Endocrine-Immune Interactions and the Immunotoxicity of Endocrine Disrupting Chemicals in Rainbow Trout (*Oncorhynchus mykiss*)

by

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Abstract

The immune system of fish provides a critical barrier to protect against infectious diseases caused by pathogens in the environment. It is known that the endocrine system can modulate immune function in fish, as can exposure to xenobiotics present in the aquatic environment. The overarching objective of the five studies presented here was to investigate the effects of endocrine hormones (17β-estradiol (E2), cortisol) and endocrine disrupting pesticides and formulants (nonylphenol, atrazine, permethrin, piperonyl butoxide, chlorothalonil, pentachlorophenol and cypermethrin) on immune function in juvenile rainbow trout (*Oncorhynchus mykiss*) using comprehensive panels of immune function assays at multiple levels of biological organization. In addition, the use of functional assays in combination with genomic techniques (quantitative polymerase chain reaction, QPCR; microarrays) was intended to elucidate some of the mechanisms through which immunotoxic effects may be occurring. Following confirmation that all 4 forms of rainbow trout estrogen receptor (ERs) mRNA was present in leukocytes, it was found that exposure of leukocytes to lipopolysaccharide (a mitogen that stimulates cellular proliferation) led to down-regulation of both ERα1 and ERα2, suggesting a role for the ER (and E2) in cell cycle control and proliferation. Exposure of fish to exogenous E2 (in water) led to several alterations in lymphocyte function, as well as differentially regulating the transcription of both ERα1 and ERα2 in leukocytes. It was also found that all of the pesticides or formulants tested, except cypermethrin, were immunotoxic or cytotoxic and may pose a risk to fish health. Nonylphenol and atrazine exposure increased disease susceptibility, and microarray analysis of liver from exposed fish showed that a broad range of immune-related functions were altered at the molecular level. Overall, the studies detailed here provide new insight (e.g. presence of ER mRNA in leukocytes, microarray data analysis revealing affected biological processes and pathways) into potential mechanisms underlying the immunomodulatory effects of both endocrine hormones and endocrine disrupting pesticides. Since many of the immunotoxic effects of pesticide or formulant exposure occurred at environmentally relevant concentrations, these chemicals may pose a risk to the immunological health of wild fish populations living in impacted aquatic environments.

Keywords: Immunotoxicity; pesticide; endocrine disrupting chemical; endocrine-immune interaction; 17β-estradiol; rainbow trout

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Chapter 1.

Introduction and Research Objectives

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The Immune System of Teleosts

The immune system of fish is a complex physiological system that has several main roles. The immune system protects the organism from infectious diseases caused by pathogens (bacteria, viruses and parasites) present in the external aquatic environment and also provides internal surveillance to identify and eliminate abnormal or non-self cells such as tumors (Magnadottir, 2010).

As in mammals, there are two main branches of the teleost immune system: the innate and adaptive immune systems (Magnadottir, 2010). The innate immune system includes all barriers (e.g. skin, mucous layers), anti-microbial proteins (lysozyme, complement) and cells (macrophages/monocytes, granulocytes, natural killer cells) that can respond to a wide range of potential pathogens in a non-specific manner (Ellis, 2001). For example, cells such as macrophages and granulocytes can engulf invading bacteria and subsequently generate reactive oxygen species during a respiratory burst, while enzymes such as lysozyme present in blood and mucus can attack and degrade the cell walls of bacteria. These activities are not specific to a particular pathogen and the cells or enzymes can respond to any pathogen they come into contact with.

In contrast, the adaptive immune system, consisting mainly of B and T lymphocytes, is responsible for generating a more specific and potent response to pathogens (e.g. generation of pathogen-specific antibodies, activation of cytotoxic T cells), with long-lasting memory and the ability to respond more quickly to a pathogen on subsequent exposures (Magnadottir, 2010). The adaptive immune response can increase the activity and efficiency of the innate immune response by producing antibodies that can opsonize pathogens, making them more visible targets for phagocytosis and activation of the complement cascade. In addition, the innate immune system plays a role in the stimulation of adaptive immune responses since macrophages/monocytes can act as antigen presenting cells for activation of T cells by displaying fragments of the destroyed pathogens on their cell surfaces in association with major histocompatibility complexes (MHC). However, given the temperature dependence of immune responses, adaptive immunity may be less important responding

to and eliminating pathogens than the innate immune system, at least in cold water fish such as salmonids (Le Morvan et al., 1998; Bowden et al., 2007).

Immunotoxicology

Aquatic environments can be contaminated by chemicals, either through direct discharges, run-off or atmospheric deposition. Both anthropogenic and natural chemicals that enter aquatic ecosystems can either remain in the water column or partition to other compartments such as sediment, air, or organisms (bioaccumulation/bioconcentration), depending largely on the physico-chemical properties of the xenobiotic (Erickson et al., 2008). Sampling of sediments and water in various areas of British Columbia has revealed contamination by various chemical classes including polycyclic aromatic hydrocarbons [PAHs; (Yunker et al., 2002)], polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers [PBDEs; (Johannessen et al., 2008)], pesticides (Harris et al., 2008; Environment Canada, 2011), and surfactants (Sekela et al., 1999) that may pose a risk to the health of fish and other organisms that inhabit or pass through these contaminated environments.

Of particular interest is the ability of xenobiotics to modulate the immune response in fish. Immunotoxicity can be defined as the alteration of immune responses as a consequence of exposure to exogenous chemicals and can result in either up-regulation (immunostimulation) or down-regulation (immunosuppression) of functions that can be measured at the molecular, biochemical, cellular or whole organism levels.

Impairment in immune function following exposure to xenobiotics has been measured in a number of ways. Because of the complexity of the immune response, it is generally agreed that the most definitive measure of a chemical's immunotoxicity is in the use of a host resistance challenge assay, where the chemical-exposed fish is subsequently or concurrently exposed to a pathogen and mortality or morbidity is measured (Arkoosh, 2005). While this assay may have the most biological relevance (i.e. it directly measures mortality due to infectious disease), it is not always performed for logistical (e.g. requires large numbers of fish, biosafety/containment issues, requirement for expertise in microbiological techniques, cost) and ethical reasons. More

frequently, measurements of innate immune responses are done to assess immunotoxicity (Kollner et al., 2002). Commonly used assessments include assays of lysozyme and complement activity or measurement of cellular function such as phagocytosis, respiratory burst, and natural killer cell activity. Occasionally, assessment of adaptive immune function has been done by measuring total antibody levels, non-specific lymphocyte proliferation (using polyclonal mitogens like lipopolysaccharide or Concanavalin A) or pre-stimulating the immune system with vaccines or other specific antigens (e.g. sheep red blood cells) and subsequently measuring adaptive immune response such as production and secretion of antigen-specific antibodies (Kollner et al., 2002).

Using these assays, a wide range of chemicals have been identified as immunotoxic in fish including PAHs (Reynaud and Deschaux, 2006), PCBs (Iwanowicz et al., 2005), PBDEs (Arkoosh et al., 2010), metals (Sanchez-Dardon et al., 1999), surfactants (Hebert et al., 2009) and pesticides (Dunier and Siwicki, 1993; Fatima et al., 2007). Complex mixtures such as pulp and paper mill effluent (Aaltonen et al., 2000) and sewage treatment plant effluent (Salo et al., 2007) have also been associated with adverse effects on the fish immune system.

Given that fish are frequently in contact with pathogens in their natural environments, proper function of the different components of the immune system is of critical importance to the health of the fish (Arkoosh et al., 2004). Although it can be difficult to conduct controlled *in situ* immunotoxicological studies of fish such as salmonids, several studies have demonstrated immune function impairment of fish living in contaminated environments compared to less contaminated reference sites. For example, English sole (*Pleuronectes vetulus*) collected from areas with high sediment contamination (PAHs, PCBs) had impaired lymphoproliferation responses (Arkoosh et al., 1996), mummichog (*Fundulus heteroclitus*) living downstream of pulp and paper mills had altered phagocytic activity (Fournier et al., 1998), and juvenile Chinook salmon (*Oncorhynchus tshawytshca*) that migrated through a more contaminated estuary (PAHs, PCBs) had increased mortality following exposure to the estuarine pathogen that causes vibriosis, *Listonella anguillarum* (Arkoosh et al., 1998). Alteration of cellular, biochemical or barrier defenses can lead to changes in disease susceptibility and population modeling has demonstrated that increased susceptibility to infectious

diseases due to immunotoxicity can negatively impact population dynamics and stability (Spromberg and Meador, 2006).

Endocrine-Immune System Interactions

Fish immune system function can be modulated by a wide range of abiotic environmental factors such as temperature, photoperiod, season, salinity, pH and water oxygen content (Bowden, 2008). Other factors such as reproductive maturity and sex (Pickering and Christie, 1980), stress (Maule et al., 1989) and social confrontation/status (Yada and Nakanishi, 2002) have also been shown to influence immune function.

The role of the endocrine system in modulating immune system function in teleosts has been recognized for years (Harris and Bird, 2000; Yada and Nakanishi, 2002). Hormones such as cortisol, androgens, estrogens and growth hormone have all been shown to alter function of various components of the immune system. The immunosuppressive effects of some of these hormones (e.g. cortisol, 17β-estradiol) have been demonstrated using host resistance challenges in the lab (Pickering and Pottinger, 1989; Wang and Belosevic, 1994; Wenger et al., 2011) and it has been noted that spawning salmonids, with their high levels of circulating steroid hormones (cortisol, androgen, estrogen), are more susceptible to infections and mortality from infectious diseases (Pickering and Christie, 1980; Currie and Woo, 2007). While it is agreed that the endocrine system modulates immune function that ultimately manifests as changes in susceptibility to infectious diseases, there is surprisingly little agreement in the literature regarding the effects of hormones (particularly estrogens) at the cellular level (see Chapter 3).

In order for hormones to exert such effects a signal transduction system must be available in target cells. For steroid hormones, traditional or classical signaling occurs through interaction of the hormone with intracellular receptors present in the cytoplasm of target cells which results in the translocation of the receptor-ligand complex to the nucleus, where it can act as a transcription factor for genes containing the specific hormone (glucocorticoid, androgen, estrogen) response element (Kime, 1998). Generally, the effects associated with this type of classical signaling pathway take longer

to become apparent (often >24 hours) and are more long lasting since changes are made in protein production. In addition, for some steroid hormones, receptors located on the cell surface that are associated with second messenger signaling systems have been identified (Thomas et al., 2010). While in some cases, activation of these receptors may indirectly lead to changes in transcription (Moriarty et al., 2006), the main effects are more rapid (within minutes to 1 hour) and do not rely on transcription.

Some hormone receptors have been identified in purified leukocytes of salmonids (Table 1-1), suggesting that these cells may be direct targets for endocrine-mediated modulation. In addition, hormone receptor transcripts have been identified in immune tissue homogenates (e.g. head kidney, spleen), particularly for estrogen receptors (Table 1-1). While these findings do suggest that the hormone receptors may be present in leukocytes, whole tissue homogenates are mixtures of multiple cell types and there is no definitive means to determine whether the transcripts are actually present in all cells or only in a sub-population of cells. This is particularly true for tissue homogenates of the head kidney, which consist of leukocytes (immune system) and supportive cell types (connective tissue, red blood cells, etc.), as well as several cell types responsible for producing hormones such as inter-renal cells (cortisol) and chromaffin cells (catecholamines) that would be expected to express hormone receptors as part of endocrine feedback mechanisms. While it seems clear that purified teleost leukocytes possess glucocorticoid and androgen receptors, the evidence for the presence of estrogen receptors in these cells is not as clear.

Endocrine Disrupting Chemicals

Kavlock et al. (1996) defined an endocrine disrupting chemical (EDC) as “an exogenous agent that interferes with the production, release, transport, metabolism, biding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes”. The adverse health effects associated with exposure to EDCs was first recognized in the early 1990’s with the acknowledgement that these chemicals in the environment may be affecting both humans and wildlife by altering physiological processes that are regulated by hormones such as development, growth, and reproduction (Colborn et al., 1993).

Concerns related to fish health have been increasing since reports in the late-1990's of reproductive impairment in fish living in impacted areas downstream of sewage treatment plants (Jobling et al., 1998; Batty and Lim, 1999).

Much of the research related to EDC toxicity has been justifiably focused on those endpoints known to be directly mediated by hormones including thyroid function (e.g. plasma thyroid hormone concentrations, thyroid structure, tumors, growth or metamorphosis), developmental processes (e.g. egg mortality, developmental abnormalities or deformities), or reproductive endpoints (e.g. reproductive success, gonadal morphology, feminization of males/masculinization of females, hormone concentrations, biomarkers such as vitellogenin and vitelline envelope proteins) (Leatherland, 1993; Kime, 1998; McMaster, 2001). However, even though endocrine-immune interaction in fish is well accepted, there has been very little research on the effects of EDCs on immune function in these organisms.

The mechanisms of immunotoxicity are not well understood in fish, although two very general pathways may be possible (Ladics and Woolhiser, 2006)(Figure 1-1). EDCs (and other xenobiotics) may exert effects directly on the immune system by directly affecting cellular functions, cytokine production, structure of lymphoid organs or expression of receptors and signaling molecules. To have direct effects requires the target cell or organ to have hormone receptors which, as discussed in the previous section, immune system tissues and cells may express. Alternatively, EDCs may also exert their effects indirectly by altering endocrine system function, which could affect immune system function as well due to the intricate signaling relationships between the two physiological systems. It may be difficult or impossible to unravel whether immunotoxic effects are direct or indirect, but ultimately both pathways may lead to immunostimulation or immunosuppression.

General Objectives of This Research

The main focus of this body of research was to investigate the immunotoxicity of pesticides and formulants (the inert ingredients included in pesticide formulations) currently in use in British Columbia, particularly those with endocrine disrupting activities.

Chemicals with various structures, physico-chemical properties and having different endocrine targets or mechanisms were investigated (Table 1-2). In addition, the effects of two hormones (cortisol and 17β-estradiol) on immune function were also investigated.

Rainbow trout (*Oncorhynchus mykiss*) were selected as a representative salmonid species for these studies. Rainbow trout are a commonly used model fish species in toxicology and are readily grown in the laboratory setting. Rainbow trout are found throughout the world and they are closely related to the other Pacific salmonid species (*Oncorhynchus* sp.), which are all important ecologically, culturally and economically in British Columbia.

The overarching hypothesis for this body of research was that estrogens and endocrine disrupting chemicals, in addition to their effects on reproductive endpoints, would also alter immune function. There were four general objectives for the research conducted in this thesis:

1. To define the functional immunological impairments at multiple levels of biological organization in fish exposed to different endocrine disrupting pesticides or formulants currently in use (Chapters 2, 4, 5, and 6);
2. To further define and clarify the immunological alterations resulting from exposure to 17β-estradiol (Chapters 2 and 3);
3. To confirm the presence of estrogen receptors in leukocytes purified from multiple tissues in rainbow trout (Chapters 2 and 3);
4. To investigate immunotoxicological mechanisms underlying the altered functional and biological effects of hormones or endocrine disrupting pesticides using molecular technologies (Chapter 2, 3, and 5).

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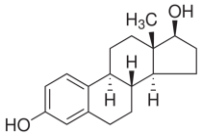
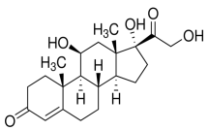
Tables

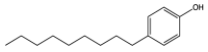
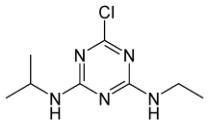
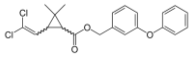
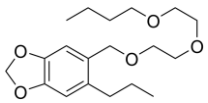
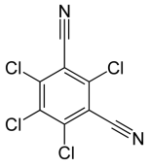
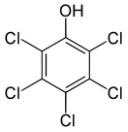
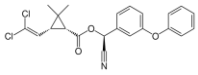
Table 1-1. Various steroid hormone receptors have been detected in leukocytes or immune system tissues of teleosts.

Hormone Receptor	Species	Tissue	Assay methodology	Reference
Glucocorticoid	Carp (*Cyprinus carpio*)	Peripheral blood leukocytes	Receptor binding assays (cortisol)	(Weyts et al., 1998)
Glucocorticoid (membrane receptor)	spotted murrel (*Channa punctatus*)	purified splenic leukocytes	Functional assays with rapid onset of effects (phagocytosis impairment)	(Roy and Rai, 2009)
Androgen	Rainbow trout (*Oncorhynchus mykiss*)	purified head kidney leukocytes	Receptor binding assays (testosterone)	(Slater et al., 1995)
Estrogen	Rainbow trout	head kidney and spleen (whole tissue homogenates)	QPCR (mRNA transcripts)	(Nagler et al., 2007)
	Rainbow trout	head kidney, spleen and thymus	QPCR (mRNA transcripts), immunohistochemistry	(Nakayama et al., 2008)*
	Channel catfish (*Ictalurus punctatus*)	purified head kidney and splenic leukocytes	Western blotting, not clearly specified	(Iwanowicz and Ottinger, 2008)*

* Refers to a conference abstract (Nakayama et al., 2008) or a book chapter (Iwanowicz and Ottinger, 2009), thus details regarding methodologies and cell purity are not provided.

Table 1-2. General information and structures for all chemicals and hormones investigated in this body of research.

Chemical or Hormone	CAS No.	log K_{ow}	Chemical class	Mechanism of endocrine disruption[1]	Chapter in thesis
 17β-estradiol	50-28-2	3.94[1]	hormone	Estrogenic	2 and 3
 Cortisol	50-23-7	1.5[2]	hormone	Glucocorticoid	2

 Nonylphenol	84852-15-3 104-40-5	3.8 - 4.8	alkylphenol, formulant	Estrogenic, anti-androgenic, thyroid hormone disruption[3]	2 and 5
 Atrazine	1912-24-9	2.8	triazine herbicide	Anti-androgenic, estrogenic, alters leutinizing hormone/prolactin, glucocorticoid disruption[3]	4 and 5
 Permethrin	52645-53-1	5.9	pyrethroid insecticide	Weakly estrogenic[3]	4
 Piperonyl butoxide	51-03-6	4.8	synergist, formulant	Listed as an EDC, but no evidence located[3]	4
 Chlorothalonil	1897-45-6	2.6 - 4.3	chloronitrile fungicide	androgenic[3]	6
 Pentachlorophenol	87-86-5	5.2	organochlorine fungicide	weakly estrogenic, androgenic[3]	6
 Cypermethrin	52315-07-8	6.1	pyrethroid insecticide	weakly estrogenic[3]	6

[1] Ying et al. (2002), [2] Cichna et al. (1995), [3] McKinlay et al. (2008)

Figure

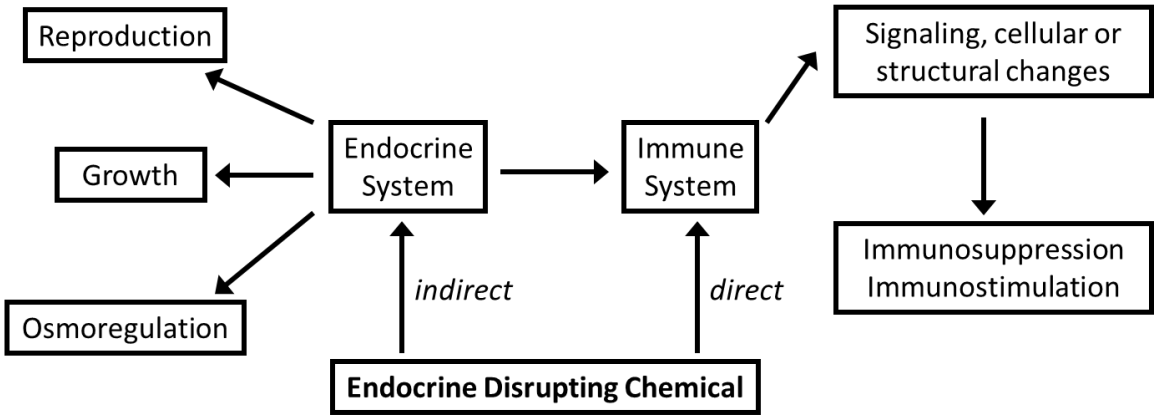


Figure 1-1. Schematic showing the general relationship between the endocrine and immune systems, the direct and indirect pathways through which endocrine disrupting chemicals can exert immunotoxic effects and the ultimate outcome of immunomodulation (alterations leading to immunosuppression or immunostimulation).

Chapter 2.

The effects of an *in vitro* exposure to 17β-estradiol and nonylphenol on rainbow trout (*Oncorhynchus mykiss*) peripheral blood leukocytes

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Abstract

While xenoestrogens are routinely detected in the aquatic environment, there is little understanding of the immunotoxicological consequences of exposure to these chemicals in fish, or of the mechanisms through which these effects are mediated. This study was conducted to determine if estrogen receptors (ERs) are present in fish leukocytes and to characterize the effects of 17β-estradiol (E2) and the xenoestrogen nonylphenol (NP) on immune system endpoints in rainbow trout (*Oncorhynchus mykiss*). QPCR was used to confirm that freshly isolated peripheral blood leukocytes (PBLs) express ERs. Following 96-h incubations with E2 or NP (1 nM to 10 μM), PBL ER transcription was again examined using QPCR and lipopolysaccharide (LPS)-stimulated proliferation was assessed using flow cytometry. While the transcription of all four forms of rainbow trout ER was unaffected by treatment with E2 or NP, transcription of ERα1 and ERα2 was down-regulated following LPS stimulation. Both E2 and NP, at concentrations of ≥100 nM and 10 nM respectively, suppressed leukocyte proliferation. This first report of ERs in rainbow trout PBLs suggests a mechanism through which E2 and other xenoestrogens can modulate immune function. These results highlight the potential for xenoestrogens to impact host resistance to pathogens in wild fish populations

Keywords

Estradiol; estrogen receptor; immune system; nonylphenol; teleost; xenoestrogen.

Introduction

Endocrine disrupting chemicals (EDCs), particularly estrogens and xenobiotics with estrogenic properties (xenoestrogens), have been documented among pesticides and their formulants, pharmaceutical and personal care products, veterinary medicines, plasticizers and natural plant products (Hewitt and Servos, 2001; Kolpin et al.,2002; Ingerslev et al., 2003). EDCs have been implicated in the complete or partial feminization of male fish, induction of vitellogenin or other egg-related proteins in male fish and juveniles, decreases in gamete quality or quantity (fecundity) and changes in sexual and mating behaviors (Rempel and Schlenk, 2008). Widespread feminization of fish in the United Kingdom has been attributed to the estrogenic properties of municipal sewage effluent (Jobling et al., 1998; Rodgers-Gray et al., 2000).

In addition to their demonstrated effects on reproduction, estrogens are also known to exert immunomodulatory effects in mammals and fish, and can suppress or down-regulate immune responses. It has been well established that, in fish, as in mammals, the endocrine system plays a role in modulating both the innate and adaptive immune system responses (Harris and Bird, 2000; Yada and Nakanishi, 2002). This interaction is partly mediated by interactions between circulating hormones and their receptors found in leukocytes. Receptors for many hormones including cortisol, androgens, and growth hormone, have been identified in fish leukocytes or tissues of the immune system (Slater et al., 1995; Weyts et al., 1998; Very et al., 2005). Although estrogen receptors (ERs) have been detected in mammalian leukocytes, they have not been fully described in teleost leukocytes (Sakazaki et al., 2002; Phiel et al., 2005). Most reports on the presence of ERs in teleost leukocytes are incomplete, inconclusive or have only considered whole tissue homogenates containing multiple cell types (Patino and Maule, 1997; Xia et al., 2000; Wang et al., 2005; Nakayama et al., 2008; Iwanowicz and Ottinger, 2009; Casanova-Nakayama et al., 2011).

Recently, it has been shown that there are four different forms of ER in rainbow trout (Nagler et al., 2007). Transcripts of ERα1, ERα2, ERβ1 and ERβ2 are all present to varying degrees in whole tissue homogenates from the head kidney and spleen, which are primary organs of the immune system. However, both head kidney and

spleen contain a number of different cell types so it is not clear whether ERs are actually present in leukocytes or the other tissues isolated from these organs.

While the downstream pathways and molecular mechanisms of action of hormones on immunomodulation remain poorly understood in teleosts, the physiological effects of a number of hormones on the immune system have been established. Studies with several teleost species have found that exposure to estrogens is generally associated with changes in leukocyte differential, suppression of phagocytosis, reduction in reactive oxygen species production, and decreased leukocyte mitogenesis (Wang and Belosevic, 1995; Law et al., 2001; Watanuki et al., 2002; Thilagam et al., 2009). Endocrine-associated changes to immune function have implications for host resistance, as elevated estradiol levels have led to increased disease susceptibility and disease-induced mortality (Wang and Belosevic, 1994; Wenger et al., 2011).

Nonylphenol (NP) is a xenoestrogen often identified in environmental samples and has been associated with sewage inputs, pesticide application and other anthropogenic discharges (Fairchild et al., 1999; Sekela et al., 1999; Soares et al., 2008). It is derived from the environmental degradation of nonylphenol polyethoxylates (NPEs), which are non-ionic surfactants used in a variety of consumer, industrial and agricultural products (Vazquez-Duhalt et al., 2005). Nonylphenol affects fish reproduction and is linked to fish feminization and abnormal vitellogenin production (Lech et al., 1996; Lahnsteiner et al., 2005; Soares et al., 2008). Nonylphenol interacts with the fish estrogen receptor (ER), albeit with lower affinity than the natural estrogen ligands, and can also disrupt additional important steps in estrogen regulation, metabolism and clearance, which may contribute to estrogenic effects (Rempel and Schlenk, 2008). While there are several studies on the immunomodulatory effects of E2 in fish, little information is available on the effects induced by NP exposure.

In the current study, QPCR was used to first determine if purified leukocytes from peripheral blood possess transcripts for the four ERs described in rainbow trout, and whether ER transcription is affected by exposure to either its natural ligand (E2) or a model xenoestrogen (NP). In addition, the *in vitro* effects of 17β-estradiol and NP on LPS-stimulated lymphocyte proliferation, an indication of B cell mitogenesis, were investigated. It was hypothesized that if ERs were indeed present in fish leukocytes,

then treatment with E2 or NP would exert immunotoxic effects as revealed through cell viability or the proliferative responses of leukocytes.

Materials and Methods

Fish

Mixed male and female rainbow trout (*Oncorhynchus mykiss*) with an average mass of 147.3 ± 5.9 g (mean ± SEM) were obtained from Miracle Springs Hatchery (Mission, BC, Canada). They were maintained in 150 L fiberglass tanks with continuously flowing water (>1 L/kg/min) at 11.0°C ± 0.7°C (mean ± SEM) and a photoperiod of 12:12 (light:dark) for at least 2 weeks prior to use in experiments and throughout the duration of the experiments. Fish were fed daily with commercial trout feed (Ewos, Surrey, BC, Canada). All work done with animals was done in accordance with a permit issued by the Simon Fraser University Animal Care Committee and in compliance with Canadian Council for Animal Care guidelines.

Chemicals

Charcoal-stripped fetal bovine serum (Gibco) was obtained from Invitrogen (Portland, OR, USA). All other chemicals were obtained from Sigma (Oakville, ON, Canada) unless otherwise indicated. Test chemicals used in the experiments were analytical grade of >95% purity and were solubilized in anhydrous ethanol (99.9%). Chemical stock solutions were prepared and aliquoted for storage in the freezer at −20 °C.

Cell isolation and in vitro culture conditions

Rainbow trout were euthanized with 0.3 g/L tricaine methansulfonate (MS222, Argent Chemical Laboratories, Redmond, WA, USA) buffered with 0.3 g/L sodium bicarbonate. Blood was aseptically collected into heparinized syringes from the caudal vessels and stored on ice for less than 30 min prior to isolation of peripheral blood leukocytes (PBLs).

Blood was diluted 1:1 with supplemented HBSS (sHBSS; 15 mM HEPES, 10% FBS, 10 U/mL heparin and 1% penicillin/streptomycin) and 1 mL transferred to several 15 mL conical Falcon tubes. PBLs were obtained using the hypotonic lysis method (Crippen et al., 2001). Briefly, 9 mL of distilled water was added and tubes were gently mixed for 30 s. Red blood cell lysis was halted by addition of 1 mL of sterile 10× PBS (0.1 M) solution. Gross cellular debris was removed, cells were centrifuged at 400 *g* for 5 min at 4 °C and then washed twice further (400 *g*, 5 min, 4 °C) in sHBSS. PBLs were resuspended in 2 mL of sHBSS for counting and viability assessment using trypan blue exclusion. Cells were then centrifuged as above and resuspended in supplemented RPMI (sRPMI; 15 mM HEPES, 10% FBS and 1% penicillin/streptomycin) at 5×10^6 viable cells/mL.

Cells were kept on ice throughout the isolation procedures. To maintain aseptic culture conditions, all solutions used in blood collection and cell culture were sterilized by syringe-filtering with a 0.22 µM filter and all disposable/consumable materials used were sterile. Cell isolations and culture plating were done in a laminar flow hood.

In vitro exposures to 17β-estradiol or nonylphenol

For each fish (n = 6 for the QPCR ER transcription experiment, n = 12 fish per test chemical for the proliferation/viability experiment), 5×10^5 viable PBLs were seeded into wells of a round bottomed culture plate. Cells from each fish received all possible treatments in a given experiment. To assess the effects of the test chemicals on ER transcription, 17-β estradiol (E2) or nonylphenol (NP) in sRPMI were added at a single concentration (10 µM), while for the proliferation and viability assays 5 concentrations (1 nM, 10 nM, 100 nM, 1 µM and 10 µM) were used. For the negative control cells (untreated), an equivalent amount of sRPMI was used in place of E2 or NP. Cortisol exposure using the same range of concentrations and exposure conditions was used as a positive control to ensure that viability and proliferation assays performed as expected (Milston et al., 2003). The solvent concentration in all treatment wells was 0.5% v/v anhydrous ethanol. Solvent was not added to the control wells since in a preliminary experiment (n = 6 fish) this concentration did not affect cell viability or proliferation (Table S2-1 in supplemental data).

In addition to the chemical treatment, half of the cells received 100 µg/mL of phenol-purified *Escherichia coli* O111:B4 lipopolysaccharide (LPS) in sRPMI to stimulate B cell proliferation (stimulated cells) while the other half received an equivalent volume of sRPMI (unstimulated cells). Total well volumes were always 200 µL and all treatments were performed in duplicate. Cells were cultured for 96 h in a humidified incubator at 15 °C and were then assessed for ER expression by QPCR or assayed for leukocyte viability and proliferation.

Estrogen receptor transcription assessment using QPCR

To determine if ERs are present in resting, purified leukocytes, unstimulated PBLs (n = 6 fish) were assessed for ER transcription on Day 0 prior to culturing. To evaluate treatment effects on ER transcription, LPS-stimulated PBLs from the same fish were assessed again after the 4 d incubation of the control and treated cells (10 µM E2 and NP). An unstimulated control group was also included in this assessment. At the end of the 4 d incubation, cells were washed twice with 0.01 M phosphate buffered saline (PBS), transferred to RLT buffer (Qiagen, Mississauga, ON, Canada) with 1% v/v β-mercaptoethanol, thoroughly vortexed for 1 min and then stored at −80 °C.

Following thawing and another thorough 1 min vortexing of the samples, total RNA was extracted using the RNeasy kit (Qiagen), following the manufacturer's instructions. Isolated RNA was precipitated overnight with 0.3 M sodium acetate and ice-cold 98% ethanol. Following several wash steps with 70% ethanol, RNA was resuspended in RNase-free water (Ambion, Austin, TX, USA) and 1 µL aliquots in duplicate were quantified using nanodrop spectrophotometry (ND1000 Spectrophotometer, ThermoScientific, Wilmington, DE, USA). Subsequently, 1 µg of total RNA was used to produce cDNA using a QuantiTect Reverse Transcription kit (Qiagen), following the manufacturer's instructions. All cDNA reactions were then diluted 15-fold prior to QPCR amplification.

The primers for ERs used in the QPCR reactions were exactly as reported by Nagler et al. (2007). The primers for L8 were as reported in Osachoff (2008) (forward primer CAGGGGACAGAGAAAAGGTG, reverse primer TGAGCTTTCTTGCCACAG). QPCR was carried out on a Stratagene Mx3000P series machine, using 96 well plates

with 2 µL of 15-fold diluted cDNA and 13 µL of mastermix (15 µL total reaction volume). Mastermixes for each gene contained the forward and reverse primers (10 pmol/reaction), 7.5 µL/reaction iTaq supermix (iTaq SYBR Green Supermix with ROX, Biorad, Hercules, CA, USA) and RNase-free water for the remaining volume. All QPCR reactions for each gene were performed in quadruplicate runs. A no template control (NTC) was prepared for each primer pair in the same manner except 2 µL of RNase-free water was used in place of a cDNA sample. All amplification reactions were done using the following instrument settings: 95 °C for 1 min followed by 40 cycles of 95 °C for 15 s, 58 °C for 25 s and 72 °C for 35 s, except for 1 replicate plate per gene where dissociation curves were generated to confirm reaction specificity. Sequencing of the 4 ER amplicons was done as part of another study and will be published separately.

QPCR data was analyzed using the MxPro software (version 4.10, Stratagene). Following the amplification reactions, data was obtained as C_t values which were averaged for each sample and converted to copy numbers using standard curves generated for each target gene. In the event that a reaction Ct value was greater than the NTC C_t value, was less than 2 C_t values away from the NTC C_t, or the calculated copy number was less than 1, indicating that the transcript was either not present or present at very low levels, a copy number of 1 was assigned for statistical purposes. L8 was found to be generally invariant and was therefore used to generate a global normalization factor, which was calculated as the average L8 copy number for every fish in the experiment/L8 copy number for each individual fish. This individual L8 normalization factor was then applied to each sample to generate an L8 normalized copy number for each of the 4 ERs.

Viability assay

Viability was assessed with propidium iodide (PI) exclusion using flow cytometry (Scharsack et al., 2001). Propidium iodide is a fluorescent dye that cannot enter viable cells with intact membranes, but can enter and stain DNA in cells with leaky, more permeable membranes, which are generally considered to be necrotic. After 4 d of incubation (Section 2.4), culture plates were put on ice for 10 min prior to gentle agitation by pipetting to ensure maximal detachment of cells. The entire contents from each well were then transferred to 5 mL glass tubes and the total volume topped up to 980 µL. To

assess viability, 20 µL of 100 µg/mL PI was added to each tube just prior to sample collection on the flow cytometer (BD FACSAria, Becton- Dickinson). A total of 10,000 cells were collected per sample. Using cells prior to PI addition (negative control), a gate was established for PI-positive cells based on the PI fluorescence histogram and the same gate was used throughout the experiments. Viable cells were calculated as 100% − % PI positive cells − % PI positive cells in the negative control (background fluorescence).

Proliferation assay

Proliferation was assessed with flow cytometry based on forward (FSC) and side scattering (SSC) properties of PBLs (Scharsack et al., 2000; Milston et al., 2003). This assay provides an indirect measure of proliferation by assessing changes in cell size and complexity that occur during lymphocyte activation and proliferation. After 4 d of incubation, culture plates were put on ice for 10 min prior to gentle agitation by pipetting to ensure maximal detachment of cells. Well contents were then transferred to 5 mL glass tubes and 10,000 events were collected per sample using a flow cytometer. Using unstimulated, untreated control cells, a gate excluding non-viable cells and debris was created on the dot plot of FSC/SSC. Within this gate, additional gates for “resting cells” were then drawn around the lymphocyte population (low FSC/low SSC) and for “blasting cells” adjacent to this population (higher FSC/higher SSC). When resting lymphocytes are stimulated with LPS to proliferate, the percent of cells in the proliferating cell gate significantly increases as cells begin dividing and become more complex and larger and the percentage of cells in the ‘blasting cell’ gate was used as a measure of proliferation (see Figure S2-1 in supplemental data). Identical gating was used for all samples throughout each experiment.

Statistics

Since cells from a single fish received all possible treatments and samples are therefore not independent, the experiment was treated as a repeated measures design. For ER transcription assessment following *in vitro* chemical exposures, L8 normalized copy number data was subjected to two-tailed paired (matched) t-tests to determine if

there were differences ($p < 0.05$) between LPS-stimulated control and E2 or NP treated cells for each of the four ER forms.

For comparisons with more than 2 groups, the fit model MANOVA platform in JMP (Version 7.0.2, SAS Institute) was used. Percentage data from the viability and proliferation assays were arcsine square root transformed prior to analysis. When significant differences between control and treatments were detected ($p < 0.05$), the F test statistic within subjects was used to determine which treatments were significantly different.

Results

QPCR for ER expression in rainbow trout PBLs

All four forms of the ER identified in rainbow trout were detected in freshly isolated PBLs (Day 0), prior to incubation in cultures (Fig. 2-1A and B). ERα2 was the most highly expressed, while ERβ1 and ERβ2 had much lower expression levels.

Interestingly, transcription of both ERα1 and ERα2 genes decreased when comparing the relative expression levels for these genes in the unstimulated control and the LPS-stimulated control PBLs after 4 d (Fig. 2-1A). ERβ1 and ERβ2 transcription did not differ between cells at Day 0 or the unstimulated and the LPS-stimulated control cells at Day 4 (Fig. 2-1B, $p = 0.0882$ and 0.0863 respectively).

Estrogen receptor transcription was not altered by 4 d incubation with either E2 or NP (Fig. 2-2), although there was considerable inter-individual variability. Although ERα2, ERβ1 and ERβ2 transcription following E2 exposure appeared to be substantially decreased relative to the control, these changes were not statistically significant (p=0.1134, 0.0586 and 0.0762, respectively).

Effects of treatment on PBL viability and proliferation

The overall viability of cells from all experiments on Day 0 prior to incubation with test chemicals was 98.8% ± 0.2% (mean ± SEM). In order to confirm that the viability and proliferation assays performed as expected, cortisol-exposed PBLs served as a

positive control (Milston et al., 2003). As anticipated, cortisol decreased the viability of both unstimulated and LPS-stimulated PBLs (Fig. 2-3A) and also markedly suppressed LPS-stimulated proliferation at all concentrations tested (Fig. 2-3B).

Following the 4 d *in vitro* incubation, E2 decreased the viability of unstimulated leukocytes at concentrations greater than 10 nM in a concentration-dependent manner (Table 2-1). Viability of E2-treated LPS-stimulated leukocytes produced a U-shaped concentration response, with decreased viability at exposure concentrations from 10 nM to 1 μM but no effects at either 1 nM or 10 μM (Table 2-1). In contrast, incubation with NP had no effect on unstimulated PBL viability (p = 0.8873) and resulted in decreased viability in LPS-stimulated cells only at the highest exposure concentration of 10 μM (Table 2-1).

In unstimulated lymphocytes, incubation with E2 or NP had no effect on the number of cells in the proliferating cell gate (Fig. 2-4A and 2-4B, p = 0.9232 and 0.1989 respectively). However, when PBLs were stimulated with LPS both E2 and NP reduced proliferation at concentrations greater than 100 nM and 10 nM respectively (Fig. 2-4A and 2-4B). Raw data for all viability and proliferation assays is provided as supplementary data (Tables S2-2 – S2-7).

Discussion

The current study presents the first report of the expression of four ER transcripts in purified PBLs of rainbow trout. This is of interest because these receptors represent a potentially important pathway through which leukocytes may be modulated by estrogens or xenoestrogens. The estrogenic effects of EDCs on reproductive endpoints in fish have long been a major concern in impacted river systems (Jobling et al., 1998; Mills and Chichester, 2005). More recent interest in endocrine-immune interactions in organism physiology has led to the study of EDCs for their immunotoxic potential (Milla et al., 2011).

Our study demonstrates that both E2 and the xenoestrogen NP exert suppressive effects on B cell proliferation at similar concentrations *in vitro*. However, the results of cell viability following chemical exposures differed, suggesting that the

mechanisms of overt cellular toxicity may not be similar between these two compounds. Nonylphenol had little effect on leukocyte viability, indicating that cytotoxicity likely did not explain the reduced proliferative responses of these cells. Similarly, for E2, the decrease in viability was subtle and was not always associated with changes in proliferation; it is unlikely that cytotoxicity accounts for the more substantial decreases in proliferation observed at concentrations >100 nM.

Relatively few studies have examined the effects of NP on the fish immune system; however nonylphenol appears to have similar effects as E2, such as suppression of phagocytosis and changes in leukocyte numbers and subpopulations (Schwaiger et al., 2000; Gushiken et al., 2002). A recent *in vivo* investigation in rainbow trout found that chronic exposure to low levels of NP (1 µg/L, ~4.5 nM) suppressed B and T cell proliferation (Hebert et al., 2009). These findings are consistent with our *in vitro* study, where the effects on proliferation began to be detectable between 1 and 10 nM. Studies of the effects of E2 on fish B cell proliferation have had mixed results. Cook (1994) showed E2 increased B cell proliferation, while others, as in our study, found suppressed proliferation at concentrations ≥1 µM (Wang and Belosevic, 1994). These divergent findings are perhaps not surprising since the effects of E2 and NP, even in *in vitro* studies, vary as a function of factors including age, sex, reproductive stage, and species of fish, as well as with differences in assay methodologies, exposure durations and exposure routes.

Of note was the diminished ERα1 and ERα2 mRNA abundance following exposure to LPS, suggesting a role for the ER in cell cycle control. Similar down-regulation of ERs following LPS treatment has been observed in mammalian microglial and endothelial cells (Sierra et al., 2008; Holm et al., 2010). In mammals, LPS interacts with either Toll-like receptor-4 (TLR-4) or radioprotective 105 (RP105) on the cell surface of B lymphocytes. Following this interaction, a number of signal transduction pathways can be activated, culminating in transcription of cell cycle progression and anti-apoptotic genes mediated by nuclear factor kappa B (NF-κB) and activating protein-1 (AP-1) (Peng, 2005).

In contrast, the molecular signaling pathways underlying LPS-induced lymphocyte proliferation in teleosts are not well understood. In several fish species, the

TLR signaling pathway has been generally conserved in macrophages (Meijer et al., 2004; Rebl et al., 2010). However, recent studies with fish have found that, while NF-κB regulated genes are induced by LPS exposure, LPS signaling does not occur through TLR-4 and that the cells may actually be responding to the presence of peptidoglycan, a common contaminant of phenol-extracted LPS preparations (Purcell et al., 2006; Sullivan et al., 2009; MacKenzie et al., 2010). Taken together, these studies suggest that TLR-4 does not have the same function in fish as in mammals and that LPS signaling might either occur through a different TLR or other receptors such as β-integrins (Iliev et al., 2005). Regardless of the stimulatory component or the transduction pathway for LPS in teleost leukocytes, it is probable that the NF-κB pathway is ultimately activated.

In mammals, NF-κB mediated transcription can be inhibited by E2–ER complexes either directly through interference with NF-κB DNA binding, or indirectly through effects on the signaling cascade (Kalaitzidis and Gilmore, 2005). Nonylphenol has been shown to also interfere, in an ER-dependent manner, with NF-κB mediated gene transcription in LPS-stimulated mouse macrophages by decreasing the amount of NF-κB transcription complex present (You et al., 2002). This modulation of NFκB activity by liganded-ER complexes would ultimately lead to decreased transcription of anti-apoptotic and cell cycle promoting genes resulting in an overall decrease in cellular proliferation. From the current study, the down-regulation of ERα1 and ERα2 transcription following LPS treatment in the control cells suggests there is diminished potential for ER-driven inhibition of NF-κB mediated gene transcription. This should permit the PBLs to proliferate more readily under LPS stimulation.

However, even with lower ERα transcript levels, the decreased proliferation following E2 and NP treatment may be due to increased abundance of ligand (and thus ligand-bound ER) exerting inhibitory effects directly, via ER-mediated gene transcription, or indirectly, via modulation of NF-κB mediated gene transcription. Exposure to E2 has been shown to have suppressive effects on cell cycle regulation in leukocytes, at least in mammals, by suppressing transcription of bcl-2 and cyclin A, which are anti-apoptotic and important for cell cycle progression, respectively (Jenkins et al., 2001) . Similarly, NP exposure can induce apoptosis by decreasing bcl-2 abundance (Mao et al., 2008). Increased apoptosis, which would not have been detected in the current study since

propidium iodide can only stain necrotic cells that have disrupted membranes, could represent a potential mechanism underlying decreased proliferation, particularly if lymphocytes were specifically targeted. In addition to direct suppressive effects of E2 and NP on cell cycle, the greater availability of ER ligand could increase the repression of NFκB mediated gene transcription by ER, contributing to decreased leukocyte proliferation. However, given the very limited information available in fish, much more work is required in order to truly understand the changes observed in the current study.

Estrogens and some xenoestrogens have been shown to exert negative effects on the immune system of fish. Since fish are regularly exposed to these chemicals in their natural environments, possible immunotoxicity may result in increased susceptibility to infectious diseases. The immunosuppressive effects of E2 and NP observed in the current *in vitro* study suggest that *in vivo* investigations are also warranted and that immunotoxicity of xenoestrogens should be considered in the context of feral fish populations in vulnerable aquatic ecosystems.

Conclusion

Overall, this study has confirmed the presence of ER mRNA in purified leukocytes, provided evidence of a role for the ERαs in cell cycle control and proliferation, and confirmed that an estrogen and a xenoestrogen impair cellular immune function in rainbow trout. It reaffirms that xenoestrogens can be immunotoxic, in addition to their well-recognized effects on reproduction. While additional research is required to substantiate the *in vivo* effects and evaluate the *in situ* implications of these results, these results clearly establish important molecular mechanisms that underlie immunosuppression related to estrogen-active compounds. Although mammalian and teleost immune systems differ in many ways, the conserved nature of many receptors between fish and mammals suggest that mammalian models can provide valuable guidance in terms of methodologies for examining the molecular mechanisms of toxic injury to the teleost immune system. With the advent of high-throughput molecular technologies, such as microarrays and proteomic techniques, such an understanding in teleosts is likely to grow substantially in the future.

Supplementary materials related to this article can be found online at doi:10.1016/j.cbpc.2011.11.006

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Table

Table 2-1. Viability of peripheral blood leukocytes from rainbow trout following a 4 d in vitro incubation with 17β-estradiol or nonylphenol. Data are shown as percent viable cells ± SEM and n = 12. Treatments significantly different from the corresponding control cells are shown as * (p < 0.05), ** (p < 0.01).

Concentration of Test Chemical	17β-Estradiol		Nonylphenol	
	Unstimulated	LPS-Stimulated	Unstimulated	LPS-Stimulated
Control	71.8 ± 1.5	78.1 ± 1.0	69.1 ± 2.5	77.4 ± 1.4
1 nM	70.7 ± 1.3	77.1 ± 0.7	69.2 ± 2.4	76.7 ± 1.4
10 nM	**68.8 ± 1.6****	**76.0 ± 0.9****	69.6 ± 2.4	76.7 ± 1.5
100 nM	**68.3 ± 1.6****	**75.9 ± 0.9****	69.5 ± 2.7	76.7 ± 1.5
1 µM	**68.7 ± 1.5****	**76.9 ± 0.8***	69.3 ± 2.6	77.1 ± 1.4
10 µM	**69.2 ± 1.3***	79.2 ± 1.2	69.0 ± 2.0	**75.9 ± 1.4***

Figures

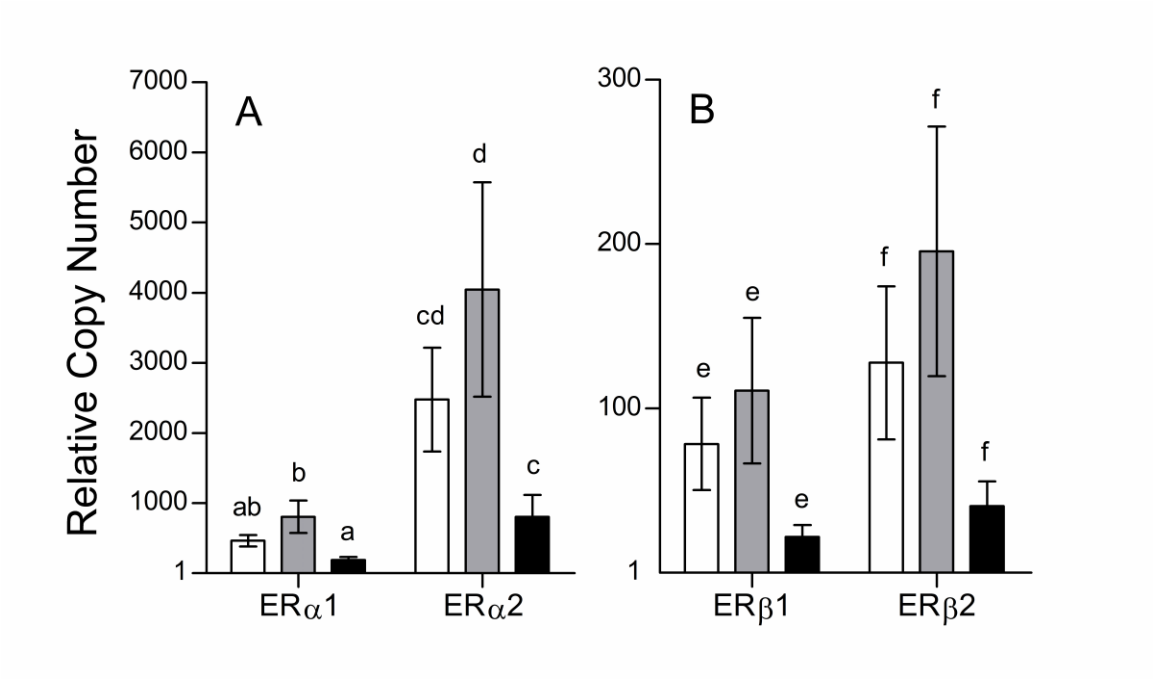

Figure 2-1. ***Four estrogen receptors (ER) were identified in freshly isolated peripheral blood leukocytes from rainbow trout (white bars). Transcription of the ERα genes (Panel A) declined after stimulation of these cells with LPS in culture for 4 d (black bars) when compared to unstimulated cells cultured for 4 d (grey bars), while ERβ genes were unaffected (Panel B). Note the difference in y-axis scale between Panel A and Panel B. Estrogen receptor transcription levels were normalized to L8 transcription and are shown as relative copy number ± SEM and n = 6. Within each ER gene, differences between treatments are denoted by the letters above the bars.***

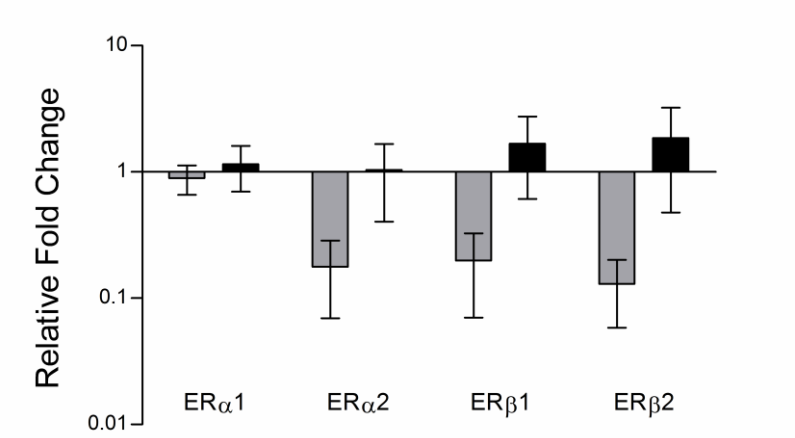

Figure 2-2. Transcription of the four forms of estrogen receptor is unaffected by 4 d incubation with either 10 µM 17β-estradiol (grey bars) or 10 µM nonylphenol (black bars) in purified rainbow trout peripheral blood leukocytes stimulated with LPS. Data were normalized to L8 expression levels and are shown on a log-scale as mean fold change ± SEM (n = 6) relative to the LPS-stimulated control cells, where a fold change of 1 indicates that that the treatment was the same as the control.

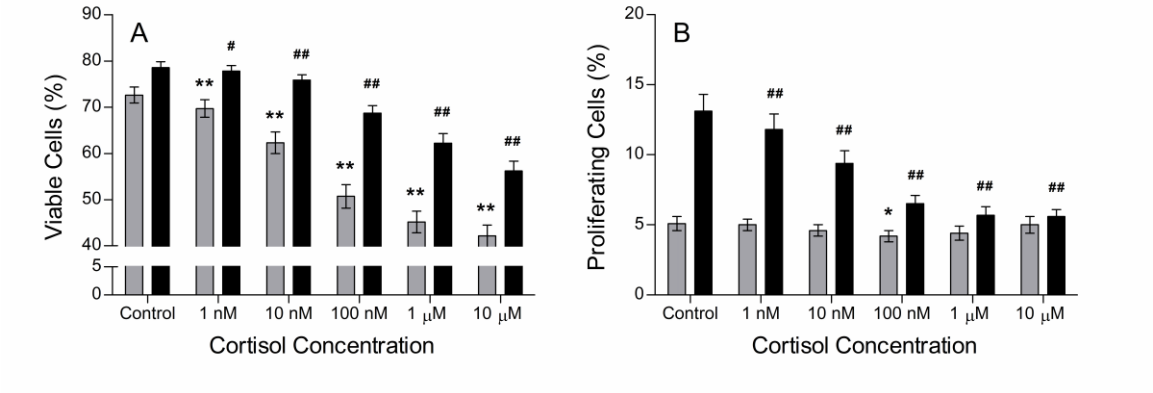

Figure 2-3. Viability (Panel A) and proliferation (Panel B) of unstimulated (grey columns) or LPS-stimulated (black columns) peripheral blood leukocytes from rainbow trout are reduced following 4-day in vitro incubation with cortisol. Data are shown as percent viable cells or percent proliferating cells ± SEM and n = 12. Treatments significantly different from the corresponding control cells are shown as * (p < 0.05) or ** (p < 0.01) for unstimulated cells or # (p < 0.05) or ## (p < 0.01) for LPS-stimulated cells.

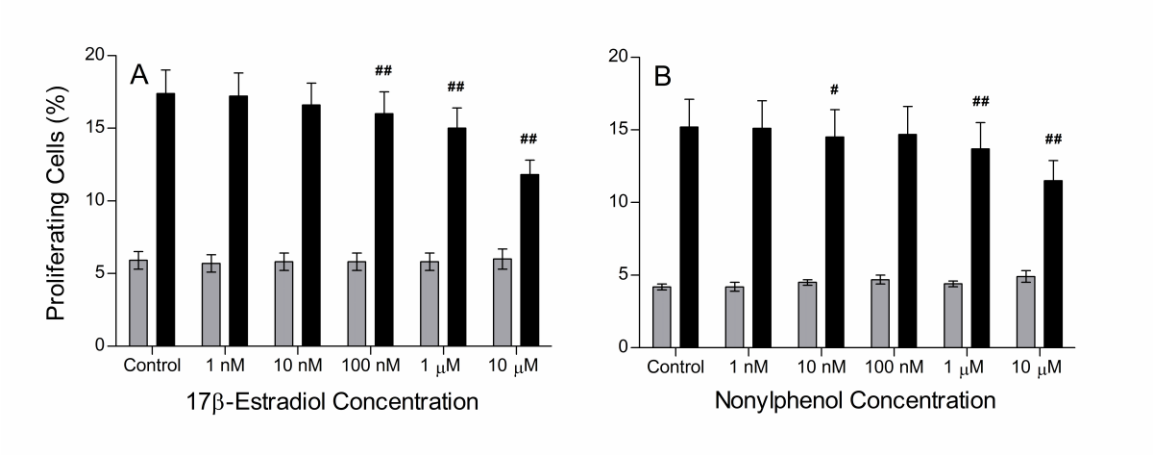

Figure 2-4. Proliferation of peripheral blood leukocytes from rainbow trout is reduced in LPS-stimulated cells (black bars) while unstimulated cells (grey bars) are unaffected following 4-day in vitro incubation with 17β-estradiol (Panel A) or nonylphenol (Panel B). Data are shown as percent proliferating cells ± SEM and n = 12. Treatments significantly different from the corresponding control cells are shown as # ($p < 0.05$) or ## ($p < 0.01$) for LPS-stimulated cells.

Supplemental Data

Table S2-1. Data from a preliminary experiment demonstrating that the ethanol solvent (0.5% v/v) had no effect on leukocyte proliferation or viability. Data is shown as mean ± SEM and represents data from 6 individual fish that received both treatments. P-value was generated using a paired, two-tailed t-test.

.Assay	Control Cells (no solvent)	Solvent Control Cells (0.5% v/v ethanol)	*p*-value
Leukocyte viability, unstimulated	63.9 ± 3.9	64.2 ± 4.2	0.8723
Leukocyte viability, LPS stimulated	74.5 ± 2.3	74.7 ± 2.3	0.8668
Lymphocyte cell gate, unstimulated	89.6 ± 0.5	88.8 ± 0.6	0.1094
Lymphocyte cell gate, LPS stimulated	84.1 ± 2.0	84.2 ± 1.8	0.6800
Blasting cell gate, unstimulated	5.8 ± 0.8	5.5 ± 0.7	0.2655
Blasting cell gate, LPS stimulated	10.8 ± 1.8	10.5 ± 1.8	0.3025

Table S2-2. Viability (in percent) of rainbow trout (Oncorhynchus mykiss) peripheral blood leukocytes following a 4 d in vitro incubation with cortisol (raw data). US = unstimulated cells, LPS = LPS-stimulated cells.

Fish	Control (%)		1 nM (%)		10 nM (%)		100 nM (%)		1 µM (%)		10 µM (%)	
	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS
1	80.7	82.7	77.7	80.4	68.5	74.6	52.2	60.4	42.7	52.4	38.9	44.9
2	75.6	82.7	74.1	81.7	66.8	80.2	57.1	74.9	52.2	69.8	50.1	63.0
3	75.7	80.6	71.7	79.9	64.9	78.2	50.6	69.8	45.1	61.0	41.0	52.5
4	71.5	79.5	68.4	79.8	63.8	79.2	54.0	73.5	47.3	68.2	40.7	61.3
5	76.1	83.2	74.5	82.4	70.4	81.1	63.4	76.8	57.0	71.9	56.2	67.8
6	59.2	75.3	53.7	73.2	41.2	69.7	29.0	61.9	26.6	54.6	27.9	56.1
7	72.0	74.2	66.7	72.8	57.9	71.2	45.7	61.8	39.0	52.8	36.5	47.2
8	72.5	78.9	70.7	77.3	64.4	75.7	55.4	72.3	50.6	69.2	48.9	63.7
9	70.3	77.8	69.3	78.8	62.7	74.9	49.0	68.9	44.4	61.5	39.4	56.4
10	81.7	83.7	77.8	82.0	68.0	80.2	54.3	67.6	48.7	60.6	46.0	54.2
11	66.7	70.8	63.8	70.9	53.7	70.3	42.2	63.6	37.1	55.4	31.5	47.9

12	69.8	73.8	68.2	74.7	65.7	74.8	56.0	73.2	51.6	69.1	49.1	60.0

Table S2-3. Proliferating cells (in percent) of rainbow trout (Oncorhynchus mykiss) peripheral blood leukocytes following a 4 d in vitro incubation with cortisol (raw data). US = unstimulated cells, LPS = LPS-stimulated cells.

Fish	Control (%)		1 nM (%)		10 nM (%)		100 nM (%)		1 µM (%)		10 µM (%)	
	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS
1	8.7	13.6	8.1	12.2	6.6	9.7	6.6	7.7	5.1	6.6	5.3	6.5
2	4.5	10.9	4.5	10.1	3.6	8.0	3.4	5.4	2.7	5.0	3.6	5.5
3	2.8	7.7	3.1	6.5	3.4	4.8	2.8	3.1	4.1	3.0	5.2	3.0
4	5.2	17.0	5.1	15.3	5.0	12.5	5.5	8.1	7.1	8.7	6.8	8.4
5	6.6	10.4	6.1	9.3	5.3	7.6	3.9	5.2	3.1	4.2	3.4	3.6
6	4.4	18.3	4.8	17.3	5.0	14.8	5.6	11.7	5.7	9.2	8.5	8.9
7	5.8	17.5	5.4	14.9	4.5	11.7	4.6	7.2	4.7	6.3	5.1	5.7
8	4.6	16.5	3.9	14.3	3.9	10.9	2.9	6.7	3.6	6.5	3.5	6.1
9	5.3	10.6	5.0	10.2	4.2	8.4	3.5	6.6	3.4	5.2	4.0	5.2
10	2.8	8.3	2.9	7.2	2.4	5.5	2.2	3.9	1.9	2.7	2.2	3.2
11	3.8	18.4	4.7	16.6	4.9	12.4	3.9	6.7	3.7	4.7	4.3	4.6
12	6.5	8.3	6.6	8.1	7.0	6.7	6.4	5.9	8.4	6.8	8.2	6.7

Table S2-4. Viability (in percent) of rainbow trout (Oncorhynchus mykiss) peripheral blood leukocytes following a 4 d in vitro incubation with 17β-estradiol (raw data). US = unstimulated cells, LPS = LPS-stimulated cells.

Fish	Control (%)		1 nM (%)		10 nM (%)		100 nM (%)		1 µM (%)		10 µM (%)	
	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS
1	70.9	75.3	73.4	75.2	69.1	74.8	69.7	73.7	67.7	72.5	62.2	72.3
2	75.3	80.8	75.7	79.6	73.8	78.7	71.5	78.9	72.2	79.6	74.8	82.3
3	66.2	72.6	65.4	74.6	63.9	72.7	62.8	73.9	62.9	76.3	64.9	85.4
4	66.7	76.4	67.8	76.1	64.3	74.7	63.7	75.3	64.1	76.3	67.5	82.0
5	80.4	85.6	79.9	82.9	80.3	84.1	81.4	83.8	80.2	83.6	77.3	82.5
6	68.4	76.7	68.0	75.8	67.4	75.3	65.5	76.4	65.5	76.8	67.0	77.7
7	71.7	81.5	69.8	76.8	67.5	76.3	67.9	76.4	70.3	78.0	66.9	80.5
8	63.8	76.3	65.2	73.7	59.3	73.2	60.6	73.4	60.7	75.1	65.0	76.8

9	74.7	76.0	72.1	76.0	74.1	74.3	72.9	72.9	70.3	74.1	70.6	73.6
10	78.4	80.6	68.9	77.4	67.8	76.9	66.2	77.4	70.1	77.8	68.5	77.1
11	68.7	77.3	67.1	77.4	66.4	73.8	66.8	73.1	68.8	76.3	72.8	82.7
12	76.1	78.3	75.3	79.8	72.5	77.6	71.2	76.3	71.8	76.7	73.5	77.2

Table S2-5. Proliferating cells (in percent) of rainbow trout (Oncorhynchus mykiss) peripheral blood leukocytes following a 4 d in vitro incubation with 17β-estradiol (raw data). US = unstimulated cells, LPS = LPS-stimulated cells.

Fish	Control (%)		1 nM (%)		10 nM (%)		100 nM (%)		1 µM (%)		10 µM (%)	
	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS
1	2.9	11.5	4.0	10.8	3.1	11.0	3.5	10.4	3.3	9.2	3.8	8.7
2	5.1	21.3	4.0	21.9	5.1	22.2	4.3	21.0	4.0	19.1	4.5	14.3
3	9.0	22.2	8.7	20.8	8.9	20.5	8.4	19.0	9.2	18.8	8.0	14.6
4	7.1	22.6	7.7	22.8	7.7	22.0	7.6	22.3	7.8	21.4	10.5	15.1
5	5.4	11.3	5.8	10.5	5.4	10.8	5.7	12.1	6.3	10.9	7.3	11.5
6	3.7	19.0	4.2	19.3	4.7	17.6	4.5	16.7	4.7	15.0	5.9	12.3
7	4.1	22.5	3.7	20.7	3.9	17.7	3.3	17.4	3.6	14.7	3.8	9.6
8	4.5	26.6	4.8	26.6	4.1	26.3	4.2	24.8	3.5	23.4	6.1	18.5
9	4.3	14.0	4.0	13.8	4.8	14.0	5.4	13.0	5.8	13.6	6.0	11.6
10	5.8	16.0	5.4	16.2	5.5	15.4	4.9	14.4	5.3	14.0	4.4	9.6
11	3.4	12.1	3.3	12.6	3.3	11.9	3.6	11.7	4.1	11.6	5.1	9.7
12	1.7	9.9	1.6	10.3	1.6	9.9	1.5	8.8	1.7	8.3	1.7	6.2

Table S2-6. Viability (in percent) of rainbow trout (Oncorhynchus mykiss) peripheral blood leukocytes following a 4 d in vitro incubation with nonylphenol (raw data). US = unstimulated cells, LPS = LPS-stimulated cells.

Fish	Control (%)		1 nM (%)		10 nM (%)		100 nM (%)		1 µM (%)		10 µM (%)	
	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS
1	74.3	78.8	73.9	78.1	73.8	77.3	73.2	76.3	73.2	76.3	70.6	75.7
2	68.1	77.2	66.2	77.2	66.9	76.9	65.7	75.7	64.7	75.7	66.6	75.4
3	76.8	82.5	77.1	81.8	75.8	84.3	77.2	82.9	80.0	82.7	76.2	80.4
4	61.6	77.4	61.3	76.9	65.3	76.8	61.8	77.8	62.8	77.9	64.7	76.2
5	58.1	66.5	58.3	66.1	58.5	66.8	57.8	65.7	57.8	66.1	67.8	67.7

6	73.3	79.5	73.9	76.3	74.8	78.6	74.6	80.0	74.8	80.1	70.0	76.9
7	63.2	74.7	63.8	75.3	64.0	74.5	63.7	76.1	64.7	75.0	64.7	75.1
8	73.6	81.4	74.1	80.3	74.2	80.7	74.4	80.3	74.2	81.1	75.7	82.2
9	77.7	83.8	77.8	83.4	79.0	82.6	79.9	83.3	78.6	83.7	80.6	83.3
10	76.9	76.9	78.5	77.8	77.8	77.1	78.0	75.5	76.4	76.8	73.3	75.9
11	74.0	79.8	72.5	77.2	73.4	77.3	76.5	78.1	73.4	79.3	63.9	75.3
12	51.2	70.1	52.8	69.9	51.7	68.0	51.0	69.4	51.0	70.8	54.7	66.5

Table S2-7. Proliferating cells (in percent) of rainbow trout (Oncorhynchus mykiss) peripheral blood leukocytes following a 4 d in vitro incubation with nonylphenol (raw data). US = unstimulated cells, LPS = LPS-stimulated cells.

Fish	Control (%)		1 nM (%)		10 nM (%)		100 nM (%)		1 µM (%)		10 µM (%)	
	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS	US	LPS
1	5.3	7.3	4.9	7.8	5.4	7.3	4.8	7.1	5.3	7.5	4.7	6.2
2	4.1	8.0	4.6	8.9	4.5	8.3	4.6	9.0	4.2	8.6	4.9	8.2
3	3.8	11.1	3.5	10.6	3.7	9.5	3.9	10.3	4.8	8.7	5.5	8.1
4	5.6	24.5	6.0	23.2	6.0	24.2	5.6	23.7	5.5	22.2	7.4	19.6
5	4.1	28.4	3.5	29.0	4.4	28.3	4.4	27.4	4.0	25.5	5.4	21.3
6	3.0	10.9	3.5	11.0	3.8	10.1	4.4	10.2	3.7	9.5	2.6	7.5
7	4.0	13.5	3.4	12.0	3.6	12.4	3.6	11.9	3.7	10.8	4.6	9.2
8	4.5	20.1	5.2	20.8	4.5	17.2	5.2	20.8	4.5	17.6	4.3	14.3
9	5.1	15.7	5.6	16.6	5.8	15.9	7.2	15.5	5.7	14.6	6.6	11.9
10	4.4	15.9	3.7	14.5	4.2	14.0	4.0	14.2	3.6	13.9	4.0	11.2
11	3.6	8.7	3.4	9.6	4.0	8.7	5.5	8.2	3.8	7.7	3.1	6.4
12	3.6	18.3	3.8	18.0	4.0	18.5	3.7	18.6	3.7	18.8	5.3	14.5

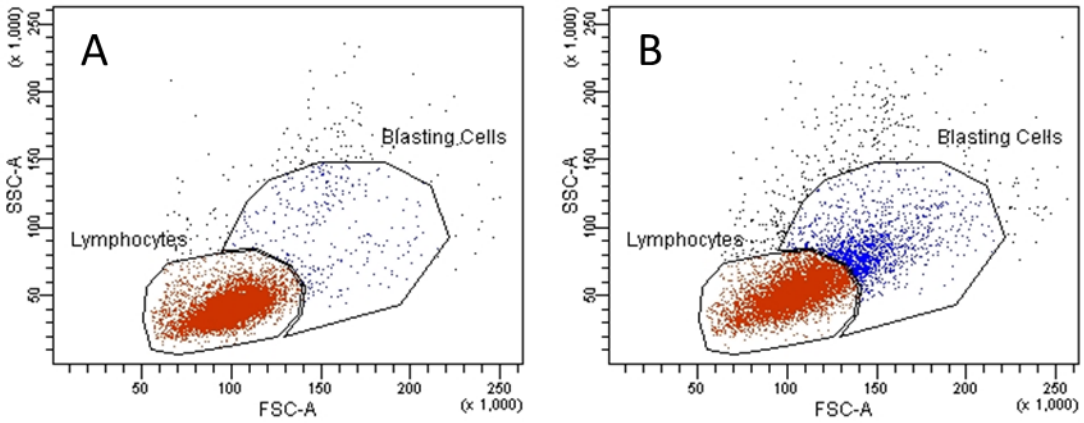

Figure S2-1. Representative example of forward scatter (FSC) / side scatter (SSC) dot plots showing the gating used throughout the flow cytometric proliferation assay. Panel A show a representative sample of unstimulated peripheral blood leukocytes following 4 d of in vitro culture, while Panel B shows cells from the same fish following 4 d of in vitro incubation with LPS. Cells within the 'blasting cell' gate were considered to be undergoing proliferative responses.

Chapter 3.

Alteration of immune function endpoints and differential expression of estrogen receptor isoforms in leukocytes from 17β-estradiol exposed rainbow trout (*Oncorhynchus mykiss*)

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Abstract

While the endocrine system is known to modulate immune function in vertebrates, the role of 17β-estradiol (E2) in cellular immune function of teleosts is poorly understood. The cellular and molecular responses of juvenile rainbow trout (*Oncorhynchus mykiss*) to E2 treatment were evaluated by exposing fish to 0.47 ± 0.02 µg/L E2 (mean ± SEM) for either 2 or 7 d, with a subsequent 14 d recovery period. After 2 and 7 d of exposure to E2, hematocrit was significantly lower than in control fish. Lipopolysaccharide-induced lymphocyte proliferation was elevated on day 2 and concanavalin A-induced lymphocyte proliferation was reduced following 7 d of E2 exposure. Four estrogen receptor (ER) transcripts were identified in purified trout head kidney leukocytes (HKL) and peripheral blood leukocytes (PBL). While the mRNA abundance of ERβ1 and ERβ2 was unaffected by treatment, ERα1 was up-regulated in HKL and PBL following 7 d of E2 exposure. ERα2 was up-regulated in HKL after 7 d of E2 exposure, but down-regulated in PBL after 2 and 7 d of treatment. All parameters that were altered during the E2 exposure period returned to baseline levels following the recovery period. This study reports the presence of the full repertoire of ERs in purified HKL for the first time, and demonstrates that ERα transcript abundance in leukocytes can be regulated by waterborne E2 exposure. It also demonstrated that physiologically-relevant concentrations of E2 can modulate several immune functions in salmonids, which may have widespread implications for xenoestrogen-associated immunotoxicity in feral fish populations inhabiting contaminated aquatic environments.

Keywords

Immune system; rainbow trout; 17β-estradiol; lymphocyte proliferation; estrogen receptor; recovery

Introduction

The immune system of teleosts provides a critical barrier function for protection against pathogens commonly found in the aquatic environment [1,37]. Its function can be up- or down-regulated by a variety of exogenous factors that include season and photoperiod, water quality parameters such as temperature and pH, as well as xenobiotic exposure [6]. In addition, hormones that guide the activity and development of normal physiological processes can also act as signaling molecules linking the perception of external conditions to internal responses. Thus circulating hormone concentrations can be influenced by exogenous factors and hormones have been shown to modulate the activities and function of immune system components [22.74]. The direct and indirect effects of altered hormone status may provide the mechanistic underpinning of altered immune function caused by such factors.

The main vertebrate estrogen hormone, 17β-estradiol (E2), has known functions in fish reproduction, growth, behaviour, differentiation and development [30]. Classical genomic pathway signaling by E2 occurs following the interaction of E2 with intracellular estrogen receptors (ERs) and associated proteins located in the cytoplasm. Translocation of the E2-ER dimer complex into the nucleus enables its action as a transcription factor for genes containing an estrogen response element [41]. Although controversial [33], E2 signaling may also occur through the interaction of E2 with transmembrane receptors such as GPER (G-protein coupled estrogen receptor, previously known as GPR30), which triggers an intracellular signaling cascade with more rapid effects [53]. Regardless of the signaling mechanism, elevated E2 concentration is mainly associated with sexual maturity and spawning, as it initiates the transcription and translation of a plethora of proteins related to gonadal maturation and the production of eggs in females [48].

In rainbow trout, four forms of ER have been identified: ERα1, ERα2, ERβ1 and ERβ2 [45]. All four forms have been detected in a variety of tissues including the liver, gonads, brain and heart, as well as tissues associated with the immune system (e.g. head kidney and spleen). The head kidney and spleen consist of a variety of different cell types, so these findings are only suggestive of the presence of ERs in leukocytes

themselves. Recent evidence has indicated that the four rainbow trout ERs are found in leukocytes from peripheral blood [46,61], providing a signaling mechanism through which E2 may exert influence over cells of the immune system.

17β-estradiol is present throughout the lifecycle of both male and female fish, with plasma concentrations in the low pg/mL range in males and immature females, and in the low ng/mL concentration range in reproductively mature females [9,49]. Estrogens can also be found in the aquatic environment in the low ng/L range as a result of sewage treatment plant effluent discharges, sewage biosolids application as soil fertilizer and runoff from agricultural livestock waste, since endogenously produced estrogens, including E2 and the other estrogens estriol and estrone, are excreted by humans and agricultural animals [26,32]. Other compounds are capable of exerting estrogen-like effects in exposed organisms; synthetic estrogens such as those used in contraceptives (e.g. 17-α-ethinylestradiol (EE2)), naturally derived phytoestrogens (e.g. genistein), and several with more limited structural resemblance to endogenous estrogens (e.g. nonylphenol, bisphenol A, and some phthalate esters) [54]. While most xenoestrogens (except EE2) have a lower binding affinity for the ER, and thus a lower biological activity than endogenous E2 [54], some of these chemicals can be present in the aquatic environment at concentrations high enough to exert physiological effects [32]. For example, sewage effluents, which consist of a complex mixture of substances, including estrogen hormones and xenoestrogens, have been associated with reproductive abnormalities [29] and immune system dysfunction [55] in exposed fish.

Chemicals that alter fish immune function and decrease pathogen resistance have important implications to fish survival and population growth [10,62]. Host resistance assays provide perhaps the most poignant evidence of the biological implications of chemical-associated immunotoxicity, since the major endpoint is survivability following exposure to a pathogen [31]. Exposure to E2 (either as an implant or through aquatic exposures) has been shown to alter susceptibility to parasitic [68] and bacterial infections [71]. Exposure to E2, therefore, is believed to affect components of the immune system that have consequences for disease survivability in individuals, and may have significant implications at the population level.

While it is clear that E2 causes immunosuppression at the organism level, considerable uncertainty exists regarding the mechanisms of E2 effects on fish immune function at the cellular level. In the current study, the immunomodulatory effects of aquatic E2 exposure in juvenile rainbow trout were examined using a panel of immune function assays and quantification of gene expression to gain insight into responses to estrogen hormone at both the cellular and molecular levels. Groups of fish were exposed to E2 for either 2 or 7 days, followed by a 14 d depuration period to assess the ability of fish to recover once E2 exposure ceased.

Materials and Methods

Fish

Rainbow trout, (mass at the time of sampling = 31.9 ± 0.7 g, mean ± SEM) were obtained from Sun Valley Trout Hatchery (Mission, BC, Canada) and maintained in fiberglass tanks with continuously flowing well water at 15 ± 1°C and a 16:8 h (light:dark) photoperiod. Prior to exposure, fish were fed a daily ration with commercial trout feed (Ewos, Surrey, BC, Canada) at 2% of their body weight per day. All experimental procedures performed with fish were done under an Animal Care Permit issued by the Simon Fraser University Animal Care Committee, and in accordance with Canadian Council on Animal Care guidelines.

Chemicals

Charcoal-stripped fetal bovine serum (FBS) was obtained from Invitrogen (Gibco, Portland, OR, USA). Unless noted, all other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada).

Experimental design and sampling

During the experiment, rainbow trout were individually housed in 68 L glass aquaria (n = 10 to 15 aquaria per treatment group per sampling day) held in temperature controlled rooms (15 ± 1°C) and were exposed to either a nominal concentration of zero (control) or 1 µg/L 17β-estradiol (E2) in well water for either 2 or 7 d. Anhydrous ethanol

(100.0%; Commercial Alcohols, Tiverton, ON, Canada) was used as a carrier for the addition of E2 to the treatment tanks and an equivalent amount of anhydrous ethanol was added to the control tanks, such that the solvent concentration in all the tanks was 0.001% v/v. Fish that were not sampled at 7 d were kept for a 14 d recovery period following E2 exposures. At the end of the 7 d exposure, these fish were transferred to new, clean aquaria containing well water and no E2. Exposures were conducted as static renewal, with water and chemical being refreshed (100% renewal) every 3-4 d throughout both the exposure and recovery periods. Water quality parameters including pH, dissolved oxygen, conductivity and temperature were monitored regularly. Composite water samples from the control or E2-treatment tanks were collected for E2 analysis at the beginning of the experiment and before and after each water change. Analysis for E2 was performed using the exact method of Furtula *et al*. [20].

At each sampling time point, individual fish were euthanized with sodium bicarbonate-buffered 0.1 g/L tricaine methanesulfonate (Argent Chemical Laboratories, Redmond, WA, USA), and then weighed. Blood was aseptically collected by caudal venipuncture into a heparinized syringe and immediately transferred to a tube containing an equal volume of sterile supplemented HBSS (sHBSS; 15 mM HEPES, 5% FBS, 10 U/mL heparin and 1% penicillin/streptomycin). Additional blood was collected following severing of the caudal peduncle into heparinized capillary tubes, which were centrifuged in order to determine hematocrit and to collect plasma samples. The head kidney was excised and placed into tubes containing 1 mL of sHBSS. All samples were stored on ice.

Hematocrit and plasma 17β-estradiol

Following centrifugation of the hematocrit tubes, hematocrit was determined based on the measured total height of the packed red blood cell column divided by the total height of the blood column. Hematocrit tubes were then cut to collect plasma, which was stored temporarily on ice until transfer for storage at -80°C. Plasma E2 was measured using a commercially available E2 ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) following manufacturer instructions.

White blood cell purification

In order to obtain single cell suspensions, head kidney samples were gently disrupted on a 75 µm nylon mesh and washed through with 2 mL of sHBSS. Purified white blood cells were then isolated from both the head kidney and blood using a hypotonic lysis method [12]. Briefly, 9 mL of sterile cold water was added to the cell suspension for 30 s to induce lysis, which was then stopped by the addition of 1 mL of 10 x PBS (0.1 M). Gross debris was removed with a sterile Pasteur pipette, samples were centrifuged at 400 x *g* for 5 min at 4°C and cells were washed twice with 5 mL of sHBSS. Cells were resuspended in 2 mL of sHBSS for counting and assessment of viability by trypan blue exclusion. Cells were then adjusted to either 1 x 10^7 viable cells/mL for head kidney leukocytes (HKL) or 5 x 10^6 viable cells/mL for peripheral blood leukocytes (PBL).

Phagocytosis and phagocytic capacity

Head kidney leukocytes were used in assays to measure phagocytosis and respiratory burst activity. Phagocytosis was assessed using flow cytometry where the ability of HKL to take up fluorescent beads is measured [8,60]. Briefly, 1 x 10^6 viable cells were incubated at room temperature in either 1 mL of sRPMI (live cells) or 1 mL of 1% paraformaldehyde in 0.01 M PBS (fixed cells, control), in duplicate. Cells were centrifuged at 500 x *g* for 5 min at 18°C, resuspended in 100 µL of sRPMI and transferred to round-bottom 96-well culture plates. Fluorescent latex beads (1 µm diameter) were added at a ratio of 100:1 (beads:cells) and samples incubated at 18°C for 18 h. Cells were then layered over a cushion of 3% BSA in 0.01 M PBS and centrifuged at 100 x *g* for 10 min at 4°C before being resuspended in 0.01 M PBS.

On the flow cytometer (Becton Dickinson FACSAria, Franklin Lakes, New Jersey, USA), a gate was set up that excluded non-phagocytosed beads and debris and 10,000 cell events were collected for this gate for each tube. Cells that had taken up beads were identified by creating a gate of bead positive cells based on the FITC histogram. The percent of cells that phagocytosed beads was calculated as percent bead positive live cells minus percent bead positive fixed cells. A phagocytic capacity (PC) based on mean fluorescence intensity was also calculated as an approximation of the number of

beads per cells, where PC is the mean fluorescence intensity of bead positive live cells divided by the mean fluorescence intensity of a single bead suspension.

Respiratory burst

Respiratory burst was measured with the nitroblue tetrazolium (NBT) method using 96-well flat-bottomed microplates [59]. This assay is based on the reduction of the soluble NBT dye to insoluble formazan by reactive oxygen species. An aliquot of 100 µL of HKL was added to quadruplicate wells and incubated at room temperature for 2 h. Non-adherent cells were removed by gently washing twice with 0.01 M PBS. Half of the cells received 100 µL of RPMI with 1 mg/mL NBT (unstimulated cells) and the other half received 100 µL of medium containing both 1 mg/mL NBT and 1 µg/mL phorbol myristate acetate (PMA) to induce the respiratory burst (stimulated cells). Cells were incubated at room temperature for 30 min before media was removed and cells were fixed by submerging plates in 70% methanol three times. Plates were air dried before adding 120 µL/well of KOH and 140 µL/well of DMSO to solubilize the formazan. Absorbance was analyzed on a Bio-Tek® PowerWave 340 microplate reader (Bio-Tek, Winooski, VT, USA) at 630 nm. Respiratory burst activity was calculated as an index of the OD of stimulated cells divided by the OD of unstimulated cells.

B and T cell proliferation

Peripheral blood leukocytes were used in an assay of B and T cell mitogen-induced proliferation. Mitogenesis was assessed by flow cytometry by comparing the proliferation of unstimulated cells to that of leukocytes following stimulation with *Eschericia coli* lipopolysaccharide (LPS) to stimulate B cell proliferation or concanavalin A (Con A) to stimulate T cell proliferation [57,58]. Briefly, 5 x 10^5 cells from each fish were added to 6 wells of a round-bottomed 96-well microplate. Duplicate wells received either 100 µL of sRPMI, 100 µL of sRPMI containing 100 µg/mL LPS, or 100 µL of sRPMI containing 10 µg/mL Con A. Plates were incubated in a humidified chamber for 96 h at 18°C before cells were transferred to glass 5 mL tubes with 0.8 mL 0.01 M PBS (total volume 1 mL) for analysis by flow cytometry.

A total of 10,000 events per sample were collected and proliferation was determined based on shifts in forward and side scatter that occur as cells begin to divide

and become more complex [57,58]. Previously, this flow cytometric method has been shown to have good positive correlation with results from ^{3}H-thymidine labeling that is frequently used to measure lymphoproliferation [57]. Gates (resting leukocyte gate and proliferating leukocyte cell gate) were defined based on a sample of unstimulated cells and the same gates were used throughout the experiment [61]. Proliferation of viable cells was determined as a stimulation index (SI), where SI = % stimulated cells in proliferating cell gate divided by % unstimulated cells in proliferating cell gate.

Evaluation of gene expression

RNA extraction and cDNA preparation

For purified HKL and PBL, total RNA extraction was performed using RNeasy Mini Spin kits (Qiagen, Mississauga, ON, Canada). To obtain 1 x 10^{6} leukocytes per sample, an appropriate volume of cells in sHBSS, was placed in 1.5 ml tubes and spun at 400 *x g* for 5 min to pellet the cells. The medium was removed and 1 ml of Buffer RLT (from the Qiagen RNeasy kit) containing 10 µl of β-mercaptoethanol was added per tube. Samples were then vortexed for 1 min to lyse the cells and stored at -80^{o}C until extraction. To extract total RNA, leukocytes were thawed, vortexed for 1 min, mixed 1:1 with 70% ethanol, loaded onto the spin column and washed as per the manufacturer's protocol, including an on-column DNase treatment (Qiagen). Immediately after extraction, samples were placed on ice and their total RNA concentrations were determined using a Nanodrop® 1000 instrument (Thermo Fisher Scientific, Toronto, ON, Canada).

Total RNA (1 µg) was transcribed into cDNA using a QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada) following manufacturer's instructions and the resultant cDNAs were diluted 1:10 with nuclease-free water.

Quantitative polymerase chain reaction (QPCR)

Gene expression of the ERs in HKL and PBL was analyzed with QPCR in E2-treated fish compared to control fish at each time point, while the set of four immune genes were analyzed only at day 2 and 7. Sequences for ER primers were obtained from Nagler et al. [45]. The primers for four immune-related genes (TNFα, IL1β, CXCR4, CCR7; see Table 3-1) were obtained from the Pacific Environmental Science

Centre (Environment Canada, North Vancouver, BC, Canada). The reference gene, ribosomal protein L8, primer sequences were obtained from Dr. Nik Veldhoen and Dr. Caren Helbing (University of Victoria, Victoria, BC, Canada). Primers were synthesized by AlphaDNA (Montreal, QC, Canada) and the sequences used are provided in Table 3-1.

Set-up of QPCR reactions occurred in triplicate, on ice, and utilized Master Mixes that contained the following per 15 µl total volume reaction: 50% iTaq™ SYBR® Green Supermix With ROX (Bio-Rad, Mississauga, ON, Canada); 20 pmoles forward primer; 20 pmoles reverse primer; 2 µl of diluted cDNA; and nuclease-free water (Ambion, Austin, TX, USA) up to the final volume. Plates were run on Stratagene MX3000P™ QPCR instruments (Agilent, Cedar Creek, TX, USA) with the program: 95°C for 1 min and 40 cycles of 95°C for 15 s, 58°C for 25 s, 72°C for 35 s. Amplification of the correct product for each gene and tissue was verified using agarose gels, melting curves and sequencing. No template controls (NTC) were performed in triplicate for each primer pair for each tissue and time point analyzed.

Standard curves for each primer pair in each tissue were prepared and used to calculate the absolute quantity (copy number) of the transcript in each sample. Criteria for the standard curve included: R^2 values > 0.99 (range was 0.992-0.999), baseline threshold levels < 0.020 (calculated as 10X the background noise level based on cycles 5 – 9), and amplification efficiencies between 80 – 120 % (actual values were 80 – 108%). The invariant ribosomal protein L8 was used as a reference gene to normalize the copy number results to account for technical variations [18].

ER sequencing

Confirmatory QPCR reactions for the 4 ERs and L8 were run on 1.5% agarose gels to visualize products for each gene target. The other 4 immune-related genes have been cloned and sequenced previously as a part of a rainbow trout microarray [73]. Bands of the correct size were excised, placed in 1.5 ml tubes, and 'freeze and squeezed' (3 cycles of 10 min in a dry-ice 100% ethanol bath, followed by 10 min in a 37°C water bath) to re-release the target amplicon, which was cloned in duplicate using Topo®-TA 2.1 cloning kits (Invitrogen, Streetsville, ON, Canada). The DNA Technologies Laboratory at the National Research Council Plant Biotechnology Institute

(Saskatoon, SK, Canada) sequenced the duplicate clones in both directions utilizing Sanger methodology and an ABI 3730xl sequencer (Applied Biosystems Inc., Streetsville, ON, Canada). Comparison of HKL and PBL ER sequences to National Centre for Biotechnology Information nucleotide collection database occurred using BLASTN (Basic Local Alignment Search Tool-Nucleotide), specifically with rainbow trout (organism #8022).

Statistics

Data for hematocrit and the immunological assays were first normalized to percent of control for each day in order to minimize any potential differences due to sampling day. Grubbs' test was used to detect and remove outliers (GraphPad Software Inc., La Jolla, CA, USA). Data were tested for equal variance and normal distribution of residuals before being analyzed using the fit model platform in JMP (v8.0.2, SAS Institute Inc, Cary, NC, USA) using a 2-way ANOVA, followed by either a Bonferroni post-hoc test (non-QPCR data) or student t-tests (QPCR data) when differences were detected ($p < 0.05$). For the four immune genes evaluated using QPCR (Table 3-1), Bartlett's test found that variances were not equal so Welch's t-test ($p < 0.05$) was used to compare control to E2-treated fish within each time point.

Results

Basic water quality parameters and water 17β-estradiol concentration

Water quality parameters were monitored regularly throughout the experiment and were: pH = 7.7 ± 0.3, dissolved oxygen = 9.7 ± 0.9 mg/L, conductivity = 479 ± 9 µS and temperature = 15.0 ± 1.0 °C. The average water concentration of E2 measured in the control treatment tanks was not detectable (< 0.005 µg/L in all samples) and in the E2 treatment tanks was 0.47 ± 0.02 µg/L (mean ± SEM) with a range of 0.40 – 0.51 µg/L. The concentration of E2 in the treatment tanks during the recovery period was also non-detectable (< 0.005 µg/L in all samples).

Plasma 17β-estradiol concentration and hematocrit

Plasma E2 concentrations were significantly elevated in E2-treated fish after both 2 and 7 d of treatment compared to control fish (Fig. 3-1), but returned to control levels following the 14 d depuration period (Fig. 1). Following E2 treatment, hematocrit was decreased on both day 2 and day 7, with recovery to control levels by 14 d post-exposure (Fig. 3-2).

Phagocytosis and respiratory burst activity

Phagocytosis and respiratory burst assays were conducted on HKL, which had a viability of 95.3 ± 0.3% (mean ± SEM) at the beginning of the assay. The percent of phagocytic cells, phagocytic capacity and respiratory burst were all unaffected by E2 treatment (data provided as supplemental information, Table S3-1).

B and T cell proliferation

B and T cell proliferation were assessed using PBL, which had a viability of 98.0 ± 0.2% (mean ± SEM) at the beginning of the assay. LPS-stimulated B cell proliferation was transiently increased on day 2 of E2 exposure, with proliferation returning to control levels by day 7 of exposure (Fig. 3-3). Concanavalin A-stimulated T cell proliferation decreased, with effects being significant by day 7 of E2 exposure and recovery to control levels by 14 d post-exposure (Fig. 3-4).

Evaluation of gene expression

QPCR analysis

All four ER transcripts were detected in both HKL and PBL at every sampling time point, with varying levels of expression (Figure 3-5). The PBL expressed greater levels of both ERα1 and ERα2 compared to HKL, while HKL expressed greater levels of both ERβ1 and ERβ2. The most prevalent transcript for both HKL and PBL was ERα1 and the least expressed transcript was ERβ1 (HKL = ERα1 > ERβ2 > ERα2 > ERβ1; PBL = ERα1 > ERα2 > ERβ2 > ERβ1).

Exposure to E2 had no effect on the mRNA abundance of ERβ1 or ERβ2 in either HKL or PBL (Supplemental Data, Table S3-2). However, treatment with E2 for 7 d

resulted in up-regulation of ERα1 expression in both HKL (Figure 3-6A) and PBL (Figure 3-6B). ERα2 in HKL was also up-regulated after 7 d of E2 treatment, but was down-regulated in PBL after both 2 and 7 d of exposure. Transcript abundance for all ERs returned to control levels following the 14 d depuration period.

For the four immune-related genes analyzed by QPCR (TNFα, IL1β, CXCR4, CCR7), all were present in the HKL and PBL at the day 2 and day 7 sampling time points. However, there were no differences in expression between the E2-exposed fish and the control fish, even though some genes exhibited more than a 2-fold change (Table 3-2).

ER sequencing

BLASTN results of ERα1 and ERα2 from HKL and PBL (Table 3-3) each had 99% similarity to the sequences reported by Nagler et al. [45], including the original ERα1 sequence GenBank IDs: AJ242740 and AJ242741 [47] and ERα2 sequence GenBank ID: DQ177438 [45]. For both HKL and PBL, the same mismatch was present in ERα1 and ERα2. The mismatch in ERα1 (nucleotide A instead of G) leads to an amino acid change (aspartate (D) instead of glycine (G)). The mismatch in ERα2 did not alter the amino acid sequence. ERβ1 and ERβ2 from HKL and PBL each had 100% similarity to the sequences reported by Nagler et al. [45], including ERβ1 sequence GenBank ID: DQ177439, and ERβ2 sequence GenBank ID: DQ248229 (Table 3-3).

Discussion

There is widespread recognition of the relationship between the endocrine system and the function of the immune system in vertebrates. Field studies have found that reproductively mature and spawning fish are more susceptible to disease, particularly ectoparasites [13,51]. During reproductive periods, circulating concentrations of a number of hormones are elevated, including cortisol, androgens and estrogens. All of these hormones have the potential to exert immunomodulatory effects, such that it remains difficult to establish which hormone, if any, are involved in increased disease susceptibility in wild populations. While the effects of cortisol on overall immunocompetence is recognized [39,52], the relationship between circulating E2

concentrations and immune system function is not as well established and existing literature regarding effects at the cellular level is often contradictory. Although the E2 in this study was exogenously delivered through aquatic exposure to juvenile rainbow trout, the resulting plasma E2 concentration (7.5 – 10.8 ng/mL) was within the range observed in mature adult females (1.5 to 45 ng/mL reported Tyler et al. [66], suggesting that the effects observed in this study may have physiological relevance.

Often used as a general health indicator in fish, hematocrit declined following E2 treatment, with effects beginning within 2 d of exposure and recovery to control levels following 14 d of depuration. Similar decreases in hematocrit have been reported by others in studies of salmon [24] and trout [56]. A decrease in hematocrit can reflect a decrease in red blood cell volume or an increase in plasma volume (hemodilution). While there is no information on the effects of E2 on erythropoiesis in fish, estradiol benzoate slowed the maturation of new red blood cells in channel catfish [21]. Elevated E2 in birds caused hemodilution by increasing plasma osmolality due to production of yolk precursors in the liver and their release to the blood, resulting in water movement into the blood to compensate [67]. Plasma vitellogenin levels are often increased in fish following E2 exposure [65], suggesting that, as in birds, hemodilution may also occur in fish.

E2 had no effect on phagocytosis or respiratory burst. There are mixed findings in the literature regarding the effects of E2 on these immune function endpoints. E2 has generally been implicated in reducing phagocytosis, both *in vitro* and *in vivo* [69,70,75]. However, this may be species-specific since one *in vitro* study of the effects of hormones on phagocytosis found that E2 was suppressive in one species of fish (common carp, *Cyprinus carpio*), but not another (tilapia, *Oreochromis* spp.) [34]. The effects on respiratory burst activity also vary, with E2 shown to stimulate [64], suppress [70] or have no effect [69,75] on respiratory burst activity. Some of the disparate observations may be due to differences in exposure protocols and pharmacokinetics among studies.

Few studies have examined the influence of E2 on B and T cell function in fish. In the present study, LPS-stimulated lymphocyte proliferation was found to be transiently elevated after 2 d of E2 exposure, while Con A-stimulated lymphocyte proliferation was decreased after 7 d. Similarly, following E2 treatment, Wang and Belosovic [74] found

that stimulating lymphocytes with phorbol myristate acetate and a calcium ionophore (which would activate both B and T cells) suppressed lymphocyte proliferation in goldfish, both *in vitro* and *in vivo*. Cook [11] found that E2 increased B cell proliferation in rainbow trout, however a more recent *in vitro* study found that E2 exposure for 4 d suppressed B cell proliferation at high concentrations (> 272 µg/L) but had no effect at lower concentrations [61] similar to the plasma concentrations measured in the current study. While the findings by Shelley et al. [61] initially seem contradictory, it is plausible that E2 exposure may not cause effects after 4 d since in the current study B cell proliferation to returned to control levels sometime between 2 and 7 d of E2 exposure.

The detection and characterization of ER transcripts in isolated leukocytes was performed in this study using QPCR (Figure 5) and represents the first report of the full complement of rainbow trout ER mRNA in purified HKL. In terms of transcript abundance, our results for HKL (ERα1 > ERβ2 > ERα2 > ERβ1) differ from those reported for the rainbow trout anterior kidney tissue ER mRNA abundance (ERβ2 > ERα1 > ERβ1 > ERα2) [45], likely due to their use of whole tissue versus our use of isolated leukocytes. For PBL, all ER transcripts were detected, which differs from Iwanowicz and Ottinger [27], who found only ERα in channel catfish PBL. The abundance of ER transcripts in our study (ERα1 > ERα2 > ERβ2 > ERβ1) is similar to that previously published for rainbow trout, except ERα1 and ERα2 are reversed (ERα2 > ERα1 > ERβ2 > ERβ1) [61], which may be related to developmental stage. In the present study with rainbow trout PBL, ERα was most abundant, consistent with both the previous studies of PBL in rainbow trout [61] and humans [50].

Previously it was shown that E2 did not differentially regulate ER transcript abundance in rainbow trout PBL [61]. However, that study was conducted *in vitro* with PBL derived from immature adult fish, used much higher E2 concentrations (~ 2 mg/L) and only assessed ER transcript abundance in activated (LPS-stimulated) PBL after 4 d of E2 exposure. In our current *in vivo* study, exposure to E2 had direct effects on HKL and PBL ERα1 and ERα2 gene expression but not ERβ1 or ERβ2 (Figure 6). There have been reports of ER transcript induction in liver tissue by xenoestrogens [19,25], including E2 treatment [4].

While differential regulation of ERα46 (but not ERβ) by E2 treatment has been reported in human macrophages [44], this is the first report of E2 treatment altering ERα1 or ERα2 gene expression in isolated fish leukocytes. This supports the hypothesis that leukocytes expressing ERs are likely direct targets of estrogens [27]. Therefore, E2-induced immunomodulation observed at the cellular level may be mediated via differential expression of ERα1 and ERα2. The ERs are nuclear transcription factors that alter the expression of genes containing estrogen-responsive elements (EREs), which have now been found in upstream regions of some cytokine genes [27]. Several microarray studies of E2-treated fish have also demonstrated that a number of immune system-related genes are differentially regulated [4,72], and it can be hypothesized that E2-mediated changes in ERα mRNA abundance may underlie at least some of those alterations in gene expression.

In an effort to further evaluate the genomic basis of immune function alteration in a teleost, QPCR was used to detect and characterize target genes that have been shown in previous studies involving E2 exposure [28,35]. The expression of these four immune-related genes (TNFα, IL-1β, CXCR4, CCR7) did not differ between control and E2-treated fish, despite some of the fold changes in transcript abundance being > 2, which is often used as a criterion for determining significant effects on gene expression (Table 2). Jin et al. [28] showed that, while TNFα and IL1β were up-regulated by exposure to 12.5 µg/L E2 in newly hatched zebrafish (*Danio rerio*), exposure to 0.5 µg/L E2 had no significant induction, which was comparable to the present results in rainbow trout exposed to 0.47 µg/L E2. Similarly, Liarte et al. [35] found that TNFα and IL-1β were up-regulated following *in vitro* exposures to 5 µg/L E2 in gilthead seabream (*Sparus aurata*) macrophages; however, this concentration is 10 fold higher than the E2 exposure in this study. Jin et al. [28] also evaluated chemokine receptor gene target(s) similar to CXCR4 and CCR7, and found no significant increases in transcript abundance following E2 treatment, which is consistent with the present results. Future studies would benefit from examining the responses of these and other immune-related genes during an activated immune response (e.g. following mitogen stimulation).

Some of the variation in outcomes, particularly in cellular assays, following E2 exposures in fish can be attributed to differences in assay methodologies and sensitivities, as well as small sample sizes [5]. Differences among studies that may

constrain comparisons also include toxicokinetic factors (uptake/absorption, distribution, metabolism, elimination) and the exposure route (*in vitro*, or *in vivo* via injection/implants, feeding or waterborne exposures) which influence the amount and form of E2 available to exert effects [27,54]. This could be overcome to some extent by comparing results based on plasma E2 concentrations; however many of the available studies do not report this. In addition, various species of fish have different ER repertoires which may be differentially expressed among cell or tissue types, resulting in the potential for an inter-species divergence of responses [27,45]. Life stage also influences which ERs are expressed at least in some tissues [16] and, although not currently demonstrated in leukocytes, this differential expression might also explain the variability in responses to E2 in the immune system. In addition, as reported here, E2 itself has been found to auto-regulate the transcription of ERs [7,38], which may change responses over time as long as E2 levels remain elevated.

Widespread reports of the effects of endocrine disrupting chemicals (EDCs) on fish have highlighted concerns about the estrogenic or anti-androgenic properties of municipal and/or industrial effluents. Much of the research has examined reproductive outcomes, with reports of reproductive abnormalities in wild fish populations living in polluted environments downstream of sewage treatment plant [3,29] and pulp mill effluent discharges [40]. Laboratory studies have further confirmed the field findings (reviewed by [15]) and uncovered some of the mechanisms underlying these effects [2,17,63].

However, the presence of ER transcripts in leukocytes and the effects of E2 on immune function suggest that the immune system may also be a vulnerable target for estrogenic EDCs [10,42]. Several recent studies have noted immunotoxicity in fish following exposure to xenoestrogens, such as those in sewage effluent [36,55] or nonylphenol [23,61]. While the estrogens and xenoestrogens present in sewage or other effluents may be degraded in the environment, their continual discharge may result in their ‘pseudo-persistence’ in impacted aquatic habitats [14] and the effects of these low-level, chronic exposures on immune system function are largely unknown. In addition, short-term pulse exposures to EDCs, such as in the current study, have been linked to long-term immunotoxicological impairment when the exposures occur during sensitive, hormone-mediated developmental stages [43]. Additional research is needed

in this area to fully understand the implications of EDC- and hormone-mediated immunomodulation in fish.

Conclusion

This study indicates that estrogen, comparable to physiological levels in adult fish, can affect components of the immune response in teleosts. The full complement of ERs is reported for the first time in purified HKLs and confirms previous reports of ER transcript presence in rainbow trout PBLs. Similar to what is observed in other tissues the ERs in both HKL and PBL are susceptible to auto-regulation by E2, which may contribute to the changes observed in immune function at the cellular level. Additionally, the altered endpoints measured here during E2 exposure appear to be transient, with cellular function and changes in ER transcript levels returning to normal levels within 2 weeks of the withdrawal of E2 exposure. Future research on the immunological effects of estrogens and xenoestrogens (EDCs) will help to clarify the implication of real-world exposures in aquatic environments influenced by urban and industrial activities.

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Tables

Table 3-1. Genes and primer sequences used in QPCR analysis

Gene Name	Gene Bank ID	Forward Primer	Reverse Primer
Tumor necrosis factor α (TNFα)	AJ277604	TGCGATGACACCTGAAGA	CCTGGCTGTAAACGAAGA
Interleukin 1β (IL1β)	AJ223954	AGGGTCTGGATCTGGAGGT	TCTGCTGGCTGATGGACTT
Chemokine (C-X-C motif) receptor 4 (CXCR4)	AJ001039	CAGCTCGGAAGAATCAGG	GTGGCAAACACTATGTCAGG
Chemokine (C-C motif) receptor 7 (CCR7)	AJ003159	ATCTGCCGTTAGGGCTTTC	TGCGGTTGTTCTGGTTACTC
Ribosomal protein L8 (L8)	AY957563	CAGGGGACAGAGAAAAGGTG	TGAGCTTTCTTGCCACAG

Table 3-2. Transcription of four immune system-related genes in rainbow trout head kidney (HKL) or peripheral blood leukocytes (PBL) is unaffected by exposure to 0.47 µg/L 17β-estradiol for 2 or 7 d. Data is expressed as mean L8 normalized copy number ± SEM and n = 10 – 12.

Gene	Tissue	Day	Control	17β-Estradiol	Fold change	*p*-value (Welch's t-test)
Tumor necrosis factor α (TNFα)	HKL	2	4836 ± 777	8006 ± 2903	1.7	0.30
		7	2811 ± 496	2740 ± 575	-1.0	0.93
	PBL	2	1974 ± 913	867 ± 156	-2.3	0.24
		7	1348 ± 559	363 ± 57	-3.7	0.15
Interleukin 1β (IL1β)	HKL	2	17372 ± 4828	23830 ± 7975	1.4	0.50
		7	20549 ± 6717	36402 ± 12985	1.8	0.28
	PBL	2	6773 ± 3711	1645 ± 390	-4.1	0.18
		7	1642 ± 896	191 ± 32	-8.6	0.19

Chemokine (C-X-C motif) receptor 4 (CXCR4)	HKL	2	3051046 ± 338752	2799715 ± 891581	-1.1	0.79
		7	1713142 ± 320363	1431502 ± 345010	-1.2	0.56
	PBL	2	768585 ± 156309	549871 ± 51704	-1.4	0.20
		7	941930 ± 356926	287109 ± 64291	-3.3	0.16
Chemokine (C-C motif) receptor 7 (CCR7)	HKL	2	527504 ± 113871	555262 ± 175119	1.1	0.90
		7	383434 ± 121355	591784 ± 142973	1.5	0.28
	PBL	2	267209 ± 57420	200520 ± 24923	-1.3	0.30
		7	186753 ± 35947	139350 ± 24818	-1.3	0.34

Table 3-3. Blast results (# of nucleotides matching and % match) of estrogen receptor (ER) sequences isolated from head kidney leukocytes (HKL) and peripheral blood leukocytes (PBL).

Target Gene	# of Nucleotides Matching	% Match	Accession # of Top Hit
HKL			
ERα1	106 / 107 #	99	AJ242741 *
ERα2	82 / 83 %	99	DQ177439
ERβ1	133 / 133	100	DQ177439
ERβ2	125 / 125	100	DQ248229
PBL			
ERα1	106 / 107 #	99	AJ242741 *
ERα2	82 / 83 %	99	DQ177439
ERβ1	133 / 133	100	DQ177439
ERβ2	125 / 125	100	DQ248229

Mismatch at position #101 (G) compared to AJ242741; HKL or PBL sequence has (A).

% Mismatch at position #1485 (T) compared to DQ177438; HKL or PBL sequence has (C).

* Same E-score (5e-50) as AF099079 and AJ242740.

Figures

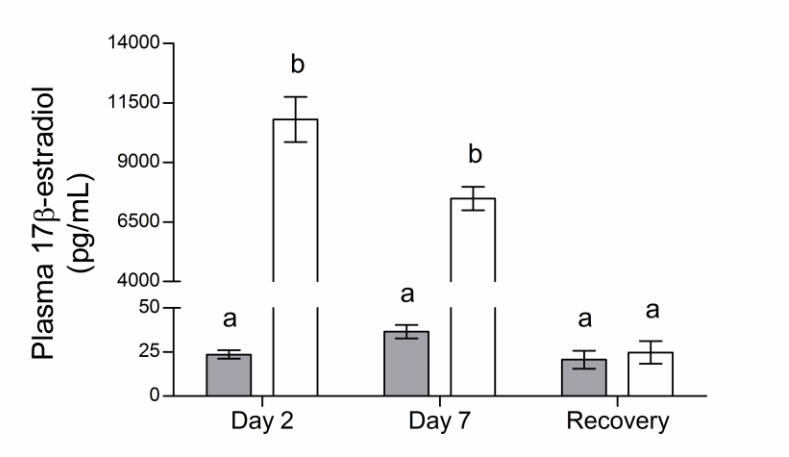

Figure 3-1. Plasma 17β-estradiol (E2) was elevated in juvenile rainbow trout treated with 0.47 µg/L E2 (white bars) after both 2 and 7 d of exposure, but returned to control (grey bars) levels after a 14 d recovery period. Data are shown as mean ± SEM, and n = 9 – 15. Differences between treatments over time were detected using a 2-way ANOVA (p < 0.05), and treatments not connected by the same letter are significantly different.

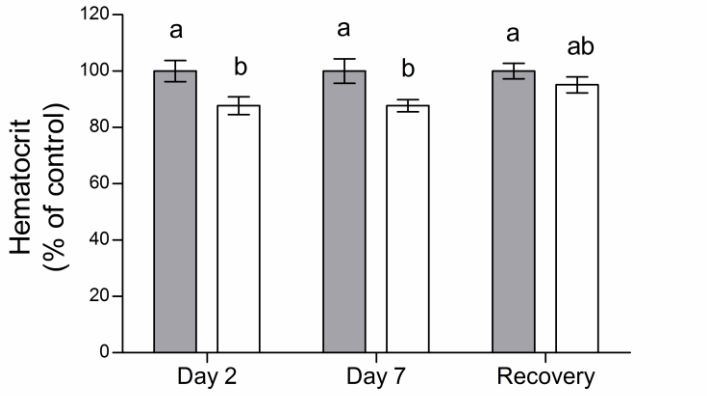

Figure 3-2. Hematocrit was decreased in juvenile rainbow trout after both 2 and 7 d of exposure to 0.47 µg/L 17β-estradiol (white bars), but returned to control (grey bars) levels after a 14 d recovery period. Data were normalized to percent of control on each day and are expressed in percent as mean ± SEM, and n = 9 – 15. Differences between treatments over time were detected using a 2-way ANOVA (p < 0.05), and treatments not connected by the same letter are significantly different.

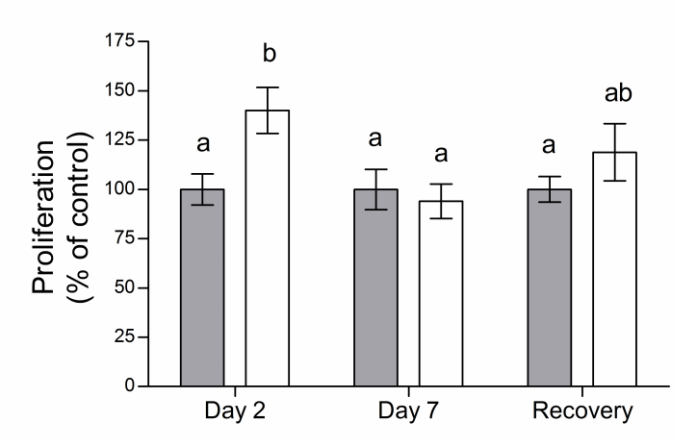

Figure 3-3. Lipopolysaccharide-stimulated lymphocyte proliferation was increased in peripheral blood leukocytes from juvenile rainbow trout following 2 d of exposure to 0.47 µg/L 17β-estradiol (white bars), but returned to control (grey bars) levels by 7 d of treatment and after a 14 d recovery period. Data were normalized to percent of control on each day and are expressed in percent as mean ± SEM, and n = 10 – 15. Differences between treatments over time were detected using a 2-way ANOVA (p < 0.05), and treatments not connected by the same letter are significantly different.

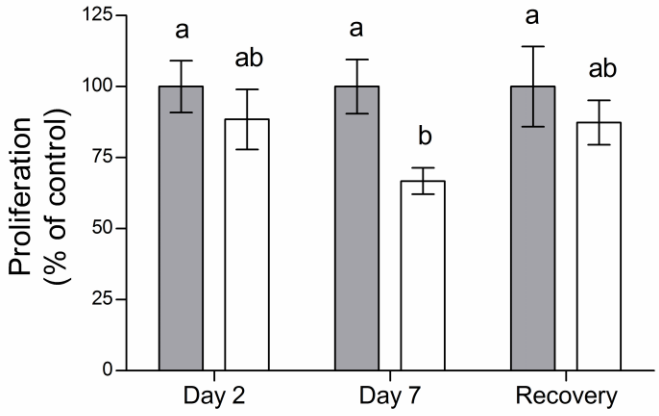

Figure 3-4. Concanavalin A-stimulated lymphocyte proliferation was decreased in peripheral blood leukocytes from juvenile rainbow trout following 7 d of exposure to 0.47 µg/L17β-estradiol (white bars), but returned to control (grey bars) levels after a 14 d recovery period. Data were normalized to percent of control on each day and are expressed in percent as mean ± SEM, and n = 9 – 15. Differences between treatments over time were detected using a 2-way ANOVA (p < 0.05), and treatments not connected by the same letter are significantly different.

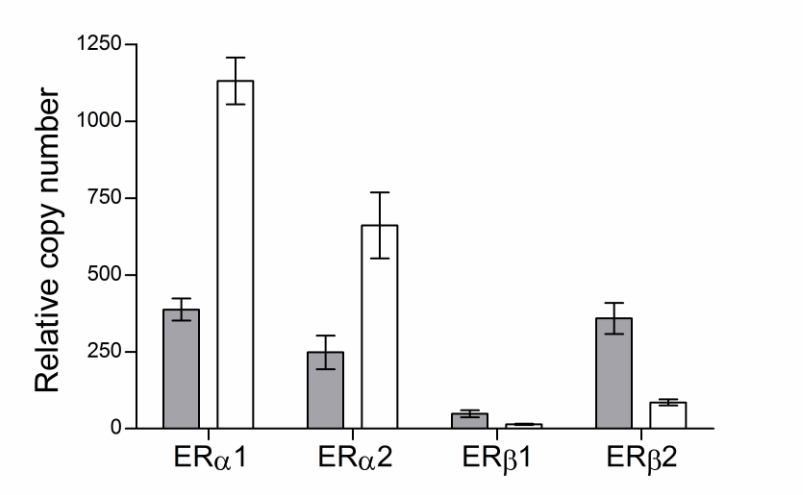

Figure 3-5. Four forms of estrogen receptor (ER; ERα1, ERα2, ERβ1 and ERβ2) mRNA are present in both head kidney leukocytes (grey bars) and peripheral blood leukocytes (white bars) isolated from juvenile rainbow trout sampled from the control group on day 2, day 7 and after the 14 d recovery period. Data are shown as mean L8-normalized copy number ± SEM, and n = 28 – 34

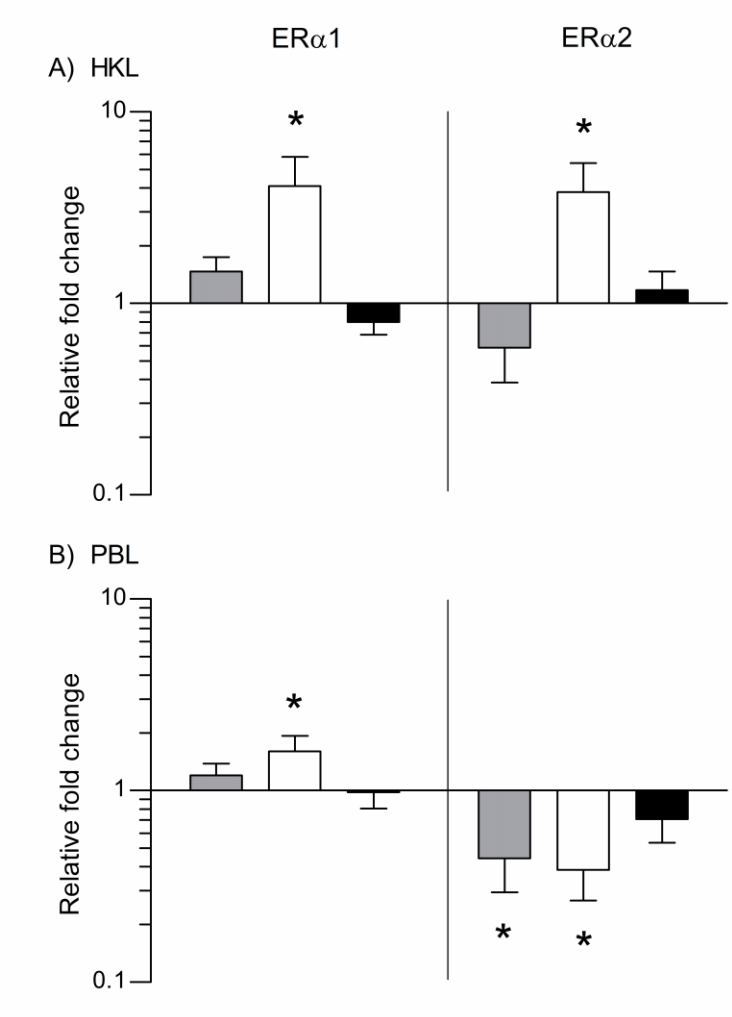

Figure 3-6. Abundance of estrogen receptors (ER) ERα1 and ERα2 mRNA was differentially regulated in A) head kidney leukocytes (HKL) and B) peripheral blood leukocytes (PBL) isolated from juvenile rainbow trout exposed to 0.47 µg/L 17β-estradiol (E2) for either 2 d (grey bars) or 7 d (white bars), but returned to control levels after a 14 d recovery period (black bars). Data are shown as fold change ± SEM in L8-normalized ER transcript abundance relative to the control (where a fold change of 1 = same as control), and n = 8 – 11. Differences between treatments over time were detected using a 2-way ANOVA (p < 0.05), and treatments that were significantly different than the control are indicated with an *.

Supplemental Data

Table S3-1. Percent phagocytic cells, phagocytic capacity and respiratory burst were unaffected by 17β-estradiol (E2) treatment. Fish were treated with 0.47 µg/L 17β-estradiol for up to 7 days, followed by a 14 day recovery period. Data was normalized to percent of control activity on each day and is expressed in percent as mean ± SEM. Data was analyzed using a 2-way ANOVA, and no significant differences were detected (p > 0.05).

Assay	Day 2		Day 7		Recovery	
	control	E2	control	E2	control	E2
Phagocytic Cells (%)	100.0 ± 15.4	84.1 ± 16.3	100.0 ± 15.2	102.7 ± 18.9	100.0 ± 11.3	76.5 ± 4.9
(n)	11	11	15	15	8	6
Phagocytic Capacity	100.0 ± 0.9	100.8 ± 0.4	100.0 ± 0.8	101.3 ± 0.7	100 ± 0.7	99.3 ± 1.3
(n)	11	11	15	15	8	6
Respiratory Burst	100.0 ± 13.9	95.7 ± 11.2	100.0 ± 17.3	96.6 ± 18.9	100.0 ± 9.4	79.4 ± 7.2
(n)	10	11	12	10	6	5

Table S3-2. Transcript abundance of estrogen receptor β1 (ERβ1) and ERβ2 in head kidney (HKL) or peripheral blood leukocytes (PBL) was unaffected by 17β-estradiol (E2) treatment. Fish were treated with 0.47 µg/L 17β-estradiol for up to 7 days, followed by a 14 day recovery period. Data are shown as L8-normalized copy number ± SEM, and n = 10-15 per treatment group per time point. Data was analyzed using a 2-way ANOVA and no significant differences were detected (p > 0.05).

	Day 2		Day 7		Recovery	
	control	E2	control	E2	control	E2
HK						
ERβ1	69.9 ± 25.8	63.7 ± 19.7	49.6 ± 18.3	76.2 ± 24.7	21.9 ± 3.0	71.5 ± 26.8
ERβ2	410.5 ± 110.4	205.2 ± 67.9	222.0 ± 30.5	540.4 ± 206.6	447.1 ± 85.4	636.8 ± 151.7
PBL						
ERβ1	14.8 ± 3.9	13.2 ± 2.9	15.3 ± 4.6	8.3 ± 1.8	12.3 ± 2.4	40.6 ± 22.9

ERβ2	95.9 ± 20.6	84.3 ± 6.6	80.1 ± 17.1	72.9 ± 11.9	78.5 ± 13.3	42.3 ± 10.6

Chapter 4.

Immunotoxic and cytotoxic effects of atrazine, permethrin and piperonyl butoxide to rainbow trout following *in vitro* exposure

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Abstract

For many current use pesticides, limited information exists on their cytotoxicity and immunotoxicity in non-target organisms such as fish. We examined the effects of atrazine, permethrin and piperonyl butoxide (PBO) exposure, *in vitro*, on rainbow trout (*Oncorhynchus mykiss*) lymphocyte viability and proliferation. Purified rainbow trout peripheral blood leukocytes (PBLs) were exposed *in vitro* to the test chemicals (0, 0.01, 0.1, 1 and 10 µM) for 96 h, with and without the mitogen lipopolysaccharide. All three chemicals caused a decrease in both lymphocyte viability and proliferation at 10 µM, while atrazine also suppressed proliferation of PBLs at 1 µM. The *in vitro* toxicity of these chemicals to this salmonid underscores the need for further investigation using *in vivo* studies and host resistance models.

Keywords

rainbow trout; pesticide; immunotoxicity; lymphocyte proliferation; cytotoxicity

Introduction

The immune system has been shown to be a sensitive target for toxic insults by xenobiotics, with impairment of the immune system associated with increased morbidity and mortality due to infectious diseases [1]. Immunotoxicity in fish has been demonstrated for a wide range of chemical classes including polycyclic aromatic hydrocarbons (PAHs) [2], polychlorinated biphenyls (PCBs) [3] and some pesticides [4]. However, for many pesticides, the potential for immunotoxicity in exposed fish has been largely unexplored or data is very limited.

Pesticides enter aquatic habitats as a result of direct use on adjacent lands, aerial drift, runoff following their applications, and/or accidental release. As a result, it is not surprising that many pesticides have frequently been detected in aquatic environments throughout North America [5, 6]. In British Columbia, Canada, alone, more than 4 million kilograms of pesticides were sold in 2003 [7], while in the United States an estimated 450 million kilograms of pesticides are used each year [5]. The heavy use of pesticides, combined with their frequent detection in the aquatic environment, suggest that there may be significant exposures and health consequences in fish residing in waterways that are adjacent to application zones.

Atrazine, a triazine herbicide, has been subjected to intense scrutiny due to its heavy use patterns, frequent identification in environmental samples, and potential for causing detrimental effects in non-target organisms [8]. Atrazine concentrations close to 700 µg/L have been reported (reviewed in [9]), and Rohr and McCoy [10] suggest that concentrations up to 500 µg/L be considered ecologically-relevant. Recent reviews have highlighted the need for more information regarding the immunotoxicity of atrazine to fish in light of the lack of information in teleosts, but notable immunotoxic effects in other organisms [9, 10].

Permethrin is a pyrethroid pesticide that is extensively used in agriculture, as well as in residential and commercial insect control [11]. As a result, permethrin is frequently identified in both sediment (up to 700 ng/g) and water samples (up to 66 ng/L) [12]. Permethrin exposure (50 ng/L) has been shown to decrease resistance to bacterial

infections in medaka (*Oryzias latipes*), despite limited effects detected on cellular immune function [13].

Piperonyl butoxide (PBO) is a common synergist added to insecticide formulations, including those containing pyrethroids. This formulant (or 'inert ingredient') acts as an anti-oxidant and impairs the metabolism of xenobiotics, thereby increasing the effectiveness of the pesticide by allowing the active ingredient to persist in the target pest. Concentrations of PBO as high as 2 – 4 µg/L have been reported in aquatic environments following pyrethroid pesticide application for mosquito control [14]. While data from mammalian studies suggest that PBO affects the function of leukocytes [15], there exist no data for fish.

In the current study, the effects of atrazine, permethrin and PBO on B cell proliferation *in vitro* were assessed using purified peripheral blood leukocytes (PBLs) from rainbow trout (*Oncorhynchus mykiss*). Lymphocyte proliferation is one of the commonly used functional endpoints for the assessment of the immunotoxic potential of xenobiotics [16] and has been used as a biomarker of immunotoxicity in fish [13].

Materials and Methods

All chemicals were obtained from Sigma (Oakville, ON, Canada) unless otherwise noted, with the pesticides being of analytical grade. Rainbow trout were obtained from Miracle Springs Trout Hatchery (Mission, BC, Canada) and acclimated at 12.7°C ± 0.1°C (mean ± SEM) in continuously flowing dechlorinated municipal water for at least two weeks prior to use. At the time of sampling, rainbow trout weighed 163 g ± 9 g (mean ± SEM). All work was conducted in compliance with an animal care permit issued by the Simon Fraser University Animal Care Committee and in accordance with Canadian Council on Animal Care (CCAC) guidelines.

Peripheral blood was aseptically collected from 12 rainbow trout by caudal venipuncture, following euthanization with 0.5 g/L bicarbonate-buffered tricaine methansulfonate (Argent Chemical Laboratories, Redmond, WA, USA). Blood was mixed 1:1 with sterile isolation medium consisting of Hank's Balanced Salt Solution (HBSS) with 15 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 10%

fetal bovine serum (FBS; Invitrogen, Portland, OR, USA), 10 U/mL heparin and 1% penicillin/streptomycin. Peripheral blood leukocytes were obtained using hypotonic lysis [17]. After washing twice, cells were assessed for viability using trypan blue exclusion. Cells from each fish were then resuspended in supplemented Roswell Park Memorial Institute medium (sRPMI) containing 15 mM HEPES, 10% FBS and 1% penicillin/streptomycin.

The cell suspension was adjusted to 5 x 10^6 viable cells/mL for each sample and 100 µL seeded into each of 20 wells of a 96 well round-bottomed plate. Cells in quadruplicate wells were cultured in the presence of the test chemical at concentrations of 0, 0.01, 0.1, 1, and 10 µM (control and 2.2 µg/L – 2.2 mg/L for atrazine; 3.9 µg/L – 3.9 mg/L for permethrin; 3.4 µg/L – 3.4 mg/L for PBO) for 96 h at 15°C. The test chemical solvent (0.5% v/v ethanol) was shown to not affect viability or proliferation in a separate study [18]. Half of the cells also received 100 µg/mL lipopolysaccharide (LPS, *Escherichia coli* O111:B4) in sRPMI to stimulate B cell proliferation while the other half received an equivalent volume of sRPMI.

At the end of the incubation period, cells were transferred to glass test tubes and cellular viability was determined with propidium iodide exclusion using flow cytometry (Becton Dickenson FACSAria, Franklin Lakes, New Jersey, USA) [19]. Proliferation of these same cells was also assessed using flow cytometry based on changes in forward (FSC) and side scatter (SSC) properties as described elsewhere [18, 19]. Gates were set up at the beginning of the experiment for resting cells (low FSC/low SSC) and proliferating cells (high FSC/high SSC), with these same gates used consistently throughout all experiments (see supplemental data, Figure S1, for representative plots). A proliferation index was calculated as the percentage of LPS-stimulated cells in the proliferating cell gate divided by the percentage of unstimulated cells in the proliferating cell gate.

Since cells from each fish received all possible treatments for a given test chemical and results are therefore not independent, the data were analyzed using a repeated-measures ANOVA with a post-hoc Dunnett's test when significant differences ($p < 0.05$) were detected.

Results and Discussion

Prior to cell culture, PBL viability was 99.1% ± 0.2% (mean ± SEM). Following the 96 h incubation, cellular viability was significantly decreased at the highest exposure concentrations of the test chemicals (10 µM) for both unstimulated and LPS-stimulated PBLs when compared to the corresponding control cells, except for PBO where unstimulated cell viability was affected at both 1 and 10 µM (Table 4-1). Permethrin had the greatest impact on viability, while atrazine and PBO had more limited, yet still significant, effects.

Similarly, lymphocyte proliferation was significantly diminished by exposure to 10 µM of each pesticide, although atrazine also elicited effects on proliferation at 1 µM (Figure 4-1). A reduction in the proliferation index may reflect either an increase in unstimulated cells or a decrease in LPS-stimulated cells in the proliferation gate. For atrazine, the effects on proliferation were evident at concentrations that may be considered ecologically-relevant [10], while for permethrin [12] and PBO [14], effects occurred at concentrations higher than those seen in the environment.

The decreased proliferation index following 10 µM permethrin or PBO exposure may be due to cytotoxicity, since viability was also affected at this concentration. Both permethrin and PBO have been found to disrupt the activity of the Ca^{2+}-ATPase present in rat leukocyte membranes at concentrations of 10 µM and 50 µM respectively, which may lead to ionic and cell signaling dysregulation, and contribute to cytotoxicity [20]. For these two treatments relative to the control group, the number of unstimulated cells in the proliferation gate was increased while the LPS-stimulated cells were unaffected (data not shown), suggesting that resting lymphocytes might be subject to preferential cytotoxicity. This is consistent with the findings of Battaglia *et al* [15], who demonstrated that resting murine splenic lymphocytes are more sensitive to cytotoxicity following PBO exposure than the overall splenocyte population.

Exposure to atrazine at concentrations of 1 µM (222 µg/L) and 10 µM (2.2 mg/L) suppressed proliferation, similar to the results of Rymuszka *et al* [21] where proliferation of rainbow trout PBLs was suppressed at ~23 µM atrazine (≥ 5 mg/L). Atrazine exposure in our study also caused a reduction in the proliferation index at a

concentration where cytotoxicity was not evident (1 µM). This suggests that, while cytotoxicity-related processes might contribute to the effects on proliferation at the higher concentration (10 µM), as has been demonstrated in cells from grass carp (*Ctenopharyngodon idellus*) [22], a different mechanism may be involved in the immunotoxicity observed at lower concentrations (1 µM). Biradar and Rayburn [23] found that exposure to 500 nM of atrazine was clastogenic in Chinese hamster ovary cells. Human hepatoma HepG2 cells exposed to atrazine (≥ 2.9 µM) exhibited an accumulation of cells in S phase and a decreased transcription of cyclins required for cell cycle progression [24]. These findings collectively suggest that, in the absence of cytotoxicity, atrazine acts through genotoxic mechanisms that prevent cell cycle progression, thereby inhibiting cellular proliferation. Further investigation would be useful to clarify the mechanisms through which atrazine suppresses proliferation specifically in immune system cells in fish.

While *in vitro* and *in vivo*-based exposures provide a basis to evaluate the mechanistic immunotoxicity of pesticides and other xenobiotics to fish under controlled conditions, host resistance studies provide an opportunity to evaluate the implications of immunotoxicity at the organismal level. Exposure of silver catfish (*Rhamdia quelen*) to 1 mg/L (~5 µM) atrazine [25] or medaka to 50 ng/L (~0.1 nM) permethrin [13] has been associated with increased susceptibility to bacterial infections following experimental challenges. The *in vivo* effects in catfish were observed at about the same concentrations as the *in vitro* effects for atrazine in the current study. However, permethrin immunotoxicity occurred at much lower concentrations *in vivo* than in our *in vitro* study, by approximately four orders of magnitude. This suggests that additional factors (e.g. bioconcentration, toxic metabolites) may influence toxicity in the *in vivo* exposure experiments that cannot be accounted for in *in vitro* studies.

This study provides additional evidence that these two currently used pesticides (atrazine and permethrin) and an “inert” ingredient commonly used in pesticide formulations (PBO) are cytotoxic to leukocytes and that atrazine in particular exhibits immunotoxicity to a test salmonid species (rainbow trout). Given these findings and the other limited data available suggesting these chemicals are immunotoxic, further research on the *in vivo* implications of exposure to these pesticides is warranted,

particularly given the heavy use of these compounds and their frequent detection in fish habitat.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsi.2012.05.020.

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Table

Table 4-1. The viability of both unstimulated and LPS-stimulated peripheral blood leukocytes from rainbow trout declined following in vitro exposure to atrazine, permethrin or piperonyl butoxide. Data is shown in percent as mean cell viability ± SEM, n = 12, and * indicates that the treatment group is significantly different from the corresponding control group (repeated-measures ANOVA with Dunnett's post hoc test, p < 0.05).

Concentration of Pesticide	Atrazine		Permethrin		Piperonyl Butoxide	
	Unstimulated	LPS-stimulated	Unstimulated	LPS-stimulated	Unstimulated	LPS-stimulated
Control (0 nM)	72.9 ± 1.6	79.0 ± 0.9	65.8 ± 2.5	69.4 ± 1.5	64.6 ± 2.8	68.5 ± 1.7
10 nM	72.0 ± 1.8	78.9 ± 0.8	65.7 ± 2.5	68.8 ± 1.5	64.3 ± 3.0	67.5 ± 1.8
100 nM	71.6 ± 1.9	78.7 ± 0.8	65.9 ± 2.8	68.8 ± 1.4	63.6 ± 3.1	67.6 ± 1.8
1 µM	73.2 ± 1.3	78.3 ± 0.8	62.8 ± 2.7	66.2 ± 1.4	62.4 ± 3.1*	67.4 ± 2.0
10 µM	69.6 ± 1.2*	77.7 ± 0.9*	47.4 ± 2.7*	53.4 ± 1.7*	59.5 ± 3.1*	65.0 ± 2.3*

Figure

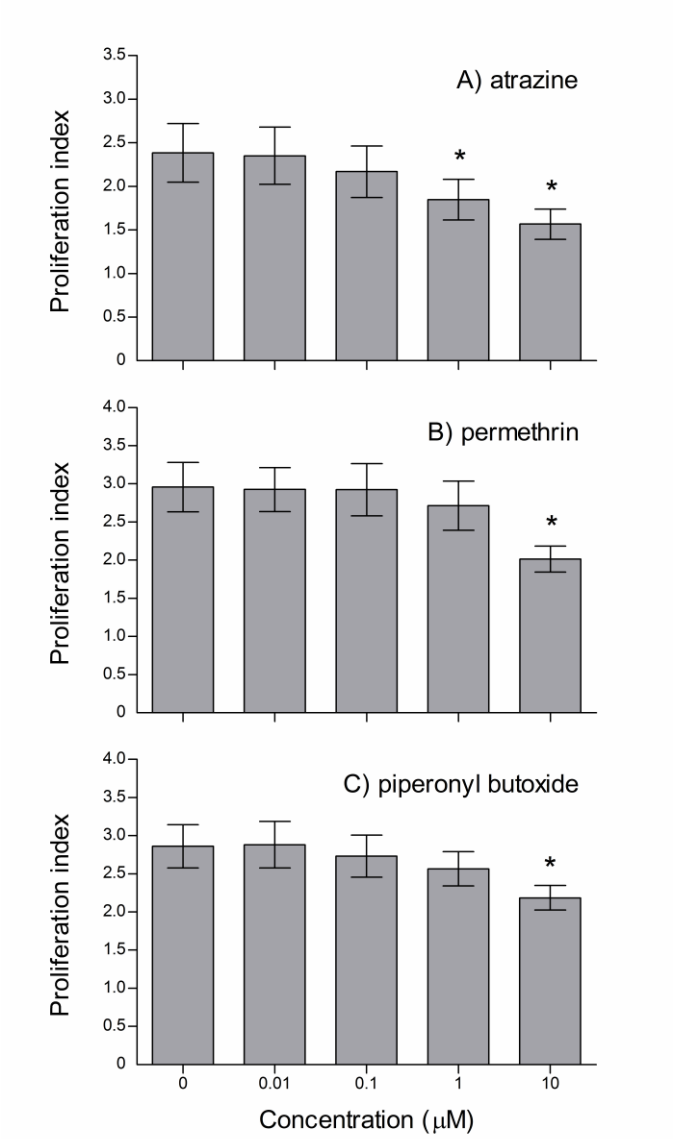

Figure 4-1. The proliferation index of peripheral blood leukocytes from rainbow trout is decreased in a dose-dependent manner following in vitro exposure to A) atrazine, B) permethrin or C) piperonyl butoxide. Data are shown as mean proliferation index ± SEM and n = 12. Differences between control and treatment groups were detected using a repeated-measures ANOVA followed by a Dunnett's post-hoc test, and * indicates that the treatment group is significantly different from the corresponding control group (p < 0.05).

Supplemental Material

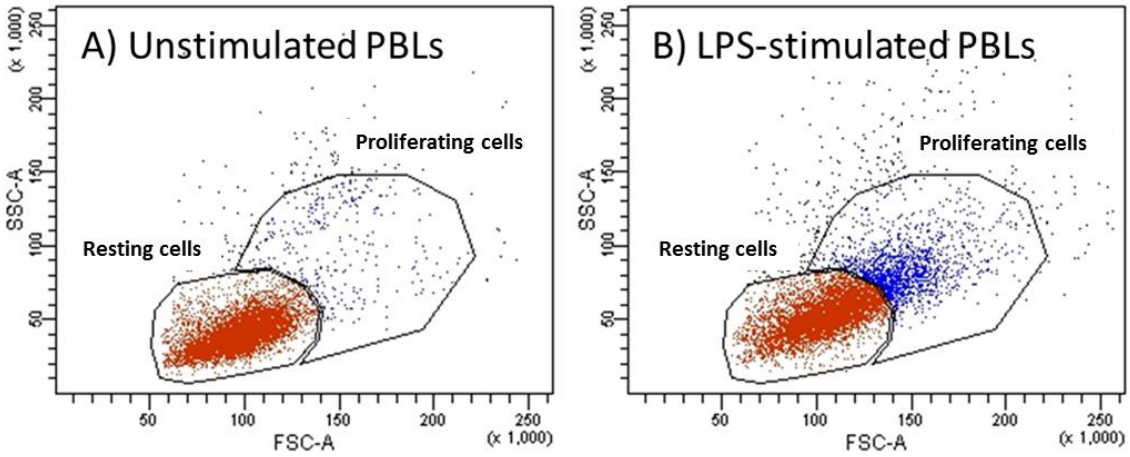

Figure S4-1 – Representative FSC/SSC dot plots showing the gating used throughout the experiment to identify cells undergoing proliferation ('proliferating cell' gate) following 96 h incubation with and without a mitogen. Plot A shows a representative sample of unstimulated peripheral blood leukocytes (PBLs), while Plot B shows cells from the same fish that had been stimulated with lipopolysaccharide (LPS) to induce proliferation.

Chapter 5.

Toxicity of atrazine and nonylphenol in juvenile rainbow trout (*Oncorhynchus mykiss*): Effects on general health, disease susceptibility and gene expression

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Abstract

Atrazine (ATZ) and nonylphenol (NP) are commonly identified contaminants in aquatic habitats; however, few studies have considered the impact of these endocrine disrupters on immune function and resistance to disease. This study examined the immunotoxicological effects of ATZ and NP at multiple levels of biological organization. Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to a solvent control (0.00625 % v/v anhydrous ethanol), or sub-lethal concentrations of ATZ (59 µg/L and 555 µg/L) or NP (2.3 µg/L or 18 µg/L) for 4 d. At the end of exposure, fish were assessed for a number of physiological endpoints, including a host resistance challenge, and liver gene expression was assessed using a salmonid microarray (cGRASP, 32K version 1). While the low ATZ and low NP treatments had no measureable effects on the physiological endpoints measured, fish exposed to the high ATZ concentration (555 µg/L) exhibited significantly elevated plasma cortisol, a decrease in SSI, and decreased lymphocytes and increased monocytes in peripheral blood, with suppression of early immune system processes apparent at the molecular level. In contrast, fish exposed to the high NP concentration (18 µg/L) showed physiological (e.g. significantly elevated LSI) and gene expression changes (e.g. induction of vitellogenin) consistent with estrogenic effects, as well as decreased lymphocytes in the peripheral blood and more limited alterations in immune system related pathways in the liver transcriptome. Fish exposed to high ATZ or NP concentrations incurred higher mortality than control fish following a disease challenge with *L. anguillarum*, while fish exposed to the lower concentrations were unaffected. Microarray analysis of the liver transcriptome revealed a total of 211 unique, annotated differentially-regulated genes (DRGs) following high ATZ exposure and 299 DRGs following high NP exposure. Functional (enrichment) analysis revealed effects on immune system function, metabolism, oxygen homeostasis, cell cycle, DNA damage, and other processes affected by ATZ or NP exposure. Overall, this study provides evidence at multiple levels of biological organization that both ATZ and NP are immunotoxic at sub-lethal concentrations and highlights the potential risk posed by these chemicals to wild fish populations.

Keywords

Immunotoxicity; atrazine; nonylphenol; rainbow trout; microarray; host resistance challenge; leukocyte differential count

Introduction

The detrimental impacts of endocrine disrupting chemicals (EDCs) on fish health are well recognized, particularly in regard to reproductive functions that are regulated by the endocrine system (Arcand-Hoy and Benson, 1998; Jobling et al., 1998; Denslow and Sepúlveda, 2007). EDCs encompass a wide range of chemical classes including phytochemicals (e.g. genistein), pharmaceuticals (e.g. ethinylestradiol, flutamide), plasticizers (e.g. dibutyl phthalate), surfactants (e.g. nonylphenol, octylphenol), and pesticides (e.g. DDT, endosulfan, atrazine) (Rempel and Schlenk, 2008). These chemicals often exert their effects through interactions with hormone receptors, as well as modifying hormone synthesis, distribution or clearance.

The immune system provides critical protective functions to an organism including defense against pathogens and internal monitoring for abnormal or cancerous cells and its function is regulated by the endocrine system (Harris and Bird, 2000). Alterations in immune system function can be measured through infectious disease susceptibility, immune cell function, or by concentrations and function of innate defense components such as antimicrobial proteins (Bols et al., 2001; Kollner et al., 2002). Laboratory studies have demonstrated that some hormones (e.g. estradiol, cortisol) cause immunomodulation in fish that is associated with decreased disease resistance to bacterial pathogens and parasites (Pickering and Pottinger, 1989; Wang and Belosevic, 1994; Wenger et al., 2011). In addition, field studies have correlated elevated levels of steroid hormones with increased disease susceptibility, notably in spawning salmonids (Pickering and Christie, 1980; Currie and Woo, 2007).

In mammals, the potential for EDCs to affect the immune system, either directly or indirectly, was recognized more than a decade ago, but only recently has the teleost immune system been considered as a potential target for EDC-related immunotoxicity (Ansar Ahmed, 2000; Casanova-Nakayama et al., 2011; Milla et al., 2011). Leukocytes of the teleost immune system express receptors for the main steroid hormones including androgens, estrogens and cortisol (Slater et al., 1995; Weyts et al., 1998b; Shelley et al., 2012). Fish leukocytes may therefore be susceptible to direct effects from EDCs that can interact with these receptors, as well as indirect effects resulting from EDC-induced

alterations in endogenous hormone concentrations or activity. Despite the documented relationships between endocrine and immune systems, the molecular mechanisms of EDC-related immunotoxicity in fish remain largely unexplored.

Microarray technology can be used to monitor the transcriptional activity of thousands of genes simultaneously, and has been applied in fish to identify molecular pathways associated with physiological responses to stress, infectious diseases and various toxicants (Krasnov et al., 2005; Hook et al., 2006; Morrison et al., 2006). Although microarrays or similar technology (e.g. suppressive subtractive hybridization) have rarely been used in fish immunotoxicology studies, alterations in immune system-related gene expression is frequently noted in more general toxicology studies using these molecular techniques (Hook et al., 2006; Williams et al., 2007; Benninghoff and Williams, 2008). However, most of these studies have not measured genomic endpoints concurrently with other biological responses, and this is particularly true for functional immunological endpoints. The importance of 'phenotypic anchoring' of genomic alterations is becoming increasingly apparent, since it is critical to link changes at the molecular level with alterations at the cellular or organism level in order to establish biological relevance (Ankley et al., 2006). One of the goals of the current study was to use a combined approach of transcriptomic analysis with relevant immunological endpoints in order to more fully assess the impact of exposure to two EDCs (atrazine (ATZ) and nonylphenol (NP)) on immune system function in teleosts.

ATZ is a triazine herbicide used in the control of broadleaf and some grass weeds in agriculture, particularly during the cultivation of corn crops. ATZ is one of the most heavily used pesticides in the world, with approximately 34,000 tonnes used in the USA in 2007 and approximately 800 tonnes in Canada in 2002 (Yao et al., 2007; US EPA, 2011). Due to its persistence and mobility in the environment, ATZ has been regularly detected in surface and ground water at concentrations in the low µg/L range (Ryberg et al., 2010; Environment Canada, 2011).

ATZ is a suspected EDC, with exposure associated with gonadal abnormalities, alterations in plasma sex hormone concentrations, and changes in developmental rate, particularly in frogs (McKinlay et al., 2008; Rohr and McCoy, 2010). The mechanisms of endocrine disruption by ATZ are unclear, but a number of direct and indirect pathways

have been suggested that involve an increase in the activity of aromatase, which converts testosterone into E2 (Fan et al., 2007). A recent review and risk assessment of ATZ by Solomon et al. (2008) concluded that ATZ did not pose a risk to aquatic species at environmental concentrations; however, the lack of data on fish immunotoxicity from ATZ exposure was specifically noted. In contrast, a meta-analysis of ATZ toxicity suggests that the herbicide poses a risk to fish and wildlife health, including immunotoxicity (Rohr and McCoy, 2010). It has been demonstrated that amphibians exposed to ATZ are more susceptible to parasitic infections in particular (Hayes et al., 2006; Rohr et al., 2008).

NP is used in the production of nonylphenol polyethoxylates (NPEs), and both NP and NPEs are commonly used as surfactants in a range of products for domestic, commercial, industrial and agricultural use. In Canada, approximately 19,000 tonnes of NP and NPEs were available for use in 1996, while an estimated 123,000 - 168,000 tonnes of NP/NPEs were used in the USA in 2008 (Environment Canada, 2001; US EPA, 2010). NPEs undergo degradation into NP, such that NP is readily detected in wastewater discharges from sewage treatment plants and other industrial sources (Soares et al., 2008). Concentrations as high as 60 µg/L in sewage treatment plant effluent and 15 µg/L in river water have been reported, although concentrations of < 1 µg/L in surface waters are more common (Environment Canada, 2001; Soares et al., 2008). NP is not considered particularly bioaccumulative, but it does tend to persist in sediments and due to its heavy use and continuous release it may exhibit 'pseudo-persistence' in the water of receiving environments (Daughton and Ternes, 1999; Sumpter and Johnson, 2008).

NP has been shown to bind to the estrogen receptor, interact with steroid hormone binding proteins in the plasma, and alter steroid hormone metabolism and clearance (Rempel and Schlenk, 2008). Exposure to NP has been associated with reproductive abnormalities and osmoregulatory deficiencies in fish; however, relatively little research has considered the effects of NP on the immune system. Hébert et al. (2009) demonstrated that very low concentrations of NP (1 µg/L) can cause *in vivo* immunotoxicity by impairing cellular immune function including effects on phagocytosis. In addition, Shelley et al. (2012) demonstrated that NP can decrease *in vitro* lymphocyte proliferation.

In the current study, the effects ATZ or NP exposure for 4 d was investigated in juvenile rainbow trout. A comprehensive suite of physiological endpoints were measured that included general health indicators (liver somatic index, spleen somatic index, hematocrit, plasma protein), plasma cortisol, and some more specific immunological endpoints (plasma lysozyme, leukocyte differential, host resistance to bacterial challenge). These physiological endpoints were combined with microarray analysis of liver gene expression (transcriptomics) to gain better insight into the toxicological effects at multiple levels of biological organization. In addition, the microarray analysis was used to generate hypotheses for potential toxicological mechanisms, as well as to direct future investigations.

Materials and Methods

Fish

Juvenile rainbow trout were obtained from Miracle Springs Trout Hatchery (Mission, BC, Canada) and maintained in continuously flowing dechlorinated municipal water for 2 weeks at 12°C ± 1°C. Fish were fin clipped to permit later identification of treatment groups and allowed an additional 3 weeks of recovery and acclimation prior to the initiation of experiments. All fish were fed at the rate of 2% of their body weight each day until the experiment started, but were not fed once the experiment began. At the time of sampling, fish were 29.6 g ± 0.4 g (mean ± SEM). All work with fish was done in accordance with a permit issued by the Simon Fraser University Animal Care Committee in compliance with the Canadian Council on Animal Care guidelines.

Chemical exposure

Unless otherwise noted, all chemicals and reagents were obtained from Sigma-Aldrich (Oakville, ON, Canada). The chemicals used for fish exposures were analytical grade. ATZ was obtained from both Sigma-Aldrich and Chem Service (West Chester, PA, USA).

The day before the experiment began, a total of 45 fish were allocated to each 125 L treatment tank and each treatment was carried out in triplicate. Treatment groups

included a solvent control group, low ATZ (59 µg/L), high ATZ (555 µg/L), low NP (2.3 µg/L) and high NP (18 µg/L), based on measured concentrations. These concentrations were selected to be sub-lethal to rainbow trout during a 4 d exposure (4 d rainbow trout LC_{50} for atrazine is 3.5 – 8.8 mg/L and 120 – 920 µg/L for nonylphenol) (Solomon et al., 1996; Environment Canada, 2001). All treatments received the same amount of solvent (anhydrous ethanol at 0.00625% v/v in the treatment tank) that was used as a carrier for ATZ and NP. Exposure to chemical lasted 4 d; this exposure duration was selected since it is commonly used in acute lethality studies of fish and, since there are relatively few studies of the sub-lethal effects of either chemical on the immune function of fish, it provides a starting point for initial screening level experiments into immunotoxicological effects. Exposures were conducted using a flow-through design, with concentrated chemical solutions from a stock reservoir (refreshed daily) introduced to the treatment tanks continuously using peristaltic pumps (Masterflex, Montreal, QC, Canada), which was mixed with additional clean dechlorinated municipal water to achieve the desired concentrations in the treatment tanks. Total flow-through for each treatment tank was 400 mL/min.

The actual concentrations of the chemicals were determined at the end of the 4 d exposures, just prior to fish sampling, by collecting a composite sample from the replicate tanks for each of the 5 treatment groups. Water samples were collected into 1 L amber glass bottles containing 12.5 mg/L of mercuric chloride as a preservative and were stored in the dark at 4°C. Samples were submitted for analysis at the Laboratories of Expertise in Aquatic Chemical Analysis (LEACA), Fisheries and Oceans Canada, Institute of Ocean Sciences, Sidney, BC.

Sampling of fish

Five fish from each treatment tank (5 treatment groups, in triplicate) were sampled at the end of the 4 d chemical exposure period. Fish were rapidly netted from the tanks, euthanized in a solution containing 0.5 g/L bicarbonate-buffered tricaine methansulfonate (MS222, Argent Chemical Labs, Redmond, WA, USA) and weighed.

Blood was collected into heparinized capillary tubes following severance of the tail at the caudal peduncle. One capillary tube was used to prepare blood smears for

leukocyte differential counts, which were preserved by submersion in 70% methanol for 5 minutes and later stained using modified Wright-Geimsa stain. Two capillary tubes were centrifuged for 3 min using a microcapillary centrifuge (International Equipment Company, Chattanooga, TN, USA) to separate plasma from cells. The capillary tubes were used to determine hematocrit and then plasma was collected from the tubes, transferred to ice and subsequently stored at -80°C in microcentrifuge tubes.

The liver was collected from each fish, rapidly weighed, and up to 100 mg of liver tissue was transferred to 1 mL of RNAlater (Ambion®, from Invitrogen, Portland, OR, USA) in RNase-free microcentrifuge tubes. Tubes were stored on ice during sampling, and then stored at 4°C for 24 h before subsequent storage at -80°C. Liver samples were later transported on dry ice to the Pacific Biological Station (Fisheries and Oceans Canada, Nanaimo, BC) where liver samples were prepared for use as the source of RNA for the microarray analysis (section 2.7). The spleen was also collected and weighed.

Health indices and biological assays

For each fish, liver somatic index (LSI) and spleen somatic index (SSI) were calculated as the weight of the organ divided by body weight x 100. Hematocrit was determined by measuring the different fractions of the centrifuged blood in capillary tubes and was calculated as the height of the red blood cell column divided by the total height of the column.

A leukocyte differential (n = 8) of the blood smears was done by manually counting at least 100 cells and identifying the numbers of lymphocytes, monocytes and granulocytes (Tierney et al., 2004). Leukocyte differential counts were only done for the control, high ATZ and high NP groups for the fish that had their liver transcriptome analyzed by microarray (n = 8 per treatment group).

Where plasma was available, cortisol concentrations, lysozyme activity and protein concentrations were measured. Plasma cortisol concentration (n = 8 per treatment group, the same fish that were used in the microarrays) was assessed using a commercially available kit (Neogen Corporation, Lansing, MI) following the manufacturer instructions. Plasma lysozyme activity (n = 7-10 per treatment group) was assessed

using a microplate method which measures the lysis of *Micrococcus lysodeikticus* over time, as previously described (Shelley et al., 2009). Plasma protein (n = 13-14 per treatment group) was measured using a kit purchased from BioRad (Mississauga, ON) based on the Bradford method (Bradford, 1976), following the manufacturer instructions.

Disease challenge

Listonella anguillarum (formerly *Vibrio*) was used as the model bacterial pathogen for assessing immune system performance following chemical exposures (Arkoosh et al., 2005). This pathogen is infectious to salmonids, causes symptoms and lethality, and the fish had been raised in freshwater and were assumed to be immunologically naïve to this bacterium. *L. anguillarum* has previously been used in other freshwater studies for this purpose (Wood et al., 1996; Shelley et al., 2009). Primary isolates (isolate number 2004-124, serotype 01) were obtained from the Pacific Biological Station in Nanaimo, BC. All work with the bacterial pathogen was done in accordance with a permit issued by the Simon Fraser University Biosafety Committee, based on guidelines from the Canadian Food Inspection Agency for aquatic pathogens.

Bacteria were grown on tryptic soy agar (VWR, Mississauga, ON, Canada) containing 1.5% NaCl w/v (TSA) for 20 h at 18°C prior to the disease challenge. On the day of challenge, bacteria were harvested from the plates, transferred to sterile peptone (BioShop Canada, Burlington, ON, Canada) saline solution and thoroughly vortexed to make a concentrated stock suspension. A serial dilution series was prepared from the stock suspension and the absorbance of each solution was measured on a spectrophotometer (Bio-tek® PowerWave Reader, Winooski, VT, USA) at 540 nm. The concentration of bacteria in the solution was estimated based on the assumption that 1 OD_{540} unit contains 1×10^9 cells. Spike solutions for the disease challenge tanks were prepared from the concentrated stock suspension that would provide 5×10^3 cfu/mL in the challenge tank and this concentration was confirmed by drop plating serial dilutions of the stock suspension (6 x 25µL drops) on TSA and counting the number of colony forming units after incubation overnight at 18°C. The actual concentration of *L. anguillarum* was determined to be 4.7×10^3 cfu/mL.

Following the 4 d chemical exposure, 40 fish from each treatment group were pooled and exposed to *L. anguillarum* by immersion challenges in aerated fiberglass tanks containing saline solution (1.5% w/v NaCl) and bacteria. The challenge was done in triplicate, with 40 fish from each of the 5 treatment groups in each replicate tank. To conduct the challenge, the water level in the tanks was dropped to $1/8^{th}$ of the total tank volume (maximum fish density of 50 g/L) and fish were maintained in the bacterial immersion for 1 h, after which the water flow was restored. Preliminary trials indicated that this exposure regime without bacteria did not cause any mortality (data not shown). Water temperature during the disease challenge was 12.5° ± 0.1°C (mean ± SEM).

Fish were monitored for mortality for a total of 14 d post-challenge. Mortalities in the disease challenge were collected and recorded 3 times per day throughout the 14 d monitoring period. Treatment group was identified by carefully examining the fin clips of fish that had died. All fish that died during the disease challenge were subsequently necropsied and aseptic swabs taken from the head kidney were smeared onto TSA plates. Fish were assumed to have died of vibriosis based on the observation of pure cultures on TSA that were consistent with *L. anguillarum* (non-pigmented, circular, cream-colored colonies), with subsequent confirmation by Gram stain of Gram negative, curved, rod-shaped bacteria.

Microarray

Due to the availability of a limited number of microarrays, liver samples from the control, high ATZ and high NP groups were used in preparation of the microarrays (n = 8 individual microarrays per treatment group). Liver samples from these treatment groups were selected since analysis of data from other physiological assays indicated that there were alterations resulting from the high ATZ and high NP exposures. Liver tissue was selected for microarray analysis because a) it is the most common tissue analyzed in fish toxicological microarray studies, b) it is involved in the production of acute phase response proteins and other components of the immune response, c) it is the primary site of biotransformation and xenobiotic metabolism in fish and d) it is a target for EDC-toxicity in fish (e.g. reproductive toxicity biomarkers), thus providing the greatest amount of useful toxicological and mechanistic data.

Isolation of total RNA, amplification and labelling

Liver sample preparation for microarray analysis was done as previously described in Miller et al. (2009) and Evans et al. (2011). Approximately 10 mg of liver was homogenized in TRI reagent (Ambion, Austin, Texas, USA) with stainless steel beads on a MM301 tissue homogenizer (Retsch, Newtown, PA, USA). A reference sample was prepared, which consisted of a pool of RNA extracted from all samples in the experiment. Homogenates (100 µL) were transferred to 96-well plates and extractions done with a Biomek NXP (Beckman-Coulter, Mississauga, ON, Canada) automated liquid-handling instrument using Magmax™-96 for Microarrays Kits (Ambion), following manufacturer instructions. Total RNA yield and quality was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA (0.5 – 5 µg) from each sample was then amplified using MessageAMP™II-96 kits (Ambion), following manufacturer instructions. Amplified RNA (5 µg) was reverse transcribed into cDNA and labeled using a SuperScript Plus Indirect Labeling Kit (Invitrogen), as described in Miller et al. (2009). The pooled reference sample was labeled with Alexa 647 (green), while the experimental samples from individual fish were labeled with Alexa 555 (red).

The 32,000 feature microarray used in this study was obtained from the Genomics Research on Atlantic Salmon Project (cGRASP 32K version 1 salmonid array, http://www.uvic.ca/bcr/grasp) and has been described and validated by Koop et al. (2008). One experimental sample and the pooled reference sample were hybridized onto each microarray slide. Hybridizations were carried out as described in Miller et al. (2009) using a Tecan-HS4800 Pro Hybridization Station (Tecan Trading AG, Männedorf, Switzerland) and were done over a 1 week period to minimize technical variation.

Microarray data collection and processing

A Tecan LS Reloaded scanner was used to collect fluorescent images, with Automatic Gain Control (AGC) setting adjusted to optimize the visualization of each image. Images were quantified with Imagene (Biodiscovery, El Segundo, CA, USA) and spots with poor quality or no signal were flagged. GeneSight (version 4.1, Biodiscovery) was used to normalize intensity data (loess normalization for background correction) and data were then $\log_2$ transformed. An intensity ratio was calculated for each spot as the

difference between the $\log_2$ transformed intensity of the experimental sample and the $\log_2$ transformed intensity of the pooled reference sample. This data, expressed as mean normalized $\log_2$ ratios, was used in all subsequent microarray statistical analyses.

In the current study, QPCR was not used to verify or validate microarray findings for two reasons. First, a number of previous studies using the cGRASP salmonid array have demonstrated good concordance between microarray and QPCR results (e.g. Miller et al., 2011; Hook et al., 2010). Secondly, the main goal of the microarray analysis was hypothesis generation for subsequent research regarding immunotoxicological mechanisms, not biomarker development, and the focus was on alterations to pathways and biological processes rather than individual genes.

Data from the microarrays was archived in accordance with Minimum Information About a Microarray Experiment (MIAME) protocols in the NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nim.nih.gov/geo/) with the experimental accession number GSE38726.

Statistics for health indices and biological assays

All statistical analysis for these assays was done using JMP (v8.0.2, SAS Institute, Cary, NC, USA). For the health indices, plasma lysozyme and plasma protein assays, no differences were detected between replicate tanks of a treatment, so data from the triplicate tanks were pooled to provide up to n = 15 per treatment group. Data were checked for normality of distribution and equal variance and was log-transformed where necessary to meet the normality and equal variance requirements. An ANOVA was used to detect differences ($p < 0.05$) between ATZ or NP treatment groups and the control group, with a post-hoc Dunnett's test to identify which groups were significantly different. When data didn't meet the criteria for an ANOVA even after being log-transformed, non-parametric analysis using the Kruskal-Wallis test was conducted ($p < 0.05$), followed by a Dunn's post-hoc test when differences were noted.

For the leukocyte differential, where only the high ATZ and high NP groups were evaluated, a t-test was used to detect differences ($p < 0.05$) between the treatment and control group. This percentage data was arc-sine square root transformed prior to analysis.

For the disease challenge data, mortality among treatment groups was assessed using Kaplan-Meier survival analysis, with the log rank test ($p < 0.05$) to determine which treatment groups were different from the control group. When differences were detected, a post-hoc t-test was used to determine on which days the NP- or ATZ-treated fish differed from the control group fish.

Microarray statistics and data analysis

To determine which genes were differentially affected by treatments, mean normalized $\log_2$ ratios were input into Gene Pattern, a publicly-available online analysis tool (Reich et al., 2006). Data was analysed with the comparative marker selection module, using the balanced permutation (50,000) approach of pairwise comparisons of control and either high ATZ or high NP groups (n = 8 per treatment group) (Gould et al., 2006). The Benjamini-Hochberg (BH) multiple test correction was applied and differentially regulated genes (DRGs) were identified as those with a BH-corrected *p*-value of < 0.05 (Benjamini and Hochberg, 1995).

Microarray data was also explored using Pathway Studio® (Ariadne Genomics, Rockville, MD, USA) to perform functional (enrichment) analysis, which identifies biological processes and pathways that are differentially affected by treatment (Nikitin et al., 2003). Since immune system function was of primary interest in this study, a significance value of $p = 0.05$ was used to identify these affected processes or pathways. An additional sub-network enrichment analysis was conducted for the DRG lists to determine if there were key transcriptional regulators that may underlie the immunotoxic responses. For more general functional analysis, a slightly more conservative significance level of $p = 0.01$ was used to gain insight into other processes and pathways affected by chemical exposure.

Results

Health indices and biological assays

LSI was significantly elevated in the high NP treatment group following the 4 d chemical exposure period compared to the control group, but was unaffected in other

treatment groups (Figure 5-1A). The SSI index was significantly decreased only in the high ATZ treatment group following chemical exposure (Figure 5-1B). Plasma cortisol concentrations were found to be significantly elevated only in the high ATZ treatment group following chemical exposure (Figure 5-1C). Leukocyte differential counts were significantly altered by both high ATZ and high NP treatment following chemical exposure (Figure 5-2). The proportion of lymphocytes was decreased by both ATZ and NP treatment, with ATZ also showing an increase in the proportion of monocytes in the blood smears. Hematocrit, plasma protein and plasma lysozyme activity were all unaffected by treatment (Supplemental Data Table S5-1).

Disease challenge

The Kaplan-Meier survival analysis indicated that both high ATZ and high NP treatments resulted in significantly increased mortality from bacterial infection, and the subsequent t-tests indicate that the elevated mortality was apparent on day 14 and day 9 post-challenge, respectively (Figure 5-3). The low ATZ and low NP treatments did not affect mortality following disease challenge (data not shown).

Microarray

General findings

Following 4 d of exposure to either high ATZ or high NP, microarray analysis of the liver revealed that 653 genes were differentially regulated for ATZ, and 845 for NP ($p < 0.05$, Table 5-1). Of these features, 211 annotated, unique genes were identified in high ATZ-treated fish and 299 for high NP-exposed fish. Of the unique genes altered by treatment, only 31 were affected by both ATZ and NP exposures. A complete list of DRGs, their fold change and basic function is provided as supplemental data for ATZ (Supplemental Data Table S5-2) or NP (Supplemental Data Table S5-3) exposed fish.

Immune system-specific gene transcription and functional analysis of microarray data

Functional analysis of the microarray data using Pathway Studio® revealed that a number of biological processes and pathways related to immune-system function were altered by high ATZ or NP exposure ($p < 0.05$, Table 5-2), with the strongest effects

apparent in the ATZ treatment. B cell activation was down-regulated in response to both chemical treatments, as were three processes associated with viral infectivity cycles. A number of processes were differentially affected by ATZ and NP exposures, such as complement activation (alternative pathway) and several STAT signaling pathways, which were up-regulated by NP exposure but down-regulated by ATZ.

In response to high ATZ specifically, innate immune response, acute-phase response, alternative complement pathway, T cell receptor signaling, defense response to virus, and several STAT signaling pathways were all down-regulated, while response to bacterium, immunoglobulin mediated immune response, and cytokine production were up-regulated (Table 5-2). A mixed response was observed for Toll-like Receptor (TLR) signaling pathways. A sub-network enrichment analysis of the DRG lists using Pathway Studio® found that some of the key transcriptional regulators of the immunotoxic response were interferon-gamma (IFNG), cytokines, interleukin-1 (IL1) family, signal transducer and activator of transcription 3 (STAT3), and interleukin 4 (IL4) (Supplemental Data, Figure S5-1).

Exposure to high NP weakly stimulated genes involved in immune response, T-cell activation and complement activation. However, NP exposure down-regulated TLR signaling pathways (MyD88-dependent and -independent pathways) and had mixed effects on the nuclear factor kappa B (NFκB) signal transduction pathway (Table 5-2). Similar to ATZ, sub-network enrichment analysis of the DRG lists for NP-exposed fish found that IFNG and cytokines were important transcriptional regulators of the immunotoxic response, although the IFNG pathway was up-regulated by NP but down-regulated by ATZ (Supplemental Data, Figure S5-2). Jun/Fos, IL6, and STAT1 were identified as other important transcriptional regulators of immunotoxicity following NP exposure (Figure S5-2).

General functional analysis of microarray data

Pathway Studio® analysis of the microarray data revealed a number of additional biological processes and pathways that were affected by chemical exposure, some of which were common to both chemical exposures ($p < 0.01$, Supplemental Data Table S5-4). A wide range of biological processes were down-regulated by both high ATZ and NP exposures, including translation and translation elongation/termination, rRNA

transcription and processing, cellular protein metabolism, hydrogen peroxide catabolism, erythrocyte development, insulin action, regulation of intestinal cholesterol absorption and aging. Only 3 processes were up-regulated by both treatments (muscle contraction, negative regulation of ubiquitin-protein ligase involved in mitotic cell cycle, and peripheral nervous system axon regeneration). Other processes and pathways were differentially regulated by the two treatments, such as down-regulation of intracellular protein transport, lipoprotein biosynthesis, phospholipid efflux, and single-strand nucleotide excision DNA repair in NP-exposed fish, but up-regulation of these processes in ATZ-exposed fish.

Unique biological processes or pathways altered by ATZ exposure included down-regulation of glycogen biosynthesis, creatine metabolism, ribosomal biogenesis, reactive oxygen species (ROS) metabolism and superoxide anion generation (Supplemental Data, Table S5-4). Up-regulated processes following ATZ treatment included pentose-phosphate shunt, protein secretion, mRNA metabolism, oxygen homeostasis, and response to metal ion. In contrast, NP exposure affected processes related to DNA damage and cell cycle control (e.g. single-strand nucleotide excision DNA repair, DNA damage response [signal transduction by p53 class mediator resulting in cell cycle arrest], positive regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle, M-G1 transition of mitotic cell cycle) and down-regulated cholesterol homeostasis, anti-apoptotic, glutathione metabolism and androgen receptor signaling pathways.

Discussion

In this study, a disease challenge coupled with a comprehensive suite of general health indicators, immune-specific endpoints, and an examination of gene expression changes were used to explore the immunotoxicity and the potential underlying mechanisms of ATZ and NP. The use of microarrays also allowed for the identification of other biological processes that are affected by exposure to these chemicals.

Fish exposed to the high ATZ (555 µg/L) treatment, but not any of the other treatments, exhibited significantly elevated plasma cortisol levels, altered leukocyte

differential, a decrease in SSI and a slight (but not significant) increase in hematocrit. Elevated cortisol levels have been previously reported in fish following ATZ exposure and stress hormones are known to induce the contraction of the spleen, resulting in a decreased SSI and elevated hematocrit (Wendelaar Bonga, 1997; Solomon et al., 2009). Elevated cortisol levels have also been associated with a decrease in circulating lymphocytes, as was observed in this study, which may be due to cortisol-induced lymphocyte-specific apoptosis or redistribution of these cells to the tissues from the blood (Weyts et al., 1998a; Davis et al., 2008). Taken together, these findings suggest that many of the physiological changes noted in high ATZ-exposed fish may be due to a generalized stress response induced by chemical exposure.

However, functional analysis of the liver microarray data indicated that the genomic signature was not entirely consistent with what would be expected during a stress response (Aluru and Vijayan, 2009). Elevation of plasma cortisol affects energy use and metabolism and is often associated with gluconeogenesis in the liver, proteolysis in the muscles and lipolysis (Mommsen et al., 1999). The synthesis and release of insulin is decreased during a stress response, which should lead to enhanced breakdown of glycogen and increases in plasma glucose. Stress effects on insulin are mediated by epinephrine, which also stimulates lipolysis and the release of fatty acids from adipose tissue. In the current study, while the insulin action pathway was down-regulated and cellular response to glucose stimulus was up-regulated in ATZ treatment as would be expected, glycogen biosynthesis, protein metabolism and lipid homeostasis were down-regulated and gluconeogenesis was not differentially affected. Thus, it is unlikely that elevated plasma cortisol is responsible for all of the effects observed here, although it could be contributing a contributing factor.

In contrast, NP treatment did not affect plasma cortisol, hematocrit or SSI. However, exposure to high NP (18 µg/L) for 4 d did lead to a significant elevation of the LSI, consistent with the effects observed for other xenoestrogens such as ethinylestradiol, which, like NP, interact with estrogen receptors (Parrott and Blunt, 2005). Elevation of the LSI due to increased liver weight may be at least partially a result of induced gene expression and production of reproductive proteins such as vitellogenin and other oocyte-related proteins that are synthesized in the liver (Arukwe and Goksøyr, 2003). In this study, microarray analysis of the liver did confirm that

transcription of both vitellogenin and zona pellucida-4 was induced (Supplemental Data Table S3), suggesting that genes containing an estrogen response element (ERE) have been activated by NP exposure.

The high ATZ and high NP treatments both altered the leukocyte differential count in peripheral blood, due to a decrease in the proportion of lymphocytes. Similar findings have been recently been reported in African catfish (*Clarias gariepinus*) following a 15 d NP exposure, although rainbow trout in the current study were more sensitive to NP-induced lymphopenia than the catfish (effects apparent at 18 µg/L in rainbow trout compared to 80 µg/L for African catfish) (Mekkawy et al., 2011). Microarray analysis revealed that immunoglobulin lambda-like polypeptide 1 was down-regulated in both high ATZ- and high NP-exposed fish (Supplementary Data Tables S2 and S3). It forms part of the pre-B cell receptor and is required for proper development of B cells in mammals; deficiency or mutation of this gene can lead to pronounced lymphopenia (Minegishi et al., 1998). The regulatory role of this gene in fish has yet to be established, but our data are consistent with a role in B cell homeostasis. Moreover, the microarray data showing down-regulation of processes associated with B cell function in both high ATZ and NP-exposed fish is consistent with the lower concentration of lymphocytes. In NP-exposed fish the Jun/Fos pathway, important for B cell activation, was also identified as a key transcriptional regulator and was down-regulated, which could compromise the activity of acquired immune system processes.

The microarray data provides insight into the differences and similarities between ATZ- and NP-induced alterations in immune gene transcription. Functional analysis in ATZ-exposed fish showing the down-regulation of innate immunity, acute phase responses, and the alternative complement pathway suggest that early immune system recognition of pathogens and associated responses may be compromised, particularly for viral pathogens. The up-regulation of B cell proliferation, Ig-mediated immune response and response to bacterium, as well as the identification of IL4 as an important transcriptional regulator, is consistent with up-regulation of humoral immunity. In general though, ATZ-exposed fish show a pervasive genomic signature consistent with systemic immune system disruption which might be expected to lead to greater susceptibility to infection. In contrast, immunosuppression was less evident at the genomic level following exposure to high NP. However, the alterations in the TLR and NFκB signaling

pathways may lead to interference with recognition of pathogen-associated molecular patterns (PAMPs), signal transduction and molecular response to pathogens, and induction of apoptosis. In addition, several key genes involved in iron homeostasis and sequestration were down-regulated at the end of the NP exposure period, just prior to the disease challenge with *L. anguillarum*, which may allow the bacterial pathogen to establish a lethal infection.

The most definitive test of immune system function with the highest level of biological relevance is the host resistance challenge assay since it measures an integrated immune response at the level of the whole organism (Kollner et al., 2002). Since fish are exposed to pathogens in the environment, any alteration in the susceptibility of the fish to pathogens can have population-level consequences in terms of mortality (Arkoosh et al., 1998; Arkoosh et al., 2004). In the current study, the high exposure groups for both ATZ (555 µg/L) and NP (18 µg/L) led to increased susceptibility to bacterial infection, while lower concentrations (59 µg/L ATZ or 2.3 µg/L NP) produced no significant alterations. Kreutz et al. (2010) recently demonstrated that channel catfish (*Ictalurus punctatus*) were more susceptible to infection caused by *Aeromonas hydrophila* following exposure to ~1 mg/L ATZ. In addition, a number of studies have also demonstrated decreased disease resistance in amphibians at low concentrations of ATZ (Rohr and McCoy, 2010). This is the first report of increased mortality due to a bacterial infection following NP exposure in fish, although it has been previously reported in shrimp (Sung and Ye, 2009).

In immunologically naïve fish, there are three main barriers to combat the agents of infectious diseases: intact skin and mucus; non-specific anti-microbial proteins and factors present in the skin, mucus and blood; and the innate immune response (Bols et al., 2001; Frans et al., 2011). ATZ down-regulated the alternative complement pathway and transcription of an iron-transporting protein (ceruloplasmin, Supplemental Data Table S5-2), both of which are demonstrated to be important for defense against *L. anguillarum* specifically (Frans et al., 2011; Liu et al., 2011). For NP-exposed fish, signaling pathways associated with NFκB and TLR were also generally down-regulated by NP exposure, which Gerwick et al. (2007) suggest are central to the defense response against *L. anguillarum*. In addition, NP exposure down-regulated transcription of two iron-transporting proteins, ferritin heavy chain and serotransferrin (Supplemental

Data Table S5-3), which have been shown to be up-regulated as part of the acute phase response following *V. anguillarum* exposure in other fish species (Liu el al., 2010; Zhang et al., 2010). NP, at similar concentrations as in the current study, has also been shown to cause histological alteration to both the structure of the epidermis and granulation pattern of the mucus-producing goblet cells of the skin in rainbow trout, which may increase disease susceptibility (Burkhardt-Holm et al., 2000). For infections caused by *L. anguillarum*, the skin may represent the most common infection route in fish, making the integrity of the skin and mucus layer a critical defensive barrier (Frans et al., 2011). While the underlying mechanisms of immunotoxicity appear to differ between ATZ and NP, the ultimate outcome in regards to increased disease susceptibility caused by *L. anguillarum* was similar; both of these chemicals were immunotoxic and led to increased mortality following bacterial infection.

Functional analysis of the microarray data also revealed a number of other, non-immune biological processes that were altered by exposure to ATZ and NP. Both chemicals strongly down-regulated translation elongation and translation termination, a response that is typically induced under stress. ATZ exposure up-regulated genes related to oxygen homeostasis, consistent with reports that ATZ exposure causes gill damage which may interfere with oxygen uptake, and down-regulated genes related to gastrulation, consistent with developmental delays and abnormalities reported in frogs (Solomon et al., 2008; Rohr and McCoy, 2009; Xing et al., 2012). NP exposure up-regulated genes related to cell cycle control, DNA damage and apoptosis, consistent with findings that NP is genotoxic and induces apoptosis (Baršienė et al., 2006). Interestingly, NP, widely known as an estrogenic EDC that interacts with the ER, was found to down-regulate the androgen receptor signaling pathway suggesting NP may also have anti-androgenic function in fish, similar to what was previously reported by Lee et al. (2003) using a yeast reporter detection system.

This study reveals that both ATZ and NP are immunotoxic, causing increased disease susceptibility, altered leukocyte differential, and a signature of immunosuppression at the molecular level. Exposure to high ATZ altered physiological parameters consistent with a generalized stress response; however, the resultant transcriptomic profile in the liver was not entirely consistent with elevated cortisol. In contrast, NP did not alter cortisol-sensitive parameters specifically, but estrogen

responsive endpoints (such as LSI) and gene expression (such as vitellogenin induction) were affected. Microarray data and the subsequent functional analysis was valuable for revealing a number of biological processes, including immune system-specific processes, that were affected by xenobiotic exposure and provided data for hypothesis generation for subsequent studies.

Conclusion

Fish are frequently exposed to pathogens in their natural environments, and immunosuppression by chemicals causing increases in infectious disease morbidity and mortality can have serious, population-level consequences. For NP in particular, decreased disease resistance occurred at environmentally-relevant exposure concentrations (18 µg/L), while for ATZ the immunotoxic exposure concentration was above that currently measured in the environment. ATZ exposure had a stronger negative transcriptional effect on genes involved in early immunity than the NP treatment, which suggests that other factors such as alterations in skin/mucus barriers are important in the pathogenesis of *L. anguillarum*. Given that NP is continually discharged to the environment through sewage treatment plants and industrial discharges (pseudo-persistence) and ATZ is heavily used and highly mobile in the environment, the immunological consequences of chronic exposures to lower concentrations should also be investigated. Additional research will be needed to fully characterize the immunotoxicological risk posed by these chemicals to wild fish populations residing in impacted environments.

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Tables

Table 5-1. Number of differentially regulated genes (DRGs) identified in the liver transcriptome of rainbow trout exposed for 4 d to either 555 µg/L atrazine or 18 µg/L nonylphenol, compared to the control group (p < 0.05 after Benjamini-Hochberg multiple test correction).

Treatment group	Total DRGs	Annotated, unique DRGs	Up regulated DRGs	Down regulated DRGs
Atrazine	653	211	118	93
Nonylphenol	845	294	141	158

Table 5-2. Altered immune system-related processes identified using Pathway Studio® (Ariadne Genomics, p < 0.05) in rainbow trout exposed to 555 µg/L atrazine (ATZ) or 18 µg/L nonylphenol (NP) for 4 d, compared to control fish. TCR = T cell receptor, Ig = immunoglobulin, TLR = toll-like receptor.

Biological processes or pathways affected by ATZ exposure		**Biological processes or pathways affected by NP exposure**	
Up-regulated	**Down-regulated**	**Up-regulated**	**Down-regulated**
Positive regulation of inflammatory response	Regulation of type I IFN-mediated signaling pathway	Regulation of defense response to virus by virus	Regulation of inflammatory response
Positive regulation of B cell proliferation	Defense response to virus	Negative regulation of B cell proliferation	I-κB kinase-NF-κB cascade
Ig mediated immune response	Innate immune response	Negative regulation of TCR signaling pathway	
Positive regulation of IL-1β secretion	Alternative complement pathway	Positive regulation of T cell activation	
TLR4/5/7/9 → NF-kB signaling	Acute-phase response		
T cell proliferation	TCR signaling pathway		
Response to bacterium	TLR signaling pathway		
IL1R → NF-kB signaling	IL1R binding		
CCR2/5 → STAT signaling	IL2R → STAT signaling		

Cytokine production	IL3R → STAT signaling		
	IL6R → STAT signaling		
	IL7R → STAT signaling		
Biological processes or pathways affected by both ATZ and NP			
Up-regulated	**Down-regulated**	**Differentially-regulated**	
PTAFR → NF-kB signaling	MyD88-independent TLR signaling pathway	MyD88-dependent TLR signaling pathway	↑ ATZ, ↓ NP
immune response	Positive regulation of NF-κB transcription factor activity	Regulation of IFN-gamma-mediated signaling pathway	↓ ATZ, ↑ NP
	B cell activation	Complement activation, alternative pathway	↓ ATZ, ↑ NP
	Viral reproduction	I-κB phosphorylation	↓ ATZ, ↑ NP
	Viral transcription	CCR1 → STAT signaling	↓ ATZ, ↑ NP
	Viral infectious cycle	CD2 → STAT signaling	↓ ATZ, ↑ NP

Figures

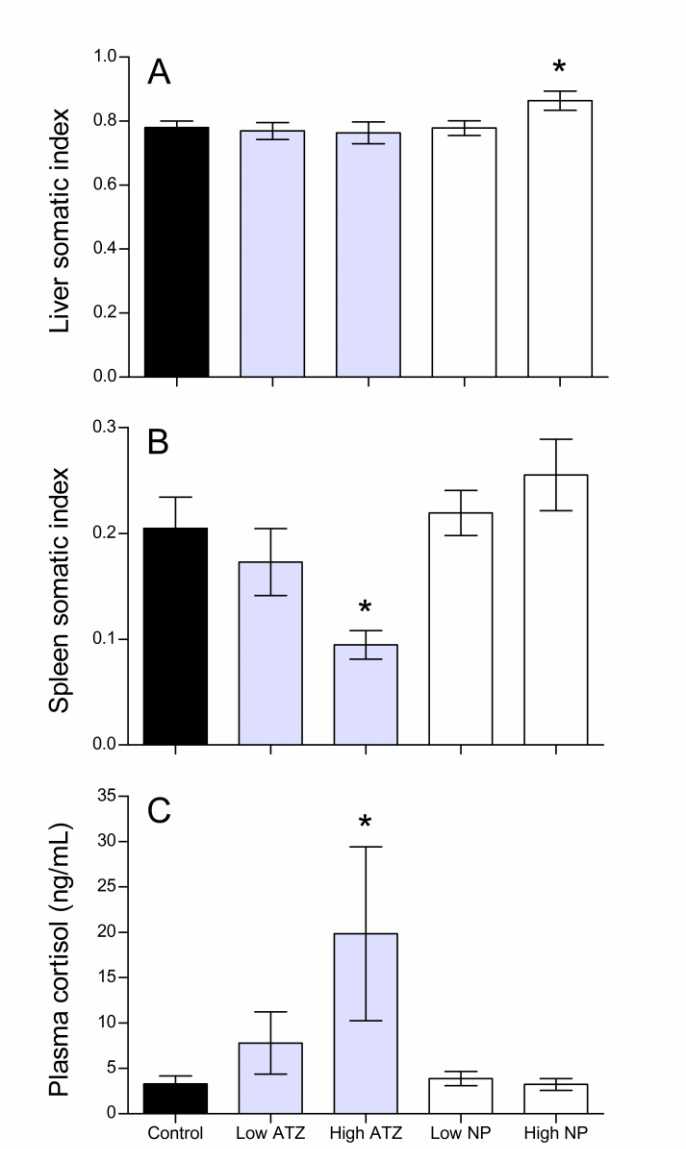

Figure 5-1. Liver somatic index (LSI, panel A), spleen somatic index (SSI, panel B) and plasma cortisol (panel C) following a 4 d exposure to 59 µg/L atrazine (low ATZ), 555 µg/L ATZ (high ATZ), 2.3 µg/L nonylphenol (low NP) or 18 µg/L NP (high NP), compared to the control group. The bars represent mean ± SEM with n = 15 per group for LSI and SSI and n = 8 for plasma cortisol. An ANOVA followed by Dunnett's post-hoc test was used to detect differences from control, which are shown as * (p < 0.05).

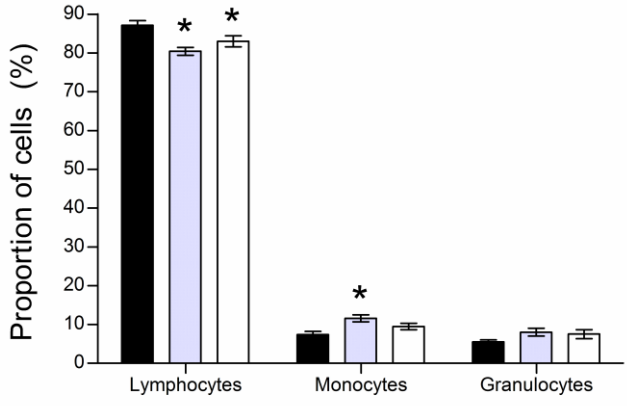

Figure 5-2. The proportion of lymphocytes was decreased and monocytes increased following a 4 d exposure to 555 µg/L atrazine (grey bars) or 18 µg/L nonylphenol (white bars), compared to the control group (black bars). The bars represent mean ± SEM and n = 8 per group. An unpaired t-test was used to detect significant differences in the atrazine or nonylphenol groups compared to the control group for each cell type, which are shown as * (p < 0.05).

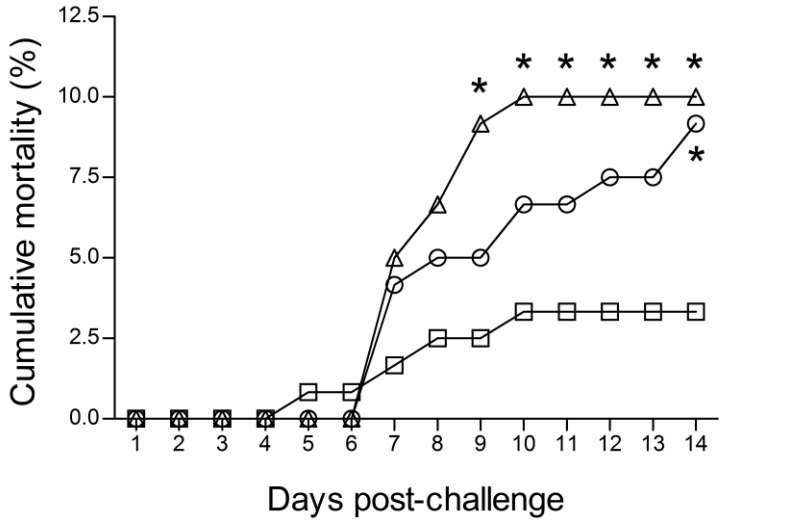

Figure 5-3. Mortality following disease challenge with Listonella anguillarum was elevated in groups previously exposed to 18 µg/L nonylphenol (NP, ∆) or 555 µg/L atrazine (ATZ, ○) for 4 d, compared to the control group (□). Error bars are omitted for clarity. Kaplan-Maier survival analysis with a log-rank test was used to determine if mortality in the ATZ- or NP-treated groups were different than control (p < 0.05) followed by a t-test to determine which days were different (n = 3 replicates, 40 fish per treatment group in each replicate), with significant differences shown as * (p < 0.05).

Supplemental Data

Table S5-1. Hematocrit, plasma protein and plasma lysozyme were unaffected by exposure to either atrazine (ATZ) or nonylphenol (NP) for 4 d, compared to the control group. Data is shown as mean ± SEM, where n = 15 for hematocrit, n = 13-14 for plasma protein and n = 7-10 for plasma lysozyme.

Treatment group	Hematocrit	Plasma protein (mg/mL)	Plasma lysozyme (µg/mL)*
Control	0.33 ± 0.01	19.8 ± 1.2	7.0 ± 1.9
59 µg/L ATZ	0.32 ± 0.01	20.9 ± 0.9	8.1 ± 2.3
555 µg/L ATZ	0.36 ± 0.02	21.3 ± 0.9	8.5 ± 2.0
2.3 µg/L NP	0.33 ± 0.01	20.5 ± 0.8	8.8 ± 2.5
18 µg/L NP	0.32 ± 0.01	22.1 ± 1.0	8.1 ± 2.1

* based on standard curve generated based on hen egg white lysozyme activity

Table S5-2. Differentially regulated genes in rainbow trout liver following 4 d of exposure to 555 µg/L atrazine.

UniProt acc. ID	Gene name	Fold change	General function
Q9Y3U8	60S ribosomal protein L36	0.33	Translation
Q6PFS5	ER lumen protein retaining receptor 3	0.37	Protein trafficking
Q9UEW8	STE20/SPS1-related proline-alanine rich protein kinase	0.38	Mediator of stress-activated signals
O15431	high affinity copper uptake protein 1	0.39	Copper transporter
P46783	40S ribosomal protein S10	0.40	Translation
P32969	60S ribosomal protein L9	0.41	Translation
P61515	putative 60S ribosomal protein L37a	0.45	Translation
Q92522	histone H1x	0.46	Chromatin organization
P62244	40S ribosomal protein S15a	0.48	Translation
P28799	granulins	0.48	Wound repair
Q567W7	ectonucleoside triphosphate diphosphohydrolase 6	0.48	Unknown
P24534	elongation factor 1-beta	0.49	Translation
P62241	60S ribosomal protein S8	0.51	Translation
O42611	protein HIRA	0.52	chromatin assembly

Q8JHJ1	60S ribosomal protein L35	0.52	Translation
P13753	BOLA class I histocompatibilty antigen, alpha chain B	0.53	Antigen presentation
Q9Y6H1	aging-associated gene 10 protein	0.53	Unknown
Q9D7P6	iron-sulfur cluster assembly enzyme ISCU, mitochondrial	0.54	Iron-containing protein modification
P19878	neutrophil cytosolic factor 2	0.54	Respiratory burst
Q8TDD5	mucolipin 3	0.54	Ion transport
Q8BIG7	catechol-O-methyltransferase domain containing 1	0.54	O-methyltransferase
P61251	40S ribosomal protein S26	0.54	Translation
Q6ZM63	ATP-binding domain 1 family member C	0.54	Unknown
P46779	60S ribosomal protein L28	0.54	Translation
P79334	glycogen phosphorylase, muscle form	0.55	Carbohydrate metabolism
Q6DHU8	lymphokine activated killer T cell originated protein kinase homolog	0.56	Unknown
O95433	activator of heat shock 90kDa protein ATPase homolog 1	0.56	Stimulates HSP90 ATPase activity
P61254	60S ribosomal protein L26	0.56	Translation
P53447	fructose-bisphosphate aldolase B	0.56	Carbohydrate metabolism
P41247	patatin-like phospholipase domain containing 4	0.57	Lipid metabolism
Q568P1	guanosine-3',5'-bis(diphosphate) 3'-pyrofphosphohydrolase MESH1	0.57	ppGpp hydrolyzing enzyme
Q6ZWV3	60S ribosomal protein L10	0.57	Translation
P62266	40S ribosomal protein S23	0.57	Translation
O42197	beta-2-microglobulin	0.57	Antigen presentation
O75643	U5 small nuclear ribonucleoprotein 200kDa helicase	0.58	mRNA processing
Q9DAC7	tetratricopeptide repeat protein 32	0.59	Unknown
Q9HDV8	uncharacterized protein P19A11.02c	0.59	Unknown
Q6PC69	60S ribosomal protein L10	0.59	Translation
P81274	G-protein signaling modulator 2	0.60	Spindle pole orientation
Q9QXE5	thymus specific serine protease	0.61	T cell development
P68246	troponin I, fast skeletal muscle	0.61	Inhibitory subunit of troponin
P34953	serine protease inhibitor Kazal type 2	0.61	Trypsin inhibitor

Q5BLI5	si:rp71-39b20.7 protein	0.62	Unknown
Q9H1K1	iron-sulfur cluster assembly enzyme ISCU, mitochondrial	0.62	Iron-containing protein modification
P12277	creatine kinase B-type	0.62	Energy transduction
Q7YRK7	cytochrome c oxidase subunit 6	0.63	Respiratory transport chain component
P35467	S100 calcium binding protein A1	0.63	Metal binding protein
Q66HZ8	leukocyte receptor cluster (LRC) member 1	0.63	Unknown
Q91ZR2	sorting nexin 18	0.63	Intracellular trafficking
Q96I99	succinyl-CoA ligase (GDP-forming) subunit beta, mitochondrial	0.64	Cofactor metabolism
Q6ZWL3	cytochrome P450 4V2	0.64	Lipid metabolism
P26325	alcohol dehydrogenase 1	0.64	Mediates chemical export
Q9QUR8	semaphorin 7A	0.64	Mediates integrin signaling
Q7ZYS1	60S ribosomal protein L19	0.65	Translation
Q6DH42	enhancer of yellow 2 transcription factor homolog	0.65	Chromatin organization
Q9Y5A9	YTH domain family protein 2	0.65	Unknown
Q96BD5	PHD finger protein 21A	0.65	Transcription regulation
P15814	immunoglobulin lambda-like polypeptide 1	0.65	B cell development
Q3ZBP1	creatine kinase S-type, mitochondrial	0.65	Energy transduction
Q5RGU1	chaperone activity of bc1 complex-like, mitochondrial	0.65	Respiratory transport chain chaperone
P26352	thymosin beta-12	0.66	Cytoskeleton organization
P12246	serum amyloid P-component	0.66	Amyloid deposits, chromatin degradation
P27918	properdin	0.66	Alternative complement pathway
Q575Z2	interleukin-1 receptor antagonist	0.66	Unknown
Q9NS23	ras association (RalGDS/AF-6) domain family member 1	0.66	Apoptosis, cell cycle control
Q9R1T1	barrier to autointegration factor	0.67	Chromatin organization
Q29524	lipoprotein lipase	0.67	Lipid metabolism
Q9ZZM6	cytochrome c oxidase subunit 1	0.67	Respiratory transport chain component
Q5K651	sterile alpha motif domain containing 9	0.67	Unknown

P45376	aldose reductase	0.67	Metabolism
Q62661	HMG-box transcription factor 1	0.68	Transcription repressor, cell cycle regulation
Q8BGS7	choline/ethanolaminephosphotransferase 1	0.69	Lipid metabolism
Q91775	fatty acid binding protein 2, intestinal	0.69	Lipid transport
Q9H3F6	potassium channel tetramerisation domain-containing protein 10	0.69	Protein degradation
Q9W6A5	green-sensitive opsin-1	0.69	Vision
Q6PBH5	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, subunit 4	0.69	Respiratory transport chain component
P62983	ubiquitin 40S ribosomal protein S27a	0.70	Protein precursor
Q9D823	60 S ribosomal protein L37	0.70	Translation
Q3T114	ribonuclease UK114	0.70	Translation inhibitor
Q96KP4	cytosolic non-specific dipeptidase	0.71	Protein metabolism
Q8IWZ6	Bardet-Biedl syndrome 7 protein	0.71	Required for ciliogenesis
Q95SX7	RNA directed DNA polymerase from transposon	0.71	Unknown
Q64471	glutathione S-transferase, theta 1	0.72	Xenobiotic metabolism
P13635	ceruloplasmin	0.72	Iron transport
Q91876	DNA replication licensing factor mcm7-A	0.72	DNA replication
Q7ZW25	charged multivesicular body protein 2a	0.73	Protein trafficking
Q9D1M7	FK506 binding protein 11	0.74	Protein modification
Q6PFJ1	neuroguidin	0.74	Translation repressor
Q71UM5	40S ribosomal protein S27-like	0.75	Translation
P82979	SAP domain containing ribonucleoprotein	0.76	Cell growth, metabolism and carcinogenesis
Q9Y239	nucleotide-binding oligomerization domain containing protein 1	0.76	Apoptosis
P51467	arrestin red cell isoform 2	0.81	Signal transduction
Q32L53	protein lifeguard 1	0.81	Apoptosis
O43166	signal-induced proliferation-associated 1 like 1	1.22	Reorganization of actin cytoskeleton
P46777	60S ribosomal protein L5	1.25	Translation
Q6PAE6	UPF0489 protein C5orf22 homolog	1.27	Unknown
P46933	amyloid beta (A4) precursor protein-binding,	1.28	Found in amyloid deposits,

	family B, member 1		chromatin degradation
P28271	cytoplasmic aconitase hydralase	1.28	Iron homeostasis
Q9QXJ1	amyloid beta (A4) precursor protein-binding, family B, member 1	1.29	Found in amyloid deposits, chromatin degradation
Q32LU7	pentatricopeptide repeat domain 3, mitochondrial	1.31	Unknown
Q9HCJ5	zinc finger, SWIM domain-containing protein 6	1.31	Unknown
Q9H9D4	zinc finger protein 408	1.31	Transcription regulation
Q4FZT8	SPRY domain containing 4	1.33	Unknown
Q8SWD4	ubiquitin	1.34	Protein signaling and trafficking
Q8TCT8	signal peptide peptidase-like 2A	1.35	Protease
Q9D2L5	inactive carboxypeptidase like protein X2	1.35	Cell-cell interaction
Q9Y2G2	caspase recruitment domain containing protein 8	1.36	Component of inflammasome
Q9UJX2	cell division cycle 23 homolog	1.36	Cell cycle control
Q9YIB9	hypoxia-inducible factor 1 alpha	1.37	Response to hypoxia
Q9NXF8	palmitoyltransferase ZDHHX7	1.37	Protein modification
Q61585	putative lymphocyte G0/G1 switch protein 2	1.38	Potential oncogene
Q9BRS8	La-related protein 6	1.38	translation
Q91971	glucagon-1	1.38	Hormone, energy homeostasis
Q8BWW9	serine/threonine protein kinase N2	1.39	Protein modification
Q2YDN6	ribosome production factor 2 homolog	1.39	Unknown
P15988	collagen alpha-2(VI) chain	1.39	Extracellular matrix
Q8WV92	MIT domain-containing protein 1	1.39	Endosomal protein transport
Q5U3F2	pre-mRNA splicing factor SLU7	1.40	mRNA processing
Q8BWY3	eukaryotic peptide chain release factor subunit 1	1.40	Translation termination
Q96KY2	2-amino-3-carboxymuconate-6-semialdehyde decarboxylase	1.40	Secondary metabolite metabolism
Q5C1C7	SJCHGC07628 protein	1.40	Unknown
Q5RI56	optineurin	1.41	Membrane trafficking, cell morphogenesis
Q5RGJ8	N-acetylglucosamine-1-phosphotransferase subunits alpha/beta	1.41	Protein modification
Q3B7H2	mitochondrial translocator assembly and	1.42	Peptide translocation

	maintenance protein 41 homolog		
Q14197	peptidyl-tRNA hydrolase ICT1, mitochondrial	1.42	Translation
Q9NQZ2	something about silencing protein 10	1.43	Gene silencing
Q8R4T5	general receptor for phosphoinositides 1-associated scaffold protein	1.45	Intracellular trafficking
Q8C8N3	brorin	1.45	Cell adhesion
Q9Y6Y8	SEC23-interacting protein	1.45	ER organization
Q96BY6	dedicator of cytokinesis protein 10	1.45	Guanine nucleotide exchange factor
P24798	sodium/potassium-transporting ATPase subunit alpha-3	1.46	Ion transporter
Q9CQ86	migration and invasion enhancer 1	1.47	Redox-related processes
P15979	class I histocompatibilty antigen, F10 alpha chain	1.47	Antigen presentation
Q8AXX2	T-box transcription factor TBX1	1.47	Developmental processes
Q15154	pericentriolar material 1 protein	1.48	Centrosome assembly and function
Q9UJX6	anaphase promoting complex subunit 2	1.48	Cell cycle control
Q9BYH1	seizure 6-like protein	1.48	ER function
Q6P6Y3	heme oxygenase (decycling) 1	1.48	Response to hypoxia
Q9BUR5	apolipoprotein O	1.48	Lipid (cholesterol) metabolism
Q864R9	multidrug resistance-associated protein 1	1.48	Mediates chemical export
P31395	stathmin 1	1.49	Microtubule organization
Q13464	Rho-associated protein kinase 1	1.49	Cytoskeleton organization
Q8K1S5	Kruppel-like factor 11	1.50	Transcription factor
Q68EH8	FAD synthase	1.50	Cofactor biosynthesis
P62316	small nuclear ribonucleoprotein Sm D2	1.50	mRNA processing
P79251	V type proton ATPase subunit G1	1.50	H+ transporter
Q6ZSZ6	teashirt homolog 1	1.52	Transcription repressor
Q7T364	tumor necrosis factor, alpha-induced protein 8-like protein 2B	1.52	Innate and adaptive immunity
Q58DV5	39S ribosomal protein L30, mitochondrial	1.52	Translation
Q3SWZ4	exosome component 9 RRP45	1.54	RNA processing
Q5M9I5	cytochrome b-c1 complex subunit 6, mitochondrial	1.54	Respiratory transport chain component

Q7ZVY5	citrate synthase, mitochondrial	1.54	Carbohydrate metabolism
Q86TI2	dipeptidyl-peptidase 9	1.55	Protein modification
Q63537	synapsin-2	1.56	Regulation of neurotransmitter release
Q67FY3	B-cell CLL/lymphoma 9-like protein	1.56	Transcription regulation
P56394	cytochrome c oxidase copper chaperone	1.56	Copper chaperone
Q6NYK8	E3 ubiquitin-protein ligase MARCH5	1.56	Mitochondrial morphology
P52742	zinc finger protein 135	1.56	Transcription regulation
O35963	Ras-related protein Rab-33B	1.57	Protein transport
Q6GQ29	plasma glutamate carboxypeptidase	1.57	Protein modification
Q7ZTV8	swelling dependent chloride channel	1.58	Ion transporter
Q8BH59	calcium-binding mitochondrial carrier protein Aralar1	1.58	Aspartate and glutamate transporter
Q6R5N8	toll-like receptor 13	1.58	Innate and adaptive immunity
Q8BG81	polymerase delta interacting protein 3	1.58	Translation
Q9Z1X4	interleukin enhancer-binding factor 3	1.59	Transcription regulation
P11413	glucose-6-phosphate 1-dehydrogenase	1.61	Carbohydrate metabolism
Q8C1F4	chondroitin sulfate N-acetylgalactosaminyltransferase 2	1.62	Condroitin synthesis
Q9VLT5	protein purity of essence	1.63	Male fertility
Q6GMV2	SET and MYND domain-containing protein 5	1.64	Unknown
Q8BHY3	anoctamin 1	1.64	Chloride channel
P54729	negative regulator of ubiquitin-like proteins 1	1.64	Protein degradation
Q8HXW3	succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	1.64	Respiratory transport chain component
Q6AXL3	leucine rich repeat containing protein 33	1.65	Unknown
Q16563	synaptophysin-like protein 1	1.65	Unknown
Q5J8M3	transmembrane protein 85	1.66	Anti-apoptotic activity
Q17433	DNaJ domain dnj-2	1.66	Unknown
P36633	amiloride-sensitive amine oxidase (copper-containing)	1.67	Degradation of signaling molecules
Q86UY8	5'-nucleotidase domain containing protein 3	1.67	Unknown
Q6DFV1	condensin-2 complex subunit G2	1.68	Chromatid organization
P49247	ribose 5-phosphate isomerase A	1.69	Carbohydrate metabolism

Q91628	origin recognition complex subunit 2 homolog	1.70	DNA replication
P12682	high-mobility group protein B1	1.72	DNA binding protein
Q9Y3R5	protein dopey-2	1.76	Protein trafficking
Q32KL5	neighbor of COX4	1.76	Unknown
O95336	6-phosphogluconolactonase	1.77	Carbohydrate metabolism
Q5TZ51	protein Mpv17	1.78	Mitochondrial homeostasis
Q5F3U9	sister chromatid cohesion protein PDS5 homolog B	1.80	Chromatid organization
P97329	kinesin-like protein KIF20A	1.80	Golgi transport
Q6MFZ5	tripartite motif-containing protein 39	1.84	Facilitate apoptosis
P79732	ribonucleoside-diphosphate reductase large subunit	1.86	Deoxyribonucleotide synthesis
Q6NV83	U2 snRNP-associated SURP motif-containing protein	1.86	mRNA processing
Q6DBY5	ubiquitin-conjugating enzyme E2 variant 3	1.90	Regulator of polyubiquitination
Q8CHG7	Rap guanine nucleotide exchange factor 2	1.93	Response to DNA damage
O54689	C-C chemokine receptor type 6	1.98	Chemokine receptor
Q9CWG1	glioma pathogenesis-related protein 1	1.98	Unknown
Q8HXX4	trifunctional enzyme subunit B, mitochondrial	2.00	Lipid metabolism
Q92974	rho/rac guanine nucleotide exchange factor 2	2.01	Exchange of GDP for GTP
Q7ZVM3	eukaryotic translation elongation factor 2, like	2.03	Translation
Q2HJ48	BTB/POZ domain-containing protein KCTD1	2.04	Transcription regulation
P53597	succinyl-CoA ligase (ADP/GDP-forming) subunit alpha, mitochondrial	2.04	Carbohydrate metabolism
Q13555	calcium/calmodulin-dependent protein kinase type II subunit gamma	2.08	Calcium transport in muscle
Q16585	beta-sarcoglycan	2.15	Cytoskeleton organization
P11147	heat shock 70kDa protein cognate 4	2.20	Response to stress
Q6P9X4	protein tyrosine phosphatase IVa2	2.23	Cell cycle control
O46629	trifunctional enzyme subunit beta, mitochondrial	2.45	Lipid metabolism
Q49LS7	Xk-related protein 8	2.46	Unknown
Q6PD62	RNA polymerase-associated protein CTR9 homolog	2.92	Unknown
Q3ZBF8	ceramide synthase 2	3.36	Lipid metabolism

Q9JKF6	poliovirus receptor-related protein 1	3.50	Cell adhesion
Q96I24	far upstream element-binding protein 3	3.79	Transcription regulation
Q96Q89	kinesin-like protein KIF20B	5.57	Cell cycle

Table S5-3. Differentially regulated genes in rainbow trout liver following 4 d of exposure to 18 µg/L nonylphenol.

UniProt acc. ID	Gene name	Fold change	General function
P61515	60S ribosomal protein L37a	0.17	Translation
O54842	nuclear protein 1	0.35	Cellular growth
Q9TTY1	metalloproteinase inhibitor 2	0.35	Enzyme inhibitor
Q5XIQ4	E3 ubiquitin-protein ligase MGRN1	0.36	Protein modification, cell signaling
Q6P9A2	putative polypeptide N-acetylgalactosaminyltransferase-like protein 4	0.37	Carbohydrate metabolism
O15431	high affinity copper uptake protein 1	0.39	Copper transporter
Q6DC93	peflin	0.39	Unknown
Q9Y3U8	60S ribosomal protein L36	0.40	Translation
Q8JHJ1	60S ribosomal protein L35	0.43	Translation
Q6SA96	40S ribosomal protein S23	0.44	Translation
Q96DB5	regulator of microtubule dynamics protein 1	0.44	Unknown
P13693	translationally-controlled tumor protein	0.44	Microtubule organization
Q6PFS5	ER lumen protein retaining receptor 3	0.44	Protein trafficking
P61254	60S ribosomal protein L26	0.45	Translation
Q5ZJF4	peroxiredoxin 6	0.45	Redox regulation
Q99733	nucleosome assembly protein 1-like 4	0.46	Nucleosome assembly
O18756	D-glucuronyl C5-epimerase	0.47	Carbohydrate metabolism
P24534	elongation factor 1-beta	0.47	Translation
Q52NJ4	ADP-ribosylation factor-like protein 3	0.48	Cell cycle
Q8BGA9	mitochondrial inner membrane protein OXA1L	0.48	Protein trafficking
O42161	actin, cytoplasmic 1	0.48	Cell motility
P59215	guanine nucleotide binding protein (G protein), subunit alpha	0.48	Cell signallng

P35579	myosin-9	0.49	Cell cycle, cell shape
Q95JC9	basic proline-rich protein	0.49	Hormone
O57524	apolipoprotein A-I-2	0.50	Lipid (cholesterol) metabolism
Q99ML5	prenylcysteine oxidase	0.50	Protein degradation
P11714	cytochrome P450 2D9	0.50	Xenobiotic metabolism
Q9BXP5	serrate RNA effector molecule homolog	0.50	mRNA processing
P46783	40S ribosomal protein S10	0.51	Translation
P62847	40S ribosomal protein S24	0.51	Translation
Q3ZC25	transmembrane protein 106B	0.51	Unknown
Q567W7	ectonucleoside triphosphate diphosphohydrolase 6 (putative function)	0.51	Unknown
Q6ZWV3	60S ribosomal protein L10	0.51	Translation
P53447	fructose-bisphosphate aldolase B	0.51	Carbohydrate metabolism
P82264	glutamate dehydrogenase, mitochondrial	0.51	Amino acid metabolism
Q9BQR3	serine protease 27	0.51	Protein degradation
P61313	60S ribosomal protein L15	0.52	Translation
Q8TDD5	mucolipin 3	0.52	Ion transport
Q7SZR5	small ubiquitin-related modifier 1	0.53	Protein trafficking and degradation
O43854	EGF-like repeat and discoidin I-like domain-containing protein 3	0.53	Cellular adhesion
O29873	p-nitrophenyl phosphatase (Pho2)	0.53	Unknown
P82159	myosin light chain 1, skeletal muscle isoform	0.53	Motor protein
P52944	PDZ and LIM domain protein 1	0.53	Cytoskeleton organization
Q8BQX5	transmembrane and coiled-coil domain-containing protein 6	0.54	Protein transport
P11142	heat shock 70kDa protein 8	0.54	Transcription repressor
P49946	ferritin heavy subunit	0.54	Iron homeostasis
O14594	neurocan core protein	0.54	Cellular adhesion
Q96S59	RAN binding protein 9	0.55	Signal transduction
Q8AY63	brain-subtype creatine kinase	0.55	Unknown
Q9CR57	60S ribosomal protein L14	0.55	Translation
Q7SXN5	dynamin 1-like protein	0.55	Membrane remodeling
Q7SIG6	development and differentiation enhancing	0.55	Regulates vesicle formation,

	factor 2		phagocytosis
Q8NCJ5	SPRY domain containing 3	0.55	Unknown
Q96AX1	vacuolar protein sorting-associated protein 33A	0.55	Protein trafficking
Q9DGK4	translationally-controlled tumor protein homolog	0.55	Microtubule organization
O75164	lysine-specific demethylase 4A	0.56	Chromatin organization
Q9NVP1	ATP-dependent RNA helicase DDX18	0.56	Transcription
Q15942	zyxin	0.56	Cellular adhesion, signal transduction
Q9D173	mitochondrial import receptor subunit TOM7 homolog	0.56	Protein transport
Q9HBW1	leucine-rich-repeat-containing protein 4	0.56	Synaptic adhesion protein
P97432	next to BRCA1 gene 1 protein	0.57	Protein degradation
P81274	G-protein signaling modulator 2	0.57	Cell cycle
Q9QZA1	pleckstrin homology-like domain, family A, member 1	0.57	Apoptosis
P21327	inositol polyphosphate-1-phosphatase	0.57	Signal transduction
Q567I4	PI-PLC X domain containing protein 1	0.57	Signal transduction
Q3SZ52	ubiquitin-conjugating enzyme E2 variant 1	0.58	Cell cycle
Q3T178	vacuolar protein sorting-associated protein 28 homolog	0.58	Protein trafficking
Q8TEA7	TBC domain-containing protein kinase-like	0.58	Unknown
O95478	ribosome biogenesis protein NSA2 homolog	0.58	Translation
Q8CG47	structural maintenance of chromosomes 4	0.58	Chromatin organization
Q8BVH9	methyltransferase-like protein 6	0.58	Methyltransferase
P31722	complement C1q subcomponent subunit C	0.58	Complement pathway
Q8BVF2	phosducin-like 3	0.59	Apoptosis
Q7KUF9	sug, isoform D	0.59	Unknown
Q6PH62	cytochrome b-245, alpha polypeptide	0.59	Unknown
Q8AYB8	homeodomain-only protein	0.59	Transcription
Q3SZY7	AN1-type zinc finger protein 6	0.59	Unknown
Q6R748	syntaxin binding protein 1	0.60	synaptic vesicle docking and fusion
Q9NPI5	nicotinamide riboside kinase 2	0.60	cell adhesion
P19823	inter-alpha-trypsin inhibitor heavy chain H2	0.60	carrier of hyaluronan in serum

Q9HAV7	GrpE-protein homolog 1, mitochondrial	0.61	protein localization
Q9Z252	protein lin-7 homolog b	0.61	cell membrane organization
Q61473	transcription factor SOX-17	0.61	transcription
Q8C669	E3 ubiquitin-protein ligase pellino homolog 1	0.61	protein modification, immune response
Q5K651	sterile alpha motif domain containing 9	0.61	Unknown
Q8RQ75	surface protein PspC	0.61	Unknown
Q5M7E9	voltage-gated hydrogen channel 1	0.61	Ion transporter
Q8JH71	fructose-bisphosphate aldolase B	0.61	Carbohydrate metabolism
Q9YGH5	somatostatin-1A	0.62	Hormone
P32368	phosphoinositide phosphatase SAC1	0.62	Cytoskeleton organization
Q8VCS6	mediator of RNA polymerase II transcription subunit 9	0.62	Transcription
Q96EX2	RING finger and transmembrane domain-containing protein 2	0.62	Unknown
P33436	72 kDa type IV collagenase	0.62	Tissue remodeling
P15924	desmoplakin	0.63	Cell membrane organization
Q9WV92	band 4.1-like protein 3	0.63	Cytoskeleton organization
Q9D7N3	28S ribosomal protein S9, mitochondrial	0.63	Translation
Q7YRK7	cytochrome c oxidase subunit 6C	0.63	Respiratory transport chain
Q6P3H7	histone binding protein RBBP4	0.63	Chromatin organization
Q96IZ7	serine/Arginine-related protein 53	0.64	mRNA processing
Q8JG54	repulsive guidance molecule A	0.64	Neuron development
P56856	claudin 18	0.64	Cell adhesion
Q8JFQ6	keratin, type I cytoskeletal 13	0.64	Cytoskeleton organization
Q8CHS8	vacuolar protein sorting-associated protein 37A	0.64	Protein trafficking
O75911	short chain dehydrogenase/reductase 3	0.65	Retinol metabolism
P13437	3-ketoacetyl-CoA thiolase, mitochondrial	0.65	Apoptosis
O15553	pyrin	0.65	Cytoskeleton organization, inflammation response
Q9NZ52	ADP-ribosylation factor-binding protein GGA3	0.65	Protein trafficking
P26352	thymosin beta-12	0.66	Cytoskeletal organization
P19218	pancreatic secretory granule membrane major glycoprotein GP2	0.66	Antigen binding

Q99JX3	golgi reassembly stacking protein 2	0.66	Golgi organization
O15541	RING finger protein 113A	0.67	Unknown
P17544	cyclic AMP-dependent transcription factor ATF-7	0.67	Transcription
P28173	amidophosphoribosyltransferase	0.68	Nucleoside metabolism
Q8BWA5	kelch-like protein 31	0.68	Transcription
Q13885	tubulin, beta 2A chain	0.68	Microtubule organization
Q61572	forkhead box protein C1	0.68	Transcription
Q9CPW3	39S ribosomal protein L54, mitochondrial	0.68	Translation
Q95SX7	RNA directed DNA polymerase from transposon BS	0.69	DNA replication
P57776	elongation factor 1-delta	0.69	Translation
Q9UI15	transgelin 3	0.69	Unknown
Q9CZ13	cytochrome b-c1 complex subunit 1, mitochondrial	0.70	Respiratory transport chain
A2AIV2	protein virilizer homolog	0.70	mRNA processing
Q9I926	fucolectin-6	0.70	Defense response
Q5XK83	DNA replication licensing factor mcm4-A	0.70	DNA replication
P35689	DNA excision repair protein ERCC-5	0.70	DNA repair
P46778	60S ribosomal protein L21	0.70	Translation
Q1JPZ7	pre-mRNA-processing factor 39	0.71	mRNA processing
P62193	26S protease regulatory subunit 4	0.71	Protein degradation
Q5XFN2	desmin	0.71	Cytoskeleton organization
Q9HBY8	serine/threonine-protein kinase Sgk2	0.71	Potassium channel regulation
Q9W669	estrogen receptor beta 1	0.72	Hormone receptor
Q9Y5B9	FACT complex subunit SPT16	0.72	Chromatin organization
Q9H2V7	protein spinster homolog 1	0.72	Transporter, apoptosis
P15814	immunoglobulin lambda-like polypeptide 1	0.72	B cell development
Q28645	podocalyxin	0.72	Cell adhesion
P41212	transcription factor ETV6	0.72	Transcription
O95671	N-acetylserotonin O-methyltransferase-like	0.73	Unknown
P55347	homeobox protein PKNOX1	0.73	Transcription
P79741	pescadillo	0.73	Translation

Q9BSD7	cancer-related nucleoside-triphosphatase	0.73	Phosphatase
Q6PBI5	40S ribosomal protein S25	0.74	Translation
Q13356	peptidylprolyl isomerase cis-trans isomerase-like 2	0.74	Protein folding
P51467	arrestin red cell isoform 2	0.75	Signal transduction
P79815	serotransferrin	0.75	Iron homeostasis
Q9UL59	zinc finger protein 214	0.75	Transcription
P11413	glucose-6-phosphate-1 dehydrogenase	0.75	Carbohydrate metabolism
Q9H3F6	potassium channel tetramerisation domain-containing 10	0.75	Protein degradation
Q9LDD8	methylcrotonoyl-CoA carboxylase beta chain, mitochondrial	0.76	Amino acid degradation
Q8BZX4	splicing factor, arginine/serine-rich 12	0.76	mRNA processing
Q8JIU7	nascent polypeptide-associated complex subunit alpha	0.78	Protein transport
Q7ZX15	RAC-beta serine/threonine protein kinase A	0.78	Signal transduction
Q8C863	E3 ubiquitin protein ligase Itchy	0.79	Inflammation processes
P19179	plastin 1	0.79	cytoskeleton organization
Q5RFH9	ATP synthase subunit b, mitochondrial	0.80	respiratory transport chain
Q9QYL8	acyl-protein thioesterase 2	0.82	lipid metabolism
Q9HBU6	ethanolamine kinase 1	0.82	Phospholipid biosynthesis
P18155	bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase, mitochondrial	1.17	carbohydrate metabolism
Q6IQ86	probable protein BRICK1	1.23	cytoskeleton organization
Q9BUZ4	TNF receptor-associated factor 4	1.25	signal transduction
Q9H8V3	protein ECT2	1.28	Signal transduction
Q7SY49	CAM kinase-like vesicle-associated protein	1.28	Signal transduction
P56382	ATP synthase subunit epsilon, mitochondrial	1.28	respiratory transport chain
Q6UB99	ankyrin repeat domain-containing protein 11	1.29	transcription
Q9Y376	calcium binding protein 39	1.29	Signal transduction
Q6PEI3	phosphatase and actin regulator 4	1.29	Unknown
Q58EJ9	MOSC domain-containing protein 1, mitochondrial	1.30	oxidoreductase
Q5E9G3	proteasome activator complex subunit 2	1.31	immunoproteasome assembly

P23492	purine nucleoside phosphorylase	1.32	Nucleoside metabolism
Q14149	MORC family CW-type zinc finger protein 3	1.32	Unknown
Q9BYD2	39S ribosomal protein L9, mitochondrial	1.32	translation
P13184	cytochrome c oxidase subunit 7A2, mitochondrial	1.33	respiratory transport chain
O75718	cartilage associated protein	1.33	Unknown
Q6PBT6	GTP cyclohydrolase 1 feedback regulatory protein	1.34	enzyme inhibitor
O95398	Rap guanine nucleotide exchange factor 3	1.34	Nucleotide exchange factor
Q5C1C7	SJCHGC07628 protein	1.34	Unknown
Q5DSV6	bloodthirsty	1.34	Erythrocyte differentiation
Q9ULV4	coronin-1C	1.35	Cytoskeleton organization
Q63753	zinc finger protein	1.36	Unknown
P29533	vascular cell adhesion molecule 1	1.36	cell-cell recognition, leukocyte adhesion
P47934	carnitine O-acetyltransferase	1.36	lipid metabolism
Q15436	protein transport protein Sec23A	1.36	protein trafficking
P58268	zinc finger protein ubi-d4	1.37	apoptosis
Q7T3S5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	1.37	glycolipid synthesis
Q6IQ96	autophagy-related 4C (yeast)	1.37	Protein degradation
P32455	interferon-induced guanylate binding protein 1	1.37	Response to interferon
Q9VVX4	mitochondrial ribosomal protein L21	1.37	Translation
Q9UJW8	zinc finger protein 180	1.38	Transcription
O88878	AN1-type zinc finger protein 5	1.38	Protein degradation, apoptosis
Q7T384	sodium-coupled monocarboxylate transporter 2	1.38	Transporter
P62258	14-3-3 protein epsilon	1.38	Signal transduction
Q8R1E7	transmembrane protein 234	1.38	Unknown
Q17477	protein B0334.5, confirmed by transcript evidence	1.39	Unknown
Q8VHT7	transcription factor IIIA	1.40	Transcription
P63329	serine/threonine-protein phosphatase 2B catalytic subunit, alpha isoform	1.40	Signal transduction
Q12952	forkhead box protein L1	1.40	Transcription

Q9CQT9	uncharacterized protein C20orf24 homolog	1.41	Unknown
Q8C437	PEX5-related protein	1.41	Signal transduction
Q96BP3	peptidylprolyl isomerase domain and WD repeat containing 1	1.42	mRNA processing
Q13464	Rho-associated protein kinase 1	1.42	cytoskeleton organization
Q5RE33	receptor expression-enhancing protein 5	1.43	olfactory receptor expression
Q5SNQ7	protein SERAC1	1.43	Protein transport
Q96NW4	ankyrin repeat domain-containing protein 27	1.43	Endosome transport
Q2LD37	uncharacterized protein KIAA1109	1.43	Cell growth
P47857	6-phosphofructokinase, muscle type	1.44	Carbohydrate metabolism
Q9HD45	transmembrane 9 superfamily member 3	1.44	Unknown
P35249	replication factor C subunit 4	1.44	DNA replication
P15988	collagen alpha-2(IV) chain	1.44	Cell adhesion
Q52NJ3	GTP-binding protein SAR1a	1.44	Protein trafficking
Q7TST5	lysosomal-associated membrane glycoprotein 3	1.44	Lysosomal function
Q6DGZ3	THO complex subunit 7 homolog	1.44	mRNA processing
Q3U6Q4	phosphoinositide 3-kinase regulatory subunit 6	1.44	Signal transduction
Q4G3H4	inhibitor of nuclear factor kappa-B kinase subunit alpha	1.45	Modulates immune response
Q9JJW5	myozenin 2	1.45	Binding protein
O14686	histone-lysine N-methyltransferase MLL2	1.45	Transcription
Q7L9L4	MOB kinase activator 1B	1.45	Cell proliferation, apoptosis
Q9Y2G2	caspase recruitment domain-containing protein 8	1.45	Apoptosis
P15979	class I histocompatibility antigen, F10 alpha chain	1.45	Antigen presentation
Q9BXM7	serine/threonine-protein kinase PINK1, mitochondrial	1.46	Autophagy, apoptosis
Q6NYY9	vacuole membrane protein 1	1.46	Unknown
P26351	thymosin beta-11	1.46	Cytoskeleton organization
Q58DV5	39S ribosomal protein L30, mitochondrial	1.46	Translation
Q91WT4	DnaJ homolog, subfamily C, member 17	1.47	Protein folding
Q8BG19	transmembrane and tetratricopeptide repeat containing 4	1.47	Unknown

Q9UTH9	tRNA-dihydrouridine synthase 3	1.47	Translation
Q8BMD7	MORC family CW-type zinc finger protein 4	1.47	Unknown
Q923J1	transient receptor potential cation channel, subfamily M, member 7	1.47	Ion transporter
Q9PVU6	hemoglobin embyronic subunit alpha	1.48	Oxygen transport
Q8R4N1	titin splice variant	1.48	Unknown
Q6AXS5	plasminogen activator inhibitor 1 RNA-binding protein	1.48	mRNA stability
P47813	eukaryotic translation initiation factor 1A, X-chromosomal	1.48	Translation
P15384	potassium voltage-gated channel subfamily A member 3	1.49	Ion transporter
Q61941	NAD(P) transhydrogenase, mitochondrial	1.49	respiratory transport chain
Q6DN14	multiple C2 and transmembrane domain-containing protein 1	1.49	Unknown
Q9H1P3	oxysterol binding protein-related protein 2	1.50	Lipid transport
P16527	myristoylated alanine-rich C kinase substrate	1.50	Cytoskeleton organization
Q9NZC3	glycerophosphodiester phosphodiesterase 1	1.50	Lipid metabolism
Q9YHZ6	cell division control protein 45 homolog	1.51	DNA replication
Q4V9P9	nucleolar protein 11-like	1.51	Unknown
Q91ZT8	ankyrin repeat and SOCS box protein 9	1.53	Protein degradation
Q9P2E9	ribosome binding protein 1	1.54	Translation
Q6DGH7	proteasome (prosome, macropain) assembly chaperone 3	1.54	Unknown
Q8TCB0	interferon-induced protein 44	1.54	Microtubule organization
Q8BUV8	protein GPR 107	1.54	Unknown
Q95KE5	39S ribosomal protein L43, mitochondrial	1.54	Translation
Q12986	transcriptional repressor NF-X1	1.55	Transcription
P39475	NADP-specific glutamate dehydrogenase	1.55	Amino acid metabolism
Q8IUN9	C-type lectin domain family 10, member A	1.55	Regulates immune response
Q14697	neutral alpha-glucosidase AB	1.55	Protein processing
Q5BJC2	protein kish-A	1.57	Secretion
Q6JWU9	coatomer protein complex, subunit alpha	1.58	Protein transport
P47727	carbonyl reductase (NADPH) 1	1.59	Xenobiotic metabolism

Q9NRR5	ubiquilin 4	1.59	Protein degradation
P62316	small nuclear ribonucleoprotein Sm D2	1.60	mRNA processing
Q9YHV3	noggin 3	1.60	Signal transduction
P22129	Ras-related protein Rab-11B	1.61	Endosome trafficking
Q5F3J5	proteasome activator complex subunit 3	1.61	Immunoproteasome assembly
P97494	glutamate-cysteine ligase, catalytic subunit	1.61	Glutathione metabolism
Q29441	cyclic nucleotide gated channel alpha 3	1.61	Transporter
P97447	four and a half LIM domains protein 1	1.62	Cell differentiation
Q6EWQ6	deoxyhypusine synthase	1.62	Amino acid metabolism
P68368	tubulin, alpha 4A	1.63	Microtubule organization
Q5JVG2	zinc finger protein 484	1.63	Transcription
P21448	multidrug resistance protein 1	1.64	Xenobiotic metabolism
Q9N4A9	protein Y77E11A.2	1.65	Unknown
Q5XIZ4	acyl-coenzyme A oxidase	1.66	Lipid metabolism
Q9JJ19	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	1.66	cell membrane organization
Q5R4Q7	leucine-zipper-like transcription regulator 1	1.67	transcription
A4D7T3	ankyrin repeat, SAM and basic leucine zipper domain-containing protein 1	1.67	spermatogenesis
P46664	adenylosuccinate synthetase isozyme 2	1.67	nucleotide metabolism
O94956	solute carrier organic anion transporter family, member 2B1	1.67	transporter
Q6PH57	transducin beta chain 1	1.68	signal transduction
P42224	signal transducer and activator of transcription 1, alpha/beta	1.69	signal transduction
P41234	ATP-binding cassette sub-family A member 2	1.73	transporter (sterol), xenobiotic metabolism
Q9P299	coatomer subunit zeta 2	1.73	protein trafficking
Q6PBN4	coenzyme Q-binding protein COQ10 homolog, mitochondrial	1.73	respiratory transport chain
Q9WTI7	unconventional myosin-1c	1.74	intracellular movement
P36412	Ras-related protein Rab-11A	1.75	Osmoregulation
Q5LQG2	AMP-binding enzyme	1.75	Unknown
P52742	zinc finger protein 135	1.76	transcription

P49798	regulator of G-protein signaling 4	1.78	signal transduction
Q5ZMD6	histone H2A.Z	1.79	chromatin organization
Q9DEX3	cathepsin d	1.81	protein degradation
O35286	putative pre-mRNA-splicing factor ATP-dependent RNA helicase DXH15	1.81	mRNA processing
Q5EAD2	D-3-phosphoglycerate dehydrogenase	1.82	amino acid metabolism
Q9VLT5	protein purity of essence	1.86	male fertility
Q2WGK2	junctional adhesion molecule A	1.87	cell adhesion
P79732	ribonucleotide-diphosphate reductase large subunit	1.96	nucleotide metabolism
P13667	protein disulfide isomerase A4	1.97	protein processing
Q5UE93	phosphoinositide-3-kinase regulatory subunit 6	1.98	signal transduction
Q9CYC5	Kinetochore-associated protein DSN1 homolog	2.01	cell cycle
Q12836	zona pellucida glycoprotein 4	2.01	response to E2, fertilization
Q91Y57	sialic acid binding Ig-like lectin 12	2.24	cell-cell interaction, receptor
Q8VIJ6	splicing factor proline/glutamine rich	2.31	mRNA processing
Q91YP3	putative deoxyribose-phosphate aldolase	2.36	nucleotide metabolism
Q499A9	acyl-CoA synthetase long-chain family member 1	2.37	Unknown
Q9H4L7	ATP-dependent helicase 1	2.46	replication
Q91994	homeobox protein OTX1 B	2.47	role in early embryogenesis
Q9BH11	zona pellucida glycoprotein 4	2.48	response to E2, fertilization
Q8JH36	vitellogenin 1	3.46	response to E2, lipid transport

Table S5-4. List of altered biological processes and pathways (excluding immune system-related processes) identified with a functional analysis (Pathway Studio®) in liver from rainbow trout exposed to 555 µg/L atrazine (ATZ) or 18 µg/L nonylphenol (NP) for 4 d, with a significance level of p < 0.01.

Effects following ATZ exposure		
Biological process or pathway	**Mean fold change**	**p-value**
negative regulation of sequence-specific DNA binding transcription factor activity	-1.21	< 0.001
glycogen biosynthetic process	-1.16	< 0.001
pentose-phosphate shunt	1.61	< 0.001

protein secretion	1.41	0.001
creatine metabolic process	-1.30	0.002
phosphatidylcholine biosynthetic process	1.02	0.003
positive regulation of lipoprotein lipase activity	-1.46	0.003
anion transport	-1.19	0.003
ribosomal small subunit biogenesis	-1.41	0.003
retrograde Golgi-ER transport	-1.16	0.003
gastrulation	-1.10	0.004
cell cycle regulation	1.04	0.004
ROS metabolism	-1.16	0.005
activation of MAPK activity	-1.15	0.005
VIPR -> CREB/CEBP signaling	1.19	0.005
middle ear morphogenesis	1.47	0.006
superoxide anion generation	-1.24	0.006
mRNA metabolic process	1.09	0.006
motor axon guidance	1.60	0.006
negative regulation of endothelial cell proliferation	-1.08	0.006
negative regulation of blood coagulation	-1.18	0.006
positive regulation of endopeptidase activity	1.32	0.007
oxygen homeostasis	1.22	0.007
positive regulation of TOR signaling cascade	1.32	0.007
cellular response to lithium ion	1.17	0.008
positive regulation of smooth muscle cell proliferation	1.10	0.008
response to metal ion	1.11	0.009
pseudouridine synthesis	1.33	0.010
axon regeneration	1.33	0.010
Effects following NP exposure		
Biological process or pathway	**Mean fold change**	**p-value**
malonate, propanoate and beta-alanine metabolism	-1.11	< 0.001
manganese ion transport	1.61	< 0.001

positive regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	-1.05	< 0.001
M-G1 transition of mitotic cell cycle	1.13	< 0.001
ubiquitin-dependent protein degradation	-1.11	< 0.001
anti-apoptosis	-1.05	< 0.001
interspecies interaction between organisms	1.00	0.001
retrograde protein transport, ER to cytosol	1.45	0.002
urokinaseR -> STAT signaling	1.54	0.003
endothelial cell morphogenesis	-1.24	0.004
negative regulation of phosphatidylinositol 3-kinase cascade	-1.10	0.004
transcription-coupled nucleotide-excision repair	-1.21	0.004
fructose 1,6-bisphosphate metabolic process	1.27	0.004
response to reactive oxygen species	-1.09	0.005
notch pathway	-1.05	0.005
DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest	1.07	0.005
histone H2B ubiquitination	-1.39	0.006
Ser/Gly/Thr/Cys metabolism	-1.04	0.006
androgen receptor signaling pathway	-1.18	0.006
cholesterol homeostasis	-1.23	0.007
de novo' posttranslational protein folding	-1.10	0.007
intestinal absorption	1.25	0.007
calcium ion transport	-1.10	0.009
positive regulation of ATPase activity	-1.33	0.009
glutathione metabolism	-1.07	0.009
tight junction assembly	1.06	0.010

Effects common to both high ATZ and NP exposure

Biological process or pathway	**ATZ mean fold change**	**ATZ *p*-value**	**NP mean fold change**	**NP *p*-value**
DOWN-REGULATED				
translational termination	-1.47	< 0.001	-1.41	< 0.001

endocrine pancreas development	-1.43	< 0.001	-1.36	< 0.001
translational elongation	-1.43	< 0.001	-1.38	< 0.001
ribosomal large subunit biogenesis	-1.41	< 0.001	-1.45	0.002
cholesterol efflux	-1.34	< 0.001	-1.34	< 0.001
hydrogen peroxide catabolic process	-1.34	0.002	-1.41	< 0.001
regulation of intestinal cholesterol absorption	-1.34	0.008	-1.56	0.003
rRNA transcription and processing	-1.27	< 0.001	-1.25	< 0.001
erythrocyte development	-1.24	< 0.001	-1.20	0.002
cellular protein metabolic process	-1.19	< 0.001	-1.18	< 0.001
gene expression	-1.11	< 0.001	-1.13	< 0.001
aging	-1.03	0.005	-1.08	0.003
insulin action	-1.02	0.003	-1.09	0.002
UP-REGULATED				
negative regulation of ubiquitin-protein ligase activity involved in mitotic cell cycle	1.07	0.005	1.07	< 0.001
peripheral nervous system axon regeneration	1.11	< 0.001	1.07	0.003
muscle contraction	1.11	0.006	1.11	0.005
DIFFERENTIALLY-REGULATED				
respiratory chain and oxidative phosphorylation	-1.09	0.010	1.07	0.003
heme biosynthesis	-1.07	0.002	1.05	0.003
response to nutrient	1.05	0.002	-1.09	0.007
intracellular protein transport	1.06	< 0.001	-1.06	0.005
single-strand nucleotide excision DNA repair	1.09	0.004	-1.21	0.002
actin-based cytoskeleton assembly	1.12	0.006	-1.03	0.002
ephrinR -> actin signaling	1.12	0.008	-1.01	0.001
lipoprotein biosynthetic process	1.29	0.001	-1.71	< 0.001
phospholipid efflux	1.29	0.002	-1.56	< 0.001
ethanol oxidation	1.36	0.006	-1.54	0.007

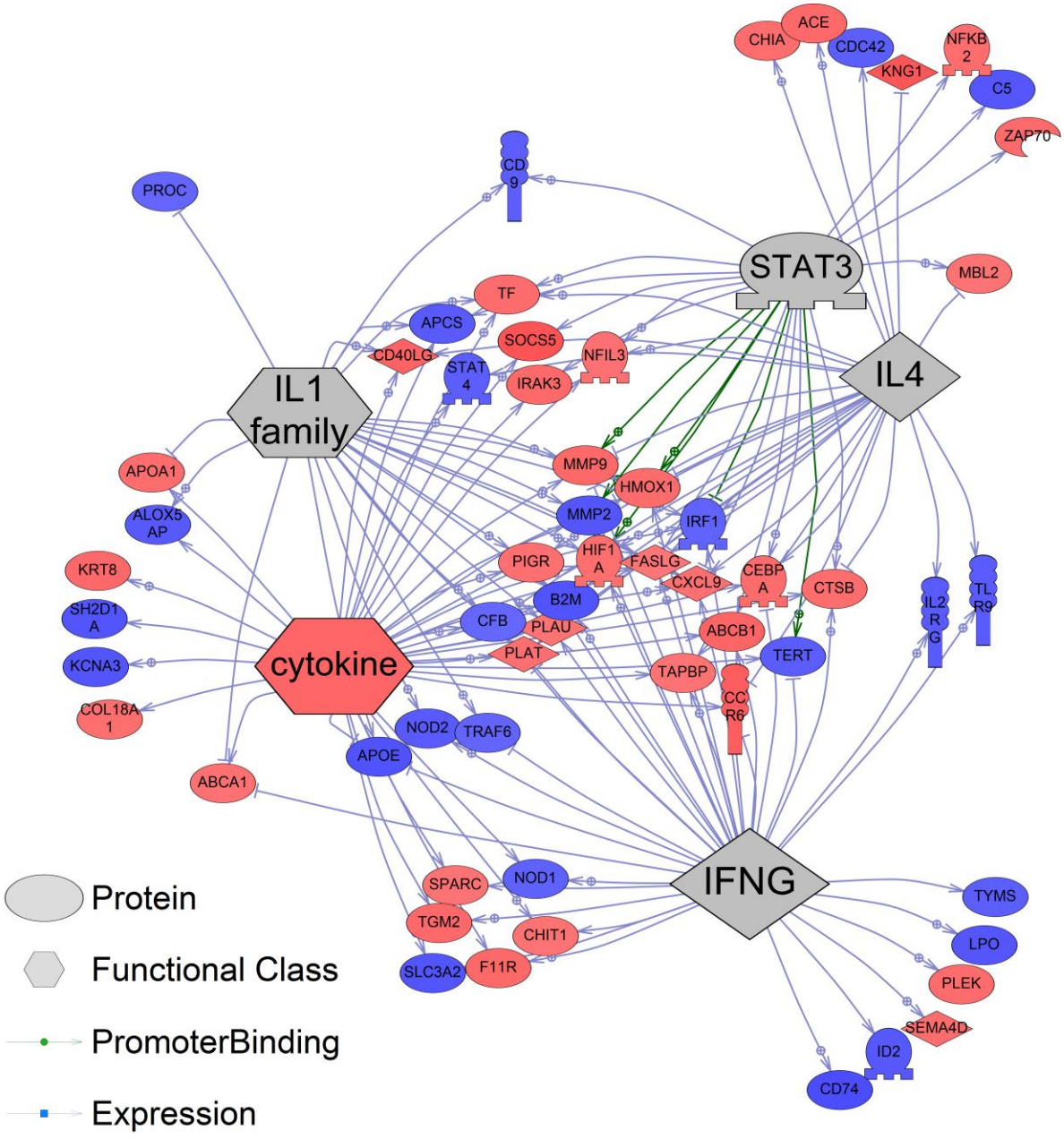

Figure S5-1. Pathway Studio® sub-network enrichment analysis of microarray data from rainbow trout exposed to 555 µg/L atrazine reveals several key transcription regulators that are important in the immunotoxic response at the genomic level, with blue symbols = down-regulated, pink = up-regulated and grey = not present on the microarray or not present in the differentially regulated gene list. IFNG = interferon-gamma, IL1 = interleukin-1, STAT3 = signal transducer and activator of transcription 3, IL4 = interleukin 4.

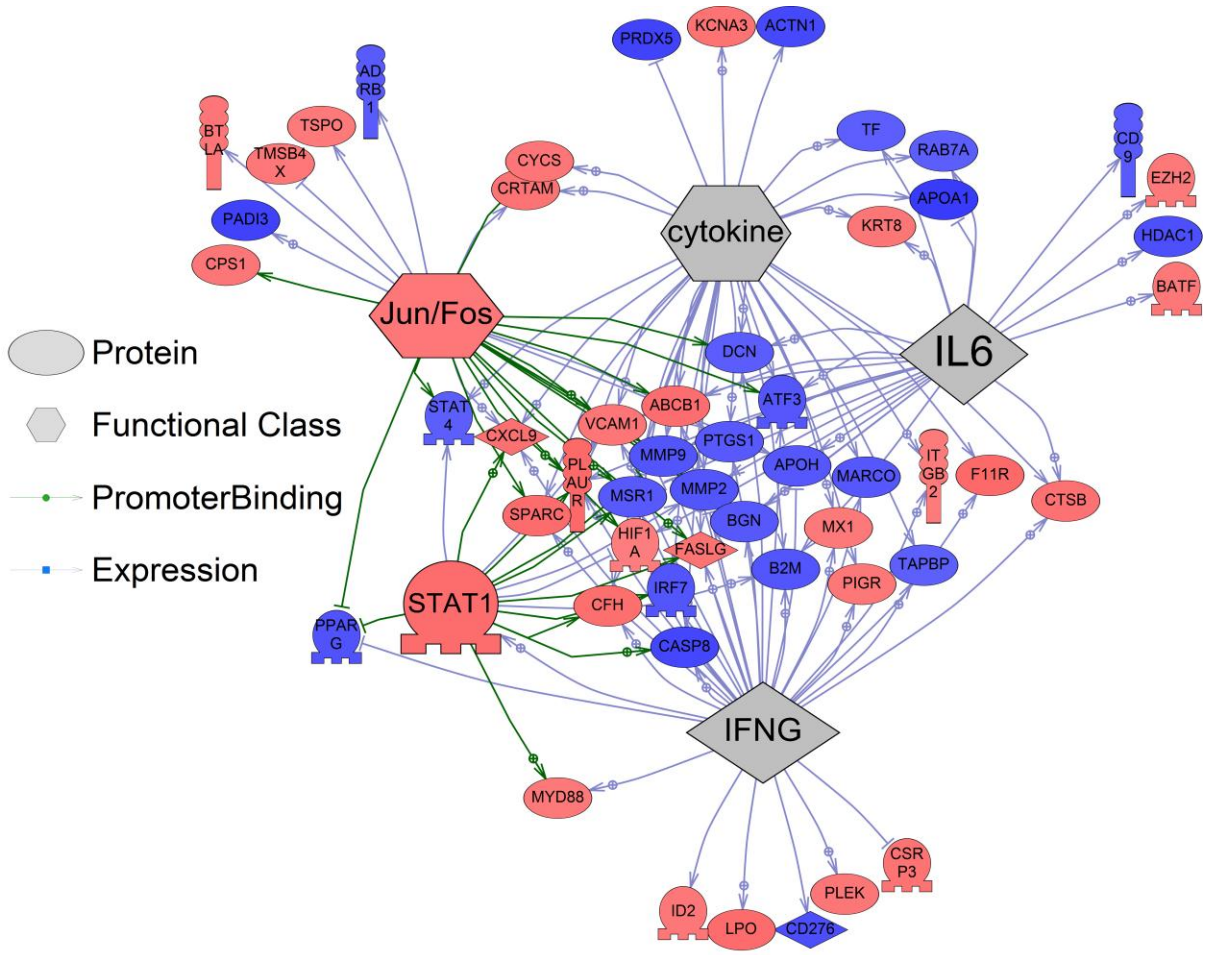

Figure S5-2. Pathway Studio® sub-network enrichment analysis of microarray data from rainbow trout exposed to 18 µg/L nonylphenol reveals several key transcription regulators that are important in the immunotoxic response at the genomic level, with blue symbols = down-regulated, pink = up-regulated and grey = not present on the microarray or not present on the differentially regulated gene list. IFNG = interferon-gamma, STAT1 = signal transducer and activator of transcription 1, IL6 = interleukin 6.

Chapter 6.

Immunotoxicological effects of a sub-chronic exposure to selected current-use pesticides in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Many current-use pesticides (CUPs) are found at increasing concentrations in aquatic environments, yet relatively little is known about their effects on the immune system of fish. We examined the *in vivo* effects of three pesticides (chlorothalonil, cypermethrin and pentachlorophenol) on the immune system of juvenile rainbow trout (*Oncorhynchus mykiss*) by assessing a suite of innate immune function tests, as well as a host resistance test using *Listonella anguillarum*. Increased activity of phagocytic leukocytes, as evidenced using flow cytometry, was observed following 28-day exposures to pentachlorophenol (1 µg/L and 2 µg/L), but not for cypermethrin or chlorothalonil, although a trend of increasing activity was noted for chlorothalonil. No recovery was observed during a 14-day post-exposure chlorothalonil experiment, as evidenced by continued elevation of respiratory burst and percent phagocytic cells at the lowest exposure concentrations (100 ng/L and 200 ng/L), suggesting a prolonged CUP-induced impact on the immune system. No effects of any pesticide on body weights, direct lethality, serum lysozyme or relative leukocyte differential were observed, suggesting that modulation of the cellular responses of the innate immune system represents a sensitive sub-lethal endpoint for these three pesticides. However, a lack of detectable effects of these CUPs on host resistance to *L. anguillarum* in our study may reflect a dose–response range that did not elicit an effect on those immune responses responsible for control and clearance of this particular pathogen. Additional research may provide more insight into the immunomodulatory effects of these and other CUPs, and the implications for host resistance against a variety of bacterial, viral and macroparasitic pathogens.

Keywords

Pesticide; rainbow trout; immunotoxicology; flow cytometry; head kidney leukocytes

Introduction

The application of many current-use pesticides (CUPs) has increased with the transition away from use of older or banned legacy pesticides that are persistent, bioaccumulative or toxic such as the organochlorine insecticides DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane) or chlordane (octachloro-4,7-methanohydroindane). Many of the CUPs are considered to be of lesser environmental concern due to the perception of generally more favorable physico-chemical properties such as shorter half-lives or decreased potential for bioaccumulation due to lower octanol–water partitioning coefficients (K_{ow}), both of which would be expected to contribute to a lower potential for toxicity in exposed organisms. While, in most cases, the concentrations of CUPs found in the environment are much lower than that expected to cause direct lethality in non-target aquatic species, there is still only limited information regarding their sub-lethal effects.

Unfortunately, as a result of their higher use levels, CUPs are being identified more frequently in environmental surface water, sediment and air samples in British Columbia, Canada and elsewhere (Harris et al., 2008; Woudneh et al., 2007; Yao et al., 2006). Pesticides can be introduced into the aquatic environment by drift, surface run-off, leaching from soil, accidental spills and atmospheric deposition and it has also been reported recently that some CUPs, similar to the legacy pesticides, have the potential for long range transport (Muir et al., 2004). Chronic exposure to xenobiotics may result in sub-lethal effects that have significant potential to impact a species at the ecosystem level since they may result in changes in reproductive ability, behaviour, growth, homeostasis or susceptibility to diseases (LeBlanc and Bain, 1997; Spromberg and Meador, 2006).

Anthropogenic chemicals may contribute to population-level declines in wild salmon stocks as a result of modulation of the fish immune system and resultant increases in mortality (Arkoosh et al., 1998a). A number of studies have found that fish sampled from chemically contaminated environments show changes in phagocytic ability or respiratory burst activity when compared to fish from relatively clean sites, which could predispose these fish to increased susceptibility to infectious diseases (Fournier et al., 1998; Rice et al., 1996; Zelikoff et al., 2000). Arkoosh et al.(1998b) reported that

field-collected wild Chinook (*Oncorhynchus tshawytscha*) salmon migrating through a contaminated urban estuary were also found to have higher mortality than those from either a non-urban estuary or hatchery following laboratory exposure to the marine pathogen *Listonella anguillarum*. Laboratory studies on fish have also found that pesticides specifically, either singly or in mixtures, may modulate immune responses at sublethal concentrations both *in vitro* and *in vivo* (Betoulle et al., 2000; Fatima et al., 2007; Harford et al., 2005).

We selected three CUPs for this immunotoxicological study: chlorothalonil (2,4,5,6-tetrachloro-isophthalonitrile), cypermethrin ((*R*,*S*)-alpha-cyano-3-phenoxybenzyl (1*RS*)-cis,trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-carboxylate) and pentachlorophenol (PCP; 2,3,4,5,6-pentachlorophenol). Chlorothalonil is a broad-spectrum fungicide with a K_{ow} of 2.64–4.28 and a water solubility of 0.9 mg/L that is widely used in both agriculture and horticulture (Caux et al., 1996). It is also used as a booster biocide in marine paints as one of the chemicals replacing the widely banned organotin fungicides such as tributyltin, resulting in greater potential for chlorothalonil contamination of marine waters and sediments (Voulvoulis et al., 2000). Chlorothalonil residues have been identified in Canadian air samples nearby to agricultural areas and also in distant Arctic areas, suggesting some potential for long-range transport and deposition (Muir et al., 2004; Yao et al., 2006).

Cypermethrin is a synthetic pyrethroid insecticide that is used in both agricultural and household pest-control applications. It is extremely toxic to fish, with a reported 4-day LC50 value in rainbow trout as low as 500 ng/L (Stephenson, 1982). The log K_{ow} for cypermethrin is 6.06, water solubility is 0.01mg/L and it has a half-life of greater than 50 days in water (Struger and Fletcher, 2007). There is increasing concern and evidence that cypermethrin and other pyrethroid insecticides are entering surface waters through residential run-off following outdoor applications and that its presence in water and sediments may pose a risk to aquatic species (Struger and Fletcher, 2007;Weston et al., 2005).

Pentachlorophenol was a widely used, environmentally persistent fungicide which has been banned or restricted for use in many countries throughout North America and Europe, although it still remains registered for restricted uses in Canada.

As recently as 2003, PCP use in British Columbia reached more than 147 000 kg, such that its persistence may still present a risk to aquatic species (ENKON Environmental Limited, 2005). It has a log K_{ow} of 5.05 (Kaiser and Valdmanis, 1981) and a water solubility of 10–20 mg/L (Arcand et al., 1995).

Immunotoxicity is best evaluated through the use of a multi-tiered approach that includes evaluation of immune status at both cellular and organismal levels (Luster et al., 1993; Kollner et al., 2002). This is due to the complex and often redundant nature of the immune response in controlling infection by pathogens. In the present study, we used a combination of *ex vivo* and *in vivo* assays to measure immunocompetence following sub-lethal continuous exposure to a single pesticide in water for 28 days. Included in the study were assessments of phagocytosis and respiratory burst, which previous studies have found to be good biomarkers of immunotoxicity (Bols et al., 2001; Fournier et al., 2000; Zelikoff et al., 2000), as well as a host resistance challenge which is useful for assessing the integrated immune system response to a particular pathogen.

Materials and Methods

Fish

Juvenile rainbow trout (*Oncorhynchus mykiss)* weighing 29.83 ± 0.188 g (mean ± S.E.M.) were obtained from Miracle Spring Hatchery (Mission, BC) and were held for acclimation in 150 L fiberglass tanks with continuously flowing (minimum flow 1 L/kg/min) dechlorinated municipal water for at least one week. Water temperatures throughout acclimation and chemical exposure periods were 12.6 ± 0.03°C (mean ± S.E.M.). Fish were fed *ad libitum* every other day with commercial salmon pellets (Ewos Pacifica, Surrey, BC). All work with animals was conducted in accordance with Canadian Council on Animal Care (CCAC) guidelines. No mortality was observed during either the acclimation period or throughout the duration of any pesticide exposure.

Chemicals

All pesticides used in exposure studies were of analytical grade (>95% purity) and were solubilized in reagent grade acetone (maximum tank concentration of acetone

was always 0.00013%, v/v). Fetal bovine serum (FBS, Gibco), 2',7'-dichlorodihydrofluorescindiacetate (DCFH-DA) and 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3)) were purchased from Invitrogen (Eugene, OR). Peptone for use in bacterial cultures was acquired from Bioshop (Burlington, ON). A Gram stain kit was purchased through VWR (Mississauga, ON). All other chemicals were obtained from Sigma (Oakville, ON).

Experimental design

Fish were exposed to a range of concentrations of a single pesticide for 28 days under flow-through conditions. Pesticide concentrations were selected to be approximately 10%, 5%, 2% and 1% of the 4-day rainbow trout flow-through LC50 (17.1 µg/L chlorothalonil (Davies and White, 1985), 1.46 µg/L cypermethrin (Davies et al., 1994), 115 µg/L pentachlorophenol (Thurston et al., 1985)). For chlorothalonil, a 14-day recovery period (post-28-day chlorothalonil exposure) was also performed. At the end of exposure or recovery periods, a total of 18 fish were randomly removed from each tank; 12 fish for the disease challenge with *L. anguillarum*, and 6 fish for the *ex vivo* assays. Each treatment for each pesticide was done in duplicate, with replicate treatments carried out on different days.

Pesticide exposure

Following acclimation, 20 fish (for pentachlorophenol and cypermethrin) or 40 fish (for chlorothalonil) per exposure concentration were weighed, fin clipped for identification and randomly allocated to treatment tanks. Nominal exposure concentrations were 1000 ng/L, 500 ng/L, 200 ng/L and 100 ng/L for chlorothalonil; 100 ng/L, 50 ng/L, 20 ng/L and 10 ng/L for cypermethrin; and 10 µg/L, 5 µg/L, 2 µg/L and 1 µg/L for pentachlorophenol. Control tanks received no chemicals and a solvent control (reagent grade acetone, 0.00013%, v/v tank concentration) was also utilized. Water flows to each tank were set at 1.5 L/min and concentrations of pesticides were maintained through continuous addition of concentrated stock solution from Mariotte bottles with flow rates of 2mL/min. Mariotte bottles were refreshed every other day with pesticide stock solutions. Mariotte bottles were made of fluorinated high density polyethylene (HDPE) to reduce pesticide adsorption. The use of HDPE containers,

0.1% acetone as a carrier, and high flow through of dechlorinated, filtered water (which minimized binding to particulate matter) would result in nominal concentrations close to expected.

Host disease challenge

The pesticide-exposed fish were experimentally infected with the marine pathogen *L. anguillarum*, via immersion challenges immediately following the 28-day pesticide exposures and 14-day recovery period (chlorothalonil only). This bacterial pathogen was selected for the disease challenges because the fish were reared strictly in freshwater and therefore were assumed to be immunologically naïve to the pathogen. *L. anguillarum* is infectious and lethal in freshwater, and there is precedent for its use as a model pathogen for disease challenges in other freshwater studies (Balfry et al., 2001; Wood et al., 1996). Mortality data therefore would provide information on the effect of pesticides on innate immunity with possible correlations between disease resistance results (i.e. mortality) and the results of the various *ex vivo* immune tests performed on the fish.

Primary isolates of *L. anguillarum* (Pacific Biological Station, Nanaimo, British Columbia, isolate number 2004-124, serotype 01) were used for all the disease challenges. *L. anguillarum* cells were grown for 18 h on tryptic soy agar (TSA supplemented with 1.0% NaCl) at 20°C, and harvested into sterile peptone-saline (PS; 0.1% peptone and 0.85% NaCl). The suspension was diluted and absorbance measured at 540 nm. An estimate of the concentration of cells in the suspension was calculated based on the assumption that 1 OD_{540} contained 10^9 cells/mL. The actual concentration of cells was subsequently determined by drop plating serial dilutions (5 × 25 µL aliquots) of the suspension onto TSA plates, incubating them overnight at 20°C, and counting the number of colony forming units.

The average challenge dose was determined to be 1.59×10^8 cfu/mL (final doses ranged from 1.12×10^8 cfu/mL to 2.13×10^8 cfu/mL). In preliminary trials this dose of *Listonella* caused 60–70% mortality in fish, a level used in previous host resistance studies (Balfry et al., 2001). Challenges were performed by pooling 12 fish per pesticide group into a large bucket containing 14 L of aerated peptone-saline (P-S), and

immediately adding 1 L of the challenge inoculum (*L. anguillarum* in P-S). After 10 min of exposure, the water and fish from the container were transferred to a 150 L fiberglass tank with flowing dechlorinated municipal water at 12.7 ± 0.6°C (mean ± S.E.M.). Mortalities were monitored and recorded three times per day for 10 days, with fish from different treatment groups identified based on fin clips. All mortalities were individually bagged and frozen at −80°C, for later necropsy and verification of fin clip. The spleen was aseptically swabbed and streaked onto TSA plates from approximately 25% of these fish. The fish were assumed to have died of vibriosis caused by *L. anguillarum*, following the observation of non-pigment producing, circular, cream-coloured colonies appearing as a pure culture on the TSA plates. The colonies were subsequently smeared onto glass slides and Gram stained. Pure cultures of Gram negative, rod-shaped, curved bacteria were observed for the final confirmation of cause of death.

Sampling for ex vivo assays

Fish were sampled from each treatment tank, euthanized in sodium bicarbonate buffered tricaine methanesulphonate (MS222; 0.5 g/L) and weighed. Blood samples were drawn by caudal venipuncture using non-heparinized syringes. Serum was obtained by allowing blood to clot at room temperature for 1 h followed by incubation on ice for 4–5 h before centrifugation at 5000 × *g* for 5 min at 4°C. Serum samples were aliquoted and archived at−80°C. Head kidney was aseptically extracted, placed into 2mL of supplemented HBSS (sHBSS; containing 15mM HEPES, 2% FBS, 10 U/mL heparin and 1% penicillin/streptomycin) and held on ice for a maximum of 3 h.

Leukocyte purification from head kidney

Leukocytes were purified from head kidney using hypotonic lysis, as modified from Crippen et al. (2001). Briefly, head kidney was gently disrupted and cells were washed through a 75 µm mesh with 2 mL of sHBSS. 9 mL of sterile deionized water was added to induce lysis of red blood cells. After 30 s, 1 mL of 10 × PBS (phosphate buffered saline, 0.1 M) was added to stop lysis. Cells were incubated on ice for 10 min and any significant debris was removed. Cells were then centrifuged at 400×*g* for 5 min at 4°C, resuspended thoroughly and washed twice in sHBSS. Leukocytes were finally resuspended in 2mL of supplemented RPMI-1640 medium (sRPMI; containing 15mM

HEPES, 5% FBS and 1% penicillin/streptomycin) for counting. Viability was assessed using trypan blue exclusion and cell concentration was adjusted to 1.0×10^7 viable cells/mL.

Partial relative leukocyte differential counts

A relative differential for lymphocytes and granulocytes was performed using flow cytometry, as modified from Inoue et al. (2002). A total of 100 µL of cell suspension was added in duplicate to 880 µL of HBSS (with 15 mM HEPES and 1% penicillin/streptomycin) plus 20 µL of DiOC6(3) for 1×10^6 cells per 5 mL glass culture tube. DiOC6(3) is a lipophilic fluorescent dye that stains both plasma and organelle membranes. It is expected that the extent of staining is based on the amount of membrane present in a particular cell, thus more membraneous cells such as granulocytes would be expected to take up more fluorescent dye than cells such as lymphocytes, resulting in the ability to distinguish populations of cells based on measurement of fluorescence intensity.

Following addition of the dye, cells were incubated at room temperature for 10 min in the dark. Within 1 h, cells were run on a flow cytometer (FACS Aria, Becton Dickinson) and 10 000 events were collected. A dot plot of FITC (DiOC6(3) fluorescence intensity) vs. SSC (representative of granularity) was generated and debris was gated out. There were two main populations discernible on the FITC vs. SSC plot; a grouping characterized by lower FITC and SSC which was expected to be lymphocytes and thrombocytes, plus a grouping with higher FITC and SSC properties which were predicted to be granulocytes. Gates were drawn around the lymphocyte/thrombocyte and granulocyte populations to determine proportions of each cell type relative to total leukocytes.

Respiratory burst assay

Respiratory burst activity was assayed by flow cytometry using the method of Bass (1983), as modified by Karrow et al. (1999). Briefly, 100 µL of cell suspension was aliquoted into round-bottom 96-well culture plates with 4 wells per sample and 1×10^6 cells/well. Each well was topped up with 100 µL of sRPMI and stored overnight on ice at 4°C. Cells were then transferred to 5 mL glass culture tubes containing 780 µL of

sRPMI. A 500 mM DCFHDA stock solution in anhydrous ethanol was added (10 µL) to each tube and incubated at room temperature in the dark for 15 min. To duplicate tubes, either 10 µL of DMSO was added for unstimulated controls or 10 µL of 10 µg/mL phorbol myristate acetate (PMA) in DMSO was added to stimulate reactive oxygen species (ROS) production. Tubes were incubated for 1 h at room temperature in the dark before being assayed by flow cytometry. A total of 10 000 events were collected for each tube and mean fluorescence was used to determine a stimulation index (SI) where SI = mean fluorescence of stimulated cells/mean fluorescence of unstimulated cells.

Phagocytosis assay

Phagocytosis was assayed by flow cytometry using the method in Karrow et al. (1999) as modified from Brousseau et al. (1998). Briefly, 1×10^6 cells were incubated at room temperature in either 1 mL of sRPMI (live cells) or 1 mL of 1% paraformaldehyde in 0.01 M PBS (fixed cells, control) in duplicate. Cells were centrifuged at 500 × *g* for 5 min at 18°C and transferred to round-bottom 96-well culture plates. Fluorescent latex beads (1 µm diameter) were added at a ratio of 100:1 (beads:cells) and samples incubated at 18°C for 18 h. Cells were then layered over a gradient containing 3% BSA in 0.01 M PBS and centrifuged at 100 × *g* for 10 min at 4°C before being resuspended in 0.01 M PBS. On FACS, a gate was set up that excluded beads and 10 000 cell events were collected for this gate for each tube. The percent of cells phagocytosing beads was determined by creating a gate of bead-positive cells based on the FITC histogram. The percent of cells that phagocytosed beads was calculated by subtracting percent bead-positive fixed cells from percent bead-positive live cells. A phagocytic capacity (PC) based on mean fluorescence intensity was also calculated as an approximation of the number of beads per cells, where PC = mean fluorescence intensity of bead-positive live cells − mean fluorescence of bead-positive fixed cells.

Lysozyme assay

Lysozyme activity was determined using the method in Litwack (1955), as modified from Ackerman et al. (2006) for microplate. In a flat-bottomed 96-well microplate, 10 µL of sample or standard were added to wells in triplicate. A 200 µL aliquot of 0.025% (w/v) *Micrococcus lysodiekticus* in 0.1 M sodium phosphate buffer at

pH 5.8 was added to each well and absorbance monitored at 450 nm with a microplate reader (Bio-Tek PowerWave 340). Lysozyme activity for each sample was determined from the change in absorbance over 20 min, based on the HEWL standard curve and results expressed in µg/mL HEWL equivalents (HEWL Eq).

Statistics

There was no significant difference between control and solvent control treatments (data not shown), so only control group data were used in subsequent comparisons and analyses. Data and results for all assays were standardized to percent of control for the day before being pooled in order to control for any differences between sampling days. A standard least squares model with sampling day as a random effect was used to analyze weight, differential, respiratory burst, phagocytosis and lysozyme data (JMP 7.0.2, SAS Institute). Data were tested for normality and equal variance of residuals and data that did not meet these conditions were square root or log transformed. Tukey's Highly Significant Differences (HSD) test was used to detect differences between groups.

In addition, JMP was used to conduct a multivariate statistical analysis using Principal Component Analysis (PCA) with datasets from each assay (excluding disease challenge) in order to identify potential interactions and dose response patterns. It was necessary to exclude the relative percentage of lymphocytes, while retaining relative percentage of granulocytes, since these two measurements are inversely correlated as cells were classified either as lymphocytes or granulocytes and inclusion of both datasets would have skewed the analysis. Data from each fish were first normalized by summing response data for all assays for a single fish then dividing each response by this total (inter-individual normalization). Data were further normalized by calculating a geometric mean based on all fish responses for an assay and then dividing each response by the geomean for that assay (geometric mean normalization). Eigenvalues were generated and factors where the eigenvalue was greater than 1 were retained for factor rotation using the varimax option in JMP. Loading and score plots were generated for principal component 1 vs. 2 and were examined for clustering of fish responses. No discernible dose–response patterns were noted as there was little clustering of fish responses, so this analysis is not discussed further here. Output (including eigenvalues,

loading plots and score plots) from the PCA for each pesticide exposure or recovery period is provided as supplementary data.

Disease challenge data (cumulative percent mortality) were analyzed by using a survival fit analysis based on the Kaplan–Meier method with a log-rank (chi-squared) test to check for differences between groups. Time to 50% mortality was calculated by extrapolation after fitting the curves to a Weibull distribution followed by an ANOVA. Statistical significance in all cases was determined at $p \leq 0.05$.

Results

Chlorothalonil 28-day exposure

Cell viability for the *ex vivo* assays was always greater than 91% (average 97.5%). Relative lymphocyte and granulocyte differential counts were unchanged by exposure to chlorothalonil as was serum lysozyme activity (Table 6-1) and change in body weight (data not shown). Respiratory burst appeared to be elevated with increasing dose, peaking in the 1000 ng/L treatment group, but this was not significant (Fig. 6-1A, $p = 0.0569$). The percent of phagocytic cells also appeared to increase in a dose dependent manner, however this trend was not significant (Fig. 6-2A, $p = 0.1494$). The phagocytic capacity of leukocytes was unchanged (Fig. 6-3A, $p = 0.6972$).

Following challenge with *L. anguillarum*, fish from the 1000 ng/L treatment group had the lowest mortality, however differences between groups were not significant (Fig. 6-4A, $p = 0.080$). Time to 50% mortality was not significant between groups ($p = 0.1015$), although the 1000 ng/L treatment group were the slowest to reach 50% mortality.

Cypermethrin 28-day exposure

There appeared to be a slight decrease in the relative lymphocyte and an increase in granulocyte proportions, however these effects were not significant (Table 6-1). Cell viability for the *ex vivo* assays was always greater than 94% (average 97.8%). Serum lysozyme appeared lower compared to control group in all treatment groups

except the 100 ng/L group, but these changes were not significant (Table 6-1, p = 0.0897). There was no change in body weight gain following exposure (data not shown). Respiratory burst, percent phagocytic cells, phagocytic capacity and mortality following *L. anguillarum* exposure were all unchanged (Fig. 6-1B, p = 0.2134; 6-2B, p = 0.62; 6-3B, p = 0.1851; 6-4B, p = 0.7646).

Pentachlorophenol 28-day exposure

Cell viability for the *ex vivo* assays was always greater than 94% (average 97.6%). PCP exposure had no effects on body weight (data not shown), relative lymphocyte or granulocyte counts or serum lysozyme activity (Table 6-1). PCP exposure resulted in increases in both respiratory burst (Fig. 6-1C, p = 0.0046) and percent phagocytic cells (Fig. 6-2C, p = 0.0488). Respiratory burst activity was significantly increased in the lowest treatment group, 1 µg/L PCP, and the percent phagocytic cells were elevated in both the 1 µg/L and 2 µg/L treatment groups. Phagocytic capacity appeared to be unchanged (Fig. 6-3C, p = 0.8943).

Following disease challenge, the 1 µg/L and 10 µg/L treated groups had lowest mortality, although no statistical differences were found between groups (Fig. 6-4C, p = 0.1384). Time to 50% mortality was also the same between treatment groups (p = 0.2325).

Chlorothalonil 14-day recovery period post-chlorothalonil exposure

An assessment of 14-day recovery period was possible for only one pesticide (chlorothalonil) due to time and financial constraints. Cell viability for the *ex vivo* assays was always greater than 93% (average 97.1%). Relative lymphocyte and granulocyte proportions and serum lysozyme were unaffected by either exposure or post-exposure recovery (Table 6-1), as was body weight (data not shown). Fish from the treated groups continued to exhibit elevated respiratory burst activity (Fig. 6-1A, p = 0.0217) with the lowest treatment group having a higher stimulation index. Percent of phagocytic cells was also increased with elevated proportions in the pesticide exposed group compared to control, particularly in the 100 ng/L and 200 ng/L chlorothalonil groups (Fig. 6-2A, p = 0.0169). Phagocytic capacity remained unchanged (Fig. 6-3A, p = 0.8419).

All groups of fish responded similarly to challenge with *L. anguillarum*, with little difference in either cumulative mortality or time to 50% mortality between groups (Fig. 6-4D, $p = 0.7307$ and $p = 0.9309$, respectively).

Discussion

Due to the concerns about increasing use of CUPs and their prevalence in the environment, in the current study we evaluated the relationship between the exposure to three CUPs and immune system effects. Our *in vivo* experiment is one of the relatively few studies to examine the immunomodulatory effects of CUPs using an assessment of commonly used innate immune system health indicators combined with a host disease challenge to assess the integrated immune system response to a pathogen. We found that exposure to the lowest concentrations of either chlorothalonil or PCP resulted in stimulatory effects on both respiratory burst activity and percent of cells that were phagocytic, with limited to no effect on any of the other immune parameters measured.

In contrast, studies carried out using *in vitro*, cell culture exposures to chlorothalonil or pentachlorophenol found reductions in respiratory burst activity in different species such as bass (*Morone saxatilus*), mummichog (*Fundulus heteroclitus*) or medaka (*Oryzias latipes*) and decreased phagocytic activity in mummichog with PCP exposure (Anderson and Brubacher, 1993; Baier-Anderson and Anderson, 1998; Roszell and Anderson, 1994). The apparent disparity between our *in vivo* findings and those from studies conducted *in vitro* may be due differences in dose, exposure or incubation time, species of test organism, laboratory methods or simply the fundamental differences between *in vitro* and *in vivo* exposures. *In vivo* studies, such as the current study, are critical because they qualitatively account for the effects of natural interactions between complex physiological systems within an organism. This highlights the importance of conducting both types of studies.

As noted, the findings also draw attention to the difficulties in comparing results generated from assays using different methods. For example, the previously cited studies examining respiratory burst following chlorothalonil or PCP exposures were conducted using either lucigenin- or luminol-enhanced chemiluminescence. The current

study used a flow cytometric technique that measures the oxidation of a non-fluorescent probe to a fluorescent probe by ROS produced inside cells (Bass, 1983). Each of these methods to assess respiratory burst measures a slightly different aspect or combination of aspects of the pathway that results in the production of ROS, complicating the direct comparison of results generated from ROS assays (Bols et al., 2001). Standardized methodologies would increase the ability to compare results from different studies (Bols et al., 2001; Brousseau et al., 1998).

In the current study, PCP was found to increase the percentage of cells engaging in phagocytosis, particularly at lower concentrations. The effects on phagocytosis are uncertain, as two earlier studies found that, following PCP exposure of whole organisms, mummichog eosinophils had decreased phagocytic activity while in carp (*Carassius auratus*), macrophage phagocytosis was unaffected (Chen et al., 2004; Roszell and Anderson, 1996). While these studies were similar to ours in that they were done using *in vivo* exposures, the lowest exposure concentrations in these studies (50 µg/L and 27 µg/L, respectively) were much greater than the highest exposure used in the current study (10 µg/L). It is possible that at higher concentrations of PCP, the rainbow trout used in the current study might have also exhibited suppressed phagocytosis similar to that observed by Roszell and Anderson (1996). Hormetic or biphasic effects in immunotoxicology are not uncommon, with xenobiotic exposure causing stimulation at low concentrations and inhibition at high concentrations (Calabrese, 2005).

The increased response of phagocytic cells in our study may be a cause for concern. Any chemical-associated modulation of the immune system, either up (i.e. immunostimulation) or down (i.e. immunosuppression), can be considered as evidence of immunotoxicity. The reduction in qualitative or quantitative aspects of immune function has been associated with dose dependent increases in mortality with some pathogens (Beaman et al., 1999; Carlson et al., 2002; Luster et al., 1993). Conversely, stimulation of the immune response following pesticide exposure can also result in detrimental effects on an organism such hypersensitivity reactions, autoimmune diseases or other damage caused by overreaction of the immune system (Blakley et al., 1999; Galloway and Handy, 2003).

For example, over-production of reactive oxygen species following stimulation of leukocytes, although potentially beneficial for the destruction of pathogens (Secombes and Fletcher, 1992), is not without other hazards. ROS produced inside macrophages or granulocytes during respiratory burst can leak out into surrounding tissues and uncontrolled or excessive production of ROS can result in oxidative stress and tissue damage such as lipid peroxidation, necrosis or cancer (Winston and Giulio, 1991). For example, Fatima et al. (2000) found that exposure to paper mill effluent caused a stimulation in ROS production by macrophages of freshwater catfish (*Heteropneustes fossilis*) which was also associated with an increase in lipid peroxidation particularly in the liver, gill and kidney. Although not measured in the current study, the predisposition of pesticide-exposed leukocytes to produce excessive amounts of oxygen radicals following antigen stimulation might cause damage to tissues other than the immune system.

The potential for lasting effects of CUP exposure was evidenced by the increase in respiratory burst and percentage of phagocytic cells in our study through at least 14-day post-exposure to chlorothalonil. Although our study design was not able to chart the timeline for recovery, the lasting effects observed post-exposure likely reflects the lag time between exposure, uptake, pharmacokinetics, and effects of chlorothalonil at an organismal level.

Changes in the immune system may, or may not, have direct bearing on host resistance to a given pathogen. Some immune parameters are more predictive of host responses to different pathogens, underscoring the importance of a tiered and multi-assay approach to assessing immunotoxicity (Luster et al., 1993). In our study, the lack of correlation between the stimulation observed in the *ex vivo* cellular assays and the response of fish to *L. anguillarum* suggests that the qualitative changes in phagocytosis and respiratory burst may not be critical for whole organism defense against this particular bacterium. Other aspects of the immune system that were not measured, such as lymphocyte function, may be more important for clearance of this organism.

Further, the importance and contribution to the integrated immune response of barrier defenses such as mucus quality and quantity, skin integrity or presence of anti-bacterial proteins in preventing infections by microbial agents cannot be ignored (Ellis,

2001). The complexity and redundancy of the immune response and the impossibility of simultaneously measuring all aspects of immune response can make it difficult to predict the outcome of pathogen exposures based on findings of a limited number of assays of internal non-barrier defenses such as those measured in the current study. This uncertainty in interpreting findings is one of the recognized challenges regarding extrapolation of cellular assays to either the whole organism or ecological levels (Bols et al., 2001).

In terms of environmental relevance, the pesticide concentrations used in the exposures, particularly at the lowest doses, represent concentrations that may be found in environmental water samples. Chlorothalonil has been identified in either fresh or marine water samples at levels of 20 ng/L up to 1130 ng/L (Voulvoulis et al., 2000; Wan et al., 2006) while our exposure range was 100 ng/L up to 1000 ng/L. Cypermethrin has recently been reported in a Canadian river at a concentration of 130 ng/L (Struger and Fletcher, 2007); however in the current study there were no significant effects of exposure to nominal concentrations up to 100 ng/L. Although PCP use has been significantly curtailed in recent years, due to its persistence and continued use in some areas of the world, environmental concentrations still remain elevated and water concentrations of 2.94 µg/L have been reported recently (Santiago and Kwan, 2007), which is within the range used in the experiment that resulted in changes to the immune system. Thus it could be expected that immunomodulatory effects observed following chlorothalonil or PCP exposures might be observed in fish in natural environments

Conclusion

Our demonstration of immunomodulation following exposure to environmentally relevant concentrations of chlorothalonil and pentachlorophenol suggests the potential sensitivity of the immune system to the effects of these CUPs. Other non-specific measures of health and condition (e.g. body weight) were unaffected by chemical exposure, and no direct lethality was observed during chemical exposure, adding support to the argument that endpoints that examine sub-lethal effects have the potential to be better and more sensitive biomarkers of environmental xenobiotic exposure. The shift in use from the legacy pesticides that are less water soluble to the CUPs that are

more water soluble has implications to issues of exposure and risk of effects on fish. Given the widespread use of several hundred CUPs in North America, coupled with their generally water-soluble properties, there is need for further research to elucidate their sub-lethal effects and mechanisms of action on aquatic species. In the context of the immune system, studies with increased sample sizes and exposures to mixtures of pesticides, assessment of a range of immunological endpoints, and challenges with multiple pathogens, should be conducted to clarify the immunomodulatory potential of this class of environmental pollutants.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2009.01.005.

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Table

Table 6-1. Relative head kidney leukocyte differential counts and serum lysozyme activity in rainbow trout following pesticide exposure or recovery period. Relative leukocyte differential counts are expressed as percent of control for sampling day for lymphocytes and granulocytes separately and serum lysozyme is expressed as HEWL equivalents in µg/mL. Values are given as mean ± S.E.M. and n = 12 for each treatment group and each assay, except cypermethrin control group for relative differential counts where n = 11. P-values provided are the probability that there are no differences between control and treatment groups.

Pesticide Concentration	Relative Lymphocyte Count (relative to control)	Relative Granulocyte Count (relative to control)	Serum Lysozyme Activity
Chlorothalonil – 28 day exposure			
Control	100.0 ± 4.2	100.0 ± 6.8	10.2 ± 1.4
100 ng/L	104.5 ± 2.1	93.9 ± 3.8	8.6 ± 1.8
200 ng/L	103.9 ± 3.7	101.9 ± 7.5	6.8 ± 1.2
500 ng/L	100.4 ± 3.2	104.8 ± 5.7	9.7 ± 1.4
1000 ng/L	96.1 ± 4.1	104.7 ± 5.8	8.7 ± 0.7
p-value	0.4651	0.7090	0.2332
Chlorothalonil – 14 day recovery			
Control	100.0 ± 3.5	100.0 ± 4.1	12.0 ± 1.0
100 ng/L	104.2 ± 2.1	94.9 ± 8.4	10.5 ± 0.7
200 ng/L	101.9 ± 2.2	97.8 ± 2.6	10.6 ± 1.0
500 ng/L	102.9 ± 2.7	96.6 ± 3.3	10.4 ± 1.0
1000 ng/L	100.4 ± 2.8	99.9 ± 3.2	10.9 ± 1.3
p-value	0.7940	0.7563	0.7561
Cypermethrin – 28 day exposure			
Control	100.0 ±3.7	100.0 ± 3.2	10.1 ± 1.6
10 ng/L	91.4 ± 2.7	107.3 ± 2.4	9.1 ± 1.0
20 ng/L	87.7 ± 3.5	110.8 ± 3.2	8.0 ± 0.8
50 ng/L	101.2 ± 5.8	99.0 ± 5.1	9.4 ± 1.2
100 ng/L	89.1 ± 3.7	109.6 ± 3.3	12.8 ± 1.5

p-value	0.0504	0.0981	0.0879
Pentachlorophenol – 28 day exposure			
Control	100.0 ± 3.1	100.0 ± 3.9	14.0 ± 1.3
1 µg/L	92.5 ± 2.2	109.3 ± 2.8	14.3 ± 1.3
2 µg/L	93.6 ± 5.4	107.9 ± 6.8	15.1 ± 1.9
5 µg/L	95.1 ± 5.0	106.0 ± 6.2	13.1 ± 1.7
10 µg/L	91.0 ± 4.0	112.5 ± 5.0	13.1 ± 1.3
p-value	0.5989	0.5346	0.8606

Figures

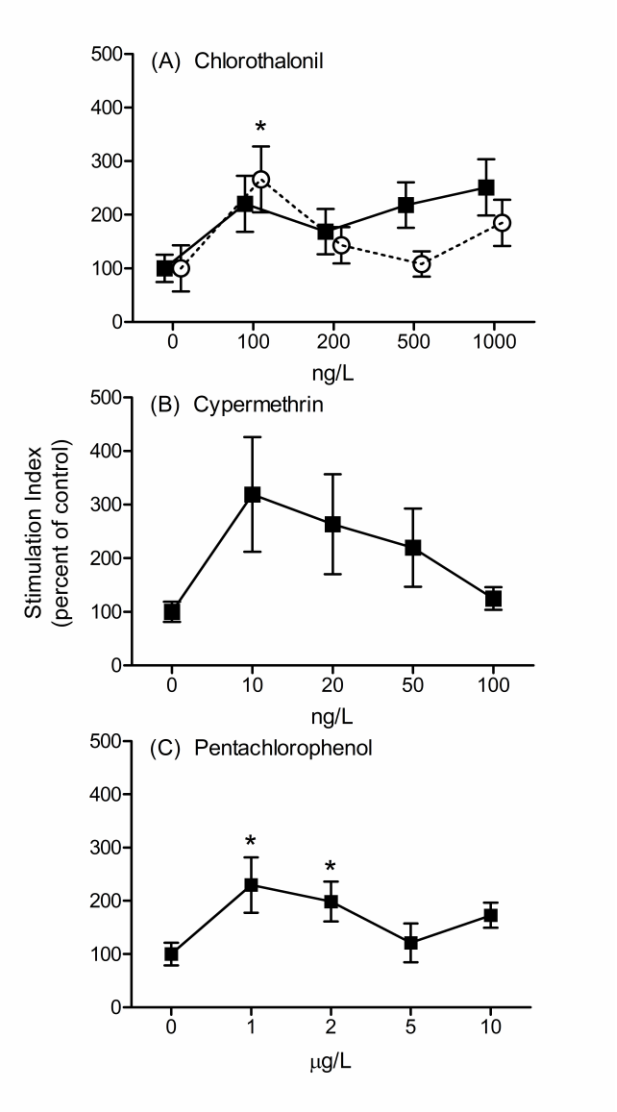


Figure 6-1. Respiratory burst activity of rainbow trout head kidney leukocytes stimulated with PMA increased following 28 days of in vivo exposure to (C) pentachlorophenol or (A, circles) a 14-day post-chlorothalonil exposure recovery period, but was not significantly changed following exposure to (A, squares) chlorothalonil or (B) cypermethrin. Data are expressed as a stimulation index which was calculated as the mean fluorescence of PMA stimulated leukocytes divided by mean fluorescence of unstimulated leukocytes. Mean fluorescence of each sample was standardized by expressing data as percent of control for the day. Points represent mean ± S.E.M., an * indicates that a treatment is significantly different than control (p-value < 0.05) and sample size is 12 per treatment group.

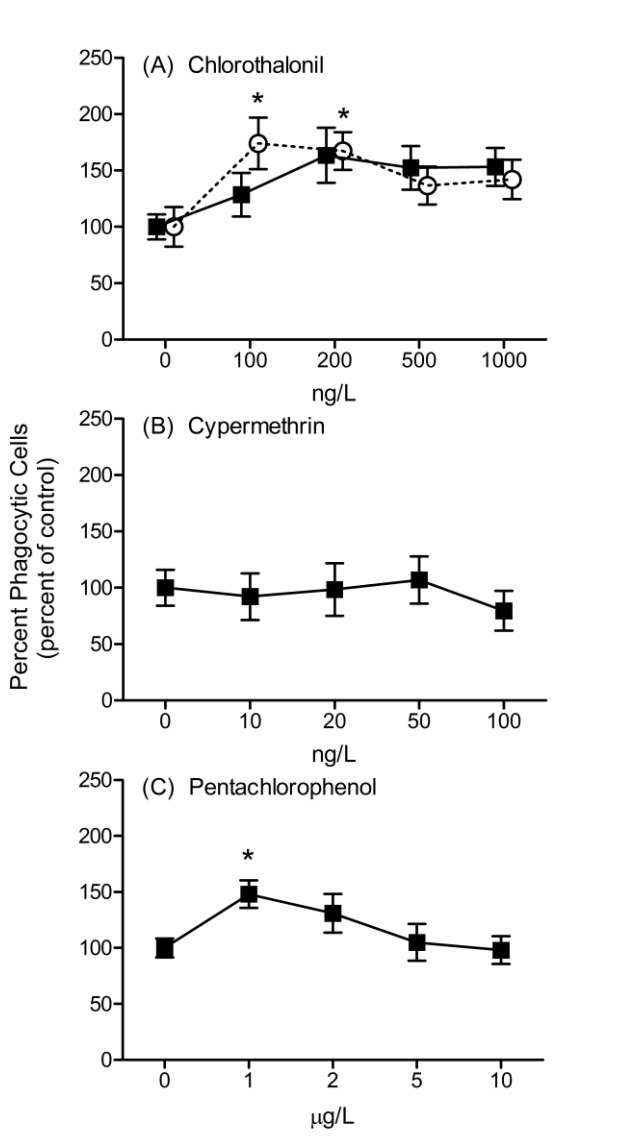


Figure 6-2. Percent of rainbow trout head kidney leucocytes that phagocytosed fluorescent latex beads was increased following 28 days of in vivo exposure to (A, squares) chlorothalonil, (C) pentachlorophenol or (A, circles) a 14-day post-chlorothalonil exposure recovery period, but was unaffected by (B) cypermethrin exposure. Percent of total leukocytes that phagocytosed beads was calculated by determining the proportion of live fluorescent cells and subtracting the proportion of fixed cells that were fluorescent (background fluorescence) for each fish. Data were standardized by expressing data as percent of control for the day. Points represent mean ± S.E.M., an * indicates that a treatment is significantly different than control (p < 0.05) and sample size (n) is 6 per treatment group for chlorothalonil 28-day exposure and n = 12 per treatment group for all others.

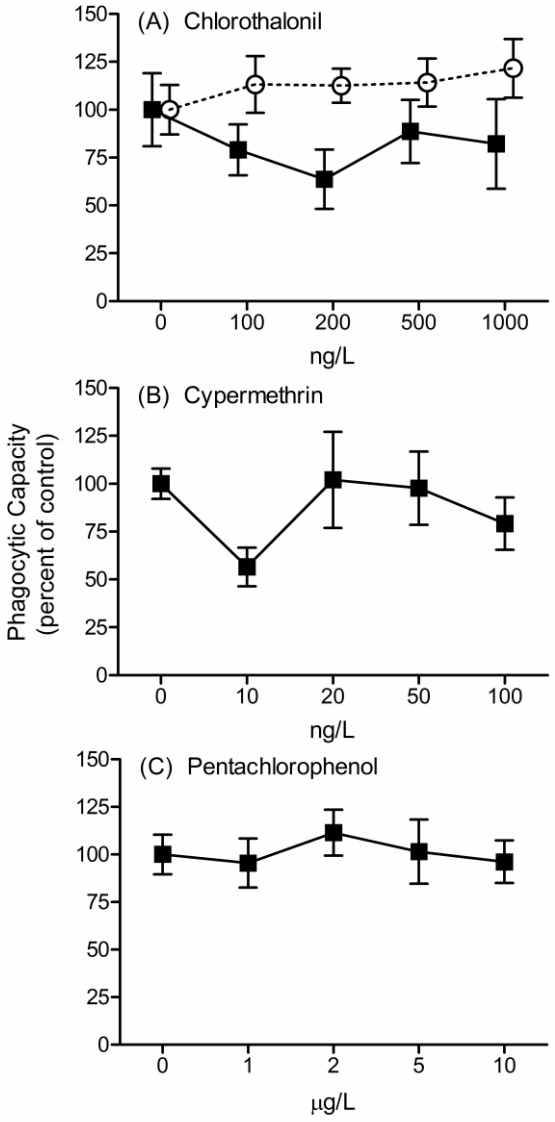


Figure 6-3. Capacity of rainbow trout head kidney leukocytes to phagocytose fluorescent latex beads following 28 days of in vivo exposure to chlorothalonil (A, squares), cypermethrin (B), pentachlorophenol (C) or a 14-day post-chlorothalonil exposure recovery period (A, circles) was unchanged, as measured by mean fluorescence of cells that phagocytosed beads. Phagocytic capacity was calculated as the mean fluorescence of live leukocytes that had phagocytosed fluorescent beads minus the mean fluorescence of fixed leukocytes that were positive for fluorescent beads (background fluorescence). Mean fluorescence of each sample was standardized by expressing data as percent of control for the day. Points represent mean ± S.E.M. and sample size (n) is 6 per treatment group for chlorothalonil 28-day exposure and n = 12 per treatment group for all others.

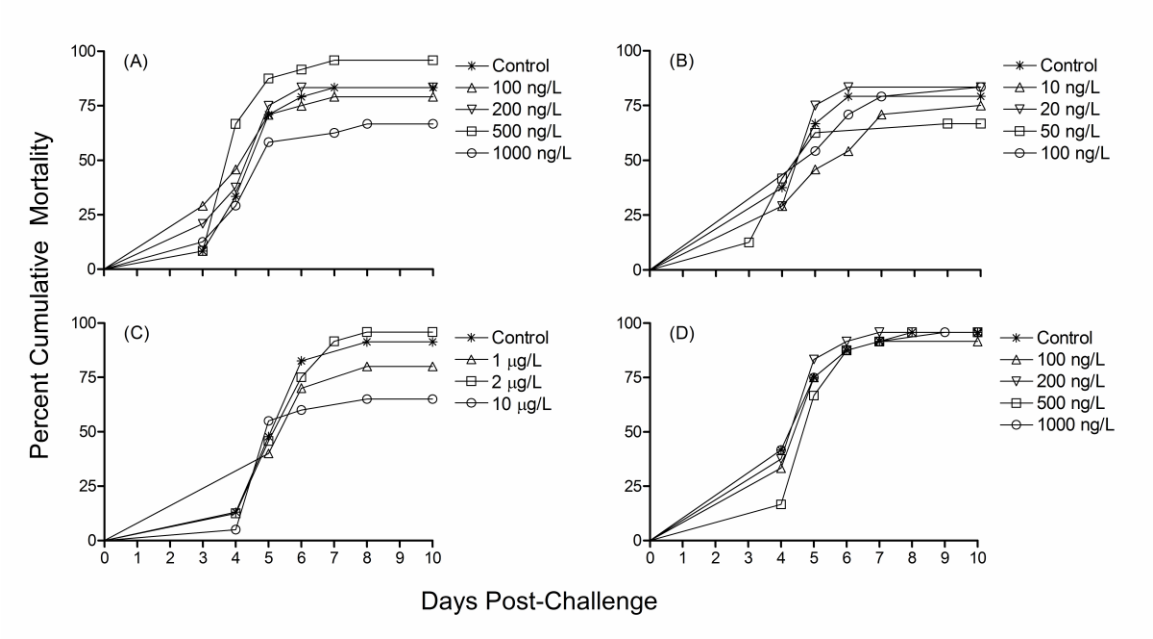
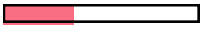
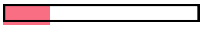
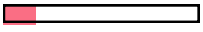
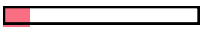
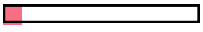
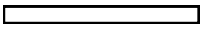
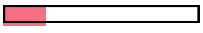
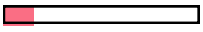
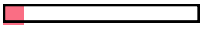
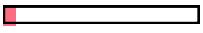
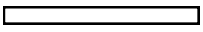
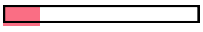
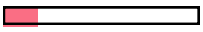
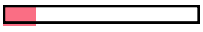
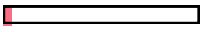
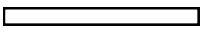
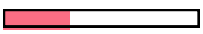
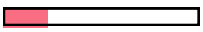
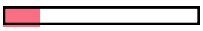
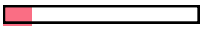
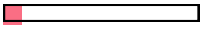
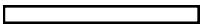


Figure 6-4. Cumulative mortality curves for rainbow trout following an immersion challenge with Listonella anguillarum following 28-day in vivo exposure to (A) chlorothalonil, (C) cypermethrin, (D) pentachlorophenol or (B) 14-day recovery period post-chlorothalonil exposure. Sample size (n) is 24 for all treatment groups except pentachlorophenol where n = 23 for control, n = 11 for 1 µg/L and n = 20 for 10 µg/L.

Supplementary Data

Table S6-1. Eigenvalues and cumulative percent contribution for each factor generated by Principal Component Analysis (PCA).

Chlorothalonil – 28 Day Exposure				
Number	Eigenvalue	Percent	Percent	Cumulative Percent
1	2.1771	36.285		36.285
2	1.4508	24.180		60.464
3	0.9925	16.541		77.005
4	0.8134	13.556		90.561
5	0.5663	9.439		100.000
6	-0.0000	-0.000		100.000
Chlorothalonil – 14 Day Recovery Period				
Number	Eigenvalue	Percent	Percent	Cum Percent
1	2.6867	44.778		44.778
2	1.3309	22.182		66.960
3	0.9256	15.426		82.386
4	0.6560	10.933		93.319
5	0.4009	6.681		100.000
6	-0.0000	-0.000		100.000
Cypermethrin – 28 Day Exposure				
Number	Eigenvalue	Percent	Percent	Cum Percent
1	2.5441	42.401		42.401
2	1.1499	19.164		61.565
3	1.0505	17.509		79.074
4	1.0102	16.836		95.911
5	0.2454	4.089		100.000
6	-0.0000	-0.000		100.000
Pentachlorophenol – 28 Day Exposure				
Number	Eigenvalue	Percent	Percent	Cum Percent
1	2.0578	34.297		34.297
2	1.3725	22.875		57.172

3	1.1093	18.488		75.661
4	0.8823	14.705		90.366
5	0.5780	9.634		100.000
6	-0.0000	-0.000		100.000

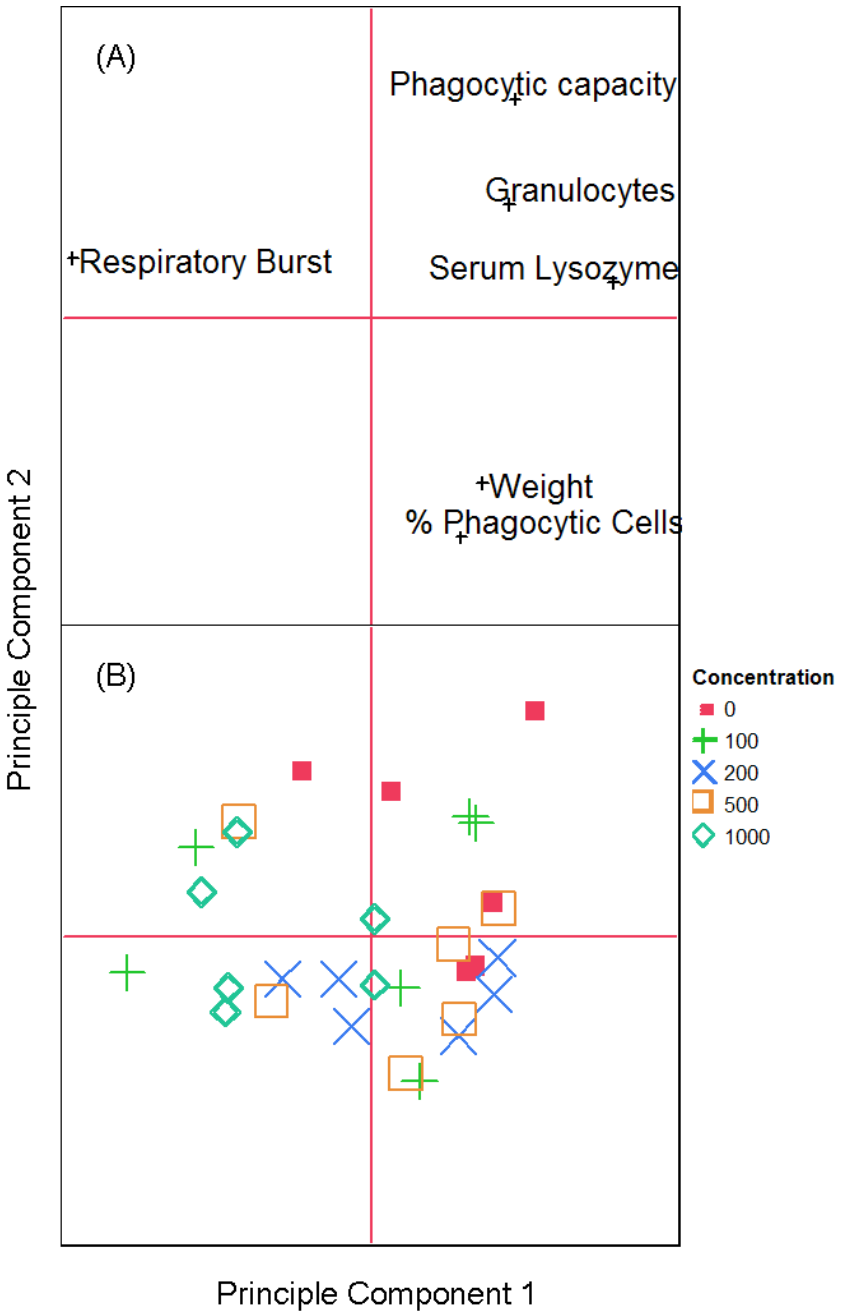


Figure S6-1. (A) Loading and (B) score plots generated from Principal Component Analysis (PCA) using normalized data from the 28 day chlorothalonil exposure experiment showing limited treatment group clustering and no clearly discernible dose response. Response data from 30 fish (n = 6 per exposure concentration) were included in the PCA for each of 6 assays. Concentrations given in the legend for the score plot are in ng/L.

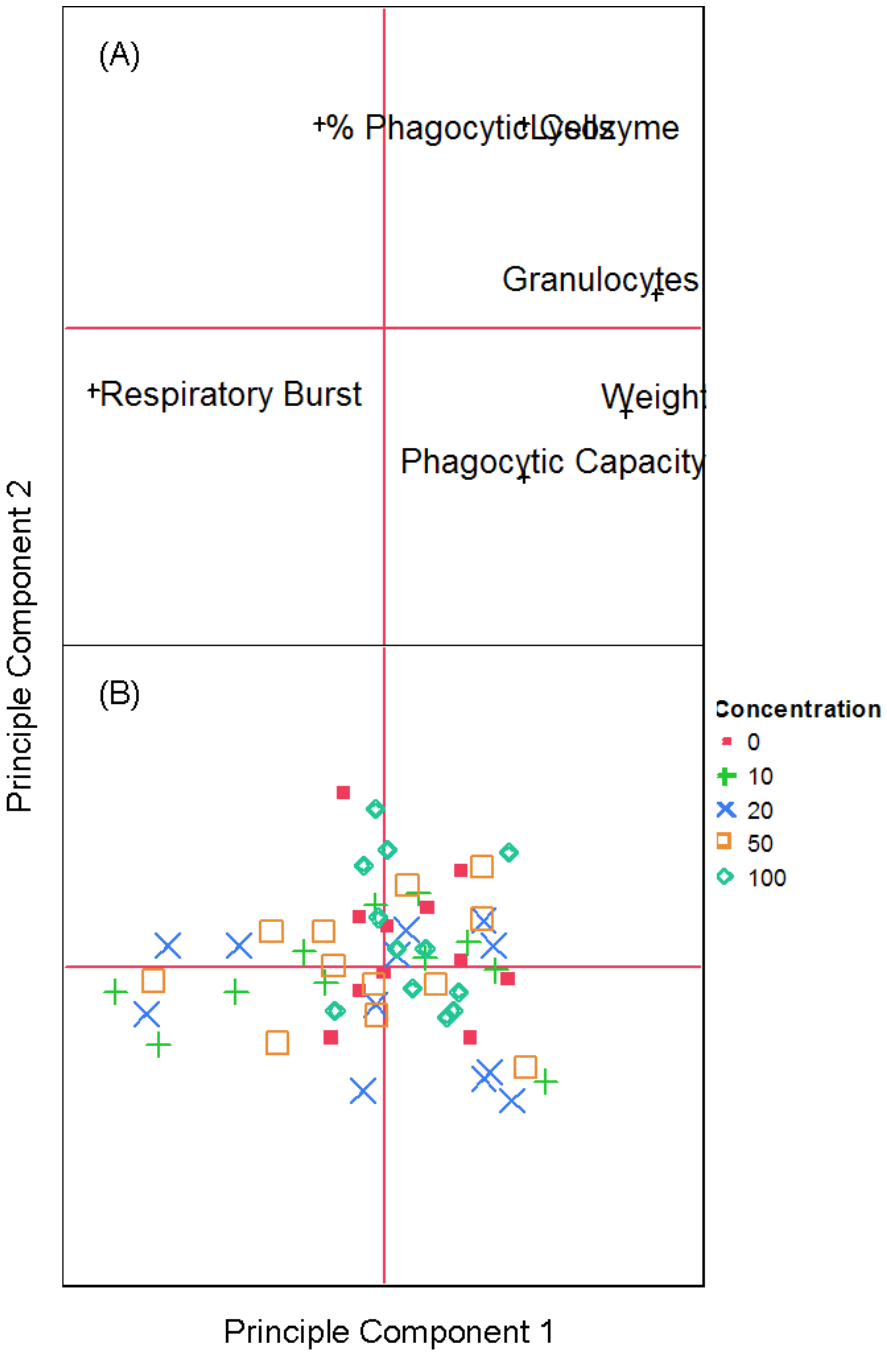


Figure S6-2. ***(A) Loading and (B) score plots generated from Principal Component Analysis using normalized data from the 28 day cypermethrin exposure experiment showing limited treatment group clustering and no clearly discernible dose response. Response data from 58 fish (n = 12 per exposure concentration except for control (0 ng/L) and 10 ng/L groups where n = 11) were included in the PCA for each of 6 innate immune system assays. Concentrations given in the legend for the score plot are in ng/L.***

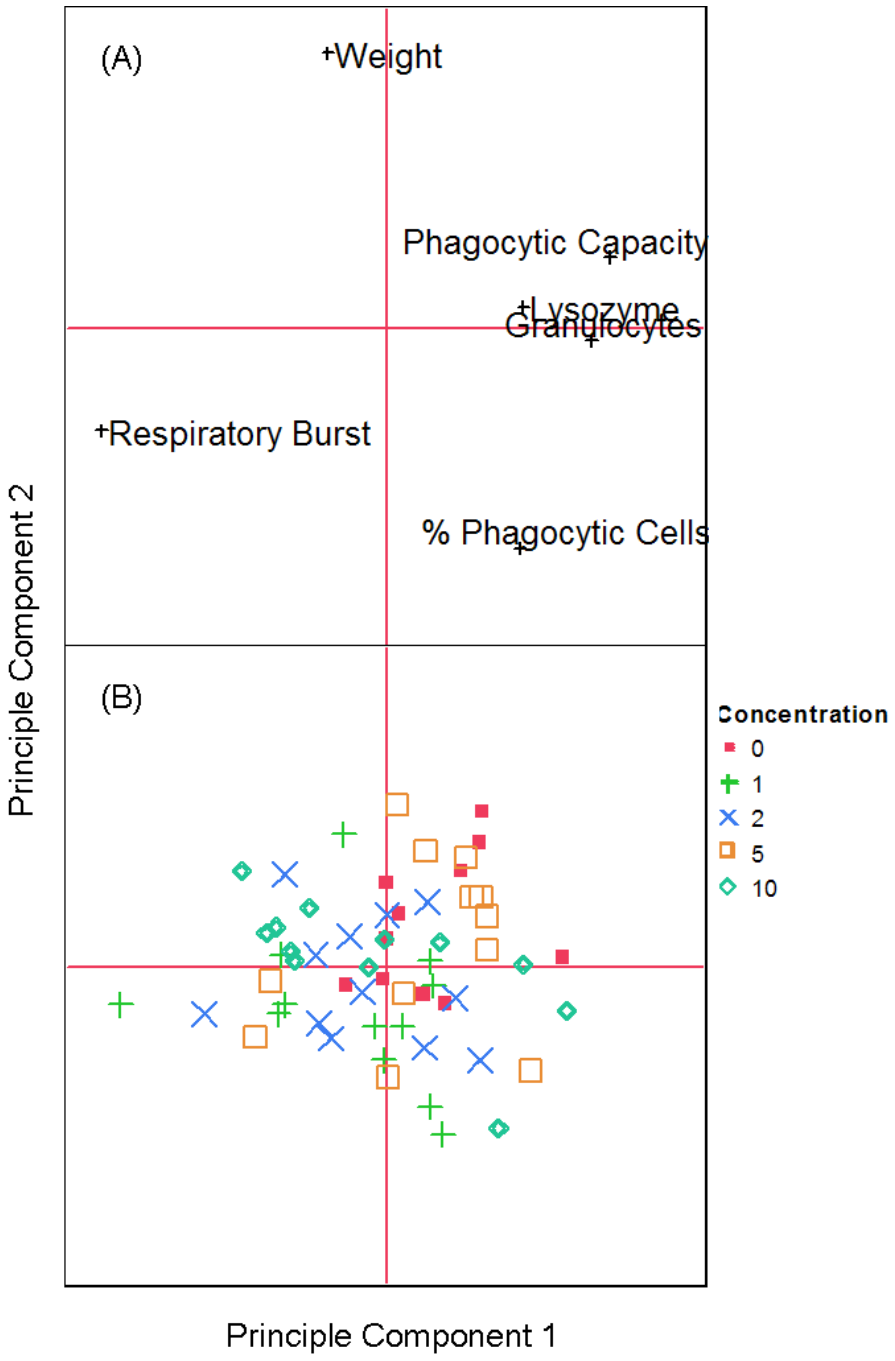


Figure S6-3. (A) Loading and (B) score plots generated from Principal Component Analysis using normalized data from the 28 day pentachlorophenol exposure experiment showing limited treatment group clustering and no clearly discernible dose response. Response data from 59 fish (n = 12 per exposure concentration except for control (0 µg/L) group where n = 11) were included in the PCA for each of 6 innate immune system assays. Concentrations given in the legend for the score plot are in µg/L.

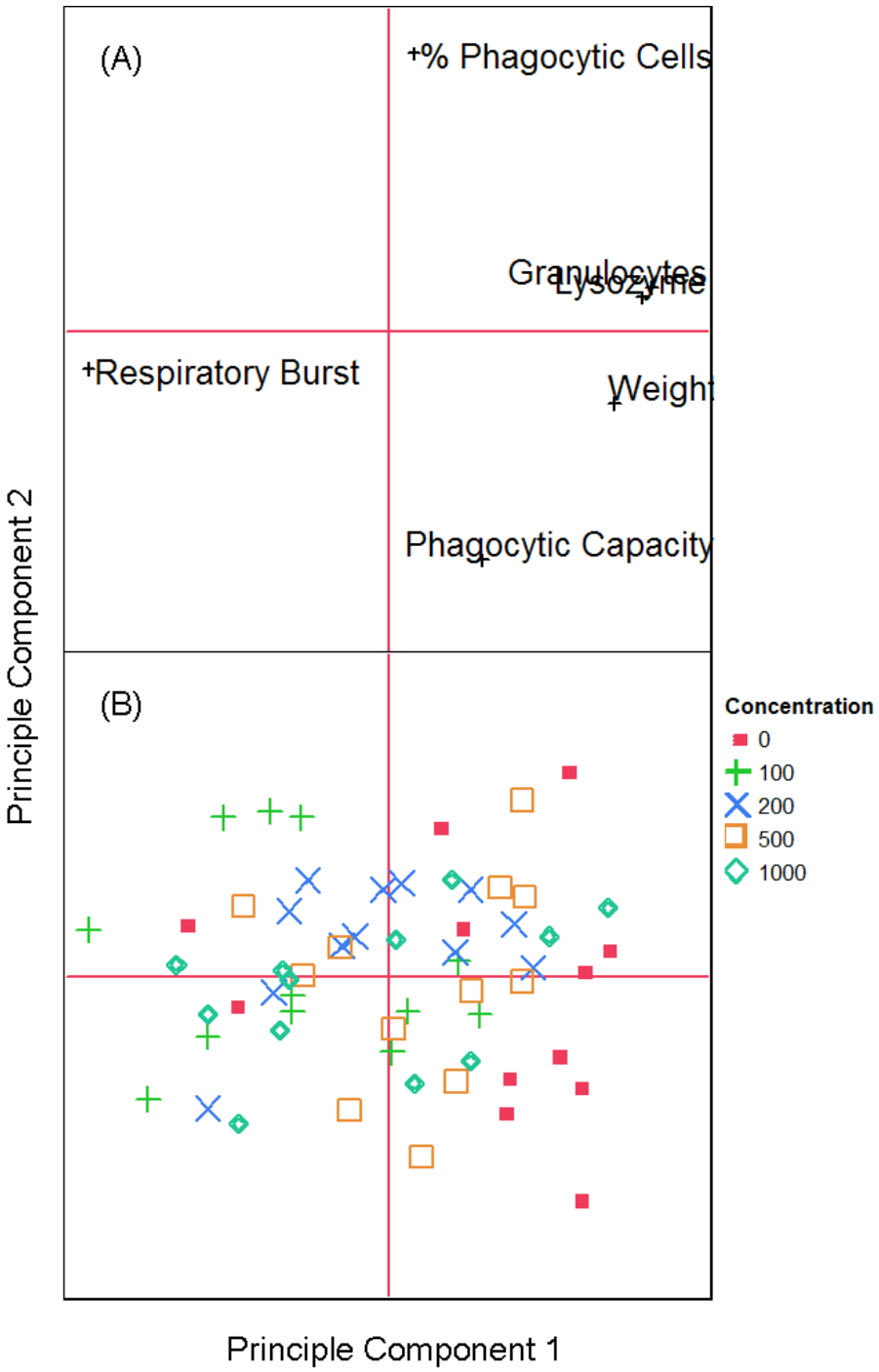


Figure S6-4. ***(A) Loading and (B) score plots generated from Principal Component Analysis (PCA) using normalized data from the 14 day recovery period following chlorothalonil exposure experiment showing limited treatment group clustering and no clearly discernable dose response. Response data from 60 fish (n = 12 per exposure concentration) were included in the PCA for each of 6 assays. Concentrations given in the legend for the score plot are in ng/L.***

Chapter 7.

Summary, Future Directions, and Conclusions

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Summary of Key Research Findings

Immunomodulation and hormesis

As expected, the studies detailed here provide additional evidence of endocrine modulation of immune system function (Ch. 2 and 3; cortisol and 17β-estradiol, E2). With the exception of cypermethrin, all of the pesticides and formulants investigated were either immunotoxic (nonylphenol, atrazine, chlorothalonil, pentachlorophenol) or cytotoxic (permethrin, piperonyl butoxide)(Ch. 2, 4, 5 and 6). The findings here highlight the range of potential immunotoxic effects, with some of the chemicals exhibiting immunostimulatory effects (chlorothalonil, pentachlorophenol) and others causing immunosuppressive effects (nonylphenol, atrazine) at the exposure concentrations tested. Immunotoxic effects were observed at all levels of biological organization (molecular, biochemical, cellular, whole organism), with two of the chemicals (nonylphenol and atrazine) causing increased disease susceptibility in exposed fish.

Hormesis refers to an atypical concentration-response relationship in which the toxic effects of chemical exposure at low concentration are opposite to the effects of the same chemical at higher concentrations. These type of concentration-response relationships are often referred to as “U”- or “J”-shaped or non-monotonic curves and are frequently observed in immunotoxicological studies, as well as in wider toxicology studies (Calabrese and Baldwin, 2001; Calabrese, 2005). For chlorothalonil and pentachlorophenol exposed-fish (Ch. 6), where immunostimulation was noted at the low concentrations used in this study, evidence from the wider literature suggested that these chemicals are immunosuppressive at higher concentrations (Anderson and Brubacher, 1993; Roszell and Anderson, 1994; Roszell and Anderson, 1996; Baier-Anderson and Anderson, 1998). This highlights some of the difficulty in assessing the risk posed by xenobiotic exposure, since traditional toxicological and risk assessment models are generally based on sigmoidally shaped concentration-response relationships.

Estrogen receptors in leukocytes

The studies presented here provide the first complete evidence of the presence of the full known complement of estrogen receptors (ERs) in purified leukocytes from both blood and head kidney of rainbow trout (Ch. 2 and 3). This confirmation is central to understanding signaling pathways and mechanisms of toxicity, since the availability of receptors is a minimum requisite for a hormone or a hormone-mimicking xenobiotic to cause alterations in target cells.

Transcription of ERα1 and ERα2 in peripheral blood leukocytes was down-regulated by exposure to lipopolysaccharide (mitogen used to stimulate leukocyte proliferation), suggesting that the ERs (and E2) may be intimately involved in cell cycle control and proliferation (Ch. 2). Exposure to E2 *in vivo* led to up-regulation of ERα1 in both head kidney and peripheral blood leukocytes and differential regulation of ERα2 in the same tissues (Ch. 3). This auto-regulation of the ERαs by E2 may underlie some of the immunomodulatory effects associated with the hormone, and represents an important step forward in understanding the mechanisms associated with endocrine-mediated immunomodulation.

Microarrays and mechanisms

Studies of the mechanisms underlying immunotoxic effects of xenobiotics in teleosts is still in its infancy, as most research focuses primarily on descriptive immunotoxicology (i.e. exposure to chemical X causes certain effects) (Ladics and Woolhiser, 2006; Pruett et al., 2006). With the increasing availability and use of molecular technologies such as those in genomics, proteomics and metabolomics, the mechanistic basis for immunotoxicity will become more apparent, particularly when the effects are 'anchored' to phenotypic changes (Ankley et al., 2006). The development and use of bioinformatics programs to aid in the interpretation of the large data volumes generated by microarrays has also contributed to the advances made in the toxicogenomics field by facilitating identification of pathways and biological processes impacted by treatments. For example, the use of microarrays and Pathway Studio®, a bioinformatics program, to assess the immunotoxic effects of nonylphenol and atrazine identified a large number of immune system-related processes and pathways that were altered by treatment and may have contributed to the increase in disease susceptibility

observed in the exposed fish (Ch. 5). This type of study combining both functional and molecular methodologies highlights the usefulness of microarray technology in furthering understanding in immunotoxicology.

Ultimately, examination of molecular pathways affected by xenobiotic exposures will allow identification of similar mechanisms of toxicity between various chemicals, such as commonalities in the effects of xenoestrogens. Molecular differences in responses will permit development of biomarkers or gene expression patterns that indicate exposure to particular classes or types of chemicals, similar to what has been done in other tissues like the liver (e.g. Kosikinen et al., 2004; Hook et al., 2006; Finne et al., 2007). These approaches will allow systems-level understanding of the mechanisms of toxicity and aid in predicting the immunotoxicity of untested chemicals.

Recommendations for Future Studies

There should be concern that, given the presence of EDCs in the aquatic environment coupled with the recognition that they may also target the immune system of fish, these chemicals have the potential to exert toxicity beyond the traditional reproductive or growth endpoints that are typically considered. The studies described here and others from the wider literature (e.g. (Wang and Belosevic, 1994; Kreutz et al., 2010; Wenger et al., 2011) have demonstrated that exposure to EDCs or exogenously applied estrogen can lead to increased disease susceptibility in fish, an outcome that can have negative population-level consequences. However, there are surprisingly few published studies investigating the effects of EDCs on immune function in teleosts and future studies may clarify the extent and degree of concern that we should have in regards to the immunotoxicity of this class of chemicals.

While some of the studies presented here have begun to delve into the mechanisms underlying immunotoxicity of xenobiotics in fish, much additional work is required in this area. Similarly, while the modulatory effects of hormones on the immune system in fish have been reasonably well described, the actual mechanisms and signal transduction pathways underlying the immunomodulatory effects have not been defined, particularly for the estrogens and androgens. Unraveling the mechanistic underpinnings

of the basic, natural physiological interaction between the endocrine and immune systems can aid in the understanding of how endocrine disrupting chemicals (EDCs) may be able to disrupt this delicate balance.

EDCs have the potential to interfere with immune function either directly (by impacting the immune system) or indirectly (by altering endocrine system function, which then affects immune function). The incorporation of hormone inhibitors into immunotoxicology experiments with EDCs may aid in determining whether the effects are direct or indirect. However, care must be taken when using inhibitors that were designed to work in mammalian systems, as the effects in fish may not be the same. For example, fulvestrant (ICI 182780), which is a pure estrogen antagonist in mammals, can act as an estrogen agonist in fish and the effects are tissue dependent (i.e. agonist in the liver but not in the testis) (Pinto et al., 2006)

Conclusion

Overall this study has demonstrated that a number of pesticides and formulants with endocrine disrupting properties are also capable of altering normal immune system function, at environmentally realistic concentrations. Several of the chemicals (nonylphenol and atrazine) are able to impair the immune system to a degree that decreases resistance to infectious disease. Genomic methodologies (QPCR, microarrays) have allowed the identification of ERs in leukocytes and the investigation of mechanisms underlying the physiological and immunotoxicological effects. Future work can address the many data gaps remaining in this field and will contribute to a better understanding of the overall risk posed by these chemicals to wild fish in their natural environments.

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