Award Number:  DAMD17-99-2-9011

TITLE:  Fish Immune Response as Biomarkers

PRINCIPAL INVESTIGATOR:  Judith T. Zelikoff, Ph.D.

CONTRACTING ORGANIZATION:  New York University School of Medicine
Tuxedo, New York 10987

REPORT DATE:  March 2001

TYPE OF REPORT:  Annual

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland  21702-5012

DISTRIBUTION STATEMENT:  Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.
Aquatic species are exposed to chemical contaminants that leach into the water from neighboring dump-sites or are directly discharged there. Heavily polluted water affects the health of aquatic life by, among other things, enhancing the incidence of infectious diseases. In light of the fact that fish are directly-exposed in the water to toxic chemicals, the immune system is an extremely sensitive indicator for detecting the effects of toxic chemicals; and that the immune responses of fish are highly-conserved phylogenetically and, thus, structurally and functionally related to that of mammals, a study is proposed to test the hypotheses that immune functions of fish can serve as biological indicators for predicting toxicological hazards associated with polluted aquatic environments, as well as serve as an alternate model for mammals for predicting human health risks. The proposed studies employ a panel of well-established immune assays to evaluate the effects of known mammalian immunotoxicants and aquatic pollutants, alone and in combination, on the immune responses of Japanese medaka (Oryzias latipes). This study provides the opportunity to better understand the toxicological hazards and human health risks associated with exposure to militarily-relevant chemicals and meets the goals of the military which are to develop and validate new and more sensitive methods for assessing the toxic effects of chemicals in the ambient environment.
Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cover</td>
<td>1</td>
</tr>
<tr>
<td>SF 298</td>
<td>2</td>
</tr>
<tr>
<td>Foreword</td>
<td>3</td>
</tr>
<tr>
<td>Abstract</td>
<td>5</td>
</tr>
<tr>
<td>I. Introduction</td>
<td>5</td>
</tr>
<tr>
<td>II. Summary of Previous Work</td>
<td>7</td>
</tr>
<tr>
<td>III. Body</td>
<td>7</td>
</tr>
<tr>
<td>IV. Key Research Accomplishments</td>
<td>12</td>
</tr>
<tr>
<td>V. Reportable Outcomes</td>
<td>13</td>
</tr>
<tr>
<td>VI. Conclusions</td>
<td>15</td>
</tr>
<tr>
<td>VII. References</td>
<td>16</td>
</tr>
<tr>
<td>Figures</td>
<td>18</td>
</tr>
</tbody>
</table>
FISH IMMUNE RESPONSES AS BIOMARKERS
(March 2000 - September 2001)

ABSTRACT
Aquatic species are exposed to chemical contaminants that are leach into the water from neighboring dump-sites or are directly discharged there. Heavily polluted water affects the health of aquatic life by, among other things, enhancing the incidence of infectious diseases. In light of the fact that: fish are directly-exposed in the water to toxic chemicals; the immune system is an extremely sensitive indicator for detecting the effects of toxic chemicals; and, that the immune responses of fish are highly-conserved phylogenetically and, thus, structurally- and functionally-related to that of mammals, a study is proposed to test the hypotheses that immune functions of fish can serve as biological indicators for predicting toxicological hazards associated with polluted aquatic environments, as well as serve as an alternate model for mammals for predicting human health risks. The proposed studies employ a panel of well-established immune assays to evaluate the effects of known mammalian immunotoxicants and aquatic pollutants, alone and in combination, on the immune responses of Japanese medaka (Oryzias latipes). This study provides the opportunity to better understand the toxicological hazards and human health risks associated with exposure to militarily-relevant chemicals and meets the goals of the military which are to develop and validate new and more sensitive methods for assessing the toxic effects of chemicals in the ambient environment.

I. INTRODUCTION
A. Statement Of Work
   A study is proposed that will test the hypotheses that fish can serve as an alternate species for mammals to predict potential human health risks. Moreover, that immune responses of fish can serve as a biological system for hazard assessment that can accurately predict toxicological risks associated with polluted aquatic environments.

   This study will examine two inter-related Objectives which are linked by their ability to provide information necessary for hazard assessment, as well as for providing a better understanding of human health risks associated with exposure to militarily-relevant chemicals (MRCs) both as single compounds and complex mixtures. Two Aims are proposed to address the aforementioned hypotheses:

   (1) To determine and compare the effects of mammalian immunotoxicants on the immune response of Japanese medaka (Oryzias latipes) using a battery of well-established immunoassays. These studies will be performed to determine how closely chemically-induced alterations in the
immune responses of fish can predict effects previously-observed in mammalian species and, provide information necessary for interspecies extrapolation and for predicting toxicological risk to both directly- and indirectly-exposed species. Effects of known mammalian immunotoxicants, as well as those lacking immunotoxic potential will be examined.

(2) To determine the immunotoxic potential of known aquatic immunotoxicants and some MRCs (alone and in combination) in Japanese medaka. Results of these studies along with those from Aim 1 will result in an immunoassay or suite of assays useful for evaluating the immunotoxic effects of MRCs (including permethrin, heavy metals, and selected mixtures) in alternative species.

B. Background

Aquatic species are exposed to mixtures of chemical contaminants that are directly discharged into the water or leached from neighboring dump-sites. Heavily polluted water can affect directly-exposed species by enhancing disease incidence leading, potentially, to death of the host; adverse health consequences on humans that consume these affected organisms have also been observed. Because of this, fish systems are currently being used for biomonitoring studies to predict toxicological hazards associated with chemical exposure (Zelikoff, 1993; Anderson et al., 1997; Barron et al., 2000). In addition to their use for hazard assessment, much emphasis has recently been placed on the development of fish as alternate models for higher vertebrates in toxicological/immunotoxicological studies (Zelikoff et al., 1991, 1998, 2000; Zelikoff, 1994; Beaman et al., 1999; Sweet and Zelikoff, 2001).

The sophistication and complexity of the immune system enables it to likely be the most sensitive and, therefore, most prominent body function to detect harmful effects from chemicals (Dean et al., 1986). Many of the same chemicals shown to alter immunoresponsiveness in mammals also act as immunotoxicants in fish, and in many cases bring about similar effects and act by like mechanisms (Carlson et al., in press) Some classes of chemicals shown to modulate immune responses of both species include: metals (i.e., cadmium, nickel, lead), pesticides/insecticides (malathion, pentachlorophenol), and polycyclic aromatic hydrocarbons (benzo[a]pyrene) (Zelikoff, 1994).

Given the: sensitivity of the mammalian immune system to respond to low levels of environmental chemicals; morphological/functional/biochemical similarities between mammalian and fish immune defense mechanisms; and, previously-observed ability of the proposed immune assays to successfully predict the immunotoxic effects of in vivo chemical exposure (Zelikoff, 1998; Beaman et al., 1999; Zelikoff et al., 2000, in press; Carlson et al., in press; Duffy et al., in press), it appears likely that results from these studies can be extrapolated to higher vertebrates for predicting toxicological risk, and that immune responses of medaka can be used successfully to predict the immunotoxic effects of aquatic environments contaminated with MRCs.
II. SUMMARY OF PREVIOUS REPORT

In the previously-submitted Annual Report (March 1999 - February 2000), we described the immunotoxic potential of the potent immunosuppressant benzo(a)pyrene (BaP) on Japanese medaka and bluegill exposed to BaP by intraperitoneal injection (IP) and in the water, respectively. These studies examined the effects of BaP on host survival, cytochrome P450 1A1 (CYP1A1) enzyme induction/activity, phagocyte-mediated oxradical production, T- and B-lymphocyte proliferation, thymic cellularity, and antibody-forming cell (AFC) numbers. Results from these studies demonstrated that BaP suppressed medaka immune responses in a manner similar to that seen in mammals. Moreover, certain immunoassays (i.e., lymphocyte proliferation and AFC numbers) appeared to be particularly sensitive to the immunotoxic effects of BaP. Results of this study are currently "in press" in Aquatic Toxicology.

In addition, data were also presented in medaka that demonstrated the immunomodulating effects of the synthetic glucocorticoid dexamethasone (DEX). Results of range-finding studies demonstrated that while IP administration of DEX concentrations > 500 μg/fish produced 100% lethality, concentrations ≤ 200 μg/fish had no effect on host survival. Moreover, IP injection of 200 μg DEX suppressed both T- and B-lymphocyte mediated immune functions; T-cell-mediated immunity appeared more sensitive to the suppressive effects of DEX than B-cell functions. Interestingly, while adaptive immune responses were suppressed, innate immune functions were enhanced by DEX exposure.

Studies examining the immunotoxic effects of malathion on medaka were also described and the resulting manuscript appended (Beaman et al., 1999. J. Toxicol. Environ. Health).

III. BODY

This Annual Report includes additional data on the immunotoxicity of BaP, as well as information from recent studies examining the effects of benzo(e)pyrene (BeP), polychlorinated biphenyl 126 (PCB-126), permethrin, nickel (Ni), and Ni in combination with another commonly-found environmental stressor (i.e., elevated temperature).

Specific Aim 1

To determine the effects of well-studied mammalian immunotoxicants (and those lacking immunotoxic potential) in Japanese medaka using a panel of well-established immune assays.

A. Benzo(a)pyrene (BaP) Studies - Studies described herein are a continuation of those cited previously (c.f. Section II). Benzo(a)pyrene, a chemical in the class of polycyclic aromatic hydrocarbons (PAH), is a ubiquitous environmental contaminant with well-known immune and endocrine disrupting potential in a variety of mammalian species.
Studies performed in the past year examined the effects of BaP on host resistance against the infectious bacterial fish pathogen, *Yersinia ruckeri*. These studies served to link previously-observed immune dysfunction with overall host immunocompetence. Figure 1 demonstrates that while no differences in *Yersinia ruckeri*-induced host mortality were observed following exposure to 2 µg BaP/g BW or 3 d following exposure to any BaP concentration (compared to the vehicle control), mortality of fish exposed to either 20 or 200 µg BaP/g BW increased significantly 14 d post-bacterial challenge. A significant increase in host mortality was also observed 7 d post-infection (compared to corn oil vehicle control), but only following exposure to the highest BaP concentration (i.e., 200 µg/g BW). Medaka exposed to 200 µg BaP/g BW and subsequently-challenged with *Y. ruckeri* had numerous external lesions consisting of small red petechiae randomly-distributed along their bodies. Such lesions were not consistently seen in the other exposure groups. Although BaP has not been shown previously in fish or mammalian models to reduce host resistance against infection, laboratory exposure of Chinook salmon to another PAH, 7,12-dimethylbenz(a)anthracene (DMBA) decreased host resistance against subsequent challenge with the marine bacterial pathogen *Vibrio anguillarum* (Arkoosh et al., 1998). In the same investigation, juvenile salmon recovered from a PAH and PCB-contaminated area of Puget Sound (Washington State) and challenged (in the laboratory) with *V. anguillarum* demonstrated increased mortality in response to infection (compared to reference site fish). Effects in medaka appear to conflict with mammalian studies which demonstrated that BaP exposure of mice had no effect upon host resistance against infection with *Listeria monocytogenes* (Dean et al., 1983). The discrepancy between results observed in medaka and those in mice may be due to difference in bacterial pathogenesis. While *L. monocytogenes* infection appears to rely primarily upon evasion of cell-mediated immunity, a compartment of the immune system not highly-sensitive to BaP-induced immunotoxicity, humoral immune defense mechanisms appear to play an important role in mediating *Yersinia* infection by producing antibodies against *Y. ruckeri* necessary for the opsonization and ultimate phagocytosis of the bacterium (Siwicki and Dunier, 1993). Since previously-observed results in rodents and those observed herein have demonstrated the ability of BaP to suppress humoral immunity, perhaps an infectious agent whose clearance/killing is mediated by humoral immune defenses is a more appropriate challenge organism for demonstrating the effects of BaP upon host resistance.

Overall, results from this study demonstrates that a single BaP injection (at concentrations having no effect upon host morbidity or mortality) can significantly compromise host resistance against infectious disease. Moreover, since BaP has been shown to suppress humoral immunity in medaka (Carlison et al., in press) and *Y. ruckeri* infection is maintained by humoral-immune defense mechanisms, it appears that immunoassays such as AFC may be a useful indicator for
demonstrating effects produced on overall host immunocompetence and susceptibility to infectious disease.

B. **Benz(e)pyrene (BeP) Studies** - Effects of the non-carcinogenic, low-affinity Ah receptor (AhR) agonist BeP was examined in these studies because of its lack of immunotoxic potential in mammalian systems. Medaka, injected (IP) with BeP at 2, 20, 200, or 400 µg/g were immunized 48 hr later with sheep red blood cells (SRBC) and effects upon AFC numbers examined 11 d later. In contrast to the dose-dependent suppressive effects produced by BaP, exposure to concentrations of BeP as high as 400 µg/g BW had no effect upon AFC numbers. Moreover, BeP failed to induce hepatic CYP1A protein expression or activity (Figure 2). The failure of BeP to produce immune suppression in this study is consistent with previous findings conducted with rodent models (White et al., 1994).

C. **Dexamethasone (DEX)** - Because of the well-recognized potent immunosuppressive effects of the synthetic glucocorticoid dexamethasone (DEX) in mammalian species, medaka were exposed by IP injection to different DEX concentrations so as to validate the medaka model as an alternate species for mammals in immunotoxicity testing and for species extrapolation modeling. In the last Annual Report the effects of DEX on innate immune functions (i.e., O₂⁻ production) and B- and T-cell proliferation were discussed. At that time, studies were underway in medaka to determine the effects of DEX on AFC numbers and host resistance against infection with *Yersinia ruckeri*. Results from these latter studies demonstrate that a single IP exposure of fish to either 200 or 300 µg DEX/fish significantly-reduced AFC numbers by ~50% compared to the saline-injected control (12.1 vs. 22.8 plaques/10⁶ cells, respectively). Dexamethasone-induced reduction in AFC numbers were in sharp contrast with the lack of effects observed on host resistance; injection with DEX at concentrations as high as 300 µg had no effect upon bacterial-induced mortality. Given that effects on AFC number appear to be highly-correlative with changes in overall host resistance, these findings are unexpected and require further study.

---

**Specific Aim 2**

*To determine the immunotoxic potential of known aquatic immunotoxicants and MRCs (alone and as complex mixtures) in Japanese medaka.*

A. **Polychlorinated Biphenyl (PCB)** - PCBs exist today as a major source of contamination in water sources worldwide. Due to its high lipid solubility, PCBs fail to be degraded and, therefore, continue to bioaccumulate throughout the environment and food chain. Exposure to PCB has been shown to depress both humoral and cell-mediated immune responses of mammals. However, despite the fact that PCBs are ubiquitous aquatic contaminants, little
information is available regarding the impact of PCBs on the immune system of residing fish species. To determine the impact of PCBs on the immune system of Japanese medaka, juvenile (4-mo-old) and aged (15-mo-old) fish were injected (IP) with corn oil (i.e., vehicle control) or 0.01 and 1.0 μg/g BW of the co-planar PCB congener 126. Effects upon humoral immunity (determined by changes in AFC numbers) and innate immune defenses (determined by effects upon intracellular O$_2^{-}$ production) were examined in both age groups of fish at 3 and 14 d post-PCB exposure.

While injection with either concentration of PCB 126 had no effect upon medaka survival or kidney cell numbers (compared to vehicle control), CYP1A1 (an enzyme marker of PCB exposure) was significantly induced in fish exposed to the highest PCB concentration 3 and 14 d post-injection (Figures 3 and 4). Exposure to PCB 126 also produced dramatic changes in AFC numbers and intracellular O$_2^{-}$ production; effects on O$_2^{-}$ production were dependent upon fish age at the time of PCB exposure. In general, O$_2^{-}$ production by unstimulated phagocytes from juvenile medaka was greater than that produced by aged fish 3 d post-exposure (Figure 5A). Treatment of juvenile fish with 0.01 μg PCB/g BW resulted in significantly greater amounts of O$_2^{-}$ production after 3 d than that produced by aged fish exposed to the same PCB dose; exposure of either age group to 1.0 μg PCB/g BW had no significant effects on basal level O$_2^{-}$ production (compared to age-matched vehicle-injected control). At 14 d post-exposure, phagocytes from aged control and PCB-treated medaka produced greater amounts of unstimulated O$_2^{-}$ than their juvenile counterparts (Figure 5B); production of O$_2^{-}$ by aged fish exposed to 0.01 μg PCB/g BW was significantly higher than that produced by similarly-exposed 4-mo-old fish. Phagocytes from 4-mo-old medaka exposed to the highest PCB concentration produced significantly greater quantities of unstimulated O$_2^{-}$ than that produced by their age-matched control or fish exposed to 0.01 μg PCB/g BW. Aged medaka exposed to 1.0 μg PCB/g BW also produced significantly greater amounts of unstimulated O$_2^{-}$ compared to the vehicle control.

At 3 d post-injection, 4-mo-old fish produced significantly-greater amounts of O$_2^{-}$ than aged fish (Figure 6A), while after 14 d, responses were reversed (Figure 6B). Exposure to both PCB concentrations significantly depressed PMA-stimulated O$_2^{-}$ production by juvenile medaka 3 d post-injection (compared to vehicle control); in contrast, juvenile fish exposed to 1.0 μg PCB/g BW and examined 14 d post-exposure produced significantly greater quantities of O$_2^{-}$ than their age-matched vehicle control. In general, juvenile fish appeared more sensitive to the effects of PCB 126 on stimulated O$_2^{-}$ production than older fish. For example, exposure of 15-mo-old fish to a PCB concentration as great as 1.0μg/g BW had no effect upon phagocyte-mediated O$_2^{-}$ production at either post-exposure timepoint.

Humoral immunity (as measured by AFC numbers) was significantly impacted in fish of both ages by PCB exposure. At 3 d post-exposure, AFC numbers in fish exposed to 1.0 μg PCB/g BW were significantly depressed (compared to vehicle-injected control); AFC response was 53 and 69%
of vehicle-injected controls for juvenile and aged fish, respectively (Figure 7A). In contrast, AFC numbers were unaffected in fish exposed to the lower PCB concentration and examined 3 d later. As post-exposure duration increased from 3 to 14 d, the impact of PCB 126 on AFC numbers became more divergent between the age groups (Figure 7B). While AFC numbers were reduced in both age groups exposed to the highest PCB dose, suppression at the lower PCB concentration was only observed in juvenile fish. Taken together, results demonstrate the sensitivity of the fish immune response for predicting PCB-induced immunotoxicity and identify age and post-exposure duration as important variables for determining adverse outcome. Moreover, it appears that enumeration of AFC numbers is a particularly sensitive immunoassay for predicting the immunosuppressive effects of this ubiquitous aquatic pollutant.

**B. Permethrin** - Permethrin, used as an insect repellent particularly during the gulf war, is a pharmacologically-active synthetic compound noted for its insecticidal properties. While effects of pyrethroids upon the nervous system have been well-studied, little information is available concerning effects of this class of compounds on the immune system. In studies previously-performed in this laboratory medaka were exposed to waterborne (alcohol-solubilized) permethrin for either 2 or 7 d at nominal concentrations between 0.01 and 15 ppb. Results from this study (i.e., effects upon host survival, lymphoid organ cellularity, plasma immunoglobulin levels, lymphocyte proliferation, $O_2^{-}$ production, host resistance, and melanomacrophage numbers/size) have been discussed in previously-submitted reports. Findings demonstrated the ability of short-term low-doses of permethrin to suppress overall host immunocompetence, particularly those immune functions important for maintaining host resistance against infectious agents. Since recent studies in this laboratory revealed the sensitivity of the AFC bioassay for demonstrating the immunotoxic effects of environmental chemicals, the same endpoint was incorporated into the suite of assays used to assess the immunotoxicity of permethrin. Results of these recently-performed studies demonstrated that exposure to 0.01 and 0.05 ppb increased AFC numbers (compared to the solvent-exposed control.) 50 and 24%, respectively, in contrast, exposure to the highest tested permethrin dose (i.e., 0.1 ppb) significantly-depressed AFC numbers by 76%; effects produced at 0.1 ppb correlates well with the previously-observed permethrin-induced decrease in host resistance.

**C. Nickel (Ni)** - To determine whether Ni, a known aquatic pollutant, mammalian immunotoxicant, and MRC altered host immunocompetence, Japanese medaka were exposed to waterborne Ni at 125 ppb for either 1, 7, or 14 d and effects upon phagocyte-mediated $O_2^{-}$ production and mitogen-stimulated T- and B-lymphocyte proliferation examined. Furthermore, to determine how a simultaneously-occurring stressor such as increased temperature (i.e., thermal
stress) might impact on Ni immunotoxicity, fish were also maintained at an elevated water temperature (i.e., 30°C) with and without Ni.

Although none of the treatments affected host survival or kidney cellularity, splenic cellularity was significantly decreased in fish exposed for 24 hr to either increased temperature or both stressors in combination. After 7 d of exposure, splenic cellularity in both treatment groups began to increase and by 14 d, cell numbers reached control levels. Results indicate that in combination with another commonly-found stressor, Ni, at a concentration below that which caused any effects by itself, can produce toxicity in exposed hosts. In regards to immune function, exposure to Ni for 14 d significantly reduced unstimulated extracellular O$_2^-\cdot$ production compared to the time-matched control and treatment-matched 1 d exposure group (Figure 8); unstimulated O$_2^-\cdot$ production by fish exposed to both stressors for 14 d or to Ni for 7 d also differed significantly from the treatment-matched 1 d exposure group. Like unstimulated O$_2^-\cdot$ production, PMA-stimulated extracellular production was also reduced in fish exposed to Ni for 14 d (compared to the time-matched control) (Figure 9). In addition, exposure to only thermal stress for 14 d also reduced PMA-stimulated O$_2^-\cdot$production.

Unlike the effects produced upon extracellular production, exposure to Ni alone had no effect upon unstimulated intracellular O$_2^-\cdot$ production (Figure 10). However, exposure to Ni in combination with thermal stress for either 1 or 7 d significantly increased intracellular O$_2^-\cdot$ production (compared to the time-matched control); similar effects were not observed in fish exposed for 14 d. Given that thermal stress alone also significantly increased basal levels of intracellular O$_2^-\cdot$, effects of combined exposure were likely due to the increased temperature rather than to Ni. Why a longer exposure to either stressor alone (or in combination) failed to produce effects similar to those seen after 1 or 7 d requires further study. As shown in Figure 11, thermal stress also altered PMA-stimulated intracellular O$_2^-\cdot$ production. However, in this case, O$_2^-\cdot$ production was depressed (rather than enhanced) and effects were only observed following a 14 d exposure. Taken together, findings show that assays which measure extracellular, but not intracellular, O$_2^-\cdot$ production are useful for assessing the effects of environmental Ni. Moreover, that thermal stress may, in fact, represent a greater risk to inhabiting species than Ni.

Innate immune responses appeared far more sensitive to the effects of Ni or thermal stress than cell-mediated immunity. As shown in Figure 12, exposure for up to 14 d to stressors alone or in combination failed to alter T-lymphocyte proliferative responses of exposed fish.

IV. KEY RESEARCH ACCOMPLISHMENTS
• Immune assays most successful for predicting chemical-induced immunotoxicity in medaka are being identified.
• The antibody-forming cell (AFC) bioassay is highly-sensitive to the immunosuppressive effects of BaP and correlates well with effects produced on overall host immunocompetence. This same assay has also proven to be a valuable endpoint for assessing the immunotoxic effects of DEX and coplanar PCBs. This model system may prove to be a valuable first tier for assessing immunotoxicity in mammalian species, including humans.

• Immune bioassays including AFC numbers and oxyradical production are sensitive indicators of the toxic effects of MRCs such as permethrin and Ni. Mixtures of two militarily-relevant stressors such as Ni and thermal stress appear to pose no greater threat than either stressor alone.

• Benzo(e)pyrene, shown to lack immunotoxicity in mammals, also fails to produce immunotoxic effects in medaka suggesting that B(a)P produces immunosuppression in fish by mechanisms similar to those described for mammals; antagonist studies performed in medaka further support this notion.

• Duration post-exposure and host age at the time of PCB exposure are important variables which need to be considered when assessing PCB-induced toxicity.

V. REPORTABLE OUTCOMES

A. Peer-Reviewed Journal Articles/Abstracts/Presentations:

1. Journal Articles


2. Abstracts


Duffy, J.E., Carlson, E., Li, Y., Prophete, C. and Zelikoff, J.T. (May 2000). Observed effects on fish immune function after exposure to a co-planar polychlorinated biphenyl (PCB) congener. Hudson-Delaware Regional Chapter of SETAC Annual Meeting


3. Presentations

Third International Meeting on Molecular Mechanisms of Metal Toxicity and Carcinogenicity - Immunodysfunction: An underlying Mechanism of Metal Toxicity in Aquatic Organisms. Sardinia, Italy. September 2001


B. Employment/Research Opportunities Supported by this Award -

Yun Li - Research Technician

Erik Carlson - Pre-doctoral candidate (completion January 2001)

Jessica Duffy - Masters student (completed September 2001).

Collete Prophete - Masters student (Pending)

VI. CONCLUSIONS

The aforementioned studies have been carried out so as to: assess how closely chemically-induced alterations in the immune responses of medaka resemble those produced in mammalian species; develop low-cost screening tools to assess health risks from environmental exposures to aquatic contaminants, MRCs, and chemical mixtures; and, to develop a single immunoassay or suite of assays in alternative species that can be used to evaluate MRCs and selected mixtures. Results of these investigations have provided information needed for interspecies extrapolation and for determining how useful assays which measure altered immune responses of fish are for predicting immunotoxicity in higher vertebrates (including humans). Moreover, immune assays most successful for predicting chemical-induced immunotoxicity in medaka are being identified.

The military is concerned with evaluating environmental effects and human health risks associated with militarily-generated/released material that is subject to remediation through installation-restoration operations. To develop these required military criteria research is needed to develop and validate new, quicker, more economical methods (i.e., biomarkers/bioindicators) for assessing the fate, effects, and toxicity of chemicals in air, water, and terrestrial ecosystems. Thus, these studies meet the research needs of the military by applying well-established immune assays to assess the toxicological impact of polluting chemicals.
The use of immune biomarkers to predict the biological effects of pollutant exposure also provides the opportunity to rapidly evaluate potential far-reaching health effects associated with pollutant exposure both to those directly-exposed aquatic species and, potentially, to humans. These types of biomarker studies offer distinct advantages over endpoints which simply assess the occurrence of toxic pollutants and fail to determine potential health risks. Thus, this study clearly meets the military's goals of evaluating environmental effects and human health risks (based on toxicological hazard) associated with militarily-generated/released materials.

VII. REFERENCES


Figure 1

BaP Exposure Increases Susceptibility to *Y. ruckeri* Infection

![Graph showing cumulative mortality](image)

Concentration of Benzo[a]pyrene (µg/g BW)

Mean (n = 3) ± SEM

* Significantly different from time-matched vehicle control (p<0.05)
Figure 2

Exposure to BeP has no Effect Upon PFC Response or CYP1A Expression / Activity

Mean (n = 3 - 5) ± SEM
* Significantly different from vehicle control (p < 0.05)
Figure 3
CYP1A Enzyme Induction 3 d Post-PCB Exposure

![Graph showing CYP1A enzyme induction 3 days post-PCB exposure.](image)

- Each bar represents the mean (n=2-4 experiments) ± SEM
- Significantly different (p < 0.05) from age-matched control
- Significantly different (p < 0.05) from 0.01 µg/g BW group

Figure 4
CYP1A Enzyme Induction 14 d Post-PCB Exposure

![Graph showing CYP1A enzyme induction 14 days post-PCB exposure.](image)

- Each bar represents the mean (n=2-4 experiments) ± SEM
- Significantly different (p < 0.05) from age-matched control
- Significantly different (p < 0.05) from 0.01 µg/g BW group
**Figure 5A**

Unstimulated $O_2^{-}$ Production 3 d Post-PCB Exposure$^a$

![Graph showing unstimulated $O_2^{-}$ production 3 days post-PCB exposure.](image)

- Each bar represents the mean ($n=3-4$ experiments) ± SEM
- Significantly different ($p < 0.05$) from 15-mo-old fish within a single exposure group

**Figure 5B**

Unstimulated $O_2^{-}$ Production 14 d Post-PCB Exposure$^a$

![Graph showing unstimulated $O_2^{-}$ production 14 days post-PCB exposure.](image)

- Each bar represents the mean ($n=3-4$ experiments) ± SEM
- Significantly different ($p < 0.05$) from 15-mo-old fish within a single exposure group
- Significantly different ($p < 0.05$) from age-matched control
- Significantly different ($p < 0.05$) from 0.01 μg/g BW group
Figure 6A
Stimulated O$_2^-$ Production 3 d Post-PCB Exposure$^a$

![Graph showing stimulated O$_2^-$ production 3 days post-PCB exposure for different PCB concentrations (0.01 and 1.0 µg/g BW) compared to controls. Each bar represents the mean (n=3-4 experiments) ± SEM.](image)

$^a$ Each bar represents the mean (n=3-4 experiments) ± SEM

$^b$ Significantly different (p < 0.05) from 15-mo-old fish within a single exposure group

$^c$ Significantly different (p < 0.05) from age-matched control

Figure 6B
Stimulated O$_2^-$ Production 14 d Post-PCB Exposure$^a$

![Graph showing stimulated O$_2^-$ production 14 days post-PCB exposure for different PCB concentrations (0.01 and 1.0 µg/g BW) compared to controls. Each bar represents the mean (n=3-4 experiments) ± SEM.](image)

$^a$ Each bar represents the mean (n=3-4 experiments) ± SEM

$^b$ Significantly different (p < 0.05) from 4-mo-old fish

$^c$ Significantly different (p < 0.05) from age-matched vehicle control

$^d$ Significantly different (p < 0.05) from 0.01µg/g BW group
Figure 7A

PFC Response 3 d Post-PCB Exposure

PCB (µg/ g BW)

\(^a\) Each bar represents the mean (n=3-4 experiments) +/- SEM
\(^b\) Significantly different (p < 0.05) from age-matched control
\(^c\) Significantly different (p < 0.05) from 0.01 µg/g BW group

Figure 7B

PFC Response 14 d Post-PCB Exposure

PCB (µg/ g BW)

\(^a\) Each bar represents the mean (n=3-4 experiments) +/- SEM
\(^b\) Significantly different (p < 0.05) from age-matched control
\(^c\) Significantly different (p < 0.05) from 0.01 µg/g BW group

23
Figure 8
Exposure to Nickel for 14 d Significantly Decreased Unstimulated Extracellular O$_2^-$ Production by Kidney Phagocytes$^a$

$^a$Mean (n =4 experiments) ± SEM
* Significantly different from time-matched control (p < 0.05)
‖Significantly different from same exposure group at 1 d (p<0.05)
Exposure to Nickel and Thermal Stress for 14 d
Depressed PMA-Stimulated Extracellular $O_2^{•-}$
Production by Kidney Phagocytes

Figure 9

![Graph showing the effect of exposure to Nickel and thermal stress on PMA-stimulated extracellular $O_2^{•-}$ production by kidney phagocytes.]

- **Mean (n = 4 experiments) ± SEM**
- * Significantly different from time-matched control ($p < 0.05$)
- ‡Significantly different from the control at 1 d ($p < 0.05$)
Figure 10
Thermal Stress and Stressors in Combination
Increased Unstimulated Intracellular O$_2^-$
Production by Kidney Phagocytes

![Graph showing the effect of thermal stress and stressors on intracellular O$_2^-$ production by kidney phagocytes.](image)

*Mean (n=4 experiments) ± SEM
* Significantly different from time-matched control (p < 0.05)
¶¶Significantly different from same exposure group at 1 d (p<0.05)
Figure 11

Exposure to Thermal Stress for 14 d Significantly Decreased Intracellular O$_2^-$ Production of PMA-stimulated Kidney Cells$^a$.

![Graph showing intracellular O$_2^-$ production over time with different treatments and statistical annotations.]

$^a$ Mean (n = 4 experiments) ± SEM
* Significantly different from time-matched control (p < 0.05)
¶ Significantly different from same exposure group at 1 and 14 d (p < 0.05)
# Significantly different from same exposure group at 1 d (p < 0.05)
Figure 12

Lymphoproliferation by ConA-Stimulated Splenocytes was not Significantly Affected by Exposure to Either Stressor$^a$

$^a$Mean (n = 4 experiments) ± SEM