 185-194.

Leppanen, H., Oikari, A., 2001. Retene and resin acid concentrations in sediment profiles of a lake recovering from exposure to pulp mill effluents. J. Paleolimnol. 25, 367-374.

MacDonald, G.Z., Hogan, N.S., Köllner, B., Thorpe K.L., Phalen, L.J., Wagner, B.D., and van den Heuvel., M.R., 2012. Immunotoxic effects of oil sands-derived napthenic acids to rainbow trout. Aquat. Toxicol.126, 95-103.

Möller, A.M., Hermsen, C., Floehr, T., Lamoree, M.H., Segner, S., 2013. Tissue specific metabolism of BaP in rainbow trout (*Oncorhynchus mykiss*): a comparison between the liver and immune organs. Drug Metab. Dispos. 42, 111-118.

Möller, A.M., Kortyar, T., Köllner, B., Schmidt-Posthaus, H., Segner, H., 2014. The teleostean liver as an immunological organ: Intrahepatic immune cells (IHIC) in healthy and BaP challenged rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol. 46, 518-529.

Nakayama, A., Riesen, I., Köllner, B., Eppler, E., and Segner, H., 2008. Surface marker defined head kindey granulocytes and B lymphocytes of rainbow trout express benzo[a]pyrene-inducible cytochrome P4501A protein. Toxicol. Sci. 103, 86-96.

Press, C.M., Evenson, O., 1999. The morphology of the immune system in teleost fishes. Fish Shellfish Immun. 9, 309-318.

Rauta, P.R., Nayak, B., Das., 2012. Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms. Immunol. Lett. 148, 23-33.

Reynaud, S., Duchiron, C., Deschaux, P., 2003. 3-methlchloranthrene inhibits lymphocyte proliferation and increases intracellular calcium levels in common carp (*Cyprinus carpio* L.). Aquat. Toxicol. 63, 319-331.

Reynaud, S., Duchiron, and C., Deschaux, P., 2004. 3-methylcholanthrene induces lymphocyte and phagocyte apoptosis in common carp (*Cyprinus carpio* L.). Aquat. Toxicol. 66, 307-318.

Reynaud, S., and Deschaux, P. 2005. The effects of 3-methylcholanthrene on lymphocyte proliferation in the common carp (*Cyprinus caropio L.*). Toxicol, 211, 156-164.

Reynaud, S., and Deschaux, P., 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: A review. Aquat. Toxicol.77, 229-238.
Reynaud, S., Raveton, M., and Ravanel, P., 2008. Interatctions between immune and biotransformation systems in fish: A review. Aquat. Toxicol. 87, 139-145.
Rice, C.D., Schlenk, D., 1995. Immune function and cytochrome P4501A activity after acute exposure to 3,3_,4,4_,5-pentachlorobiphenyl (PCB 126) in channel cat fish. J. Aquat. Animal Health. 7,195–204.
Schindler, D.W., 2014. Unravelling the complexity of pollution by the oil sands industry. Proc. Natl. Acad. Sci. 111, 3209-3210.
Secombes, C.J., Tahir, A., Stagg, R., 1997. Immunocompetence in flatfish as a measure of the biological effects of exposure to sewage sludge or hydrocarbon contaminated sediments. Ecotoxicology: Responses, Biomarkers and Risk Assessment. SOS publications, Fair Even, NJ, 281–292.
Seeley, K.R., Week-Perkins, B.A., 1991. Altered phagocytic activity of macrophages in oyster toadfish from a highly polluted estuary. J. Aquat. Anima lHealth. 3, 224–227.
Smith, D.A., Schurig, G.G., Smith, S.A., and Holladay, S.D., 1999. The hemolytic plaque-forming cell assay in tilapia (*Oreochromis niloticus*) exposed to benzo[a]pyrene: Enhanced or depressed plaque formation depends on dosing schedule. Toxicol. Methods. 9, 57-70.
Stockinger, B., Hirota, K., Duarte, J., Veldhoen, M., 2011. External influences on the immune system via activation of the aryl hydrocarbon receptor. Semin. Immunol. 23, 99-105.
Stosik, M., Detula, W., Travnicek, M., Baldy-Chudzik, K., 2002. Phagocytic and bactericidal activity of blood thrombocytes in carps (*Cyprinus carpio*). Vet. Med. Czech. 47, 21-25.
Tahir, A., Secombes, C.J., 1995. The effects of diesel oil-based drilling mud extracts on immune responses of rainbow trout. Arch. Environ. Contam. Toxicol. 29, 27–32.
Uribe, C., Folch, H., Enriquez, R., Moran, G., 2011. Innate and adaptive immunity in teleost: a review. Vet. Med-Czech. 10, 486-503.
Weeks, B.A., Warinner, J.E., 1984. Functional evaluation of macrophages in fish from a polluted estuary. Vet. Immunol. Immunopathol. 12 (1–4), 313–320.
Weeks, B.A., Warinner, J.E., Mason, P.L., McGinnis, D.M., 1986. Influence of the toxic chemicals on the chemotactic response of fish macrophages. J.FishBiol.28,653–658.
Zhou, H., Wu, H., Liao, C., Diao, X., Zhen, J., Chen, L., Xue, Q., 2010. Toxicology mechanism of the persistent organic pollutants (POPs) in fish through AhR pathway. Toxicol. Mech. Methods. 20. 279-286.
Zwollo, P., Cole, S., Bromage, E., Kaattari, S., 2005. B cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. J. Immunol. 175, 6608-6616.

Chapter 2

Phalen, L.J., Köllner, B., Leclair, L.A., Hogan, N.S., van den Heuvel, M.R. 2013. **The effects of benzo[a]pyrene on leukocyte distribution and antibody response in rainbow trout (*Oncorhynchus mykiss*)**. Aquat. Toxicol. 147, 121-128.

2.1 Abstract

Polycyclic aromatic hydrocarbons (PAHs) are a group of compounds with immunotoxic and carcinogenic potential that may pose a threat to fish populations. This study aims to utilize a newly developed fish immunotoxicology model to determine the immune tissue/cell population level effects of PAHs on rainbow trout, using benzo[a]pyrene (BaP) as a representative immunotoxic PAH. Intraperitoneal injection of 25 or 100 mg/kg BaP resulted in sustained exposure as indicated by biliary fluorescence at BaP wavelengths for up to 42 d. A new flow cytometry method for absolute counts of differential leukocyte distributions in spleen, blood, and head kidney was developed by combining absolute quantitative counts of total leukocytes in the tissue (3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) dye) with relative differential counts using monoclonal antibodies for B cells, T cells, myeloid cells, and thrombocytes. Experiments indicated dose- and time-dependent decreases in the absolute number of B cells, myeloid cells, or T cells in blood, spleen, or head kidney after 7, 14 or 21 d of exposure. There was no change in the absolute numbers of erythrocytes or thrombocytes in any tissue. When rainbow trout were exposed to inactivated *Aeromonas salmonicida* (*A.s.*) after a 21

d exposure to 100 mg/kg BaP, circulating antibody concentrations were decreased by 56 %. It was concluded that BaP has a cell lineage-specific toxic effect on some immune cells of rainbow trout, and causes a decrease in circulating antibody levels.

2.2. Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent one of the most ubiquitous groups of toxicants in the environment; they are formed through incomplete combustion of organic materials, namely from petrochemical sources. It has been known for nearly a century that PAHs are carcinogenic (Waldron, 1983) and more recent evidence suggests some of these compounds can be immunomodulators in fishes (reviewed by Reynaud and Deschaux, 2006, 2008). Understanding the mechanism of these immune effects is vital in being able to predict and mitigate potential population level effects in the wild.

Exposure to PAHs has demonstrated a number of effects on the immune function of fishes in laboratory studies. Decreased lymphocyte proliferation was observed in Japanese medaka (*Oryzias latipes*) dosed intraperitoneally (i.p.) with benzo[a]pyrene (BaP) (Carlson *et al.*, 2002) as well as in bluegill sunfish (*Lepomis macrochirus*) following dietary exposure to three different PAHs (Connelly and Means, 2010). Altered *in vitro* proliferation of leukocytes from common carp was observed after i.p. injection of 3-methylcholanthrene (3-MC); the nature of this effect depended on presence or absence of a mitogen (Reynaud and Deschaux, 2005). A number of studies with fishes have shown that exposure to PAHs affects phagocyte function demonstrated by decreased oxidative burst or lysozyme activity (reviewed by Reynaud and Deschaux, 2006). Rainbow trout exposed *in situ* to oil sands contaminated water were observed to have decreased leukocyte counts in blood, and decreased circulating antibody in response to a

pathogen associated molecular pattern; PAHs were concluded to be a potential causative agent for this effect (McNeill *et al.*, 2012). More detailed studies are needed to understand the mechanism of effect on specific immune cell types in fishes.

The study of immune toxicity at the cellular level in fishes has been limited by inavailability of antibodies specific for cell surface markers, a key tool in mammalian toxicant models. It is now feasible to characterize the majority of leukocyte populations in rainbow trout using monoclonal antibodies (mAbs) (MacDonald *et al.*, 2012). Recently, a model involving rainbow trout (*Oncorhynchus mykiss*) and immune challenge with formalin inactivated *Aeromonas salmonicida* (i*A.s.*) has been developed (Hoeger *et al.*, 2004a; 2004b; 2005; Hogan *et al.*, 2010; McNeill *et al.*, 2012). We have designed a staggered toxicant exposure-immune challenge design, reducing biosafety and animal welfare concerns associated with use of live pathogens. Combined with mAbs, this allows for examination of how pre-exposure to a toxicant affects the immune system at the cellular distribution level, *in vivo*, in multiple tissues. With an interest in PAHs, we have decided to use BaP as a model compound as it is accepted as being among the most biologically potent of the PAHs, and is used in much of the supporting literature on this topic.

There are few *in vivo* studies investigating the effects of BaP on the rainbow trout immune system. With this study, we aim to add information at a different biological level of organization than many studies in this field have provided. The focus of this study is to elucidate the time course of effects of i.p. injected BaP on the immune system of the model fish species rainbow trout (*Oncorhynchus mykiss*). Our hypothesis is that BaP will selectively reduce the number of certain leukocyte populations in a tissue-specific

manner. Secondly, it is hypothesized that this toxicity will lead to a reduction in antibody production because adaptive immune response requires the coordination of a number of leukocyte types. Finally, we seek to further develop the rainbow trout immune toxicity model for use in environmental toxicology studies. In the present study, we use tissue specific absolute leukocyte counts obtained by flow cytometry to determine what dose and duration of i.p. injected BaP could cause a detectable change in B cell, T-cell, myeloid cell, and thrombocyte populations from blood, spleen and head kidney. Subsequently, antibody production in response to an immune challenge, i*A.s.*, was evaluated after a pre-exposure to the most effective dose and duration of BaP exposure.

2.3 Materials and Methods

2.3.1 Experimental design

Included in this study are three distinct experiments. In Experiment 1 rainbow trout were exposed to one of 3 doses of BaP (Sigma Aldrich, Oakville, On, Canada; 0, 25, 100 mg/kg) and lethal sampling was conducted at three time-points (7, 14, and 21 d); the goal of this study was to determine a dose and duration of BaP exposure that would cause a detectable change in the immune cell profile of blood, spleen, and head kidney. Experiment 2 exposed rainbow trout to one of 6 concentrations of i*A.s.*; the goal of this was to determine the dose needed to induce a detectable antibody response using this strain of i*A.s.* In experiment 3 rainbow trout were exposed to 3 doses of BaP for 21 d followed by exposure to i*A.s.* for 21 d; the goal of this experiment was to determine the effect that these doses of BaP had on the ability of the fish to produce antibodies in response to the i*A.s.* challenge.

Rainbow trout were acquired from Ocean Trout Farms, Brookvale, Prince Edward Island, Canada. For all experiments, fish were allowed to acclimate in experimental tanks for 1 week prior to exposure. Fish were fed commercial trout food at 0.75% of their wet body weight daily, except for fasting one day prior to sampling. Upon injections or sampling, fish were anaesthetized using 0.1 g/L tricaine methanesulfonate (MS-222) (Argent, WA, USA). Tanks used in this study were between 240 and 312 L and had a flow through system that allowed 99% replacement of water within 3-7 h. The ranges of water quality parameters over acclimation and exposure periods were as follows: dissolved oxygen > 90%, pH 7.80-8.10, temperature11.3-11.7°C, conductivity 912-960 $\mu S/cm^3$.

2.3.2 Experiment 1 – BaP effect – dose and duration investigation

The effect of two intraperitoneally (i.p.) injected doses of BaP on the un-stimulated rainbow trout immune system was investigated at three times points: 7, 14, and 21 d. Doses were chosen based on preliminary experiments (unpublished data) that indicated 25 and 100 mg/kg BaP would cause a change that was detectable within this time frame using these methods. To begin this experiment, rainbow trout were removed from their holding tanks and randomly placed into 6 tanks; each tank received 24 fish. A total of 6 sampling d were included in this experiment: 3 time-points each repeated in two replicate trials. Each sampling day had fish that were housed in separate tanks in order to avoid sampling stress. Trials were staggered by 1 day, so trial 1 injections and sampling occurred the day before trial 2 for the same time-point. Upon exposure, fish were anaesthetized and injected i.p. with the one of two doses of BaP in 100 µL of corn-oil carrier, or a control dose using a 23 gauge, 1 mL syringe (BD, NJ, USA). Therefore, each

tank contained 3 groups (8 fish per group): control (corn-oil), low dose (25 mg/kg BaP), and high dose (100 mg/kg BaP) with separate groups identified by the use of visual implant elastomer (VIE) tags of various colours injected under the transparent skin posterior to the eye (Northwest Marine Technologies, WA, USA). The concentrations of BaP necessary to achieve these doses were based off an average wet fish weight ± SEM of 32 ± 0.6 g. Complete cell and isolated leukocyte suspensions were analyzed from blood, spleen and head kidney, and bile metabolite analysis was performed to evaluate exposure.

2.3.3 Experiment 2 – Optimization of iA.s. exposure concentration

To test these BaP concentrations on animals with an activated immune system, the concentration of i*A.s.* necessary to induce detectable antibody production in rainbow trout was first investigated. The i*A.s.* was administered by i.p. injection with 100 µL of phosphate buffered saline (PBS) carrier and 6 groups of 10 fish were exposed to one of 6 doses using a 300 µL insulin syringe (BD). The treatment groups were differentiated with VIE tags. Doses were as follows: PBS control, 1×10^6, 1×10^7, 1×10^8, 1×10^9, and 1×10^{10} cells i*A.s.*/ kg wet fish weight. The average wet fish weight of 17.2 g was used to calculate the dilution needed for each dose. Fish were held in a flow-through tank for 21 days before sampling. Complete cell and isolated leukocyte suspensions were analyzed from blood and spleen, and antibody induction was measured using an enzyme linked immunosorbent assay (ELISA).

2.3.4 Experiment 3 – BaP affected immune response to iA.s. challenge

The ability of BaP to modulate the rainbow trout immune response to i*A.s.* was investigated. To begin this experiment, 60 fish were moved from their holding tank into

one of two experimental tanks (30 fish per tank), each tank representing a separate replicate trial. This experiment involved a 21 d exposure of rainbow trout to one of two doses of BaP or a control injection of corn oil followed by a subsequent 21 d exposure to i*A.s.* (a total of 42 d before sampling). Exposure of rainbow trout to BaP was carried out in similar manner as described for Experiment 1. Differences included an increased average fish weight (86.1 ± 1.6 g), a delivery volume of 200 µL of corn-oil carrier to account for increased fish weight, and 10 fish per sampling group per trial. All fish were injected i.p. with 1×10^8 particles/kg of i*A.s* using 100 µL of PBS carrier. Complete cell and isolated leukocyte suspensions were analyzed from 8 of 10 fish/group in trial 1 and from 10 of 10 fish/group for trial 2. Enzyme-linked immunosorbent assay (ELISA) and bile metabolites analysis were performed for all fish for both trials.

2.3.5 Sampling

Upon sampling, rainbow trout were anaesthetized before being bled via caudal puncture. For experiments 2 and 3, a blood sample (50-100 µL) using a non-heparinized 21 gauge syringe (BD) was taken first and placed in a non-heparinized microtainer SST (BD). These microtainers contain silica and gel, initiating clotting and allowing for serum separation. Subsequently, 300-500 µL of blood was taken via the same puncture using a 1 mL, 21 gauge heparinized syringe (BD) and placed immediately into a heparinized vacutainer (BD). A 5 µL subsample of the heparinized blood for complete cell analysis was then placed into 1 mL of PBS/ ethylenediaminetetraacetic acid (EDTA) (5 mM, pH 7.4), while the rest was diluted into 3 mL of L15 media (Invitrogen, ON, Canada). The fish were killed by a blow to the head before further sampling, as per Canadian Council on Animal Care guidelines. Fork length and wet weight of each fish

was recorded and the spleen, liver, gall bladder, and head kidney were removed and weighed. Spleen was placed in 3 mL of L15 media, head kidney in 3 mL of PBS/EDTA. Liver and bile were placed into liquid nitrogen and stored at -80°C pending analysis. In order to prepare a cell suspension, spleen and head kidney were homogenized with 5 passes of a Ten Broeck glass mortar and pestle tissue grinder (Millville, New Jersey, USA) and filtered using 100 µm Nitex mesh.

2.3.6 Fluorescence assisted cell sorter (FACS) analysis of complete cell suspensions

Sub-samples from each cell suspension were taken in order to allow for absolute counting of total leukocytes in cell suspensions. A volume of 5 µL of blood (during sampling) and 50 µL of head kidney and spleen suspensions (after homogenization) were diluted into 1 mL PBS/EDTA. The cell suspensions were then prepared for flow cytometry according to the 3,3-dihexyloxacarbocyanine ($DiOC_6$) staining method of Inoue *et al.* (2002) whereby 5 µL of $DiOC_6$ was added to the cell suspension and allowed to stain for 15 min. After staining, 250 µL of the cell suspension was placed into a 20 mL plastic tube (BD) containing 20 µL of CountBrite© counting beads containing 20,000 multicolour beads (Invitrogen). The cell suspensions were analyzed by flow cytometry, using fluorescent assisted cell sorter (FACS)Calibur© (BD) flow cytometer equipped with 488 and 635 nm lasers. The flow cytometer was set to count all events up until the point that 1000 CountBrite beads were detected as determined by a gate set in channel 4 (FL4, far red). Erythrocytes and leukocytes were counted in channel 1 (FL1, green) using a plot of side scatter versus FL1. Absolute cell counts were calculated based on the concentration of CountBrite beads.

2.3.7 FACS analysis of isolated leukocyte suspensions

To analyze the composition of immune cell populations, leukocytes were isolated from cell suspensions with the use of a density gradient (Secombes, 1990). Head kidney, spleen, and blood cell suspensions were placed onto Lympholyte H (1.0770 g/cm3) (Cedarlane, ON, Canada) and centrifuged at 4°C for 40 minutes at 400 × g. The leukocytes were then washed in 5 mL of PBS/EDTA, centrifuged at 4°C for 8 min at 800 × g, and re-suspended in 0.5-1.0 mL of L15 media.

Before staining with mAbs, the cells in the suspension were quantified using a hemocytometer and the volume that contained 4×10^5 leukocytes was calculated. This volume was placed into four wells of a 96-well round bottom plate (Griener, Maybachstrasse, Germany) and subsequently one of two mAb mixtures in 100µL of L15 media, or just media (unstained) was added to the wells. There were four primary mAbs used in this experiment: mAb 1.14 is specific against IgM^+ B cells, mAb D30 against T cells, mAb 42 against thrombocytes, and mAb 21 against cells of myeloid lineage. Note that henceforth, cell types identified by these mAbs will be referred to simply as B cells, T cells, thrombocytes, and myeloid cells, respectively. Further detail on each of these mAbs can be found in Table 2.1 and were described in MacDonald *et al.* (2012). MAbs 1.14 and 42 are both primarily labeled with fluorescent labels (BD) and they were combined in the same well during staining. MAbs D30 and 21 are not primarily labeled, and therefore secondary mAbs X56 and X57 (BD; Table 2.1) were used to label them; they were also combined during staining.

Cell suspensions were incubated for 1h in the dark at 4°C and pelleted by centrifugation (250 × g, 4 min, 4°C). MAb mixture D30 and 21 then had their secondary,

fluorescently mAb added and were incubated for an additional 1 h. After staining, all cell suspensions were washed and re-suspended in 400 µL of PBS/EDTA. Cell suspensions were then analyzed using a FACSCalibur (BD) flow cytometer. Antibody, fluorescent label, and laser combinations were as follows: mAb D30 labeled with X57 had its fluorescent label (PE) excited at 488 nm and emission was read at 585 nm; mAb 21 labeled with X56 had its fluorescent label (APC) excited at 635 nm and emission was read at 661 nm; mAb1.14 had its fluorescent label (APC) excited at 635 nm and emission was read 661 nm; mAb 42 had its fluorescent label (ATTO488) excited at 488 nm and emission was read 530 nm. The FACSCalibur was set to read 5000 counts, and appropriate gates were applied to count desired cells.

2.3.8 ELISA – blood serum antibody analysis

To assess the amount of antibody specific to the i*A.s.* antigen in blood serum, an ELISA was performed according to methods adapted from Köllner and Kotterba (2002). After sampling, blood samples in microtainers were spun at 13500 × g for 25 min and blood serum removed and stored at -80◦C until further analysis. In preparation for analysis, plates were coated with antigen solution of concentration 6.4×10^7 particles/mL i*A.s.* (in PBS) at 4°C overnight. Plates were then blocked using protein free blocking reagent (Thermo Scientific, IL,USA), incubated for 1h at 25°C, and washed with phosphate buffered saline (pH 7.4) containing 0.05% tween 20 (Invitrogen). The samples were then thawed and serially diluted in a 1:1 ratio to produce an 8 point dilution into the coated wells, incubated for 1h at 4°C and washed twice. To detect bound i*A.s.*, a monoclonal mouse-anti trout IgM antibody (mAb 4C10) was applied and allowed to incubate for 1h at 4°C, washed, and a goat anti-mouse-IgG-horse radish peroxidase

conjugated antibody (1:1250 dilution; BioLegend, CA, USA) was applied and incubated for 1h at 4°C. After washing twice, a substrate solution was applied (Sigma) and incubated for 30 min at 25°C. The substrate-horse radish peroxidase reaction was stopped by the addition of 100 µL/well of 3N hydrochloric acid (HCl), and plates were read using an EL_X800UV microplate reader (Bio-Tek instruments, Vermont, USA) at 490 nm.

Table 2.1: Mouse anti-trout antibodies used to label immune cell populations, for analysis by flow cytometry.

	B cell	T cell	Myeloid cell	Thrombocyte
Primary Antibody	Mouse IgG	Mouse IgG1 - D30	Mouse IgG2 - 21	Mouse IgG1 - 42-1
Primary Antibody Label	ATTO488	--	--	ATTO488
Primary Antibody Target Epitope	IgM heavy chain	Possible CD2 or CD3	Possible CD11	Possible CD41
Concentration of Primary Antibody (ng/4 × 10^5 cells)	40	20	20	40
Primary Antibody Reference	DeLucia *et al.*, 1983	Unpublished (Köllner)	Unpublished (Köllner)	Köllner *et al.*, 2004
Secondary Antibody	--	X56	X57	--
Secondary Antibody Label	--	APC	PE	--

2.3.9 High performance liquid chromatography (HPLC) analysis of bile metabolites

Bile samples were diluted by a factor of either 200 or 2000 in high performance liquid chromatography (HPLC) grade water (Fischer Scientific, ON, Canada), filtered with 13 mm polypropylene syringe filters (0.45 µm pore size; Pall, Ville St. Laurent, Canada), and placed into glass auto-sampler vials. A Varian Prostar model 240 HPLC pump, a model 410 auto-sampler, and a model 363 fluorescence detector was used to perform the bile metabolite analysis. A 150 mm×4.6 mm Varian Microsob-MV C18 column was employed to separate bile metabolites at a flow rate of 1 mL/min at 35 ºC. Solvent elution profile was from 5% acetonitrile (Caledon Laboratories, Georgetown, Canada) and 95% HPLC grade water (Caledon) to 98% acetonitrile and 2% water gradually over 25 min, and was held for 5 min. Excitation and emission wavelengths used were that of BaP (380 nm and 430 nm, respectively). The BaP equivalent concentration was derived by summing the area of all peaks that eluted during 3-15 min of the run and dividing by the slope of a BaP standard curve. It should be noted that peaks indicative of BaP parent compound were not observed in the time frame or doses used in any experiments.

2.3.10 Statistical analysis

The ELISA data was expressed as the negative logarithm of the antibody titre. The titre value was determined by the lowest dilution of serum at which antibody could be detected. The threshold for detection was defined as the next highest dilution that had an absorbance greater than twice the value of the most dilute in the dilution series (all antibody titres were diluted to background levels). Those data points that didn't have detectable antibody within the range of dilutions tested were expressed as twice the

lowest dilution factor (0.2). Cell counts from analysis of both complete and isolated leukocyte suspensions were transformed to absolute cell numbers (cells/mL or cell/g). Complete cell suspension numbers were based on CountBrite bead concentration, while isolated leukocyte suspension percentages were multiplied by the total leukocyte counts for that tissue.

Levene's and Brown Forsythe tests were used to determine homogeneity of variance of data, while normality was tested using visually normal probability plots. Data that did not conform to the appropriate parametric assumptions based on these tests were log transformed prior to statistical analysis. Extreme outliers (greater or less than the upper or lower quartile plus or minus 3 times the difference between the upper and lower quartile, respectively) were removed from all leukocyte data. All data (except experiment 2) were analyzed using full factorial 2-way analysis of variance tests (ANOVAs) with dose and trial as the independent variables. For BaP and i*A.s.* doses, significant differences from control were tested *a posteriori* using Dunnett's test. Body weight, liver weight and spleen weight were analyzed using analysis of covariance (ANCOVA) with logarithmically transformed values using length (weight) or body weight (liver, spleen), plus the categorical treatment variables. Statistical differences from the control were determined using Dunnett's test. Somatic data were expressed as indices, liver somatic index (LSI), condition factor, or spleen somatic index (SSI) for presentation purposes using the least square means and covariate means from the ANCOVA. All statistics were performed in STATISTICA v. 8.0 using an experiment-wise alpha of 0.05.

2.4 Results

2.4.1 Experiment 1 - BaP effect of dose and time

No mortalities were observed in any of the BaP trials. Statistical analysis revealed a significant increase in liver size at 7, 14, and 21 d in fish exposed to high dose (100mg/kg). A significant decrease in spleen size at low (25 mg/kg) and high dose (100 mg/kg) was observed in the 21 d experiment (Table 2.2). Bile metabolites showed that a single injection of BaP in corn oil resulted in a sustained exposure to BaP for the 21 d of the experiment (Fig. 2.1). At the high dose (100 mg/kg), approximately two orders of magnitude increase over the controls was observed and levels did not appear to decrease over the 21 d. At the low dose (25 mg/kg), bile metabolites also remained elevated over the controls, but did show a decreasing trend over time (Fig. 2.1).

There were no significant differences between the numbers of erythrocytes found in control fish and exposed fish (evaluated in blood and spleen). For ease of interpretation, comparisons of total leukocyte counts are displayed as percentages of the appropriate control; all absolute cell counts can be found in supplemental data in the appendix (Table A.1). At 14 d, total blood leukocytes were 65.6 ± 6.5 and 46.2 ± 4.2 % of control values among fish exposed to low and high dose respectively. At 21 d, fish exposed to high dose had significantly lower total blood leukocytes than the controls (50.2±4.8 % of control values). At day 14 and 21, total spleen leukocytes in the fish exposed to high dose were significantly reduced to 58.3±6.1 and 43.2±6.2 % of control values, respectively. Total spleen leukocytes on days 14 and 21 among fish exposed to low dose were 67.5±9.0 and 41.4±0.1 %, respectively. Among high dose they were 58.8±3.3, 67.5±9.0, and 41.4±0.1 % on days 7, 14, and 21 respectively.

Table 2.2: Significant differences in condition factor (CF), liver somatic index (LSI), and spleen somatic index (SSI) of rainbow trout exposed to one of two doses of BaP analyzed at one of three days post exposure (D. P.E.) and then compared to control. Asterisks (*) indicate significant difference from the corresponding control.

	D. P.E.	Dose (mg/kg)		
		0	25	100
CF	7	1.04 ± 0.01	1.03 ± 0.01	1.05 ± 0.01
	14	1.06 ± 0.01	1.08 ± 0.02*	1.12 ±.02*
	21	1.10 ± 0.01	1.11 ± 0.01	1.12 ± 0.01
	42	1.12 ± 0.02	1.09 ± 0.02	1.13 ± 0.02
LSI	7	1.06 ± 0.03	1.16 ± 0.03	1.23 ±0.03*
	14	1.38 ± 0.07	1.46 ± 0.08	1.77 ±0.09*
	21	1.21 ± 0.06	1.40 ± 0.08	1.67 ± 0.09*
	42	1.32 ± 0.06	1.17 ± 0.05	1.13 ± 0.07
SSI	7	0.15 ± 0.01	0.14 ± 0.01	0.13 ± 0.01
	14	0.17 ± 0.03	0.15 ± 0.02	0.14 ± 0.02
	21	0.21 ± 0.03	0.14 ± 0.02*	0.13 ± 0.02*
	42	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01

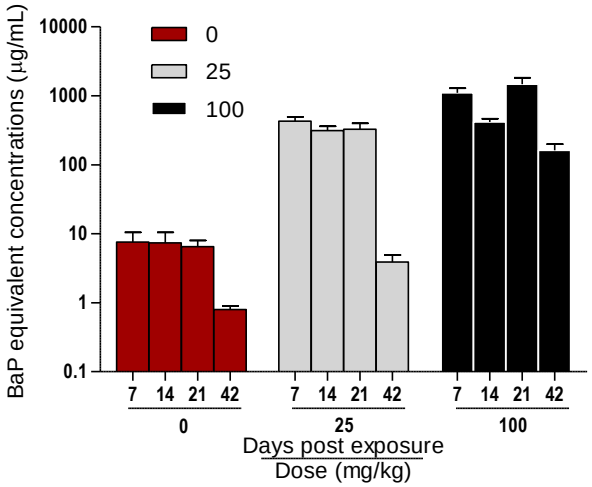

Fig. 2.1: Benzo[a]pyrene fluorescent metabolites in the bile of rainbow trout exposed intraperitoneally to benzo[a]pyrene as determined by high performance liquid chromatography. Error bars indicate the standard error of the mean. For 7, 14, 21 d N = 13-16 except control group 14 d where N = 8. 42 d N = 18-20.

Isolated leukocyte counts revealed cell type-specific depletion in leukocytes in the three organs examined (Fig. 2.2). Data are expressed as percentage of the appropriate control; absolute cell counts can be found in supplemental data in the appendix (Table A.1). B cells are highly prevalent in all three organs examined (approximately 50%, 30%, and 20% of blood, spleen, and head kidney leukocytes, respectively) and were among the most impacted of the cell types. The highest dose of BaP caused significant reductions in B cells in all organs, at all time periods, with the exception of spleen at 7 d. Thus, there appeared to be a delay in the response in spleen as compared to the other organs and the most severe depletion of B cells was observed in the spleen at 21 d with an approximately 80% reduction compared to control values. Results for B cell counts with the 25 mg/kg dose were inconsistent, with the exception of a significant 60% reduction of B cells in the head kidney at 21 d.

Myeloid cells represent approximately 30% of head kidney leukocytes and around 5% of blood and spleen leukocytes. The myeloid cell populations in head kidney appeared to respond rapidly with significant differences at both doses for all time periods. There were no significant patterns of response in myeloid cells in blood; however, myeloid cell numbers were reduced in spleen at both BaP doses at 21 d.

In contrast to B cells and myeloid cells, T cells and thrombocytes did not show clear or consistent patterns of response to BaP. T cells were highest in spleen with ~10-30% of the leukocyte distribution, and ~5-15% in head kidney and blood. There were a number of significant reductions in T cell counts, particularly in head kidney at 7 d at both doses, though these trends were not significant in that organ at 14 and 21 d. At the high dose of BaP, the blood did appear to manifest reductions in T cells at 14 and 21 d. Thrombocytes represent approximately 20% of the leukocytes in blood and spleen, while they are a very minor (~3%) component of head kidney. There were no significant changes in thrombocyte absolute counts in any organ, at any time point within the experiment.

2.4.2 Experiment 2 - Optimization of iA.s. exposure concentration

The highest dose of i*A.s.* (10^{10} particles/kg) caused mortality in 7 of 10 fish within 24 h of injection. The three highest doses (10^{10}, 10^{9}, and 10^{8} particles/kg) showed an elevated blood serum antibody titre as compared to the control (Fig. 2.3). No significant differences between i*A.s.* doses were found in absolute leukocyte counts (data not shown). Based on this experiment, a dose of 10^{8} was chosen for experiment 3.

2.4.3 Experiment 3 - BaP affected immune response to iA.s. challenge

No mortalities occurred during this experiment and there were no significant differences between body or organ indices of control and treated fish (Table 2.2). By 42 d post BaP injections, there were decreases in the BaP bile metabolites as compared to the 21 d values measured in Experiment 1 (Fig. 2.1). At 42 d, the difference in bile metabolites between 25 and 100 mg/kg doses were more apparent than in the time points examined in the previous experiment as the low dose BaP bile metabolites were more than an order of magnitude lower than the high dose. There were dose-dependent decreases in the blood serum antibody in those fish that were pre-exposed to BaP, however, the decrease of 56% at high dose was the only statistically significant finding (Fig. 2.4). Analysis of complete cell and isolated leukocyte counts showed no significant differences with BaP dose; all mean counts are tabulated in the supplemental data in the appendix (Table A.1).

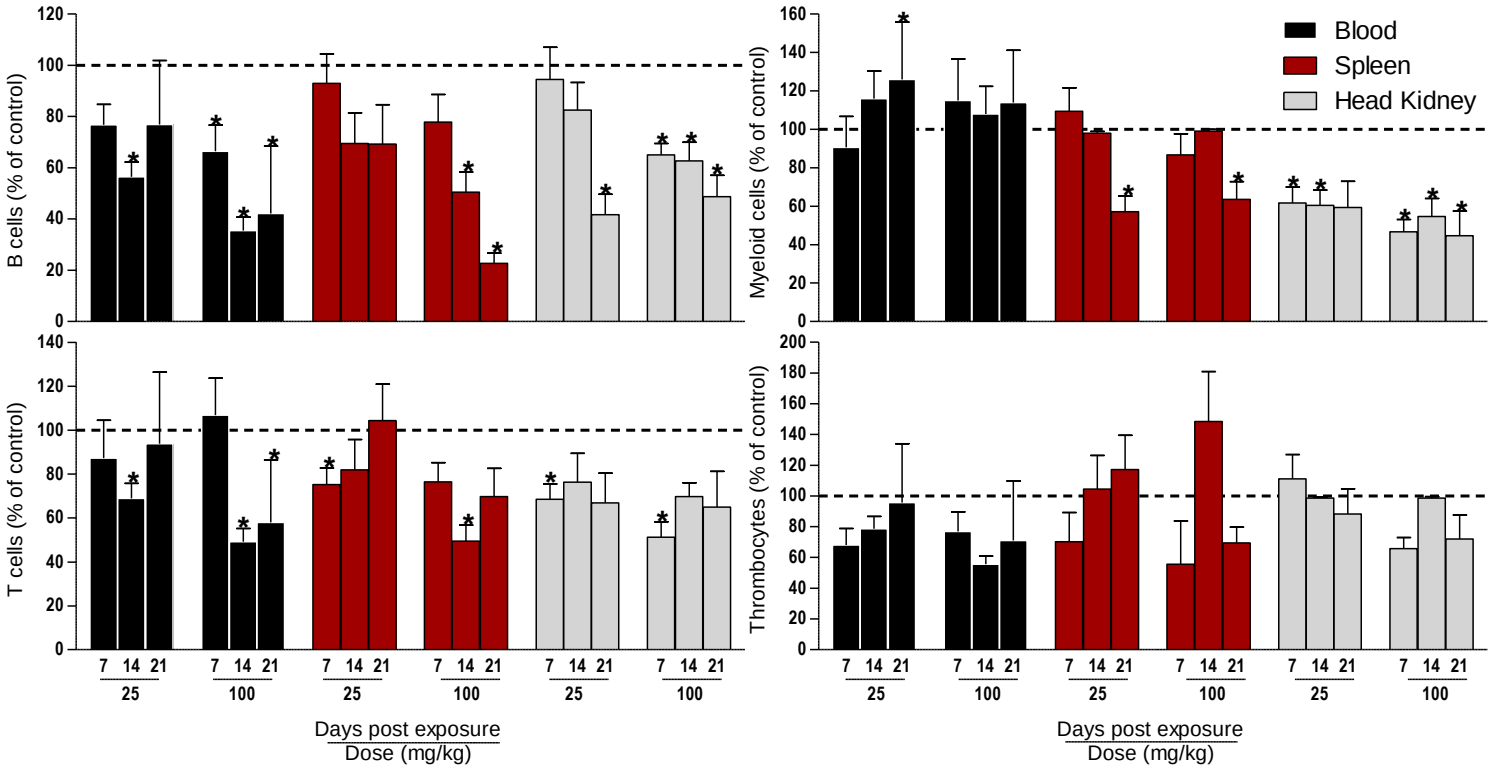

Fig. 2.2: Mean leukocyte counts expressed as percentage of trial control group in lymphatic organs of rainbow trout analyzed after intraperitoneal injection of benzo[a]pyrene. Isolated leukocytes were stained with labeled monoclonal antibodies and analyzed with a fluorescence assisted cell sorting flow cytometer. Asterisks (*) indicate significant difference as compared to control as determined by two-way ANOVAs with Dunnett's test. Error bars indicate the standard error of the mean. N= 16 for each group.

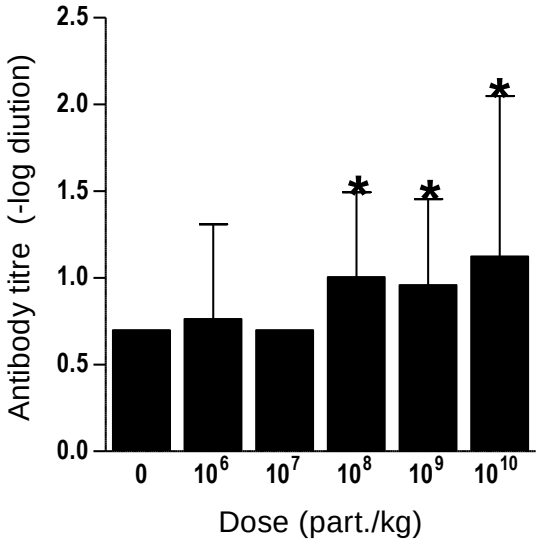

Fig. 2.3: Mean anti-*Aeromonas salmonicida* antibody titres in rainbow trout serum analyzed by enzyme-linked immunosorbent assay. Serum was taken 21 ds after an intraperitoneal injection of antigen; doses were based on average fish weight. Asterisks (*) represent significant difference from control as determined by two-way ANOVA and Dunnett's test. Error bars indicate the standard error of the mean. N = 10 for all groups except dose 10^{10} where N = 3.

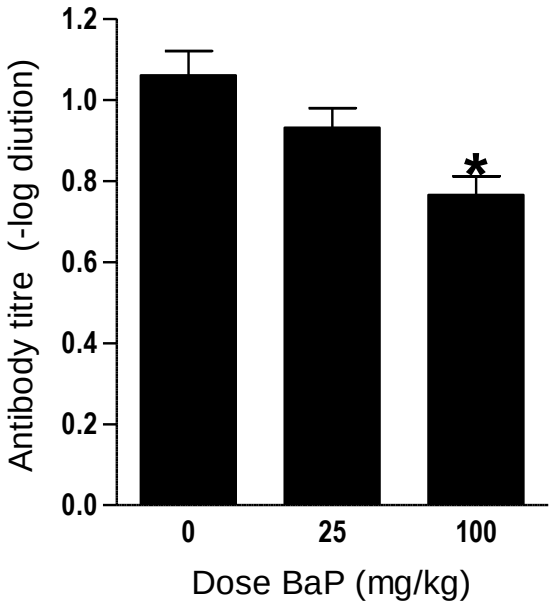

Fig. 2.4: Mean serum anti-*Aeromonas salmonicida* antibody titre in BaP exposed rainbow trout analyzed by enzyme-linked immunosorbent assay. Serum was taken 42 ds after an intraperitoneal injection of benzo[a]pyrene and 21 ds after an intraperitoneal injection of antigen. Asterisks (*) represent significant difference from control as determined by two-way ANOVA and Dunnett's test. Error bars indicate the standard error of the mean. N= 18-20.

2.5 Discussion

Bile metabolite analysis indicated that a single i.p. injection of BaP resulted in sustained excretion of BaP and its metabolites for 21 d and substantial excretion was occurring even at 42 d. Rainbow trout leukocytes including myeloid cells, B cells, and T cells were reduced in absolute numbers in a time-dependent manner in blood, spleen, and head kidney after this BaP exposure. Reductions were not seen in erythrocytes or thrombocytes, indicating that BaP has a selective effect on particular cell types. It was also found that a BaP pre-exposure of 21 d caused a decrease in the titre of circulating antibody following antigen challenge.

Single dose i.p. injection, though it is not representative of exposure in the environment, appeared to provide a relatively continuous exposure of BaP over 21 d. In the environment, aquatic animals are typically exposed to toxicants in a chronic manner through food or water. While intraperitoneal injections with other PAHs have been observed to display a rapid peak in excretion and effect (Hogan *et al.*, 2010), in the study at hand BaP exposure appeared to be sustained for at least 21 d. Effects on leukocytes were apparent by 21 d; this temporal effect was also observed in a study done by Danion *et al.* (2011) which showed decreased leukocytes due to lymphopenia in sea bass after a 21 d water borne exposure to a mixture of PAHs.

BaP-induced effects on the B cell population were observed regardless of tissue type, supporting selective toxicity towards this cell type. Control values also demonstrate prevalence of this cell type in various immune organs of the rainbow trout, suggesting importance in immune function. The effects demonstrated herein are supported by a study done by Carlson *et al.* (2002) where Japanese medaka exposed by i.p. injection to similar concentrations of BaP for 48 h showed decreased splenic lymphocyte proliferation in response to the B cell mitogen lipopolysaccharide *in vitro*; the short time frame of this effect is not reflected in our study. This finding is also supported by the study from Smith *et al.* (1999) where i.p. injection of BaP caused decreased plaque formation in tilapia 21 d after a sheep red blood cell immunization followed 7 d later by a comparable BaP injection; B cells are assumed to be responsible for antibody production and therefore partially for plaque formation. The capacity of BaP to reduce the numbers of T-dependent antigen specific antibody has also been demonstrated in mammalian models (White *et al.*, 1994). Finally, BaP induced effects specific to rainbow

trout B cells are supported by the knowledge that these cells express inducible CYP1A (Nakayama, 2008), an enzyme that may be involved in mediating PAH immune toxicity. Heidel *et al.* (2000) found that CYP1B1 enzymes were required in order to manifest immune toxicity of the PAH 7,12-dimethylbenz(a)anthracene (DMBA) in cultured mice leukocytes. The inducible expression pattern of cytochrome genes is not as well characterized in rainbow trout immune cells although recent investigations show a complex tissue specific pattern of induction by 3,3',4,4',5-pentachlorobiphenyl (PCB126) of CYP1A, CYP1B, and CYP1C (Jönsson *et al.*, 2010) in gill and liver tissue.

The current study also demonstrated a toxic effect on T cells, albeit at an earlier time point than B cells. This is the first report of BaP effects directly on T cells in fishes, as there is no mAb commercially available for T cells, and there are no cell-type-specific assays such as plaque forming assays available. The study by Carlson *et al.* (2002) supports this finding through a decrease in Concavalin A stimulated splenic lymphocyte proliferation. Davila *et al.* (1996) demonstrated that BaP, 3-MC, and 7,12-dimethylbenz(a)anthracene (DMBA) suppress human peripheral blood T cell mitogenesis and that BaP toxicity to these cells could be reversed using α-naphthaflavone, an AhR agonist. A study done by Gao *et al.* (2005) demonstrated that exposure to DMBA by oral gavage in mice resulted in a decrease in CYP1B1-dependent T cell mitogenesis in spleen cells.

Myeloid cells in the head kidney represent approximately 30% of the leukocyte population, making this a good tissue to demonstrate effects on this cell type. There does not appear to be any time-dependent effect of BaP on myeloid cells, but may show dose dependence in spleen. It is difficult to make conclusions on the relative sensitivity of this

cell population to BaP exposure because myeloid cells represent several cell types which may not all show the same profile of toxicity. No mAbs are available for specific types of rainbow trout myeloid cells but effects on cellularity and functional end points attributed to this cell type have been demonstrated. Smith *et al.* (1999) reported decreased cellularity in head kidney 14 d after an i.p. injection of BaP. Carlson *et al.* (2004) showed decreased intracellular superoxide production in head kidney cells isolated from Japanese medaka after i.p. injection of BaP. Many studies on fish species sampled from PAH contaminated waters also show decreases in phagocytosis and chemotaxis (reviewed by Reynaud and Deschaux, 2006). The BaP induced effect on myeloid cells is further supportive of a CYP1A-dependent effect, since rainbow trout granulocytes (a major sub-population within the myeloid lineage) express inducible CYP1A (Nakayama, 2008).

The decrease in circulating antibody observed in this study could be mediated by the BaP-induced toxicity on any of the three cell types affected (B cells, T cells, myeloid cells) since antibody is a product of many cell types working in concert; but ultimately, it is B cells that generate antibody and are the most affected of studied cell types. Observation of decreased antibody is corroborated by both previously discussed studies (Carlson *et al.*, 2002; Smith, 1999) through the decreases in plaque formation, and Carlson *et al.* (2002) also indicate a decreased ability to defend against a bacterial pathogen which is mainly dealt with via humoral antibodies. An *in situ* experiment performed in Alberta oil sands experimental ponds (PAH contaminated water) with rainbow trout also showed a decrease in circulating antibody in response to an i*A.s.*immune challenge (McNeill *et al.*, 2012). It is worth noting that although antibody production was impaired, circulating antibody titre was still 56-78% of control values,

suggesting that this may not be the mechanism leading to increased disease in animals exposed to that same environment (van den Heuvel *et al.*, 2000).

The reductions in leukocyte cell numbers observed in this study could be attributable to movement of cells to unstudied parts of the animal, apoptosis, inability to proliferate, or necrosis. For example, we have observed that a large number of B cells die in the peritoneum within the first few days after BaP injection (Phalen, Köllner, and van den Heuvel, unpublished data). This, however, does not provide mechanistic insight into the long term depletion of B cells from immune organs seen in this study. In a study done by Reynaud and Deschaux (2005), common carp demonstrated increased peripheral blood leukocyte proliferation *in vitro* after harvesting of cells post-3-MC i.p. injection. This result was contrasted by a decrease in proliferation of leukocytes exposed to a mitogen *in vitro*. Our *in vivo* study also shows a decrease in leukocytes when exposed to both toxicants and, in our case, pathogen associated molecular pattern (PAMP). Given that a decrease in leukocytes is observed in the BaP exposure lacking i*A.s.*, it seems possible that we are seeing a depletion *in vivo*. Additional studies examining leukocyte depletion is necessary to understand the mechanisms of BaP toxicity. Given that a period of three weeks was required to produce the most severe effects on B cells it is likely that the mechanism of toxicity is relatively complex, or involves the gradual movement of B cell to other tissues.

Several observations in the literature allow speculation on the mechanism of immune toxicity of PAHs. While apoptosis has been observed in both mammals and fishes exposed to PAHs, the precise pathways remain unknown. Human HepG2 cells have displayed apoptosis in response to exposure to BaP metabolites as measured by

caspase activity and the toxicity of one of those metabolites was shown to be mediated by the AhR (Chen *et al.*, 2003). Page *et al.* (2002, 2004) suggest an apoptotic mechanism of the PAH DMBA in pre-B cells that is TNF-α dependent, and they speculate that metabolism by CYP1B1 maximizes toxicity.

There is also evidence that PAH-induced immunosuppression is due to apoptosis initiated by the formation of DNA adducts. Curtis *et al.* (2011) demonstrated that chronic exposure of rainbow trout to 3 µg BaP/fish/d in feed increased DNA damage in blood at 14 d. Gao *et al.* (2008) suggest that the immunosuppression induced by DMBA in mice is caused by DNA damage and is p53-dependent and AhR- independent. 3-MC was found to induce apoptosis in carp lymphocyte and phagocyte primary cell cultures; this effect may have been caused by an alteration in intracellular calcium levels (Reyanud *et al.*, 2004). Overall, the mechanisms of action of BaP in fish is understudied, and existing literature is not enough to draw conclusions except that known mammalian mechanisms may be a good place to start investigating (reviewed by Reynaud and Deschaux, 2006).

In conclusion, we determined i.p. exposure to BaP causes tissue level changes via specific immune cell populations in rainbow trout, and that these changes can be detected using fluorescence assisted cell sorting methods. Additionally, the functional endpoint of antibody production in response to i*A.s.* was impaired. This study provides precedence for the use of rainbow trout as a model species for the investigation of BaP immune toxicity mechanisms. Future studies in our lab will focus on determining the cellular level mechanism of BaP toxicity, with a focus on determining the role that AhR and CYP enzymes play in the respective cell types. Beginning to elucidate these effects of PAH

representative BaP will support future functional and environmentally relevant experimentation on PAH immunotoxicity in fish species.

2.6 Literature Cited

Carlson, E.A., Li, Y., and Zeilkoff, J.T., 2002. Exposure of Japanese medaka (*Oryzias latipes*) to benzo[a]pyrene suppresses immune function and host resistance against bacterial challenge. Aquat. Toxicol. 56, 289-301.

Carlson, E.A., Li, Y., and Zeilkoff, J.T., 2004. Suppressive effects of benzo[a]pyrene upon fish immune function: Evolutionarily conserved cellular mechanisms of immunotoxicity. Mar. Environ. Res. 58, 731-734.

Chen, S., Nguyen, N., Tamura, K., Karin, M., and Tukey, R.H., 2003. The role of the Ah Receptor and p38 in Benzo[a]pyrene-7,8-dihydrodiol and Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide-induced apoptosis. The Journal of Bio. Chem. 278, 19526-19533.

Connelly, H., and Means, J.C., 2010. Immunomodulatory effects of dietary exposure to selected polycyclic aromatic hydrocarbons in the bluegill (*Lepomis macrochirus*). Int. J Toxicol. 29, 532-545.

Curits, L.R., Garzon, C.B., Arkoosh, M., Collier, T., Myers, M.S., Buzitis, J., and Hahn, M.E., 2011. Reduced cytochrome P4501A activity and recovery from oxidative stress during subchronic benzo[a]pyrene and benzo[e]pyrene treatment of rainbow trout. Toxicol. Appl. Pharmacol. 254: 1-7.

Danion, M., Le Floch, S., Kanan, R., Lamour, F., and Quentel, C., 2011. Effects of *in vivo* chronic hydrocarbons on sanitary status and immune system of sea bass (*Dicentrarchus labrax* L.) Aquat. Toxicol.105, 300-311.

Dean, J., Luster, M., Munson, A., and Amos, H., 1994. Immunotoxicity of PAHs. In Immunotoxicology and Pharmacology, second ed., Raven Press, New York, NY, pp.123-142.

Galván, N., Page, T.J., Czuprynski, C.J., and Jefocoate, C.R., 2006. Benzo[a]pyrene and 7, 12-dimethylbenz[a]anthracene differentially affect bone marrow cells of the lymphoid and myeloid lineages. Toxicol. Appl. Pharmacol. 213: 105-116.

Gao, J., Lauer, F.T., Dunwat, S., and Burchiel, S.W., 2005. Cytochrome P450 1B1 is required for 7,12-dimethylbenz(a)anthracene (DMBA) induced spleen cell immunotoxicity. Toxiocol. Sci. 86: 68-74.

Gao, J., Mitchell, L.A., Lauer F.T., and Burchiel, S.W., 2008. P53 and ATM/ATR regulate 7,12-dimethylbenz[a]anthracene-induced immunosuppression. Mol Pharmacol. 73: 137-146.

Heidel, S.M., MacWilliams, P.S., Baird, W.M., Dashwood, W.M., Larsen, M.C., Czuprynski, C.J., and Jefcoate, C.R., 2000. Cytochrome P4501B1 mediates induction of bone marrow cytotoxicity and preleukemia cells in mice treated with 7,12-dimethylbenz[a]anthracene. Cancer Res. 60, 3454-3460.

Heidel, S.M., Holston, K., Buters, J.T., Gonzalez, F.J., Jefcoate, C.R., and Czupyrynski, C.J., 1999. Bone marrow stromal cell cytochrome P4501B1 is required for pre-B cell apoptosis induced by 7,12-dimethylbenz[a]anthracene. Mol Pharmacol 56:1317–1323.

Hoeger, B., van den Heuvel, M.R., Hitzfeld, B.C., and Dietrich, D.R., 2004a. Effects of treated sewage on immune function in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol.70, 345-355.

Hoeger, B., Köllner, B., Kotterba, G., van den Heuvel, M.R., Hitzfeld, B., and Dietrich, D.R., 2004b. Influence of chronic exposure to treated sewage effluent on the distribution of white blood cell populations in rainbow trout (*Oncorhynchus mykiss*). Toxicol. Sci.82, 97-105.

Hoeger, B., Hitzfeld, B., Köllner, B., Dietrich, D.R., and van den Heuvel, M.R., 2005. Sex and low-level sampling stress modify the impacts of sewage effluent on the rainbow trout (*Oncorhynchus mykiss*) immune system. Aquat. Toxicol.73, 79-90.

Hogan, N.S., Lee, K.S., Köllner, B., and van den Heuvel, M.R., 2010. The effects of the alkyl polycyclic aromatic hydrocarbons retene rainbow trout (*Oncorhynchus mykiss*) immune response. Aquat. Toxicol.100, 246-254.

Inoue, T., Moritomo, T., Tamura, Y., Mamiya, S., Fujino, H., and Nakanishi, T., 2002. A new method for fish leukocyte counting and partial differentiation by flow cytometry. Fish Shellfish Immunol.13, 379-390.

Jönsson, M.E., Gao, K., Olsson, J.A., Goldstone, J.V., and Brandt, I., 2010. Induction patterns of new CYP1 genes in environmentally exposed rainbow trout. Aquat. Toxicol. 98, 311-321.

Köllner, B. and Kotterba, G., 2002. Temperature dependent activation of leucocyte populations of rainbow trout, *Oncorhynchus mykiss*, after intraperitoneal immunization with *Aeromonas salmonicida*. Fish Shellfish Immunol. 12, 35-48.

MacDonald, G.Z., Hogan, N.S., Köllner, B., Thorpe K.L., Phalen, L.J., Wagner, B.D., and van den Heuvel., 2012. Immunotoxic effects of oil sands-derived napthenic acids to rainbow trout. Aquat. Toxicol.126, 95-103.

McNeill, S.A., Arens, C.J., Hogan, N.S., Köllner, B., and van den Heuvel M.R., 2012. Immunological impacts of oil sands-affected waters on rainbow trout evaluated using an *in situ* exposure. Eco. Tox. and Env. Safety. 84, 254-261.

Nakayama, A., Riesen, I., Köllner, B., Eppler, E., and Segner, H., 2008. Surface marker defined head kindey granulocytes and B lymphocytes of rainbow trout express benzo[a]pyrene-inducible cytochrome P4501A protein. Toxicol. Sci. 103, 86-96.

Page, T.J., O'Brien, S., Jefcoate, C.R., and Czuprynski, C.J., 2002. 7, 12-dimethylbenz[a]anthracene induces apoptosis in murine pre-B cells through caspase-8-dependent pathway. Mol. Pharmocol. 62: 131-319.

Page, T.J, MacWIlliams, P.S., Suresh, M., Jefcoate, C.R., and Czuprynski, C.J., 2004. 7, 12-dimethylbenz[a]anthracene-induced bone marrow hypocellularity is dependent on signaling through both the TNFR and PKR. Toxicol. Appl. Pharmacol. 198: 21-28.

Reynaud, S., Duchiron, and C., Deschaux, P., 2004. 3-methylcholanthrene induces lymphocyte and phagocyte apoptosis in common carp (*Cyprinus carpio* L.). Aquat. Toxicol. 66, 307-318.

Reynaud, S., and Deschaux, P. 2005. The effects of 3-methylcholanthrene on lymphocyte proliferation in the common carp (*Cyprinus caropio L.*). Toxicol, 211, 156-164.

Reynaud, S., and Deschaux, P., 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: A review. Aquat. Toxicol.77, 229-238.

Reynaud, S., Raveton, M., and Ravanel, P., 2008. Interatctions between immune and biotransformation systems in fish: A review. Aquat. Toxicol. 87, 139-145.
Secombes, C.J., 1990. Isolation of salmonid macrophage and analysis of their killing activity. In Techniques in Fish Immunology (Van Muiswinkel, W.B.), second ed., SOS Publication, Fair Haven, NJ, pp. 101-103.
Smith, D.A., Schurig, G.G., Smith, S.A., and Holladay, S.D., 1999. The hemolytic plaque-forming cell assay in tilapia (*Oreochromis niloticus*) exposed to benzo[a]pyrene: Enhanced or depressed plaque formation depends on dosing schedule. Toxicol. Methods. 9, 57-70.
van den Heuvel, M.R., Power, M., Richards, J., MacKinnon, M., and Dixon, D.G., 2000. Disease and gill lesions in yellow perch (*Perca flavescens*) exposed to oil sands mining-associated waters. Ecotoxicol. Environ. Saf. 46, 334-341.
Waldron, H.A., 1983. A brief history of scrotal cancer. Br. J. Ind. Med.40, 390-401

Chapter 3

CYP and AhR Gene Expression in Immunomagnetically Isolated Rainbow Trout Immune Cells

3.1 Abstract

The mechanism underlying immune toxicity of polycyclic aromatic hydrocarbons (PAHs) in fishes remains unclear. Some evidence supports the requirement for metabolism to more toxic metabolites, and many studies report a decrease in cell numbers or cell proliferation following exposure. It has been previously observed that B cells are affected in several tissues, while thrombocytes remain unaffected. In this study, we examined whether intraperitoneal exposure of rainbow trout to BaP caused leukocyte mRNA expression changes in five cytochrome P450 (CYP) enzymes; to their transcription factor, AhR; or to an extrinsic pathway apoptosis checkpoint, p53. mRNA expression was analyzed in immunomagnetically isolated B cells and thrombocytes from blood, spleen, or head kidney in an effort to clarify the tissue and cell specific toxicity of BaP. There was significant inductions of CYP1A1 in liver, blood B cells, and blood thrombocytes; CYP1B1 in blood B cells, blood thrombocytes; CYP1A3 in liver, blood and spleen B cells; and AhR was induced in spleen thrombocytes. No statistically significant changes were found in CYP1C1, CYP1C2, or p53. Increased mRNA expression was observed 14 d after exposure, indicating a prolonged physiological effect of a single BaP injection. Although there were differences in expression, it was concluded that variations in the presence or induction of CYP enzymes is not enough to explain the difference in toxicity observed between B cells and thrombocytes. An

induction of AhR in thrombocytes is interesting, but difficult to explain given current knowledge on endogenous AhR regulation and the limited toxicity in thrombocytes.

3.2 Introduction

A growing body of literature supports the hypothesis that polycyclic aromatic hydrocarbons (PAHs) are toxic to the immune system of fishes (Bado-Nilles *et al.*, 2009; Danion *et al.*, 2011; McNeill *et al.*, 2012; Phalen *et al.*, 2014; Reynaud and Deschaux, 2006). Exposure of fishes to petrogenic mixtures that include PAHs has been observed to cause opportunistic disease outbreaks (van den Heuvel *et al.*, 2000) and it has been suggested that fish population collapses have resulted from immune impairment and disease outbreak following an oil spill (Marty *et al.*, 1998). In such studies, it is often difficult to attribute immune toxicity to particular groups of compounds such as PAHs where organisms are exposed to complex mixtures. Determining cellular level mechanisms behind immunotoxic effects provides an important basis to draw causal linkages between PAHs and immunological effects in wild populations.

PAHs bind to and activate the aryl hydrocarbon receptor (AhR), a transcription factor that regulates expression of cytochrome P450 (CYP) enzymes involved in the excretion of organic substances (Zhou *et al.*, 2010). Immune toxicity of PAHs in mammalian lymphocyte lines has been shown to depend on metabolic conversion to more reactive products by a CYP enzyme (CYP1B1) (Heidel *et al.*, 1999; Gao *et al.*, 2005). Whether metabolism by CYP enzymes is required for PAH toxicity in fishes has not been shown definitively. AhR-mediated PAH toxicity has been shown to persist even after knockdown of CYP1A (Incardona *et al.*, 2006, Scott 2010). However, the complexity of CYP enzymes in fishes has only recently been recognized; several CYP

enzymes other than CYP1A are implicated in toxicant metabolism. Jönsson *et al.* (2010) identified that rainbow trout express several other subfamilies of CYP enzymes (1A1, 1A3, 1B1, 1C1, 1C2, and 1C3) in liver and gill, and their results suggest that they are all AhR regulated. CYP expression in rainbow trout immune cells is not well characterized, though subpopulations of B cells and granulocytes in rainbow trout head kidney expressed inducible CYP1A (Nakayama, 2008).

PAHs are capable of affecting immune cell numbers of fishes. Several studies report a decrease in lymphatic cell numbers after exposure to PAHs in both *in vivo* and *in vitro* settings (Carlson *et al.*, 2002a,b; Connolley and Means, 2010; Reynaud and Deschaux, 2005; McNeill *et al.*, 2012, MacDonald *et al.*, 2012). Phalen *et al.* (2014) concluded that B cells, T cells, and myeloid cells are differentially depleted in multiple tissues after an *in vivo* exposure to benzo[a]pyrene (BaP). B cells were the most universally affected, whereas thrombocytes showed no significant changes in cell numbers. Furthermore, the most severe depletion of leukocytes did not occur until three weeks after the administration of a single intraperitoneal (i.p.) injection of BaP, suggesting a complex systemic effect of this PAH on immune tissue.

There are a number of mechanisms that could result in decreased immune cell numbers including: apoptosis, necrosis, lack of proliferation, or in the case of *in vivo* studies – movement out of a particular tissue. Following *in vivo* exposure to BaP, tilapia (*Oreochromis niloticus*) head kidney had an increased number of apoptotic cells in the leukocyte fraction (Holladay *et al.*, 1998). Lymphocytes and phagocytes isolated from carp (*Cyprinus carpio* L) and exposed to 3- methylcholanthrene (3-MC) demonstrated characteristics of apoptotic cells (Reynaud *et al.*, 2004). BaP metabolites cause DNA

damage via adduction in rainbow trout (Schnitz and O'Connor, 1992); in humans and mice, the protein p53 will begin to accumulate and become activated in response to DNA damage, signalling for DNA repair, cell cycle arrest, and apoptosis (Caspari, 2000; Hickman *et al.*, 2002).

In this study, we investigated changes in mRNA expression of CYP enzymes (CYP 1A1, 1A3, 1B1, 1C1, and 1C2), AhR, and p53 in immunomagnetically purified rainbow trout B cells and thrombocytes after a 14 d *in vivo* exposure to 100 mg/kg BaP. These cells were isolated from blood, spleen, and head kidney. Whole liver tissue was analyzed as a reference, because this suite of CYP enzymes has already been characterized after exposure to an AhR agonist (Jönsson *et al.*, 2010). We hypothesized that B cell expression profiles would differ from those in thrombocytes, potentially explaining the differential effect on cellular toxicity observed in Phalen *et al.* (2014), and helping to elucidate the manner by which BaP causes immune toxicity.

3.3 Materials and Methods

3.3.1 Experimental design

Rainbow trout were injected with a single dose of 100 mg/kg BaP and tissues sampled after 14 d. The dose and duration were chosen to represent the maximum toxic effects of BaP as determined in a previous study (Phalen *et al.*, 2014). Rainbow trout (mean wet body weight of 82.8 ± 4.7 g) were injected intraperitoneally (i.p.) with 1 mL of 35.5 mg/mL BaP in corn oil or 1 mL of corn oil alone to serve as a carrier control. Both treatment groups were held in the same tank, and differentiated by the use of visual implant elastomer (VIE) tags (Northwest Marine Technology, Shaw Island, WA). A total of 20 animals were injected: 10 animals per treatment group separated into 5 replicate

trials of 4 animals each. Each trial was performed on separate, consecutive days, each housed in a separate tank.

Upon sampling, rainbow trout were anaesthetized (0.1 g/L tricaine methanesulfonate (MS-222) (Argent, WA, USA)) before being bled via caudal puncture. Approximately 3 mL of blood was taken using a 21 gauge heparinized syringe (BD, NJ, USA) and placed into a heparinized vacutainer (BD) before being diluted in 21 mL of L15 media (Invitrogen, ON, Canada). Animals were killed by a blow to the head, and then fork length and wet weight were measured. Tissues were excised and placed in appropriate medium: liver in liquid nitrogen before storage at -80°C, spleen in 21 mL of L15 media, and head kidney in 21 mL of phosphate buffered saline containing 5 mM ethylenediaminetetraacetic acid (PBS/EDTA; Sigma Aldrich, Oakville, ON).

3.3.2 Leukocyte isolation and immunomagnetic separation

Spleen and head kidney were homogenized with 5 passes of a Ten Broeck glass mortar and pestle tissue grinder (Millville, New Jersey, USA) and filtered using 100 µm Nitex mesh. Leukocytes were isolated from cell suspensions with the use of a density gradient (Secombes, 1990). Head kidney, spleen, and blood cell suspensions were placed in aliquots of 3 mL onto 3 mL of Lympholyte H (1.0770 g/cm3) (Cedarlane, ON, Canada) and centrifuged at 4°C for 40 minutes at 400 × g.

After enumeration of leukocytes using a haemocytometer, dilutions were performed such that 1 mL contained 2×10^7 cells. Blood and spleen samples had two such dilutions made, one destined for staining with fluorescently labelled mouse anti-trout monoclonal antibody (mAb) 1.14 (specific against trout IgM^+ B cells) (DeLucia *et al.*, 1983) and the other with mAb 42 (Köllner *et al.*, 2004). Head kidney samples were

only stained with mAb 1.14. Both antibodies were labelled with APC, which excites at a wavelength of 635 nm and is read at emission wavelength 661 nm. Staining occurred at a concentration of 200 ng/mL for 1h at 4℃ in the dark. Cells were pelleted at 800 × g for 8 min at 4℃ and washed in 1 mL PBS/EDTA. The pellet was then re-suspended in 160 µL of PBS/EDTA/BSA (7.5 % BSA, Sigma) and stained with 20 µL of anti-mouse IgG microbeads (Milteny Biotech, San Diego, CA) for 15 min at 4℃ in the dark. Cells were once again pelleted and washed before being re-suspended in 500 µL of PBS/EDTA/BSA.

This cell suspension was then passed through an MS column placed onto an OctoMACS separator as per manufacturer instructions (Milteny Biotech). Thrombocyte samples were passed through two columns to increase purity. A subsample was then removed to check for purity; samples were read on channel FL4 using a fluorescence assisted cell sorter (FACS)Calibur© (BD). Only samples with a purity ≥ 90% were considered for qPCR analysis (Fig. 3.1). Remaining cells were pelleted and re-suspended in 30 µL PBS/EDTA before addition of 300 µL of RNA*later* (Sigma), stored at room temperature for 1 d, and held at 4℃ until processing.

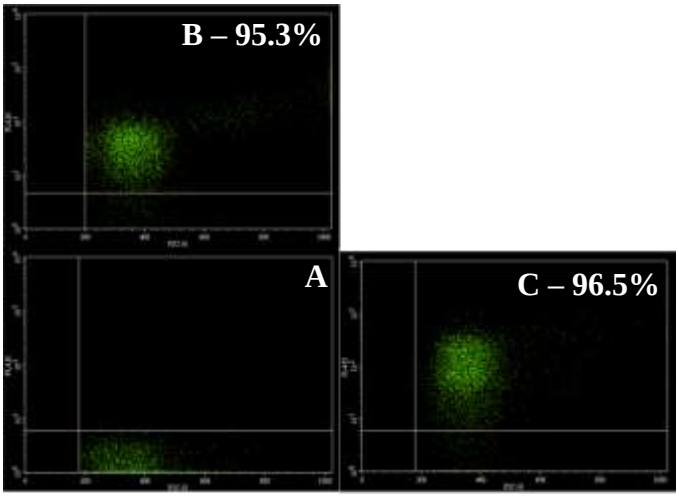

Fig. 3.1: FACS analysis of purity of immunomagnetically isolated rainbow trout blood cell populations. Y-axis represents magnitude of fluorescent staining with APC labelled antibody, x-axis represents size of particle. A) unstained blood leukocytes B) stained blood thrombocytes C) stained blood b cells.

3.3.3 RNA Extraction, cDNA synthesis

All RNA extractions from isolated cell populations were performed using RN*easy* Plus Universal Mini Kits (Qiagen, Toronto, ON) according to manufacturer's instructions for cell suspensions (incliding DNase step), with modifications: A portion of RNA*later* preserved liver between 10-15 mg was weighed out and placed in 600 µL lysis buffer (Buffer RLT, Qiagen) containing β-mercaptoethanol and homogenized with the use of a mixer mill (Retsch MM40, Germany). Cell suspensions from immunomagnetic separation were pelleted at 5000 × g for 3 mins. Following aspiration of the RNA*later*, pellets were re-suspended in the remaining fluid before addition of 600 µL of the same lysis buffer. Thrombocyte samples were further lysed by sonication for 5 s with a Branson SLPe sonicator set to 15%. After isolation, RNA was re-eluted in 30 uL DNase, RNase, pyrogen free H_2O. All samples were quantified using a Nanovue (General Electric, Peterborough, ON), visualized on a 1% agarose gel, and stored at -80℃.

Synthesis of cDNA was performed using a qScript cDNA synthesis kit (Quanta Biosciences, MD, USA) according to manufacturer's protocol. For β-actin, elongation factor 1α (EF1α), p53, CYP1A1 and CYP1A3 all samples had 0.05 µg RNA added to the reaction. Due to high Ct values, more RNA template had to be used in CYP1C1, CYP1C2, CYP1B1 and AhR analysis: 1 µg RNA from liver, 0.5 µg RNA from B cells, and 0.05 µg RNA from thrombocytes (thrombocytes were not analyzed for CYP1C1 and CYP1C2). Samples were stored at -80℃ until qPCR analysis.

3.3.4 qPCR

QPCR and analysis was performed using MxPRO3000 equipment and software (Strategene, La Jolla, CA). Reaction mixture for all reactions contained: 10 uL PerfeCTa SYBR Green Fastmix, Low Rox (Quanta Biosciences), 2 uL of 20 × dilute cDNA (β-actin , EF1α, p53, CYP1A1 and CYP1A3) or undiluted cDNA (CYP1C1, CYP1C2, CYP1B1 and AhR), 2 uL of primer mix (yielding 500 nM final concentration), and 6 uL of H_2O. Cycling protocol for all primers followed: 30 s @ 95℃ once, 40 cycles of: 5 s 95℃, 15 s @ elongation temperature, 10 s @ 72℃. Primers were ordered from Invitrogen; further details can be found in Table 3.1.

Table 3.1: Technical details of the primers used in qPCR analysis on liver tissue, isolated IgM^+ B cells, and thrombocytes from spleen, head kidney, and blood of rainbow trout exposed to BaP.

Primer	Elongation Temp. (◦C)	Forward	Reverse	Reference
EF1α	57	GCAGGTACTACGTCA CCATCAT	CACAATCAGCCTGAG ATGTACC	Jönsson *et al.*, 2010
β-actin	63	GGCTTCTCTCTCCACC TTCC	AGGGACCAGACTCGT CGTAC	Lo *et al.*, 2007
AhR	62	CAGCGAGGGGAGCG GTAA	TGGACCCGGCCAGTG ATA	Abnet *et al.*, 1999
P53	61	GAATCAAACGTGCTA TGAAGGA	CCCTCGAATCTGAAG AGTGTAG	Bobe *et al.*, 2004
CYP1A1	58	GGAAACTAGATGAGA ACGCCAACA	GTACACAACAGCCCA TGACAG	Jönsson *et al.*, 2010
CYP1B1	60	CATTCTGATACTTGTG AGGTTTCC	CAACTGAGACTGGTC TTCCAT	Jönsson *et al.*, 2010
CYP1C1	64	GCAGCACAGAGAAAC CTTCAAC	GTCCTTTCCGTGCTCA ATCACA	Jönsson *et al.*, 2010
CYP1C2	60	GAGCACAGGGAGAC ATTTGAC	GGTATCACTGTCCGC CTTG	Jönsson *et al.*, 2010

3.3.5 Data Transformation and Statistics

Relative copy number was determined based on a standard curve that had efficiency between 90 and 110% and R^2 value between 0.96 and 1.00. All sample values were adjusted within plate standards and normalized to the housekeeping genes using the geometric mean of β-Actin and EF1α relative copy numbers. This final relative quantity was used in statistical analysis. Levene's and Brown Forsythe tests were used to determine homogeneity of variance of data, while normality was tested using visually normal probability plots. Data that did not conform to the appropriate parametric assumptions based on these tests were log transformed prior to statistical analysis. Data were analyzed using 2-way analysis of variance tests (ANOVAs) with dose and tissue and separately with dose and trial as independent variables. Significant changes were tested post hoc using Dunnett's test. Body and tissue weights were analyzed using analysis of covariance tests (ANCOVAs) using length or body weight along with dose. Liver somatic index, spleen somatic index, and condition factor were calculated using the least square means and covariate means generated by the ANCOVA. All statistics were performed in STATISTICA v 8.0, and an experiment-wise alpha of 0.05 was chosen.

3.4 Results

Of the 20 fish sampled during this experiment, only one mortality was observed. No effect of BaP was found on fish weight as it covaries with length. The mean ± standard error of the mean (SEM) for condition factors were: 1.17±0.022 and 1.23±0.02 for 0 mg/kg and 100 mg/kg dose respectively. Neither liver nor spleen size, as they covaried with body weight, was different between treatment groups. Liver somatic index

means ± SEM were 1.53±0.11 and 1.74±0.13 for 0 and 100 dose respectively. Spleen somatic index means ± SEM were 0.22±0.02 for both doses.

Isolated subpopulations of leukocytes had the following mean purity (represented as the % stained of whole isolated fraction) ± standard error: blood B cells - 95.8±0.5%; blood thrombocytes - 98.1±0.4%; spleen B cells - 95.1±0.9%, spleen thrombocytes - 97.2±0.5%; head kidney B cells - 92.8±0.5%. No effect of dose was observed for either of the reference genes using any of the tissues or cell types examined (Fig. 3.2).

CYP1A1 was significantly induced in the liver (11-fold), blood B cells (10-fold), and blood thrombocytes (9-fold) by the treatment as compared to their respective controls (Fig. 3.3). CYP1B1 was significantly induced in blood B cells (14-fold) and blood thrombocytes (8-fold), and had notable inductions in liver, spleen B cells, and spleen thrombocytes but these were not statistically significant (Fig. 3.3). CYP1A3 was significantly induced in liver (10-fold) as well as blood and spleen B cells (10-fold and 8-fold respectively) in treatment group as compared to their controls (Fig. 3.3). CYP1C1 and CYP1C2 showed no significant changes when comparing the treatment group to the control (Fig. 3.3).

Spleen thrombocytes had a significant induction (7-fold) of AhR in the treatment group as compared to control, and liver may have been induced but there was no statistical difference (Fig. 3.4). P53 had no statistical changes from the control, although the mean relative copy number from liver treatment group was less than half that of the control.

Overall, CYP gene expression was variable depending on cell type and tissue, although a fairly consistent induction was seen in liver tissue despite lack of statistical

significance. B cells clearly had less relative CYP1A1 induction as compared to thrombocytes, but 1A3 had a high er relative increase. Thrombocytes had greater relative increases in the expression of CYP genes in response to BaP in spleen than was found in blood as compared to their respective controls. Effect of treatment on CYP1B1 was similar in both B cells and thrombocytes (Fig. 3.3).

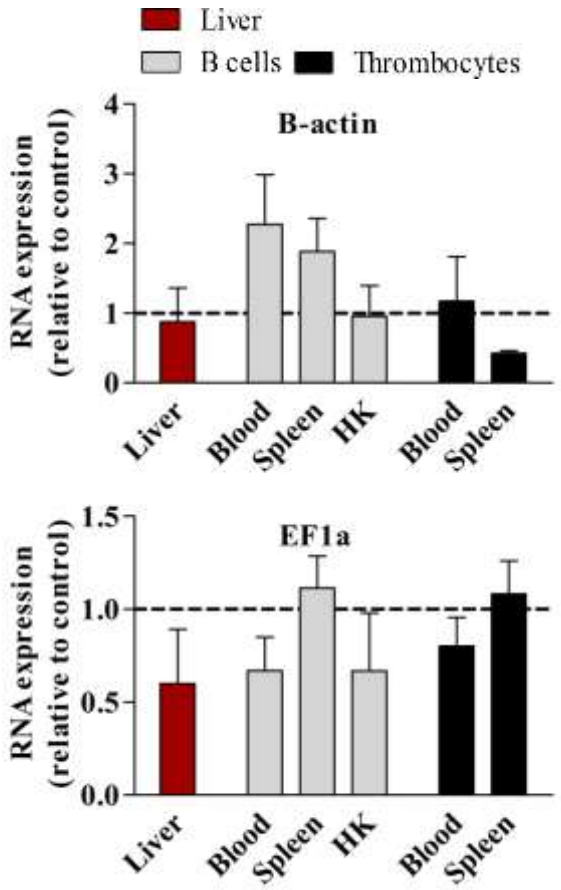

Fig. 3.2: Rainbow trout liver and immunomagnetically isolated cells 14 d after single intraperitoneal injection of 100 mg/kg benzo[a]pyrene in corn oil carrier. Dotted line represents the mean of the respective control group, bars represent standard error. N= 3-5 for liver and 5-10 for isolated cells.

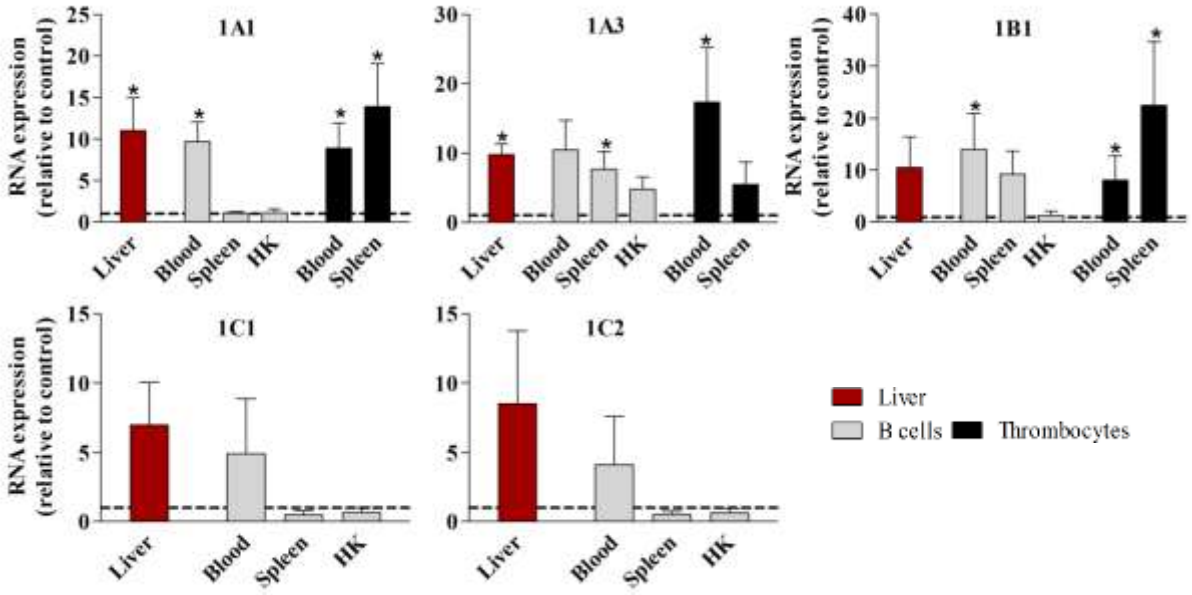

Fig. 3.3: Rainbow trout liver and immunomagnetically isolated cells 14 d after single intraperitoneal injection of 100 mg/kg benzo[a]pyrene in corn oil carrier. Dotted line represents the mean of the respective control group, bars represent standard error. Asterisks indicate a significant difference from the control group, with a p-value < 0.05 as determined by a 2-way ANOVA. N=4-10.

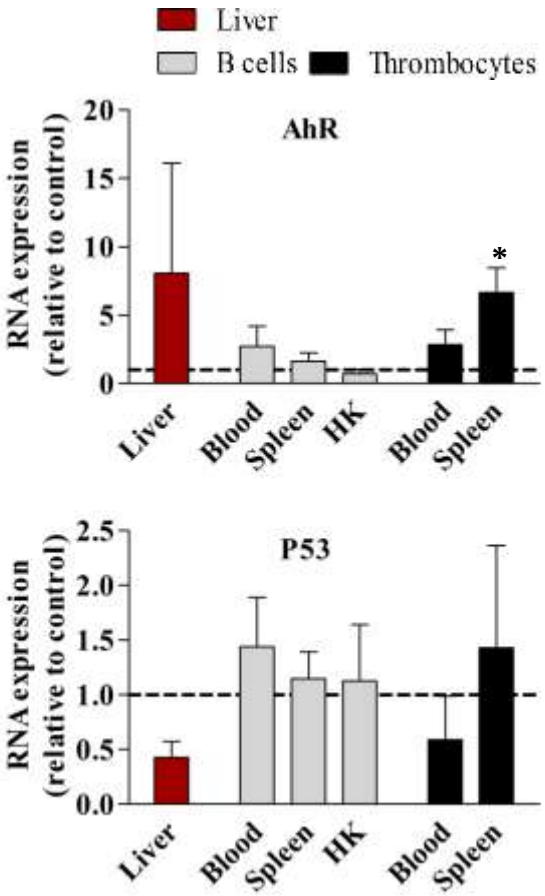

Fig. 3.4: Rainbow trout liver and immunomagnetically isolated cells 14 d after single intraperitoneal injection of 100 mg/kg benzo[a]pyrene in corn oil carrier. Dotted line represents the mean of the respective control group, bars represent standard error. Asterisks indicate a significant difference from the control group, with a p-value < 0.05 as determined by a 2-way ANOVA. N=4-10.

3.5 Discussion

Immunomagnetically separating B cells and thrombocytes from the whole leukocyte population was successful, with purity values averaging above 95%. To our knowledge, this is the first time that gene expression of several CYP enzymes after exposure to a PAH has been analyzed in isolated B cell and thrombocytes in a fish species. Both cell types exhibited an induction in CYP enzymes following exposure to

BaP. That induction occurred in both cell types is contrary to what we expected. However, the profiles of expression differ between cell types and tissues. These data indicated that even 14 d after a single intraperitoneal exposure, BaP was still having an effect on leukocytes. We found that expression of a traditional indicator of DNA damage leading to apoptotic signalling, p53, was not affected in either cell type. In spleen, expression of AhR was induced in thrombocytes following exposure, but not in B cells.

Our study demonstrates that changes in expression of CYP enzymes in response to toxicant exposure can vary from enzyme to enzyme and between tissues for a given enzyme which is consistent with the several other studies (Cao *et al.*, 2000; Gao *et al.*, 2011; Hook *et al.*, 2010; Dorrington *et al.*, 2012; Tuan *et al.*, 2014). Variations in CYP gene expression profiles suggests that each enzyme may be regulated independently. Hook *et al.* (2010) concluded that using a single gene as an indicator of AhR mediated toxicant exposure may be inappropriate. Gao *et al.* (2011) suggest that higher CYP gene expression corresponds to higher numbers of dioxin-response elements upstream of the gene. Little is known about any endogenous functions that may help explain their differential regulation and expression. Phylogenetic analysis suggests that CYP1B1 and CYP1C1 and C2 are more closely related than the CYP1As (Jönsson *et al.*, 2010). A study on zebrafish report high CYP1B1expression in the eye, reflecting findings that it is important for mammalian eye development (Jönsson *et al.*, 2007). CYP1C gene homologs have not been found in mammals. No studies indicate functions of these enzymes in fish immune cells specifically. Clarification of these roles may help to illuminate the differences in toxic response demonstrated by B cells and thrombocytes.

The lack of change in p53 expression observed in this study may not rule out its involvement in BaP toxicity, as it is highly regulated at the protein level. For example, an AhR agonist that does not produce toxic metabolites, 2,3,7,8-tetrechlorodibenzodioxin (TCDD), has been shown to induce apoptosis, but not change protein or mRNA levels of p53 (Chopra *et al.*, 2011). Liu *et al.* (2011) concluded that p53 may not be a useful indicator for DNA damage induced apoptosis in rainbow trout as it is in mammals. In mammals, p53 regulates cellular apoptosis by inducing transcription of factors important in several apoptotic pathways, including the Fas death receptor ligand, Fas/apo1 (Hickman *et al.*, 2003). There is evidence that activated AhR increases the apoptotic signal of this pathway (Stolpmann *et al.*, 2012), and that mice lacking AhR via gene knockout have a reduction in the magnitude of apoptosis in response to this signal (Park *et al.*, 2005), potentially linking PAH exposure to alterations in cell cycle regulation. If the extrinsic apoptotic pathway (Fas pathway) is part of the PAH mechanism in fish leukocytes, it may be more useful to directly examine the surface expression of the Fas receptor, or of Fas ligand using flow cytometry.

Relatively few studies focus on change in AhR mRNA expression, however our observation of increased AhR mRNA expression in thrombocytes is reflected in studies of other cell types. Wiseman and Vijayan (2007) found that exposure of rainbow trout liver cells in primary culture to β-napthoflavone (β-NF) (an AhR agonist that does not interact with DNA) yielded an increase in both AhR mRNA and protein. Allan and Sherr (2005) report an upregulation of AhR mRNA in antigen activated human B cells, but found little or no AhR in resting cells. Similarly, Prigent *et al.* (2007) found an immediate upregulation of AhR in human T cells after exposure to an antigen. Qiu *et al.*

(2011) demonstrate that innate lymphoid cells of the mouse gut display increased apoptosis and reduced IL-22 expression in AhR null mice. These studies suggest that AhR mRNA regulation may be important in mediating response to toxicity and antigens by immune cells.

The functional effects caused by a change in AhR expression as observed in this study may be important; several recent reviews focus on the role of AhR in subjects as diverse as inter-cell contact and tumour regulation (Dietrich and Kaina, 2010), immunological regulation in resting and activated cells (Stockinger *et al.*, 2014), and its involvement in the regulation of programmed cell death (Chopra and Schrenk, 2011). Much of this research is inspired by the effects of the protypical AhR agonist, TCDD, exposure to which has induced effects that cannot be explained by a toxic metabolite. Unravelling the importance of AhR induction in fish immune cells will require much more study of the protein in models specific to this context. For example, does exposure to PAHs causes changes in regulation at the protein level, how are mRNA expression and protein activity related, and are there additional roles of AhR besides as a transcription factor of CYP enzymes? These details are currently unknown.

Overall, it seems that differences in the CYP suite of enzymes examined in this study does not clarify the specificity of chronic BaP toxicity on rainbow trout B cells that was observed in previous work. Difficulties inherent in isolating cell populations makes studies of this nature at multiple time points challenging and costly. However, the time point chosen here was based on when maximum cell toxicity occurs, and the signals that lead to that toxicity could certainly arise before the impacts are seen in leukocytes. Given the complexity of AhR activities described in mammalian literature, the up regulation of

AhR in thrombocytes seen in this study, and the long time frame of effect on rainbow trout immune cells (Phalen *et al.*, 2014), it seems likely that a complex mechanism of effect is at play much more similar to the systemic effects of TCDD than to short-term toxicity due to reactive metabolites. While a number of studies have shown the importance of reactive metabolite generation to toxicity, it is possible that short-term acute toxicity in cell culture may be entirely different from the mechanisms occurring *in vivo*. The extrinsic apoptotic pathway may also require T or NK cells to cause mortality, thus such a mechanism would not be apparent in culture. The extensive and diverse literature on AhR activity suggests it is possible that this receptor could regulate immune cell specific toxicity of PAHs in a manner more complex than transcribing enzymes of the CYP family.

3.6 Literature Cited

Abnet, C.C., Tanguay, R.L., Hahn, M.E., Heideman, W., Peterson, R.E, 1999. Two forms of aryl hydrocarbon receptor type 2 in rainbow trout (*Oncorhynchus mykiss*) – evidence for differential expression and enhancer specificity. J. Biol. Chem. 274, 15159-15166.

Allen, L.L., Sherr, D.H., 2005. Constitutive activation and environmental chemical induction of the aryl hydrocarbon receptor/transcription factor in activated human B lymphocytes. Mol. Pharmacol. 67, 1740-1750.

Bado-Nilles, A., Quentel, C., Thomas-Guyon, H., Le Floch, S., 2009. Effects of two oils and 16 pure polycyclic aromatic hydrocarbons on plasmatic immune parameters in the European sea bass, *Dientrarchus labrax* (Linné). Toxicol. *In Vitro*. 23, 235-241.

Bobe, J., Nguyen, T., Jalabert, B., 2004. Targeted gene expression profiling in the rainbow trout (*Oncorhynchus mykiss*) ovary during maturational competence acquisition and oocyte maturation. Biol. Reprod. 71, 73-82.

Cao, Z., Hong, J., Peterson, R.E., Aiken, J.M., 2000. Characterization of CYP1A1 and CYP1A3 gene expression in rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 49, 101-109.

Carlson, E.A., Li, Y., and Zeilkoff, J.T., 2002 a. Exposure of Japanese medaka (*Oryzias latipes*) to benzo[a]pyrene suppresses immune function and host resistance against bacterial challenge. Aquat. Toxicol. 56, 289-301.

Carlson, E.A., Li, Y., and Zeilkoff, J.T., 2002b. The Japanese medaka (*Oryzias latipes*) model: applicability for investigating the immunosuppressive effects of the aquatic pollutant benzo[a]pyrene (BaP). Mar. Environ. Res. 54, 565-568.

Caspari, T., 2000. Checkpoints: How to activate p53. Curr Biol. 10, R315-R317.

Chopra, M., Schrenk, D., 2011. Dioxin toxicity, aryl hydrocarbon receptor signalling and apoptosis – Persistant pollutants affect programmed cell death. Crit. Rev. Toxicol. 41, 292-320.

Connelly, H., and Means, J.C., 2010. Immunomodulatory effects of dietary exposure to selected polycyclic aromatic hydrocarbons in the bluegill (*Lepomis macrochirus*). Int. J Toxicol. 29, 532-545.

Danion, M., Le Floch, S., Kanan, R., Lamour, F., and Quentel, C., 2011. Effects of *in vivo* chronic hydrocarbons on sanitary status and immune system of sea bass (*Dicentrarchus labrax* L.) Aquat. Toxicol.105, 300-311.

DeLucia, D., Wilson, M., Warr, G.W., 1983. Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. Eur. J. Immunol. 13, 546-551.

Dietrich, C., Kaina, B., 2010. The AhR in the regulation of cell-cell contact and tumor growth. Carcinogenesis. 31, 1319-1328.

Dorrington, T., Zanette, J., Zacchi, F.L., Stegeman, J.J., Bainy, A.C.D., 2012. Basal and 3-MC induced expression of cytochrome P450 1A, 1B and 1C genes in the Brazilian guppy, *Poecilia vivipara*. Aquat. Toxicol. 124-125, 106-113.

Gao, K., Brandt, I., Goldstone, J.V., Jonsson, M.E., 2011. Cytochrome P450 1A, 1B, and 1C mRNA induction patterns in three-spined stickleback exposed to a transient and a persistent inducer. Comp. Biochem. Phys. 154, 42-55.

Gao, J., Lauer, F.T., Dunwat, S., and Burchiel, S.W., 2005. Cytochrome P450 1B1 is required for 7,12-dimethylbenz(a)anthracene (DMBA) induced spleen cell immunotoxicity. Toxiocol. Sci. 86: 68-74.

Heidel, S.M., Holston, K., Buters, J.T., Gonzalez, F.J., Jefcoate, C.R., and Czupyrynski, C.J., 1999. Bone marrow stromal cell cytochrome P4501B1 is required for pre-B cell apoptosis induced by 7,12-dimethylbenz[a]anthracene. Mol. Pharmacol. 56:1317–1323.

Hickman, E.S., Moroni, M.C., Helin, K., 2002. The role of p53 and pRB in apoptosis and cancer. Curr. Opin. Genet. Dev. 12, 60-66.

Holladay, S.D., Smith, S.A., Besteman, E.G., Deyab, A.S.M.I., Gogal, R.M., Hrubec, T., Robertson, J.L., Ahmed, S.A., 1998. Vet. Immunol. Immunop. 64, 69-82.

Hook, S.E., Lampi, M.A., Febbo, E.J., Ward, J.A., Parkerton, T.F., 2010. Hepatic gene expression in rainbow trout (*Oncorhynchus mykiss*) exposed to different hydrocarbon mixtures. Environ. Toxicol. Chem. 29, 2034-2043.

Incardona, J.P., Day, H.L., Collier, T.K., Scholz, N.L., 2006. Developmental toxicity of 4-ring polycyclic aromatic hydrocarbons in zebrafish is differentially dependent on AH receptor isoforms and hepatic cytochrome P4501A metabolism. Toxicol. Appl. Pharmacol.

Jönsson, M.E., Gao, K., Olsson, J.A., Goldstone, J.V., and Brandt, I., 2010. Induction patterns of new CYP1 genes in environmentally exposed rainbow trout. Aquat. Toxicol. 98, 311-321.

Köllner, B., Fischer, U., Rombout, J.H.W.M., Taverne-Thiele, Hansen, J.D., 2004. Potentil involvement of rainbow trout thrombocytes in immune functions: a study using a panel of monoclonal antibodies and RT-PCR. Dev. Comp. Immunol. 28, 1049-1062.

Liu, M., Tee, C., Zeng, F., Sherry, J.P., Dixon, B., Bols, N.B., Duncker, B.P., 2011. Caracterization of p53 expression in rainbow trout. Comp. Biochem. Phys. 154, 326-332.

Lo, J.H., Chiou, P.P., Lin, C.M., Chen, T.T., 2007. Molecular cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*) CCAT/enhancer binding protein genes and their responses to induction by GH *in vitro* and *in vivo*. J. Endocrinol. 194, 393-406.

MacDonald, G.Z., Hogan, N.S., Köllner, B., Thorpe K.L., Phalen, L.J., Wagner, B.D., and van den Heuvel., 2012. Immunotoxic effects of oil sands-derived napthenic acids to rainbow trout. Aquat. Toxicol.126, 95-103.

Marty, G.D.,Freiberg,E.F.,Meyers,T.R.,Wilcock,J.,Farver,T.B.,Hinton,D.E.,1998. Viral hemorrhagic septicemia virus (*Ichthyophonus hoferi*) and other causes of morbidity in Pacific herring (*Clupea pallasi*) spawning in Prince William Sound. Alaska USA. Dis. Aquat. Org.32,15–40.

McNeill, S.A., Arens, C.J., Hogan, N.S., Köllner, B., and van den Heuvel M.R., 2012. Immunological impacts of oil sands-affected waters on rainbow trout evaluated using an *in situ* exposure. Eco. Tox. and Env. Safety. 84, 254-261.

Nakayama, A., Riesen, I., Köllner, B., Eppler, E., and Segner, H., 2008. Surface marker defined head kindey granulocytes and B lymphocytes of rainbow trout express benzo[a]pyrene-inducible cytochrome P4501A protein. Toxicol. Sci. 103, 86-96.

Park, K.T., Mitchell, K.A., Huang, g., Elferink, C.J., 2005. The AhR predisposes hepatocytes to Fas-mediated apoptosis. Mol. Pharmacol. 67, 612-622.

Phalen, L.J, Köllner, B., Leclair, L.A., Hogan, N.S., van den Heuvel, M.R., 2014. The effects of benzo[a]pyrene on leukocyte distribution and antibody response in rainbow trout (*Oncorhynchus mykiss*). Aquat. Tox. 147, 121-128.

Prigent, L., Robineau, m., Jouneau, S., Morzadec, C., Louarn, L., Vernhet, L., Fardel, O., Sparfel, L., 2007. The AhR is functionally upregulated early in the course of human T-cell activation. Eur. J. Immunol. 44, 1330-1340.

Qiu, J., Heller, J.J., Guo, X., Chen, Z.M.E., Fish, K., Fu, Y.X., Zhou, L., 2011. Immunity. 36, 92-104.

Reynaud, S., Duchiron, and C., Deschaux, P., 2004. 3-methylcholanthrene induces lymphocyte and phagocyte apoptosis in common carp (*Cyprinus carpio* L.). Aquat. Toxicol. 66, 307-318.

Reynaud, S., and Deschaux, P. 2005. The effects of 3-methylcholanthrene on lymphocyte proliferation in the common carp (*Cyprinus caropio L.*). Toxicol, 211, 156-164.

Reynaud, S., and Deschaux, P., 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: A review. Aquat. Toxicol.77, 229-238.

Reynaud, S., Raveton, M., and Ravanel, P., 2008. Interatctions between immune and biotransformation systems in fish: A review. Aquat. Toxicol. 87, 139-145.

Schnitz, A.R., O'Connor, M., 1992. *In vivo* DNA/RNA adduction of 7,12-DMBA and BaP in th liver of rainbow trout (*Oncorhynchus mykiss*). J. Environ. Pathol. Tox. 11, 229-233.

Scott, J.A., Incardona, J.P., Pelkki, K., Shepardson, S., Hodson, P.V., 2010. AhR2-mediated, CYP1A-independent cardiovascular toxicity in zebrafish (*Danto rerio*) embyos exposed to retene. Aquat. Toxicol. 101, 165-174.

Secombes, C.J., 1990. Isolation of salmonid macrophage and analysis of their killing activity. In Techniques in Fish Immunology (Van Muiswinkel, W.B.), second ed., SOS Publication, Fair Haven, NJ, pp. 101-103.

Stockinger, B., Meglio, P.D., Gialitakis, M., Duarte, J.H., 2014. The AhR: Multitasking in the immune system. Ann. Rev. immunol. 32, 403-432.

Stolpmann, K., Brinkmann, J., Salzmann, S., Genkinger, D., Fritsche, E., Hutzler, C., Wajant, H., Luch, A., Henkler, F., 2012. Activation of the AhR sensitises human keratinocytes for CD95L- and TRAIL- induced apoptosis. Cell Death Dis. 3, 1-11.

Tuan, T., Kaminishi, Y., Funahashi, A., Hassanin, A.A.I, Itakura, 2014. Afr. J. Biotechnol. 13, 2028-2040.

van den Heuvel, M.R., Power, M., Richards, J., MacKinnon, M., and Dixon, D.G., 2000. Disease and gill lesions in yellow perch (*Perca flavescens*) exposed to oil sands mining-associated waters. Ecotoxicol. Environ. Saf. 46, 334-341.

Wiseman, S.B., Vijayan, M.M., 2007. Aryl hydrocarbon receptor signaling in rainbow trout hepatocytes: Role of Hsp90 and the proteasome. Comp. Biochem. Physiol. 146, 484-491.

Wu, R., Zhang, L., Hoagland, M.S., Swanson, H.I., 2007. Lack of the AhR leads to impaired activation of AKT/Protein kinase B and enhanced sensitivity to apoptosis induced via the intrinsic pathway. J. Pharmacol. Exp. Ther. 320, 448-467.

Zhou, H., Wu, H., Liao, C., Diao, X., Zhen, J., Chen, L., Xue, Q., 2010. Toxicology mechanism of the persistent organic pollutants (POPs) in fish through AhR pathway. Toxicol. Mech. Methods. 20. 279-286.

General Conclusions

The objective of these studies was to learn more about the ability of PAHs to specifically affect the immune system of rainbow trout, while simultaneously developing and validating tools for use in fish immune toxicology. Exposing rainbow trout intraperitoneally to BaP dissolved in corn oil consistently resulted in prolonged exposure in all tissues examined as determined by analysis of bile metabolites, CYP induction measured by catalytic activity, and mRNA expression. This method of exposure also resulted in observable effects on the immune system at as little as 7 d and as many as 42, as determined by altered cell numbers and circulating antibody titres. FACS analysis was capable of detecting effects in numbers of leukocyte subpopulations at both low and high dose of BaP at 7,14, and 21 d. Attempts to physically separate B cell and thrombocyte subpopulations from the entire leukocyte fraction from important immune organs including blood, spleen, and head kidney were very successful resulting in an average purity above 95%. Together, these results represent a promising experimental model for studies in *in vivo* fish immune toxicology. Further, this work supports the use of BaP as a model for studying the immune toxicity of PAHs.

In Chapter 2, we tested the hypotheses that BaP exposure would selectively reduce the number of certain leukocyte populations in a tissue-specific, dose dependent manner, and that BaP toxicity would lead to a decreased ability to generate antibody against an antigen. We observed that decreases in cell numbers were found in B cells, T cells, and myeloid cells, but not in thrombocytes or erythrocytes, confirming the prediction of cell specific toxicity. As predicted, the higher dose caused greater reductions in cell numbers. Severity of effects did not show a clear relationship with

time: for example, affected cells of head kidney demonstrated similar reductions across time points especially in the high dose, whereas spleen B cells demonstrated greater reductions with time, and T cell numbers appeared to recover with time, particularly at the low dose. Circulating anti-*A.s.* antibody titre after pre-exposure to high dose BaP was significantly reduced, and this effect appeared to be dose dependent.

In the third chapter, we aimed to explain what allowed differential toxicity between cell types. B cells and thrombocytes were chosen for further study as it is possible to physically isolate them using immunomagnetic separation. We hypothesized that the known AhR-CYP pathway would be different between cell types, expecting that if less CYP enzymes were transcribed there would be less generation of toxic BaP metabolites. Analysis of mRNA after 14 d of BaP exposure yielded results that conflicted with this hypothesis. Both cell types showed inductions of multiple CYP genes, particularly 1A1, 1A3, and 1B1, in multiple tissues. However, these inductions were not identical between cell types: 1A1 clearly had greater induction in spleen thrombocytes than spleen B cell, while 1A3 was higher in spleen B cells than spleen thrombocytes. 1B1 was induced in both cells types in both tissues, but the magnitude of change was greater in spleen thrombocytes. Notably, induction of the AhR was observed in spleen thrombocytes, but not at all in B cells. From this, we concluded that differences in CYP expression after exposure to BaP is not likely to be a robust enough explanation for the differences in cell number alterations observed in Chapter 2.

Our overarching hypothesis is that a cellular mechanism underlying BaP toxicity will in some way explain observed immune specific toxicity. Differences in cell number alterations between cell types and tissues, the long time frame that it takes to induce this

effect, that the effects continue to occur over a long time frame, and the differences in CYP induction between cell types and tissues are all indicative of a complex mechanism of regulation, supporting this hypothesis. Determining what exactly the mechanism is is challenging, as intra-cellular functions of fish immune cell subtypes is not well characterized.

In this study, we demonstrate that the AhR-CYP-metabolism pathway is likely not the full story, but that does not eliminate involvement of AhR entirely. This receptor has been suggested to have other roles, including in cell cycle regulation and immune cell activation. PAH activation of AhR could alter these functions and lead to toxicity without the involvement of toxic metabolites. Before hypotheses of how PAHs may affect the role of AhR in cells can be generated, the action of this protein must first be known. Further work in this field would benefit from characterizing the intra-cellular interactions of AhR in rainbow trout immune cells, although, there is currently no commercially available antibody for rainbow trout AhR subtypes. Additionally, examinations into the nature of PAH induced apoptosis in isolated immune cells may help identify which cellular signalling cascades are affected, providing a starting point for further inestigations.

Appendix

A.1: Chapter 1 Supplementary data

Table A.1: Lymphatic organs of rainbow trout analyzed after intraperitoneal injection of benzo[a]pyrene; doses were based on average weight. Isolated leukocytes were stained with DiOC6 dye (leukocytes and erythrocytes) or with labeled monoclonal antibodies and analyzed with a fluorescence assisted cell sorting flow cytometer. Asterisks (*) indicates that 42 d exposure was part of a separate experiment that involved exposure to the immune challenge inactivated *Aeromonas salmonicida* at 21 d post toxicant exposure. Numbers are expressed in units of cells/mL or g of tissue.

	7 d						14 d						21 d						42 d*					
	0		25		100		0		25		100		0		25		100		0		25		100	
BLOOD	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE	M	SE
Leukocytes	30.9	3.4	22.4	2.8	24.2	3.7	49.3	3.1	32.3	3.2	22.8	2.1	39.0	2.6	34.1	1.4	19.6	1.9	37.8	4.3	36.2	2.1	32.6	2.9
B cells	13.3	1.3	10.2	1.0	8.8	1.4	25.0	2.7	14.1	1.4	8.9	1.3	23.0	1.9	17.6	1.7	9.7	1.5	18.0	2.9	17.1	1.5	13.9	2.2
T cells	2.9	0.5	2.6	0.5	3.1	0.5	5.4	0.7	3.7	0.4	2.7	0.3	4.5	0.5	4.2	0.4	2.6	0.3	5.1	0.7	4.4	0.5	4.1	0.5
Myeloid cells	1.8	0.3	1.6	0.3	2.1	0.4	1.5	0.2	1.8	0.2	1.6	0.2	1.9	0.1	2.4	0.2	2.2	0.1	2.3	0.4	2.3	0.3	2.9	0.4
Thrombocytes	11.5	1.3	7.8	1.2	8.8	1.5	14.2	1.2	11.2	1.1	7.9	0.8	8.2	0.6	7.9	0.9	5.9	0.8	10.2	0.9	8.3	0.8	8.4	0.8
Erythrocytes	1.7	0.1	1.5	0.1	1.5	0.1	1.4	0.0	1.5	0.0	1.4	0.0	1.4	0.0	1.4	0.0	1.4	0.0	1.4	0.1	1.3	0.0	1.3	0.1
SPLEEN																								
Leukocytes	308	20.8	248	22.1	245	22.8	260	29.9	200	33.6	151	15.8	469	70.3	365	60.2	203	28.9	223	20.0	271	21.1	205	24.2
B cells	128	18.4	119	14.9	99.8	12.9	133	23.0	92.7	15.9	67.3	10.6	255	42.8	177	39.0	57.8	10.2	95.0	13.9	128	27.5	90.1	22.8
T cells	108	8.2	81.1	8.1	82.3	7.1	77.7	9.1	63.7	10.7	38.5	5.7	132	18.6	137	21.7	91.8	17.2	80.9	10.8	100	9.3	72.4	9.9
Myeloid cells	15.7	2.3	17.2	1.9	13.6	1.9	10.9	2.4	8.5	1.4	9.9	1.4	28.0	2.6	16.0	2.3	17.8	2.6	18.7	5.3	20.5	2.3	18.3	2.8
Thrombocytes	28.5	4.7	29.8	3.9	30.2	3.0	37.6	5.7	36.4	5.6	33.2	3.8	44.9	4.7	32.8	4.9	31.1	7.0	27.8	3.4	22.4	2.9	23.8	3.7
Erythrocytes	1.2	0.3	0.8	0.2	0.6	0.2	0.4	0.1	0.5	0.1	0.7	0.1	1.1	0.2	1.3	0.3	0.8	0.1	0.5	0.1	0.7	0.2	0.7	0.1
HEAD KIDNEY																								
Leukocytes	4.9	0.3	4.4	0.4	2.9	0.2	178	16.9	112	10.5	120	16.0	375	29.5	220	29.3	155	19.1	239	23.9	203	16.0	206	13.9
B cells	1.9	0.2	1.8	0.2	1.2	0.1	58.9	4.5	48.6	6.4	37.0	4.2	192	35.9	80.4	15.2	93.8	16.1	65.8	9.9	58.8	5.4	54.4	5.7
T cells	0.6	0.1	0.4	0.0	0.3	0.0	18.8	1.2	14.3	2.5	13.1	1.2	48.2	7.0	32.3	6.5	31.3	7.9	28.8	4.4	26.0	2.1	20.9	3.1
Myeloid cells	2.6	0.2	1.6	0.2	1.2	0.2	81.4	8.9	49.3	6.5	44.5	7.6	127	18.3	75.7	17.3	57.0	16.4	136	17.8	95.4	7.4	107	11.1
Thrombocytes	0.3	0.0	0.3	0.0	0.2	0.0	7.8	1.3	5.7	0.5	6.0	0.7	20.6	3.1	18.2	3.4	14.8	3.2	11.5	2.3	17.3	4.3	15.9	3.4

A.1 Primary cell cultures

Using *in vivo* models has many benefits in attempting to establish whether a toxicant has an effect on the whole organism. However, challenges inherent to this type of model also exist. There are cost and ethics considerations with using whole animals, and although systems of concern can be identified, determining a mechanism of effect using an *in vivo* model can be difficult due to an inability to control and manipulate variables. For these reasons, we aimed to develop a protocol that allowed for the use of primarily cultured cells in experimentation that would complement the work already presented in this thesis.

Rainbow trout were acquired from Ocean Trout Farms, Brookvale, Prince Edward Island, Canada and held in a 2000 L holding tank that had a flow through. Fish were fed commercial trout food at 0.75% of their wet body weight daily.

Upon sampling, fish were removed from holding tank and anaesthetized using 0.1 g/L tricaine methanesulfonate (MS-222). Following anaesthetization, fish were bled via caudal puncture with a 21 G needle. Blood was immediately placed in a heparinized vacutainer before being diluted with 20 mL of L15 media (with L-glutamate) (Sigma) and placed on ice. Fish were then killed by a blow to the head. Diluted blood was layered in 3 mL aliquots onto 3 mL of Lympholyte-H (Cedarlane, ON, Canada) before centrifugation at 600 × g for 30 min at 4 ℃. The cells remaining at the interface were removed and washed in 8 mL of PBS/EDTA before being pelleted at 800 × g for 8 min at 4℃. The pellet was re-suspended in complete media (L15 media with L-glutamate) containing 7.5% fetal bovine serum (Sigma), 7.5 µg/mL streptomycin, and 75 units penicillin and cell concentration was determined with the use of a hemocytometer. Cells were plated at a concentration of 1.5×10^5 cells/mL (80-90%) confluence). For all *in vitro* experiments, cells were maintained in complete media.

Where cells were analyzed by FACS with fluorescein diacetate (FDA) and propidium iodine (PI) stain, the following procedure was observed: Entire cell pellets were re-suspended in 250 µL of PBS/EDTA (1 packet PBS and 2 g of EDTA disodium salt in 1 L of sterile water) and maintained on ice. A 1 µL volume of PI stock solution (0.5 mg/mL) was added to the suspension before storage at 4 ℃ for 5 min in the dark. Next, 2 µL of FDA working solution (0.5 mg/mL FDA) was added before holding samples at 37℃ for 15 min in the dark. For those experiments in which cells were quantified, 20 µL of CountBrite Beads containing 20,000 multicolour beads were added before FACS analysis. The cell suspensions were analyzed by flow cytometry, using FACSCalibur© (BD) flow cytometer equipped with 488 and 635 nm lasers. The flow cytometer was set to count all events up until the point that 1000 CountBrite beads were detected as determined by a gate set in channel 4 (FL4, far red). Cells capable of metabolizing FDA (i.e.: live) and therefore containing fluorescent breakdown compound acetate were visualized in channel 1 (FL1, green) using a plot of side scatter versus FL1. Cells with permeable membranes (ie: dead) were visualized in channel 3 (FL3, red) using a plot of FL3 vs side scatter. Cell concentrations were calculated based on the number of CountBrite beads visualized in channel 4 (FL4, far red) using a plot of side scatter vs FL4.

A total of 4 biological replicates represented by 2 technical replicates each were exposed to BaP in DMSO (a total of 0.1% DMSO) 0 (DMSO carrier), 500, 5000, 50 000,

or 500 000 nM BaP for 24 h. Plates were stored at room temperature, sealed, but uncovered from light. This experiment was repeated except doses of 0 (DMSO carrier control), 6250, 12 500, 25 000, or 50000 were used, and these plates were stored in the dark. Cell viability was analyzed by staining with propidium iodide (PI; which indicates decreased cell membrane integrity) and fluorescein diacetate (FDA; which becomes metabolised to the fluorescent acetate by metabolically activated cells) using FACS analysis as described above.

Results of these experiments demonstrated that when left in the light, BaP doses 5000, 50 000, and 500 000 nM caused decreased FDA staining, increased PI staining, and increased events classified as debris. FDA staining decreased by as much as ~70%, while PI staining increased by as much as 45%, and events classified as debris increased by as much as double (Fig. A.1). In all cases, the 50 000 nM dose had the greatest effect. The second trial that had a narrower BaP dose range did not show similar trends.

It was concluded from these experiments that the light caused metabolises of BaP (a known phenomenon) and the breakdown metabolites of this process caused cellular toxicity, resulting in a decrease in viability. It was likewise concluded that rainbow trout leukocytes do not have the ability to metabolise BaP sufficiently in this setting to generate toxicity. Finally, it was observed that 500 000 nM of BaP precipitates, which may explain the reduced effect compared to two lower doses.

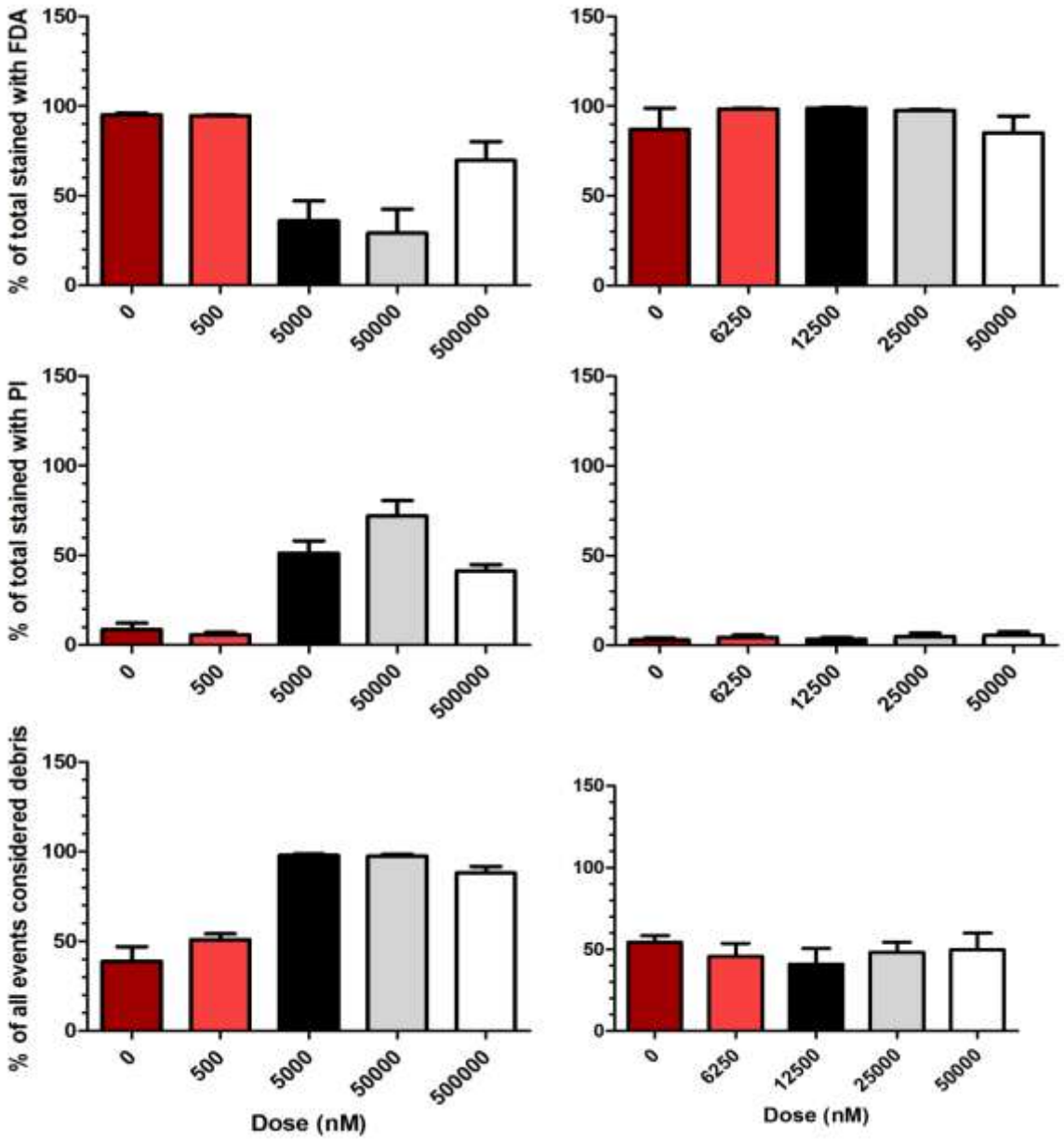

Fig. A.1: Isolated leukocytes from rainbow trout exposed to BaP in DMSO and analyzed by staining with FDA and PI staining. Stained cells and debris were determined by gating using FACS Quant software.

In order to generate toxic metabolites *in vitro* we attempted to design an extracellular metabolising system (EMS). To get a concentrated source of CYP enzymes rainbow trout were exposed to 2 intraperitoneal injections (1 day apart) of 100 mg/kg β-naphthaflavone (βNF) and subsequent harvesting of the liver after 1 week. Microsomes were isolated by first homogenizing tissues as described in EROD methods and then spun at 40 000 rpm for 60 min at 4°C. Microsomes were aliquotted and stored at -80°C. The other important part of an EMS is a source of energy for the enzymes. CYP enzymes use

NADPH for energy, but this source runs out quickly and is expensive to buy. Therefore, we tested the use of a ReadiUse NADPH regenerating kit (AAT BioQuest, CA, USA).

A reaction including 125 µL of NADPH regeneration reagent (mixed as per manufacturer's instructions), 15 uL microsome suspension, 107.7 µL complete media and either 10 or 100 nM of BaP in 2.5 µL was set up. There was also a no NADPH control, and a no protein control. To determine the efficacy of these reaction components to metabolise BaP to toxic metabolites, subsamples were taken 0, 5, 10, 20, 30, 40, 60, 80, and 100 min after the reaction began. Reaction was halted by use of acetonitrile, samples were filtered and analyzed by HPLC as described in Chapter 2.

It was found that the EMS successfully metabolized BaP parent compound, with an increase in total metabolites increasing from 1.2 – 7.3 BaP equivalents/mL. This formation of these metabolites seemed to peak around 30 mins, and a slow decrease in metabolites was seen after 60 minutes (Fig. A.2.). I was concluded that the EMS was functional, and has potential to be used in a primary cell culture setting.

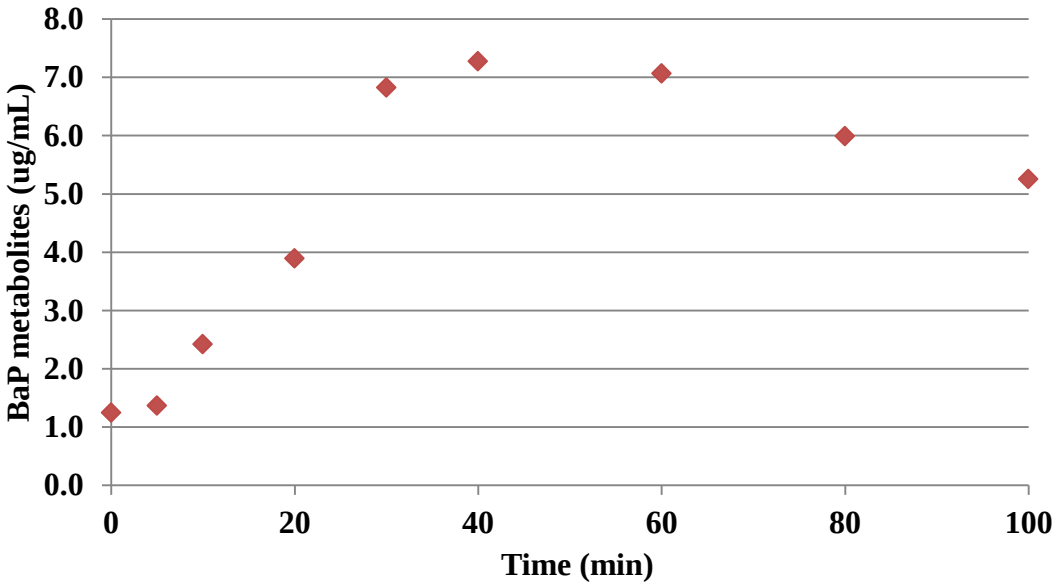

Fig A.2: HPLC analysis of BaP metabolites generated by a reaction containing rainbow trout liver microsomes, NADPH regenerating reagent, BaP, and media. BaP metabolite units are relative to a BaP parent standard curve.

With a successful EMS described, the next step was to see if including it with primary cell cultures would decrease cell viability by way of exposure to toxic metabolites. Two biological replicates had cells isolated and exposed to the following conditions:

1. No treatment
2. 0.1% DMSO (carrier control)
3. 5000 nM BaP in DMSO
4. 50 000 nM BaP in DMSO

Steps 1-4 were duplicated except with inclusion of the EMS: half the media is replaced by the NADPH reagent, and 20 uL/mL of microsome suspension is added. FBS and

pen/strep concentrations remain the same. Once plated, all samples were allowed exposure to BaP and any metabolites produced for 2 h before removal of the media by aspiration, and re-application of equivalent volume of complete media. Cells were collected at 2, 4, 6, and 18h after media switch.

The results of this experiment demonstrated that there is considerable variability in the number of cells collected. The EMS containing cultures seemed to have more cells stained with FDA, as well as more stained with PI, despite not having more cells in the unexposed, media-only contexts (Fig A.3.). The trends represented by these data are not what one would expect if the cells were undergoing BaP metabolite dependent toxicity.

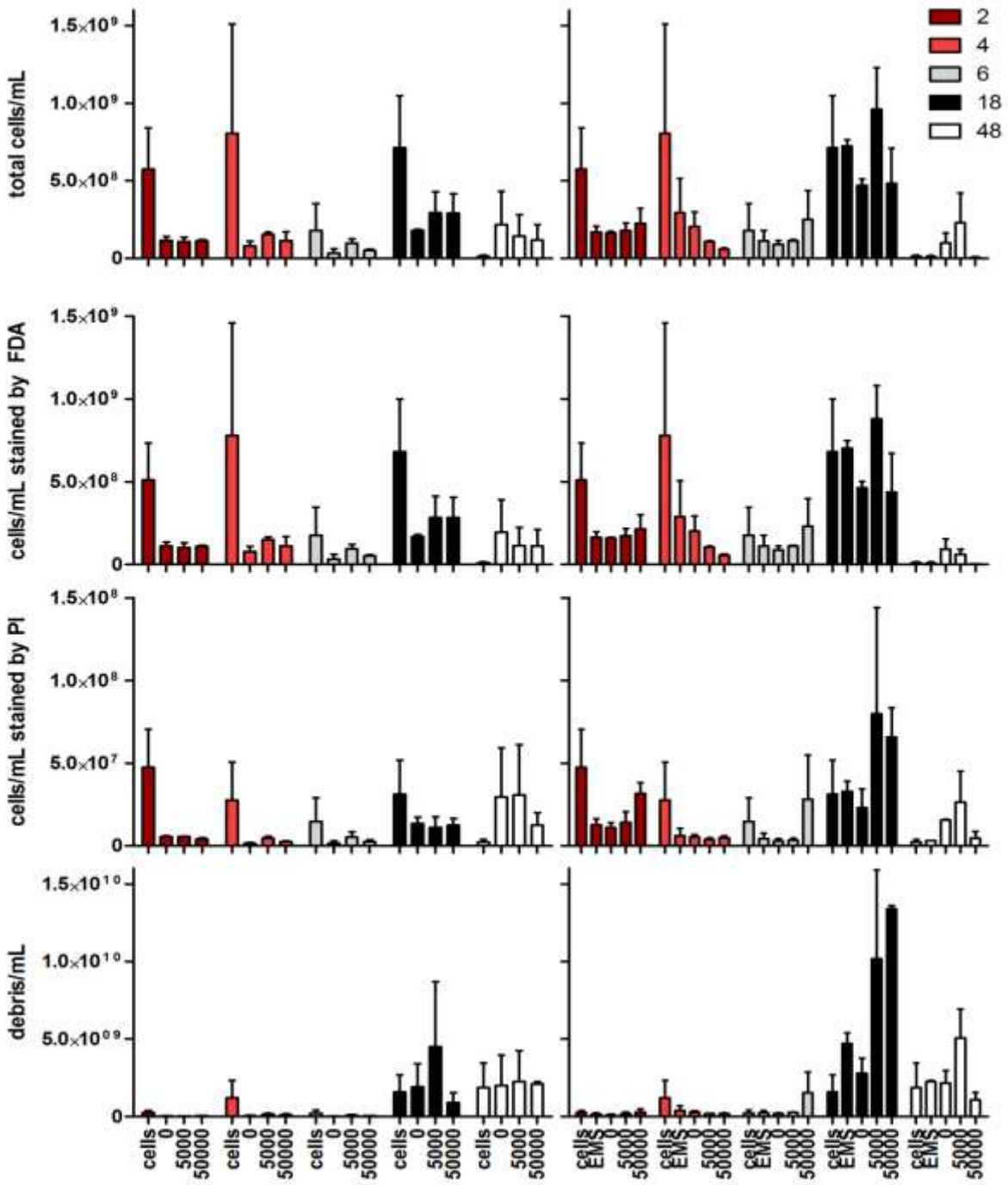

Fig A.3: Isolated leukocytes from rainbow trout blood exposed to either no treatment, or varying doses of BaP in DMSO. The first column represents cultures with no extracellular metabolizing system, while the second contains EMS in all but the untreated group. Total cells were determined using CountBrite beads and different groups were differentiated using FL2, FL4 and SSC of Cell Quest software.