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Effects of Concurrent Phenathrene and Mycobacterium marinum Exposure in the Zebrafish (*Danio rerio*)

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Effects of Concurrent Phenanthrene and *Mycobacterium marinum*
Exposure in the Zebrafish (*Danio rerio*)

A Dissertation
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia
In Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

by
Christopher M. Prosser
2011

ABSTRACT

As a way to gain better understanding of how aquatic organisms respond within their natural environment, this dissertation set up a series of controlled laboratory experiments to investigate the effects of chemical and biological stressors on the zebrafish (*Danio rerio*). I performed a series of studies in which animals were simultaneously exposed to the toxicant phenanthrene, and infected with the bacterium *Mycobacterium marinum*. Unexpectedly, we found that at a high dose of phenanthrene ($284 \mu\text{g L}^{-1}$) results in an antagonistic relationship between stressors with dually challenged fish having significantly higher survivorship than those exposed solely to phenanthrene. Dually challenged fish also had reduced phenanthrene metabolism, suggesting higher mortality in phenanthrene only exposed fish may be due to increased toxicity from phenanthrene metabolites. We postulated metabolic reduction was due to inflammatory cytokines suppressing metabolism. However, in latter experiments using a more environmentally relevant dose of phenanthrene ($157 \mu\text{g L}^{-1}$), dually challenged fish had significantly higher mortalities than all other treatments, and there was no disruption in phenanthrene metabolism. Our second study clearly showed an additive effect: the summation of mortalities for each stressor individually is approximate to what is observed in dually challenged fish. My third experiment was designed to better elucidate the effects of dose and exposure order on stressor interaction. I used a matrix of dose and order such that two phenanthrene doses were used ($157 \mu\text{g L}^{-1}$ and $86 \mu\text{g L}^{-1}$), with a staggered order of exposure. Significantly higher mortalities in dually challenged fish for the high dose of phenanthrene versus the low dose, regardless of exposure order, demonstrated phenanthrene concentration, not exposure order is a significant factor. This study also showed activation of the cytochrome P450 pathway by phenanthrene. These studies highlight the exceptionally complex interactions between multiple stressors and how minor alterations in experimental design can produce dramatic changes in stressor interaction. I have concluded that toxicant dose plays a significant role in this interaction causing an antagonistic interaction at high concentrations; however, at lower doses an additive effect is seen. Additionally, I have shown the importance of phenanthrene metabolites in toxicity. Finally I have clearly demonstrated that phenanthrene can induce the cytochrome P450 pathway.

In addition to dual-stressor experiments, I conducted a comparative study between two *Mycobacterium* spp.: *M. marinum* and *M. pseudoshottsii*. The goal of this study was to find a bacterium to model disease recrudescence. Despite the close relationship between these two species, we observed dramatically different virulence and pathology. *M. marinum* infected fish had <10% survivorship over a 4-week exposure; however *M. pseudoshottsii* infected fish had ~98% survivorship. Additionally, *M. marinum* infected fish displayed a classic granulomatous inflammation with bacilli sequestered within, or in immediate proximity to, well formed granulomas. In contrast, *M. pseudoshottsii* infected fish displayed little granuloma formation, instead having large area of diffuse inflammation and cellular necrosis. *M. pseudoshottsii* were seen disseminated both extra and intra-cellularly throughout areas of inflammation, a phenomenon not seen with *M. marinum*. I postulate that these differences are due to a unique mycolactone toxin secreted by *M. pseudoshottsii*. *M. ulcerans* is the only other *Mycobacterium* spp. to produce a mycolactone toxin, and produces pathologies similar to what we observe here for *M. pseudoshottsii*.

APPROVAL SHEET

This dissertation is submitted in partial fulfillment of

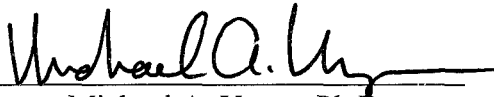
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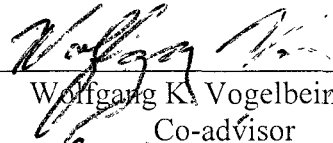


Christopher M. Prosser

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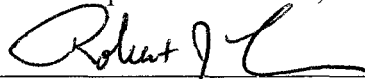
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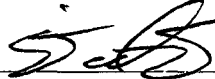
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Dedication

I wish to dedicate this work to my parents for their collective support but especially my father. Without his years of advice and guidance I would not be where I am today.

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ABSTRACT

As a way to gain better understanding of how aquatic organisms respond within their natural environment, this dissertation set up a series of controlled laboratory experiments to investigate the effects of chemical and biological stressors on the zebrafish (*Danio rerio*). I performed a series of studies in which animals were simultaneously exposed to the toxicant phenanthrene, and infected with the bacterium *Mycobacterium marinum*. Unexpectedly, I found that at a high dose of phenanthrene (mean daily concentration: $284 \mu\text{g L}^{-1}$) results in an antagonistic relationship between stressors with dually challenged fish having significantly higher survivorship than those exposed solely to phenanthrene. Dually challenged fish also had reduced phenanthrene metabolism, suggesting higher mortality in phenanthrene only exposed fish may be due to increased toxicity from phenanthrene metabolites. I postulated metabolic reduction was due to inflammatory cytokines suppressing metabolism. However, in latter experiments using a more environmentally relevant dose of phenanthrene (Mean daily concentration: $157 \mu\text{g L}^{-1}$), dually challenged fish had significantly higher mortalities than all other treatments, and there was no disruption in phenanthrene metabolism. Our second study clearly showed an additive effect: the summation of mortalities for each stressor individually is approximate to what is observed in dually challenged fish. My third experiment was designed to better elucidate the effects of dose and exposure order on stressor interaction. I used a matrix of dose and order such that two phenanthrene doses were used (mean daily concentrations: $157 \mu\text{g L}^{-1}$ and $86 \mu\text{g L}^{-1}$), with a staggered order of exposure. Significantly higher mortalities in dually challenged fish for the high dose of phenanthrene versus the low dose, regardless of exposure order, demonstrated

phenanthrene concentration, not exposure order is a significant factor. This study also showed activation of the cytochrome P450 pathway by phenanthrene. These studies highlight the exceptionally complex interactions between multiple stressors and how minor alterations in experimental design can produce dramatic changes in stressor interaction. I have concluded that toxicant dose plays a significant role in this interaction causing an antagonistic interaction at high concentrations; however, at lower doses an additive effect is seen. Additionally, I have shown the importance of phenanthrene metabolites in toxicity. Finally I have clearly demonstrated that phenanthrene can induce the cytochrome P450 pathway.

In addition to dual-stressor experiments, I conducted a comparative study between two *Mycobacterium* spp.: *M. marinum* and *M. pseudoshottsii*. The goal of this study was to find a bacterium to model disease recrudescence. Despite the close relationship between these two species, we observed dramatically different virulence and pathology. *M. marinum* infected fish had <10% survivorship over a 4-week exposure; however *M. pseudoshottsii* infected fish had ~98% survivorship. Additionally, *M. marinum* infected fish displayed a classic granulomatous inflammation with bacilli sequestered within, or in immediate proximity to, well formed granulomas. In contrast, *M. pseudoshottsii* infected fish displayed little granuloma formation, instead having large area of diffuse inflammation and cellular necrosis. *M. pseudoshottsii* were seen disseminated both extra and intra-cellularly throughout areas of inflammation, a phenomenon not seen with *M. marinum*. I postulate that these differences are due to a unique mycolactone toxin secreted by *M. pseudoshottsii*. A mycolactone toxin secreted by *M. ulcerans* causes immuno-suppression and cell necrosis. It is therefore tempting to postulate that the

pathologies seen with *M. pseudoshottsii* (i.e. lack of an organized immune response) are also due to secretion of a mycolactone toxin.

Chapter 1

Introduction

Effects of Multiple Stressors on Aquatic Organisms

The interactions between multiple stressors within host organisms are crucial to better understanding complex environmental connections and population level effects. Stressors are either the extrinsic or intrinsic stimuli (chemical, biological or physical) that threaten homeostasis within an organism (Chrousos and Gold 1992). However, traditional laboratory based exposure studies often focus on single exposures with other parameters (temperature, water quality, pH etc.) kept within optimal ranges (Holmstrup et al. 2010). Natural settings however frequently subject populations to sub-optimal conditions. Thus these studies fail to realize the potential for additive, synergistic or antagonistic effects associated with multiple stressors and therefore, may not accurately estimate the “true” risk to a species or population.

Although still nascent, multiple stressor studies represent an increasingly important field of study within environmental science. A large number of multiple-stressor studies have been field oriented and focused on changes in the population-level as a function of growth, reproduction or mortality (Power 1997). However, the inherent variability within the environmental framework makes establishing causation extremely difficult (Adams et al. 2005). Further complicating quantification of multi-stressor interaction is how a stressor may impact multiple physiological and biochemical pathways simultaneously (Schulte 2007). This underscores the importance for robust laboratory studies investigating the effects of multiple stressors on a species and

understanding the mechanisms involved over both spatial and temporal frameworks (Adams et al. 2005).

Among the potential stressors affecting aquatic organisms are a multitude of hazardous chemicals. Pollutants in the environment are not maintained at a constant level, but vary as a function of anthropogenic influences (point/nonpoint sources) and environmental factors (e.g. wind, tides, river flow, season). These factors can influence both the concentration and duration of an organism's exposure. Chemical stressors can induce a suite of biological effects including alterations in enzyme function, growth, reproduction, immune status and survival (Smolders et al., 2005; Loge et al., 2005; Jacobson et al., 2003; Lawrence and Hemingway 2003; Anderson et al., 1998; Anderson et al., 1996; Sniezko 1973). The timescale for these effects may be immediate in the case of acute toxicity or greatly extended in the event of low level chronic exposures (DiGiulio and Hinton 2008; Lawrence and Hemingway 2003). Although immediate effects on growth and survival may be easily observed, subtle impacts of chronic stress such as reduced fecundity, fitness or immunity may be cryptic and not become evident for years.

A major impact of many chemical stressors in aquatic organisms is now widely accepted to be immunosuppression (DiGiulio and Hinton 2008; Reynaud and Deschaux 2006; Carlson et al., 2004; Bols et al., 2001; Arkoosh et al., 1998; Anderson et al., 1998; Collier et al., 1998; Anderson et al., 1996; Chu et al., 1994; Crawshaw 1979). Decreased resistance to pathogens has been reported in several teleost species following chemical exposure (Arkoosh et al., 2001; Carlson et al., 2002). Despite extensive studies on the topic, the exact role that chemical contaminants play in modulating expression and

outcome of concurrent or subsequent infectious disease in aquatic animals is still not well understood. The complex interplay between a chemical's direct toxic effects in target tissues, its ability to initiate an integrated stress response and its potential to modulate immunity during an infectious disease process require additional investigation.

Although modulation of immune function is a widely accepted sequel to toxicant exposure, modulation of toxicant metabolism, as a function of the innate inflammatory response, has also been widely reported. Samaras and Dietz (1953) first demonstrated that up-regulated immune function could influence drug metabolism by down regulating the cytochrome P450 (CYP) pathway (Reviewed in Renton 2001). CYP is a super family of structurally similar heme proteins that represent one of the main mechanisms whereby vertebrates metabolize lipophilic toxicants. Modulation of this pathway will alter the bioaccumulation and depuration capabilities of an organism. Although in select instances this has been suggested to prevent chemical activation into more hazardous, or carcinogenic compounds (Reynaud et al. 2008), the general perception is that modulating the biotransformational pathway will incur detrimental effects on organismal health (Wessel et al. 2010; Varanasi et al. 1986).

Acute phase inflammation in vertebrates, including fishes, is a vascular and cellular response to injury and infection mediated by a suite of proinflammatory cytokines, including interleukins 1 and 6 (IL1, IL6) and tumor necrosis factor alpha (TNF α) (Bayne and Gerwick 2001). Several immune system mediated mechanisms of CYP down regulation have been proposed and IL1 and TNF α are considered to be intimately involved. Nicholson and Renton (1999) demonstrated that CYP activity was depressed in rat astrocytes when these cells were exposed to lipopolysaccharide, a highly

toxic cell wall component of gram negative bacterial pathogens that stimulates production of IL1 and TNF α *in vitro*. Similarly, carp, *Cyprinus carpio*, exposed to 3-methylcholanthrene, a known CYP inducer, showed a marked increase in the phase II enzyme, glutathione S transferase (GST); however, subsequent injection of IL1 and TNF α significantly reduced GST activity (Reynaud et al. 2005). Marionnet et al. (2006) hypothesized that macrophages, activated by dextran sulfate or LPS, are capable of down regulating some CYP isoforms through reactive oxygen species generation. They suggested that because IL1 and TNF α are potent macrophage activators, they may therefore also act indirectly in the modulation of the cytochrome P450 pathway.

The overall goal of my dissertation research is to develop a piscine model for controlled laboratory studies investigating the adverse health impacts and interactive effects of dual-stressor exposure. Specifically, my aim is to develop a better understanding of the specific host response mechanisms during simultaneous exposure to phenanthrene, a polycyclic aromatic hydrocarbon (PAH) of environmental concern, and the bacterial pathogen, *Mycobacterium marinum*. *Mycobacterium marinum* is one of the most commonly reported mycobacterial pathogens of fishes worldwide (Hedrick et al., 1987; Kent et al., 2004; Watral and Kent 2007; Gauthier and Rhodes 2008). The zebrafish was selected as our model species because it is widely accepted by the National Institutes of Health as a model for human disease (Henken et al. 2004) and is rapidly becoming a toxicological model (Spitsbergen and Kent, 2003). The small size of the zebrafish allows for robust statistical analysis and ease of maintenance. In addition, it is a natural host for *M. marinum* (Watral and Kent, 2007).

Preliminary Objectives:

To my knowledge, this was the first study designed to investigate potential interactions (e.g., synergy, antagonism, additivity, etc) between a chemical stressor and a chronic infectious disease. The null hypothesis (H_0) was that no interaction between stressors will be evident. The alternate hypothesis (H_a) was: a significant interaction between stressors will be evident. These hypotheses were tested by examining survivorship, cellular pathology (basic histology), toxicant body burden, and cytochrome P450 induction. The results from the first combined exposure study were then used to guide subsequent experiments. The high degree of uncertainty and potential for changes in direction as the investigation progressed are illustrated in Figure 1.

Preliminary Experiments:

Zebrafish Colony

I have established a viable zebrafish colony at VIMS that provides experimental animals for the proposed experiments. This colony is housed in two Aquatic Habitats[®] (Apopka, FL) recirculation systems, and consists of two racks measuring 5ft x 6ft x 1ft. Each rack holds 60 3L tanks capable of housing 30 fish per tank, with a total capacity for 3600 broodstock. Five wild type breeding pairs were obtained from the Zebrafish International Resource Center (ZIRC; Eugene, OR), with additional adults purchased from a commercial supplier. When stocked with 2500 adults the system is capable of producing approximately 4 million embryos per month.

Mycobacterium marinum range finding experiment

The objective of this study was to find a dose of bacteria inducing 50% mortality in approximately 14 days. Mortality range finding experiments were conducted in six-10 gallon glass aquaria using dechlorinated tap water for 15 days. Because this was a preliminary study, no replicate treatments were used. Each tank held 25 fish, all of which received the same bacterial dose by intracoelomic (IC) injection. Water quality parameters were monitored daily to ensure unintended stress was not placed on the animals. These included DO (Oxyguard handy polaris), ammonia (Salifort box kit), pH (Orion 250A with Thermo pH electrode). Temperature was kept at 28°C and water quality maintained using box filters. Water quality parameters followed those previously established (Lawrence 2007). Fish were fed once daily, with excess food siphoned out as needed. All tanks were examined at 12 hour intervals with dead animals being removed.

The M30 strain of *M. marinum* originally isolated from Chesapeake Bay striped bass was used in this study. This strain has been previously shown to induce mortality in zebrafish (Ostland et al. 2008). The IC injection had a total volume of 30µl fish⁻¹. The range finding study consisted of 5 bacterial doses at 25 fish per dose. Based on previous research (Ostland et al 2008), a reasonable high dose for injection was estimated to be 1x10⁶ bacteria fish⁻¹. Each subsequent dose was one order of magnitude lower. Twenty five fish given IC injection of phosphate buffer with Polysorbate 20 (Tween 20) solution served as the control. At the end of the study all remaining animals were euthanized with an overdose of MS-222 (400mg/L) and placed into Z-fix preservative for histological examination. All animal care and euthanasia protocols were approved by the Institutional Animal Care and Use Committee (IACUC) for the College of William and Mary.

Phenanthrene Toxicity Range Finding

The purpose of these experiments was to establish a range of phenanthrene concentrations that resulted in both chronic and acute toxicity to zebrafish. The objective was to establish the acute dose that would provide an LC₅₀ of less than two weeks and a more chronic dose to resemble an environmentally relevant concentration that would result in no mortalities after two weeks. Three target concentrations were initially chosen to be 750µg L⁻¹ (“High”), 500µg L⁻¹ (“Med”) and 250µg L⁻¹ (“Low”).

Animals were held in static 5-gallon (10 per tank) aquaria containing 8-liters dechlorinated tap water and maintained at 28°C with aeration, as described by Watral and Kent (2006). Similar to the *M. marinum* range finding study, no replicates were used because of the preliminary nature of the experiment. Water changes were performed once daily to add fresh exposure water. Feeding occurred once daily, just prior to the water change. The following water quality parameters were monitored daily to ensure unintended stress was not placed on the animals. These include DO (Oxyguard handy polaris), Ammonia (Salifort box kit), pH (Orion 250A with Thermo pH electrode). All water quality parameters were maintained within the normal physiological range for zebrafish (Lawrence 2007)

Phenanthrene solutions were prepared in 4L solvent bottles by spiking 4mL of phenanthrene standard (in an acetone carrier) into each jug. The control group fish received an equivalent amount of acetone spike into the water. At 12 hours post phenanthrene spike, a 60% loss of phenanthrene from the system was assumed based off previous studies (Unger et al. 2007), and an additional phenanthrene spike in 5 mL acetone was added to each tank. Spike concentrations were 900µg L⁻¹, 600µg L⁻¹, and 300µg L⁻¹, for the “High”, “Med” and “Low” treatments respectively.

Water was analyzed for phenanthrene using high performance liquid chromatography (HPLC) with a fluorescence detector (SpectraSYSTEM® P400 Controller, SpectraSYSTEM® AS3000 Autosampler [San Jose, CA, USA], Waters 474 Fluorescence Detector [Milford, MA, USA] and Restek® Allure C18 column [Bellefonte, PA, USA]) by methods used previously (Unger et al. 2007). In brief: a solvent gradient of water/acetonitrile was used with the gradient shifting from 100% water to 80% acetonitrile and back to water over a time of 35 minutes and a flow rate of 1 mL min⁻¹. The fluorescence detector was set to an excitation wavelength of 265nm and an emission wavelength of 370nm. Data were collected using ChemStation (Hewlett Packard Santa Clara, Ca, USA) software. Calibration of the machine was done prior to analysis of experimental samples. Calibration used a 9 point calibration curve and an internal standard of 1-methylnaphthalene (1-MN) (0.40µg mL⁻¹).

Range Finding Results:

Mycobacterium marinum range finding study

Infected fish showed a dose dependent mortality response. Those infected with the highest dose displayed mortality starting at day five with 71% mortality by day 14 (Figure 2). In contrast, the low dose (1.5x10² bacteria/fish) did not exhibit any mortality until day 12, with only 33% mortality by day 14. Fish that were injected with 1.5x10³ bacteria/fish showed 54% mortality at day 14. This dose approximates the desired criteria of an LC₅₀ of two weeks so no formal statistics were performed to generate an LC₅₀.

Histological analysis was performed to qualitatively assess the location and degree of granulomatous inflammation. Granulomas were observed in all infected fish at the end of the study. Granuloma organization ranged from a loose association of inflammatory cells with no defined organization, to highly structured granulomas with epithelioid cells surrounding a highly eosinophilic necrotic core. Number of granulomas present was directly proportional to the dose. Histological examination for the lowest two treatments showed granulomas primarily located in pancreatic and hepatic tissue. Fish infected with large amounts of *M. marinum* also displayed granulomas in the kidney, muscle tissue, cardiac tissue and nervous tissue.

Phenanthrene Toxicity Range Finding Study

HPLC data showed average aqueous phenanthrene for each group was below predicted values, with mean concentrations (\pm Standard Deviation) for the three exposure groups at $360\mu\text{g L}^{-1}$ ($183\mu\text{g L}^{-1}$), $215\mu\text{g L}^{-1}$ ($112\mu\text{g L}^{-1}$), and $117\mu\text{g L}^{-1}$ ($50\mu\text{g L}^{-1}$) (Figure 3). Mortality data showed a dose dependent response with deaths reaching 63.4% for the highest concentration at day 18 of the study. This is contrasted by 20% mortality and 10% mortality for the two lower concentrations, respectively (Figure 4). Based on these values, Probit analysis was used to calculate a 14-day LC_{50} of $293\mu\text{g L}^{-1}$ (95% confidence interval $216\mu\text{g L}^{-1}$ - $366\mu\text{g L}^{-1}$). Fish in all exposure tanks showed behavioral signs consistent with a narcotic mode of action including lethargy, disorientation, hyperventilation, and reduced feeding. The severity of observed narcotic effects occurred in a dose dependent manner.

Future Dual-stressor Experiments:

Using the data from the range finding studies, I designed a set of experiments with the goal of gaining a better understanding of the interactions between toxicant and bacterial pathogen in the zebrafish.

Experiment 1: Dual-stressor Interactions in the Zebrafish (*Danio rerio*): Concurrent Phenanthrene Exposure and *Mycobacterium marinum* Infection

The goal of my initial study was to elucidate the interactions between stressors in the host organism. Fish were exposed to either a high or low dose of phenanthrene, infected with *M. marinum* or received a combination exposure of toxicant and bacteria. As previously mentioned, the potential results from this study were varied, and so this study was designed specifically to investigate a broad range of potential outcomes and ultimately help design future experiments to more closely examine specific aspects. For that reason fish were sampled for survivorship, body burden analysis, and classic histology. Additional tissue samples were obtained for use in a qPCR assay specific for *M. marinum*. Unfortunately, the qPCR assay never came to fruition, and so no data were obtained. Fish sampled for histology were also retroactively used for immunohistochemical analysis.

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Experiment 2: Phenanthrene and *Mycobacterium marinum* exposure in the zebrafish (*Danio rerio*): additive mortality from dual stressors

Through conversations with Dr. Charles Rice (Clemson University) the unexpected results from experiment 1 were put into the context of exposure order, with

the idea that the biotransformational and inflammatory immune pathways interact. The hypothesis being that the first pathway activated is able to significantly disrupt the other. The objective of this study was then to examine the effects on organismal health when stressor order was reversed. Additionally, I opted for a longer study duration to fully allow the chronic nature of each stressor to manifest. Because of this, phenanthrene concentration had to be reduced to avoid potential starvation in toxicant exposed fish. Fish were exposed to 250 $\mu\text{g L}^{-1}$ phenanthrene, infected with *M. marinum* or received a combination exposure for 21 days. Results were again evaluated using survivorship analysis, toxicant body burden and classic histology. In addition, based on the immunohistochemical analysis from the first study, CYP1A quantification via western blotting was also performed. Plasma glucose analysis was also added as a surrogate for measuring the integrated stress response.

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Prosser, C., M.A. Unger and W. Vogelbein. Concurrent phenanthrene and *Mycobacterium marinum* exposure in the zebrafish (*Danio rerio*): Additive mortality from dual stressors. Submitted for review in *Environmental Chemistry and Toxicology*

Experiment 3: Comparative pathogenicity of *Mycobacterium pseudoshottsii* and *Mycobacterium marinum* in the zebrafish (*Danio rerio*)

As our initial two experiments were conducted I became interested in the long term affects of a chronic infection and toxicant stress, specifically if phenanthrene exposure could ultimately lead to disease recrudescence in *M. marinum* infected zebrafish. The ultimate goal was to use this as a model for tuberculosis. However, the M30 strain that had been used in our previous work was too pathogenic, and a chronic

infection could not be established. Alternatively, I chose to investigate whether the closely related *M. pseudoshottsii* would induce the chronic granulomatous infection needed. Therefore, the goal of this study was to examine comparative pathogenicity between *M. pseudoshottsii*, and the better characterized *M. marinum* in the zebrafish (*Danio rerio*) model. *M. marinum* and *M. pseudoshottsii* are closely related sharing >99% of the 16S rRNA gene sequence. One strain of *M. pseudoshottsii* (L15) and two strains of *M. marinum* (M30 and ATCC BAA-535) were used. Fish were given 18 / μ L (~1500 bacteria/fish) IC injection of bacterial inoculum or sham injection of phosphate buffer. Fish were monitored daily for survivorship and at 28 days were sacrificed for histological examination.

Experiment 4: Concurrent phenanthrene exposure and *M. marinum* infection induces a dose-dependent, additive effect in the zebrafish *Danio rerio*

Several factors, specifically the contradictory results from the first two experiments (antagonism vs. additivity in dually challenged fish), lead to a final experiment. Because of differences in toxicant dose and study duration, the first two dual-stressorexperiments were not directly comparable, requiring an additional study. The goal of this study was to definitively determine how toxicant dose and/or order of exposure impact organismal health. We exposed zebrafish to one of two concentrations of phenanthrene (250 μ g L⁻¹ or 150 μ g L⁻¹) plus *M. marinum*. However, treatments were ordered so that half received toxicant first followed 48 hours later by bacterial infection, and the other half received bacterial injection first followed by phenanthrene exposure 48 hours later.

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Figure 1: Flow diagram illustrating possible avenues of experimentation based upon possible results from scoping and full length exposure studies.

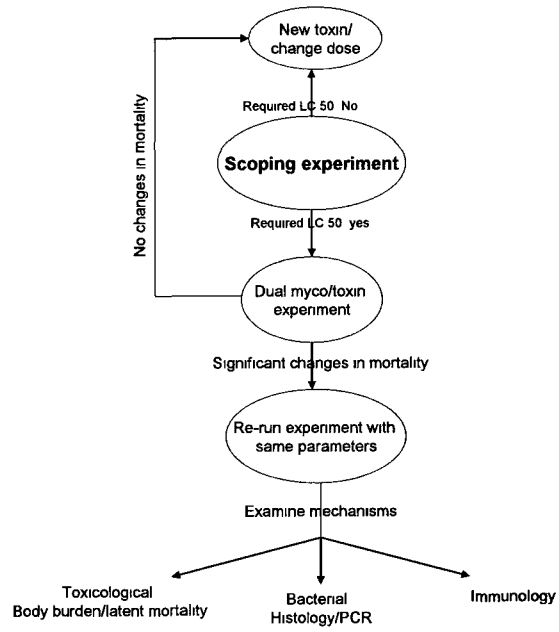


Figure 2: Range finding study showing cumulative mortality in *D. rerio* challenged by IC injection with *M. marinum* (Strain M30) over five doses. The goal of this study was to establish an LC₅₀ of approximately 14 days.

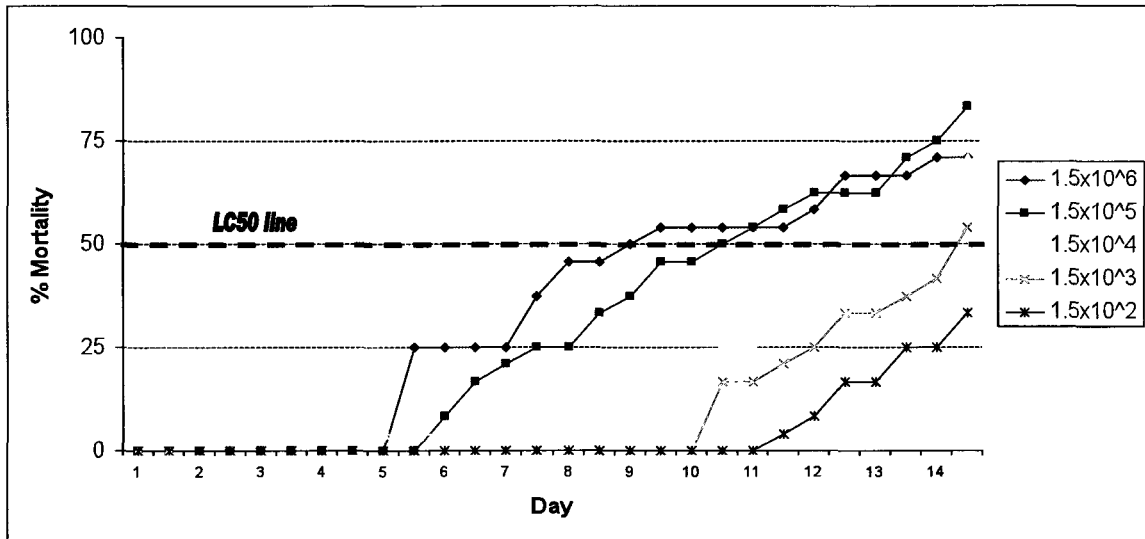


Figure 3: Daily fluctuations in phenanthrene water concentrations ($\mu\text{g L}^{-1}$) over the duration of phenanthrene range-finding experiment. Complete water changes were performed every 24 hours with additional phenanthrene spikes in acetone at time 12 hours of each day. Nominal Concentrations: High: $750 \mu\text{g L}^{-1}$; Med: $500 \mu\text{g L}^{-1}$; Low: $250 \mu\text{g L}^{-1}$

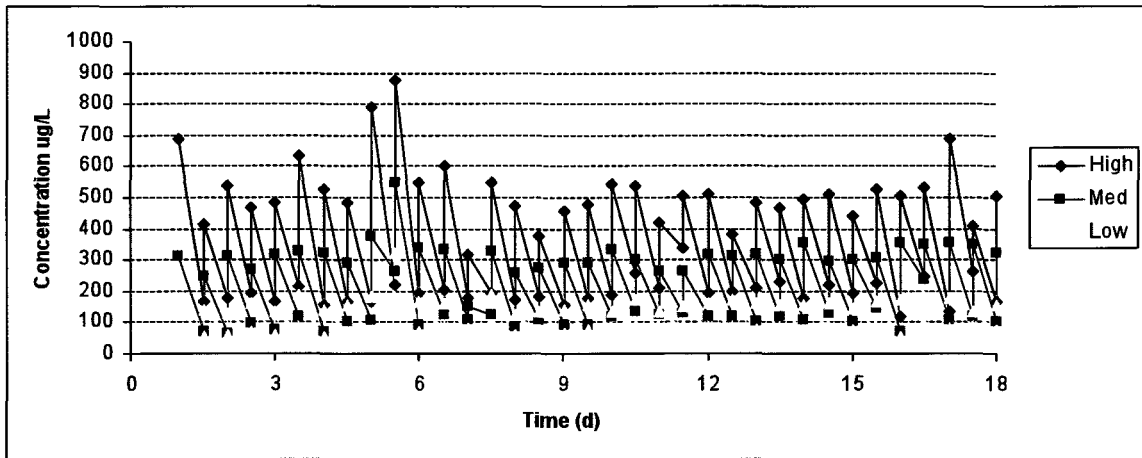
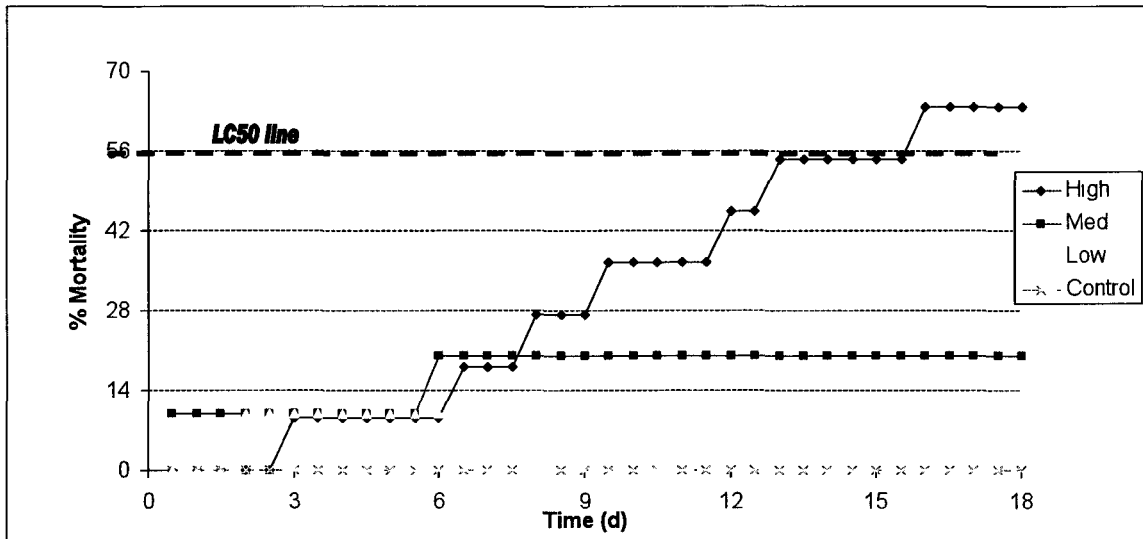


Figure 4: Results from a range finding experiment showing percent mortalities of *D. rerio* exposed to three different concentrations of phenanthrene over an 18 day period. High: 750 $\mu\text{g L}^{-1}$; Med:500 $\mu\text{g L}^{-1}$; Low:250 $\mu\text{g L}^{-1}$.



CHAPTER 2

Dual-stressor Interactions in the Zebrafish (*Danio rerio*): Concurrent Phenanthrene Exposure and *Mycobacterium marinum* Infection

Introduction

Infectious disease is a significant force that threatens biodiversity by causing population declines and extinctions in both aquatic and terrestrial systems (Harvell et al. 2002). Over the past several decades marine disease outbreaks have been increasing in both frequency and severity. Infectious diseases of corals in most tropical seas (Harvell et al. 1999), pilchard in Australia and New Zealand (Whittington et al. 1997), Caspian seals in the Caspian Sea (Kennedy et al. 2000), sea urchins in Panama (Lessios 1988), and abalone in California (Lafferty and Kuris 1993) are just a few examples of emerging disease issues that highlight the global scale of this problem and the diversity of aquatic animals affected. Losses from infectious disease outbreaks can alter entire animal communities with the affected populations never fully recovering to prior levels (Rosenberg and Ben Haim, 2002). Emerging aquatic animal disease outbreaks have recently been linked to anthropogenic influences such as climate change, chemical exposure, and the global transport of infectious agents (Dasak et al. 2001; Rosenberg and Ben Haim, 2002; Harvell et al. 2004; Lafferty et al. 2004). As many bacterial agents are considered to be opportunistic pathogens that cause disease in aquatic organisms only in association with stress (Arkoosh et al. 1998; Gauthier and Rhodes, 2009), a greater understanding of the interactions between stress and disease is needed.

Decreased resistance to infectious disease agents has been reported in aquatic organisms, and immunosuppression is now widely perceived to be a consequence of

contaminant exposure. Arkoosh et al. (1998) found that Chinook salmon (*Oncorhynchus tshawytscha*) inhabiting a polluted estuary in Puget Sound, Washington displayed increased mortality (e.g., lower disease resistance) following challenge by *Vibrio anguillarum*, than salmon inhabiting nonpolluted estuaries. Carlson et al. (2002) reported decreased resistance to the bacterial pathogen *Yersinia ruckeri* in Japanese medaka (*Oryzias latipes*) exposed by IC injection to the polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene. Laboratory studies using Chinook salmon have also shown a decrease in the number of plaque forming cells, a measure of B cell function, when exposed to the PCB mixture Aroclor 1254 (Jacobson et al. 2003). However, reviews of studies examining the mechanisms of toxicant induced immunosuppression have not fully elucidated the implications for overall fish health (Bols et al. 2001; Reynaud et al. 2006).

Aquatic organisms are constantly exposed to a wide variety of foreign compounds. The specific immune response in vertebrates is composed of a vast repertoire of receptors capable of specifically detecting foreign antigens and eliciting a complex, chemically mediated response designed to neutralize them. In parallel, organisms have evolved mechanisms to detect and metabolize exogenous and endogenous chemicals. It is not surprising then that these two systems are closely related and able to interact. Samaras and Dietz (1953) first demonstrated that heightened immune function could influence drug metabolism by suppressing the cytochrome P450 (CYP) pathway (Reviewed in Renton, 2001). CYP is a super family of structurally similar heme proteins that represent one of the main mechanisms whereby vertebrates metabolize lipophilic toxicants. CYP isoforms catalyze a phase I oxidative response in which compounds are made more water soluble, and thus more easily excreted.

Additionally, these enzymes aid in phase II reactions by conjugating oxygenated metabolites to smaller molecules, such as glutathione or sugars, thereby even further increasing their water solubility (Lawrence et al. 2003). Perturbations in this enzyme cascade will interfere with the ability of an organism to adequately metabolize and excrete toxicants, leading to their increased bioaccumulation and subsequent impacts on organism health (Wessel et al. 2010; Varanasi et al. 1986).

Acute phase inflammation in vertebrates, including fishes, is a vascular and cellular response to injury and infection mediated by a suite of proinflammatory cytokines, including interleukins 1 and 6 (IL1, IL6) and tumor necrosis factor alpha (TNF α) (Bayne and Gerwick, 2001). Several immune system mediated mechanisms of CYP down regulation have been proposed and IL1 and TNF α are considered to be intimately involved. Nicholson and Renton (1999) demonstrated that CYP activity was depressed in rat astrocytes when cells were exposed to lipopolysaccharide, a highly toxic cell wall component of gram negative bacterial pathogens that stimulates production of IL1 and TNF α in vitro. Similarly, carp, *Cyprinus carpio*, exposed to 3methylcholanthrene, a known CYP inducer, showed a marked increase in the phase II enzyme, glutathione S transferase (GST); however, subsequent injection of IL1 and TNF α significantly reduced GST activity (Reynaud et al. 2005). Marionnet et al. (2006) hypothesized that macrophages, activated by dextran sulfate or LPS, can down regulate some CYP isoforms through generation of reactive oxygen species. They suggested that because IL1 and TNF α are potent macrophage activators, they may therefore also act indirectly in the modulation of the cytochrome P450 pathway.

The overall aim of this study was to gain a better understanding of the interactive effects between a chronic infectious disease process characterized by immune mediated granulomatous inflammation and a chemical toxicant in an aquatic vertebrate model. I therefore conducted a combined challenge study of zebrafish (*Danio rerio*) using the bacterial pathogen, *Mycobacterium marinum*, and the polycyclic aromatic hydrocarbon (PAH) phenanthrene. The zebrafish is widely accepted by the National Institutes of Health as a model for human disease (Henken et al. 2004) and is rapidly becoming a toxicological model (Spitsbergen and Kent, 2003). The small size of the zebrafish allows for robust statistical analysis and ease of maintenance. In addition, *D. rerio* is a natural host for *M. marinum* (Watrall and Kent, 2007). I chose phenanthrene, a 3 ring PAH, because it is ubiquitous in coastal environments, has a relatively high water solubility ($1280 \mu\text{g L}^{-1}$), and is toxic to vertebrates, causing both Type I narcosis (Sikkema et al 1994) and dose dependent oxidative stress (Sun et al. 2006; Yin et al. 2007). I chose *M. marinum* because it causes a chronic infection characterized histologically by prominent granulomatous inflammation (Gauthier and Rhodes, 2009). To my knowledge, this is the first study designed to investigate potential interactions (e.g., synergy, antagonism, additivity, etc) between chemical exposure and a chronic infectious disease such as mycobacteriosis.

2 Materials and Methods

2.1 Experimental Animals

Adult fish (wild type AB strain) were originally obtained from the Zebrafish International Resource Center (ZIRC) (Eugene, OR). Fish were maintained in an Aquatic Habitats © (Apopka, FL) recirculating system. Reverse osmosis (RO) water was

adjusted to a conductivity of approximately 1000 $\mu\text{S}/\text{cm}$ and a pH of approximately 7.5 using Instant Ocean[®] (Madison, WI) salts. Adult animals were actively bred according to standard procedures (Westerfield, 2000). Additional animals were purchased from a local pet store “Luv-a-pet” (Virginia Beach, VA). Experimental animals not bred in house were quarantined for one month. Prior to introduction into the colony, ten individuals were randomly selected, euthanized, necropsied and evaluated microscopically for the presence of granulomatous inflammation to prevent the introduction of mycobacteria to the system.

2.2 Preliminary Range Finding Experiments

Prior to conducting a concurrent exposure with toxicant and pathogen, range finding experiments were conducted to establish appropriate concentrations for each stressor. Two week exposure duration was selected to allow sufficient time for mycobacterial disease progression. My goal was to find a phenanthrene concentration that generated an LC_{50} in less than 14 days (high), a concentration generating an LC_{50} in longer than 14 days (low), and an LD_{50} for *M. marinum* of 14 days. These three very distinct mortality curves would then be compared to the curves obtained from the dual stressor exposures. Based on the preliminary range finding studies, I calculated a 14 day phenanthrene LC_{50} of $293 \mu\text{g L}^{-1}$ (95% confidence intervals $216 \mu\text{g L}^{-1}$ - $366 \mu\text{g L}^{-1}$) and therefore set the desired high nominal concentration at $450 \mu\text{g L}^{-1}$ and the low nominal concentration at $150 \mu\text{g L}^{-1}$. A 14 day LD_{50} was established for *M. marinum* at 2.3×10^3 bacteria/fish.

2.3 Experimental Design

To examine the effects of combined stressors, zebrafish were simultaneously exposed to *Mycobacterium marinum* and the toxicant phenanthrene. 635 zebrafish were maintained in one of eighteen 30L glass aquaria at 28° C receiving aeration. Fish were randomized and distributed into six experimental treatments, with each treatment run in triplicate: 1) control, 2) *M. marinum* infected (Myco), 3) low dose phenanthrene only exposure (Low), 4) high dose phenanthrene only exposure (High), 5) *M. marinum* + low dose phenanthrene exposure (Myco+Low) and 6) *M. marinum* + high dose phenanthrene exposure (Myco+High).

Target nominal phenanthrene concentrations were 450ug L⁻¹ for the high dose treatments, and 150ug L⁻¹ for the low dose treatments. All fish received an intracoelomic (IC) injection (20µL) of phosphate buffer (pH 7.4) (Controls) or *M. marinum* suspended in phosphate buffer (2.3x10³ bacteria/fish) (Treatments). In order to account for and eliminate variations due to post injection mortality, all bacterial injections were done 24 hours prior to initiation of phenanthrene exposure, and only surviving, healthy fish were used. The experiment was conducted as an aqueous static renewal, with approximately 95% of exposure solutions renewed every 24 hours. An additional 5 mL spike of phenanthrene in acetone (1260 µg mL⁻¹ (high dose) and 540 µg mL⁻¹ (low dose)) was added at 12.1 hours post renewal to make up for losses due to volatilization, sorption and metabolism. Control groups received a spike of acetone only. Toxicant dosing was continuous for 14 days. Fish were monitored for an additional 7 days post phenanthrene exposure to examine latent mortality.

Fish were subdivided within each tank using a screened partition. One group (n=20) was used for survivorship analysis and monitored throughout the day for

mortalities. The second group (n=35) was sampled temporally, every four days starting at day one post phenanthrene exposure, for histology and phenanthrene body burden analyses.

2.4 Preparation of Bacterial Cultures

Mycobacterium marinum (strain M30) was originally isolated from Chesapeake Bay striped bass (*Morone saxatilis*) using standard aseptic necropsy protocols in a BSL2 cabinet and cultured by standard bacteriological methods. This strain was recently shown to be pathogenic, causing high mortality in zebrafish (Ostland et al. 2008). Archived samples were inoculated aseptically into Middlebrooks 7H9 broth with OADC enrichment and incubated at 30°C for two weeks. The culture in log growth phase was centrifuged at 10,000 x g for 15 minutes to obtain a cell pellet. The supernatant was aspirated and the pellet was resuspended in 2 mL Butterfield's phosphate buffer (PB) (pH 7.4) with 0.05% Tween 80. To reduce adhesion and clumping of bacteria, the culture was vortexed for approximately 30 seconds with sterile glass beads. An additional 5 mL of PB were added and the optical density was measured at 590 nm. The desired OD of 0.1, representing approximately 5×10^4 bacteria/ μL , was achieved by adjusting with additional PB. Serial ten fold dilutions were prepared to obtain the desired bacterial density ($75 \text{ bacteria } \mu\text{L}^{-1}$). Plate counting was used to quantify actual bacterial densities.

2.5 Generation of Phenanthrene Solutions

Aqueous phenanthrene dosing solutions were made fresh every 24 hours during the experiment using a generator column. The generator column design and procedure to produce a saturated aqueous phenanthrene solution has been described previously (Unger et al. 2007). Briefly, sand coated with phenanthrene (97%; Acros Organics ©

New Jersey, USA) was dry packed into a 7.5cm x 59.2cm aluminum column. RO water was passed through the column, against gravity, at a rate of 2.0L min⁻¹. This flow rate was reported to be sufficient to achieve a saturated aqueous solution (Unger et al. 2007). RO water was conditioned with 0.3 g/L artificial sea salt (Instant Ocean ®) and used to dilute the saturated phenanthrene solution to the desired dosing concentrations.

2.6 Phenanthrene Analysis of Water Samples

Water was analyzed for phenanthrene using high performance liquid chromatography (HPLC) with a fluorescence detector (SpectraSYSTEM® P400 Controller, SpectraSYSTEM® AS3000 Autosampler [San Jose, CA, USA], Waters 474 Fluorescent Detector [Milford, MA, USA] and Restek® Allure C18 column [Bellefonte, PA, USA]) (Unger et al. 2007). In brief: a solvent gradient of water/acetonitrile was used with the gradient shifting from 100% water to 80% acetonitrile and back to water over a time of 35 minutes and a flow rate of 1mL min⁻¹. The fluorescence detector was set to an excitation wavelength of 265nm and an emission wavelength of 370nm. Data were collected using ChemStation (Hewlett Packard Santa Clara, Ca, USA) software. Calibration of the machine was done prior to analysis of experimental samples. Calibration used a 9 point calibration curve and an internal standard of 1-methylnaphthalene (1-MN) (0.40µg/mL).

Water samples (1.0 mL) were removed from each tank and spiked with 0.030 mL 1-MN in acetonitrile. Samples were collected at time 0 hours, 12 hours, 12.1 hours and 24 hours each day. Samples were analyzed immediately after collection, or refrigerated to minimize contaminant loss. Blanks of acetonitrile were run at the start of every new sample set, and replicates were taken every ten samples and analyzed to document

sampling and analytical precision. In addition, a calibration standard was run at the start of each day to ensure analytical accuracy.

2.7 Water Quality

Exposure tanks were monitored daily for temperature, pH, dissolved oxygen (DO) and total ammonia. DO and temperature were monitored using an Oxyguard® Handy Polaris (Birkerød, Denmark). Salifort® box kits were used for analysis of ammonia and pH was measured with an Orion 250A with Thermo pH electrode (Waltham, Ma, USA).

2.8 Histology

Standard histological techniques were used (Prophet et al. 1992). Briefly, moribund fish were euthanized by overdose with MS 222 (500mgL⁻¹). A small mid ventral incision was made into the abdomen to allow for fixative penetration and the entire fish was placed into Z-Fix, a buffered zinc formalin fixative (Anatech LTD) for at least 48 hours. Fish were subsequently decalcified overnight in a 50:50 formic acid: sodium citrate solution. Fish were then rinsed in running tap water for 3 hours, bisected sagittally using a single edge razor and placed into uniquely labeled tissue cassettes. Tissues were then dehydrated through a graded ethanol series, cleared and infiltrated in hot paraffin wax in a Shandon Excelsior Tissue Processor (Miami FL, USA), after which they were embedded on a Microm EC350-1 tissue embedding center (Microm Inc. [Mound MN, USA]). Sections (5µm) were cut on an Olympus rotary microtome 4055, and stained with hematoxylin and eosin, in a Varistain Gemini ES automatic slide stainer [Thermo Scientific Miami, FL, USA].

Histologic tissue sections were scored semi-quantitatively for diffuse leukocytic infiltration (none, low, medium and high), stage of granuloma organization (none,

diffuse, epithelioid without a necrotic core, and epithelioid with necrotic core). Total number of granulomas per fish was also quantified. Each category was assigned a rank number from 0-3 with 0 representing no difference from the controls, and 3 having the most severe indications of disease. Granuloma number was not ranked, but reported as total number observed per fish. To avoid bias towards experimental groups, samples were randomly selected and scored blind. Values were then averaged for each experimental group and assessed for statistical significance (See below).

2.9 Phenanthrene Tissue Concentrations (Body Burdens)

Analysis of phenanthrene in fish tissues followed a modified version of those reported previously (Unger et al. 2007) on PAH burdens in grass shrimp (*Palaemonetes pugio*). Briefly, individual fish were thawed, rinsed in deionized water, weighed and homogenized using Kontor® glass tissue homogenizers (Vineland, NJ, USA) in 1mL deionized water and 500ng deuterated (d10) phenanthrene surrogate standard. Homogenates were then transferred to 50 mL Teflon centrifuge tubes with 2 mL of concentrated hydrochloric acid, sonicated for ten minutes, after which two 2 mL aliquots of hexane were used to extract the aqueous homogenate. Samples were centrifuged between hexane extractions. Extracts were reduced in volume under a gentle stream of nitrogen until the desired volume of 0.1 mL was attained. An internal standard of p-terphenyl was added (0.8 µg) before analysis on a 4D ion trap mass spectrometer (Varian, Walnut Creek, Ca, USA). Analytes and ions measured were p-terphenyl internal standard [152+230], phenanthrene D10 [187-189], phenanthrene [176-179], 1-hydroxyphenanthrene [165+194] and 3-hydroxyphenanthrene [165+194]. A seven point calibration curve was generated for all target analytes. Identification was based on

matching retention time and spectra to standards (1-hydroxyphenanthrene (EQ Laboratories Inc. Augsburg, Germany) and 3-hydroxyphenanthrene (Cambridge Isotope Laboratories, Andover MA, USA) or to those in the NIST 05 mass spectral library.

Samples were normalized to hexane extractable lipids to compensate for differences in contaminant uptake between individuals. After mass spectrometric analysis, sample vials were left open and the hexane was allowed to evaporate off to constant weight. Weight differences between original vial weight and final vial weight were determined to be hexane extractable lipid.

2.10 *Statistical Analysis*

The dual exposure study was analyzed for differences in time to death using the Kaplan Meier method. This method estimates survival function of an exposed group of individuals (Newman, 1995) and allows comparison of two or more survival curves and evaluation of treatment effects. The log rank test was used to establish if there were significant deviations between survivorship curves. LC_{50} 's and associated 95% confidence intervals were generated using Probit analysis. Bioaccumulation data, phenanthrene/metabolite ratios and semi quantitative histological data (e.g., severity of granuloma formation) were analyzed by the nonparametric Mann Whitney U test. ANOVA was used to evaluate if toxicant water concentrations differed significantly between replicates. ANOVA was also used to test the significance in toxicant water concentration between groups exposed to the high dose of phenanthrene, and between groups exposed to the low dose of phenanthrene. All statistical analyses were performed using SAS software [Cary NC, USA].

3 Results

3.1 Water Chemistry

Water quality data are presented in Table 1. The mean values (+/- standard deviation) for dissolved oxygen, pH, temperature and total ammonia were 6.5 +/- 0.95 mgL⁻¹, 6.2 +/- 0.26, 27.8 +/- 1.36, and <0.25 respectively. All water quality parameters remained within the physiological range of zebrafish and none were considered to be stressful (Lawrence 2007).

3.2 Phenanthrene Water Concentration

Mean daily concentrations (+/- standard deviation) were 284 +/- 187 µg L⁻¹ and 285 +/- 187 µg L⁻¹ for the “High” and “Myco+High” groups, respectively (Figure 1A). Both the “Low” and the “Myco+Low” groups had a mean daily value of 85 +/- 44 µg L⁻¹ (Figure 1B). Average 12 hour loss of aqueous phenanthrene from all treatment groups was 49%. There were no significant differences in phenanthrene concentrations between replicate tanks for any treatment allowing replicates to be combined for survival analysis. Additionally, there were no significant differences between related experimental groups (i.e the “High” versus the “Myco+High” group). Phenanthrene concentrations in all control tanks were repeatedly below detection limits (1 µg L⁻¹).

3.3 Fish Mortality

Narcotic effects from phenanthrene exposure were evident in fish exposed to both the high and low doses regardless of mycobacterial infection. Clinical signs included erratic swimming and disorientation and were most pronounced in the groups receiving the high dose of phenanthrene. Two days after termination of phenanthrene exposure, all exposure groups returned to normal swimming patterns showing rapid recovery. This behavior is consistent with post-exposure to Type I narcotics.

Fourteen day survivorship was significantly lower in fish exposed to 450 µg/L of phenanthrene alone (High group) than in any other treatment group, including the “Myco+High” group ($p<0.01$) and when compared to all other groups individually ($p<0.001$) (Figure 2). Survivorship in the “Myco+High” group did not significantly differ from fish exposed to *M. marinum* only (“Myco”) ($p=0.1$). Survivorship in the “Myco+Low” group was not significantly different from the “Myco” group, although both groups differed significantly from the controls ($p<0.05$). Survivorship in the “Low” group was not significantly different from that of the controls.

3.4 Tissue Concentrations

Phenanthrene tissue concentrations were significantly higher ($p<0.05$) in the “Myco+High” group than in the “High” group (Figure 3). Tissue concentrations in the “Myco+Low” fish did not significantly differ from those in “Low” group ($P=0.1$).

Mass spectrometry confirmed the presence of two phenanthrene metabolites, 3-hydroxyphenanthrene and 1-hydroxyphenanthrene. Metabolite concentrations were summed, and a ratio of phenanthrene to metabolites was calculated in order to normalize differences in phenanthrene uptake between individuals. The ratio for the “Myco+High” treatment was significantly higher on sample day one than the “High” group ($p<0.01$), but not on days five, nine or thirteen (Figure 4). Hydroxyphenanthrene concentrations in the “Low” fish were often below the detection limit and therefore not quantified ($DL<1\mu\text{g mL}^{-1}$).

3.5 Histology

Fish were examined histologically to evaluate disease severity (granuloma number, stage and degree of leukocyte infiltration) and phenanthrene associated

pathology. Only fish infected with *M. marinum* displayed inflammation and granulomas (Figure 5). By day nine every experimentally infected fish examined showed some degree of granulomatous inflammation. Inflammation (granuloma organization) ranged from diffuse accumulations of leukocytes with no defined organization, to highly organized granulomas comprised of epithelioid cells surrounding a highly eosinophilic necrotic core. No significant differences in number of granulomas or degree of organization were evident between any groups exposed to *M. marinum* (Table 2). The Ziehl Neelsen stain confirmed the presence of acid fast mycobacteria only in experimentally infected fish (Figure 6). Fish were also examined for pathologies associated with phenanthrene exposure. No fish examined in this study displayed histopathological changes attributable to toxicant exposure.

4 Discussion

4.1 Survivorship

Zebrafish concurrently challenged by high dose phenanthrene and chronic bacterial disease exhibited significantly higher survival than zebrafish exposed only to high dose phenanthrene. This observation was unexpected given our working hypothesis that mortality will be higher in fish challenged by concurrent dual stressors than mortality in fish exposed to a single stressor alone. This was clearly not the case in this study and suggests an antagonistic interaction between phenanthrene toxicity and mycobacteriosis.

The mechanisms underlying the reduced mortality (increased survivorship) in the dually exposed fish are presently not understood. However, analysis of phenanthrene metabolites in fish tissues does provide some insight into possible causes. In addition, survivorship for “Myco+Low”, “Myco+High” and “Myco” exposure groups did not

differ significantly from one another. Even though the “Low” dosed fish do appear to be metabolizing phenanthrene at an increased rate as compared to the “Myco+Low” fish (Figure 4), the concentration of metabolites produced at this low dose appears insufficient to induce increased mortality. Thus, I feel that mortality in the “Myco+Low” group is driven by *M. marinum*. This is supported by the nearly identical survivorship curves between the “Myco” and “Myco+Low” exposure groups. Histological analysis also did not show any significant differences in disease progression between any groups. This further supports the hypothesis that for each group infected with *M. marinum*, mortality was the result of the disease process and was independent of toxicant exposure. This result is surprising as previous researchers have found PAHs to increase susceptibility of fishes to bacterial challenge. Carlson et al. (2002) showed a reduced resistance of the Japanese medaka (*Oryzias latipes*) to the bacterium, *Yersinia ruckeri*, following IP injection of benzo[a]pyrene. Similarly, Arkoosh et al. (2001) showed higher mortalities in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) challenged with *Vibrio anguillarum* plus ten high molecular weight PAHs versus *V. anguillarum* challenge alone.

It should however be noted that survivorship curves between the “Myco” and “Myco+High” groups, although not significantly different ($p=0.1$), did begin to diverge from one another at day 14 (Figure 2). For that reason it is possible that the study duration (14 days) was not sufficient for differences between these two treatments to become apparent, and that an additive effect may have been seen had our study duration been longer. *M. marinum* is widely recognized as the causative agent of chronic granulomatous disease affecting fishes. Therefore future work will require additional

time to fully examine the interactions between phenanthrene and this chronic disease state.

4.2 Phenanthrene Toxicity

I measured tissue phenanthrene concentrations using GC/MS to provide some insight into the underlying mechanisms responsible for the unexpectedly higher survival observed in dually challenged zebrafish. In addition to the characteristic peaks attributable to phenanthrene, we observed additional peaks in the chromatograms representing two hydroxylated metabolites of phenanthrene: 1-hydroxyphenanthrene and 3-hydroxyphenanthrene. The ratio of parent phenanthrene:metabolites (1-hydroxyphenanthrene plus 3-hydroxyphenanthrene), was significantly higher in dually challenged fish than in fish exposed only to high dose phenanthrene at day 1, suggesting reduced phenanthrene metabolism in fish receiving dual challenge compared to fish exposed only to high dose phenanthrene. I hypothesize that 1) phenanthrene metabolism either produces toxic metabolites or causes oxidative stress by production of toxic oxygen species, increasing mortality in the “High” exposure group and 2) the reduced phenanthrene metabolism in dually exposed fish may be caused by release of pro-inflammatory cytokines following *M. marinum* induced inflammation (Section 4.3).

Phenanthrene concentrations for my study were selected to produce desired LC₅₀s, and as such are not of direct environmental relevance. However, the mean values (+/- standard deviation) of 284 +/- 187 µg L⁻¹ and 85 +/- 44 µg L⁻¹ for groups exposed to the high and low doses respectively were similar to those reported in other investigations. Jee et al (2004), Olivera et al (2008) and Wenju et al. (2009) all used concentrations

ranging between $17.8 \mu\text{g L}^{-1}$ and $480 \mu\text{g L}^{-1}$ when performing phenanthrene exposure studies with teleosts.

As previously stated, my finding that the highest mortality occurred in fish exposed to phenanthrene alone was unexpected. I initially expected to observe the highest mortality in dually exposed fish due to potential synergistic effects of stressors resulting in immunosuppression and/or increased phenanthrene body burden. However, the results suggest that the parent phenanthrene molecule is not the only causative agent of toxicity, but that metabolites are major contributors. This is supported by the highest mortality rate in the “High” exposure group. These fish displayed a reduced phenanthrene:metabolite ratio as compared to the dually exposed fish, suggesting that this lower ratio is due to more efficient metabolism of the parent compound and increased toxicity. This is further supported by a preliminary immunohistochemical assay to assess the levels of CYP1A present in the cells (See Appendix A).

Phenanthrene metabolites may be directly toxic or toxicity may result from the production of free radicals generated during metabolism. Although there is no evidence to suggest that either 3-hydroxy or 1-hydroxyphenanthrene are directly toxic, previous researchers have demonstrated increased toxicity with increased phenanthrene metabolism. Hawkins et al. (2001) demonstrated higher mortality in rainbow trout (*Oncorhynchus mykiss*) fry simultaneously exposed to phenanthrene ($100\mu\text{g L}^{-1}$) and β naphthoflavone ($10\mu\text{g L}^{-1}$) (a cytochrome P450 inducer) than in trout exposed to phenanthrene alone ($100\mu\text{g L}^{-1}$). However, they did not determine if metabolites were directly toxic or if toxicity was due to oxidative stress resulting from increased metabolism. Oxidative stress was shown to occur in the liver of goldfish (*Carassius*

auratus) exposed to 50 $\mu\text{g L}^{-1}$ phenanthrene for seven days (Sun et al. 2006). Similarly, Yin et al. (2007) showed oxidative damage to hepatocytes in as little as four days when goldfish were exposed to phenanthrene concentrations ranging from 50-1000 $\mu\text{g L}^{-1}$. Mortalities were not reported in either of these studies. However, toxicant concentrations were lower and/or study durations were significantly shorter than those used in this study. In addition, I replenished phenanthrene every twelve hours in my study as opposed to twenty four hours in these two studies. These differences may account for the observation of reduced survivorship in the “High” exposure group whereas Yin et al. (2007) and Sun et al. (2006) did not observe any mortality.

As noted in section 4.1, the survivorship curves between the “Myco” and “Myco+High” exposure groups begin to diverge at day 10, although this divergence is not statistically significant. This suggests that a longer timeline may be required to detect potential differences between these treatments. Because the fish in the “Myco+High” group were still producing metabolites, albeit at a reduced rate, I postulate that the metabolites could still induce increased mortality compared to the “Myco” treatment alone.

In this study, I did not observe histopathological changes directly attributable to toxicant exposure. Pathological changes, if they occurred, may have been subcellular, and not evident at the histological level, requiring electron microscopy in future work.

4.3 Proposed Cytochrome P450 Inhibition

My second hypothesis is that the reduced phenanthrene metabolism in dually exposed fish may be caused by release of pro-inflammatory cytokines following *M.*

marinum induced inflammation. This hypothesis finds support in prior studies showing that the proinflammatory cytokines, IL1 and TNF α , inhibit the CYP1A biotransformational pathway in mammals (Nicholson and Renton 2002; Nicholson and Renton 1999; Renton 2001) as well as teleost fishes (Best et al. 2002; Chambras et al. 1999; Marionnet et al. 2006; Reynaud et al. 2008; Reynaud et al. 2005). The role of inflammatory cytokines in suppression of cytochrome P450 (CYP) activity has been known for over thirty years (reviewed in Morgan 2001). More recent work by Morgan et al. (2002) using lipopolysaccharide (LPS) to induce inflammation in rats showed two distinct pathways of CYP suppression. They found both a nitric oxide dependent pathway, resulting in proteolytic degradation of CYP enzymes, and a nitric oxide independent pathway in which post transcriptional mRNA levels are depressed; although the specific mechanisms have not yet been identified.

Even though the exact mechanisms controlling CYP pathway suppression during PAH exposure are still unclear, direct inhibition of the CYP pathway in association with inflammation has been observed previously in the laboratory. Stimulation of the inflammatory response using LPS (50 μ g mL⁻¹) resulted in a marked decrease in CYP1A1/2 activity in rat astrocytes as measured by a decrease in ethoxyresorufin O deethylase (EROD) (Nicholson and Renton 1999). These researchers also demonstrated that despite a decrease in enzyme (EROD) activity, the amount of CYP1A1/2 protein remained unchanged suggesting the enzymes were functionally inhibited. Additional work using direct addition of cytokines (IL1 β and TNF α) to rat astrocyte isolates resulted in CYP1A suppression; however the addition of dexamethasone (a cytokine inhibitor) reversed this effect (Nicholson and Renton, 2002).

Although most research on the relationship between toxicant metabolic pathways and inflammation has been driven by human health and thus limited to mammalian models, several studies have been performed on teleost species. To directly assess the effects of proinflammatory cytokines on the CYP pathway, Reynaud et al. (2005) intravenously injected carp (*Cyprinus carpio*) with IL1 β and TNF α . This study showed a significant reduction in CYP protein content in both the liver and head kidney of fish exposed to 3 MC. 3 MC induced GST activity was also depressed in the liver, head kidney and spleen. Marionnet et al. (2006) hypothesized that IL1 β and TNF α , activate macrophages, ultimately resulting in increased production of reactive oxygen species (ROS), and that these compounds are responsible for depression of CYP1A activity. Although there is evidence of this mechanism in mammals (Proulx and Souich 1995; Morel and Barouki 1998), no definitive studies have been performed in teleosts.

5 Conclusion

This dual stressor study shows an interaction between *M. marinum* infection and high dose phenanthrene exposure in the zebrafish. Survivorship was significantly lower in animals exposed to the high dose of phenanthrene alone than in animals comprising all other experimental groups, including those dually challenged by phenanthrene and *M. marinum*. The higher survivorship for fish exposed to *M. marinum* and high dose phenanthrene suggests an antagonistic interaction between stressors. I have demonstrated here that phenanthrene does not affect disease progression in zebrafish within the temporal framework of this study. This is evident by the lack of significant differences in survivorship curves between the “Myco”, “Myco+High” and “Myco+Low” exposure groups and no differences in granuloma number or stage of organization between

experimental groups. However, the timeline of this study may not be sufficient for differences between experimental groups to become apparent. Body burden data, which show significantly higher phenanthrene:metabolite ratios in fish receiving combined exposure than in fish receiving phenanthrene only, suggests a disruption of the biotransformational pathway in fish mounting a granulomatous inflammatory response to a bacterial pathogen. I hypothesize that this is due to the release of proinflammatory cytokines as part of the immune response to infection. In addition, the relationship between reduced metabolite production and increased survival in fish receiving dual exposure indicates that phenanthrene metabolism may be a significant contributor to mortality either through direct toxicity or indirectly through oxidative stress resulting from production of toxic free radicals. However, the specific mechanisms by which biotransformation are altered, or how phenanthrene metabolism increases toxicity were not definitively identified in this study. For these reasons this work is considered preliminary and additional studies should be directed at further elucidating these interaction.

Unlike single stressor challenges, this study provides some insight into the complex interactions between dual stressors that may more closely resemble what actually happens in the natural environment. Alterations in the biotransformational pathway such as those suggested by this study, have broad implications to aquatic animal health. Although the results indicate that xenobiotic metabolism can ultimately lead to mortality, other toxicants which are not adequately eliminated from the body may pose serious risk to the health of the organisms. For this reason long term studies need to be

conducted to evaluate how both toxicant exposure and chronic, non lethal, infection interact. Additional work will examine this issue.

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Table 1: Water quality data

	Mean	+/- range
Dissolved Oxygen (mg/L)	6.5	0.95
pH	6.2	0.26
Temperature (C°)	27.8	1.36
Ammonia (mg/L)	<0.25	0

Table 2: Granuloma counts and semi quantitative histological data for Day 1, 5, 9 and 13. Granuloma numbers were counted as the number of visible granulomas per fish. Other categories were ranked from 0-3 with 0 indicating no pathology observed and 3 indicating a high degree of pathology observed. Table values are the mean rank for each experimental group (St. Dev). No significant differences were calculated between any of the experimental groups for a given day (n=3).

Day	Number of granulomas				Granuloma stage				Inflammation severity			
	1	5	9	13	1	5	9	13	1	5	9	13
Control	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
High phenanthrene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 2(2)	1 3 (1 1)
Low phenanthrene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 7(1 2)	1(0)	1(1 4)	0 (0)
<i>M. marinum</i>	0 (0)	4 3(4 5)	0 5(0 7)	7 5(10 6)	0 (0)	1 (0)	0 (0)	2(0)	0 2(2)	1 2 (8)	5 (7)	1 5 (7)
<i>M. marinum</i> + High phenanthrene	33 (58)	67 (58)	2 3(1 5)	3 3(4 2)	67 (1 2)	1 (1)	1(0)	1 (1 4)	5 (5)	67 (58)	1 (0)	1 (1 4)
<i>M. marinum</i> + Low phenanthrene	0 (0)	33 (58)	3 5(0 7)	1(1 4)	0 (0)	1(1)	2(0)	2 (1 4)	0 (0)	1(0)	7 5 (3 5)	5 (7 1)

Figure 1: Daily fluctuations in phenanthrene concentration for A) high dose groups (“High”-x-, “Myc+High” --▲--) and B) low dose groups (“Low” --x--, “Myc+Low” -●-). Phenanthrene solutions were made fresh every 24hours with additional phenanthrene spikes added at time 12 hours each day. Error bars represent standard deviations. No significant differences were observed between experimental groups.

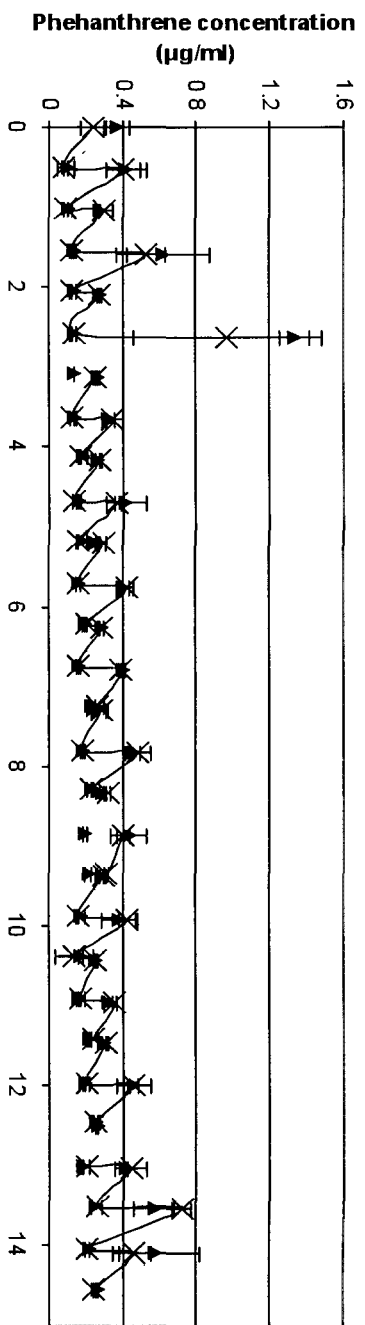
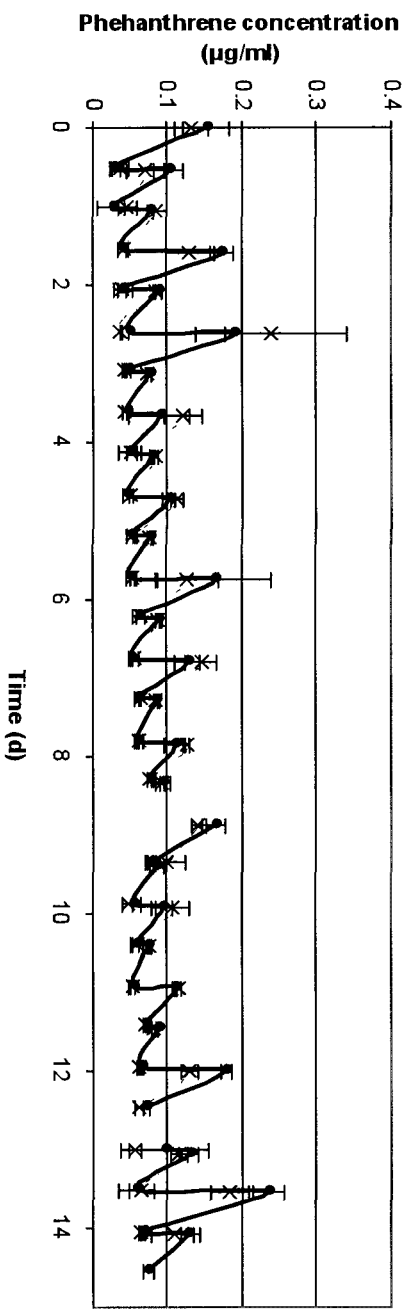
A**B**

Figure 2: Kaplan-Meier survivorship curves for zebrafish, *Danio rerio*, exposed to phenanthrene and *M. marinum*. Survival in fish exposed to high dose phenanthrene was significantly lower than in fish exposed to high dose phenanthrene+*M. marinum*. Error bars: standard error of the mean. Key: “High” (-X-), “Low” (--x--), “Myco” (--□--), “Myco+High” (--▲--), “Myco+Low” (--●--), Control (-■-). * denotes $p < 0.01$

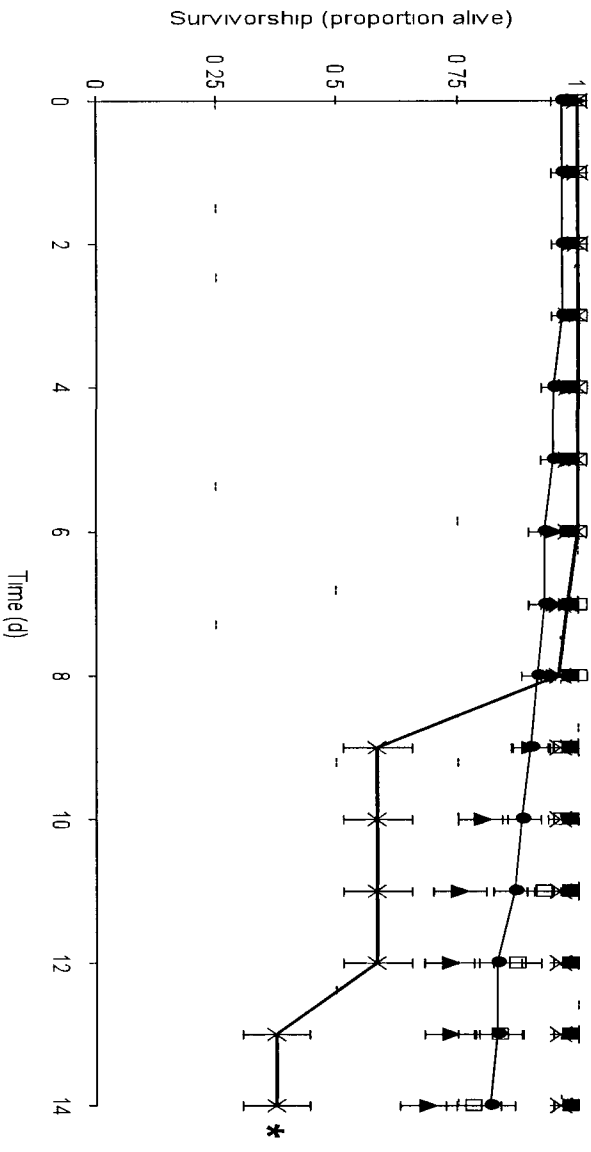


Figure 3: Mean phenanthrene concentrations for each experimental group in μg phenanthrene/g hexane extractable lipid. Phenanthrene concentrations for all unlisted treatments were below detection. (DL: $1\mu\text{g L}^{-1}$). Error bars represent 95% confidence intervals. Key: “High”=high dose phenanthrene exposure, “Low”= low dose phenanthrene exposure, “Myc+High”= simultaneous high phenanthrene+*M. marinum*, “Myc+Low”= simultaneous low phenanthrene +*M. marinum*

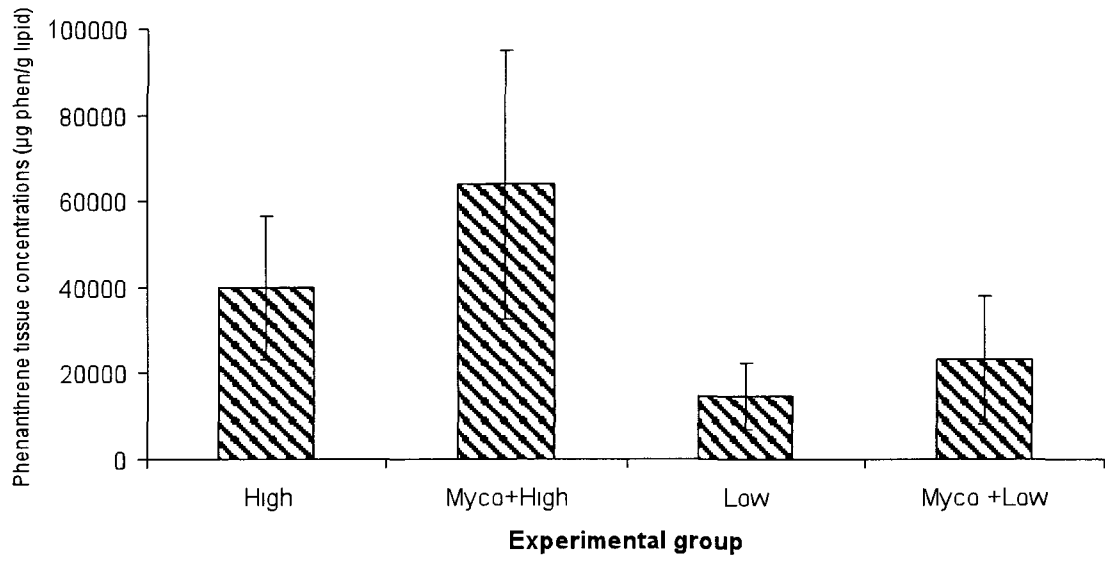


Figure 4: Mean phenanthrene:hydroxyl-phenanthrene ratios in zebrafish, *Danio rerio*, exposed to phenanthrene and *M. marinum* over 13 days. Error bars represent 95% confidence limits. * indicates significantly higher ratio vs. other group for that day ($p < .01$). Y axis is log scale. Key: Horizontal patterning represents the “High” group. Slanted patterning represents the “Myco+High” group.

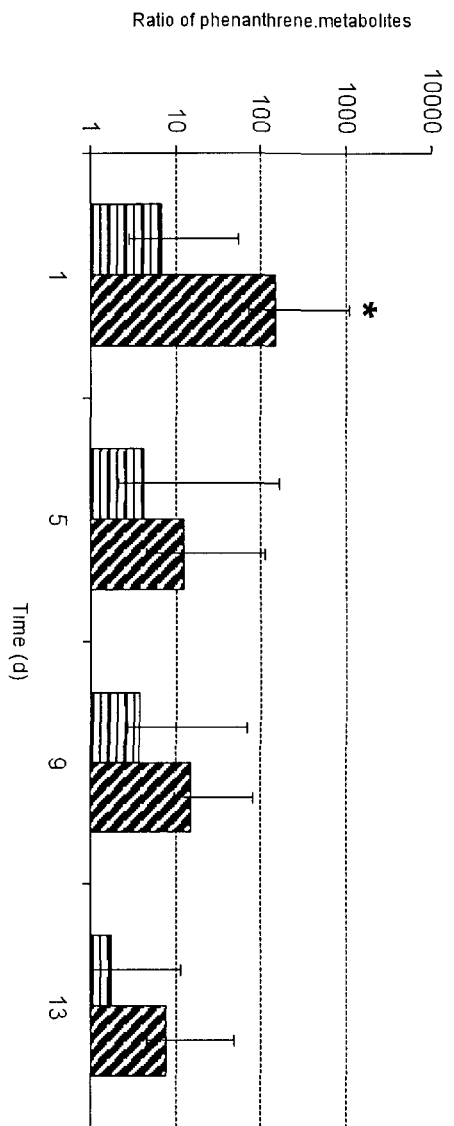
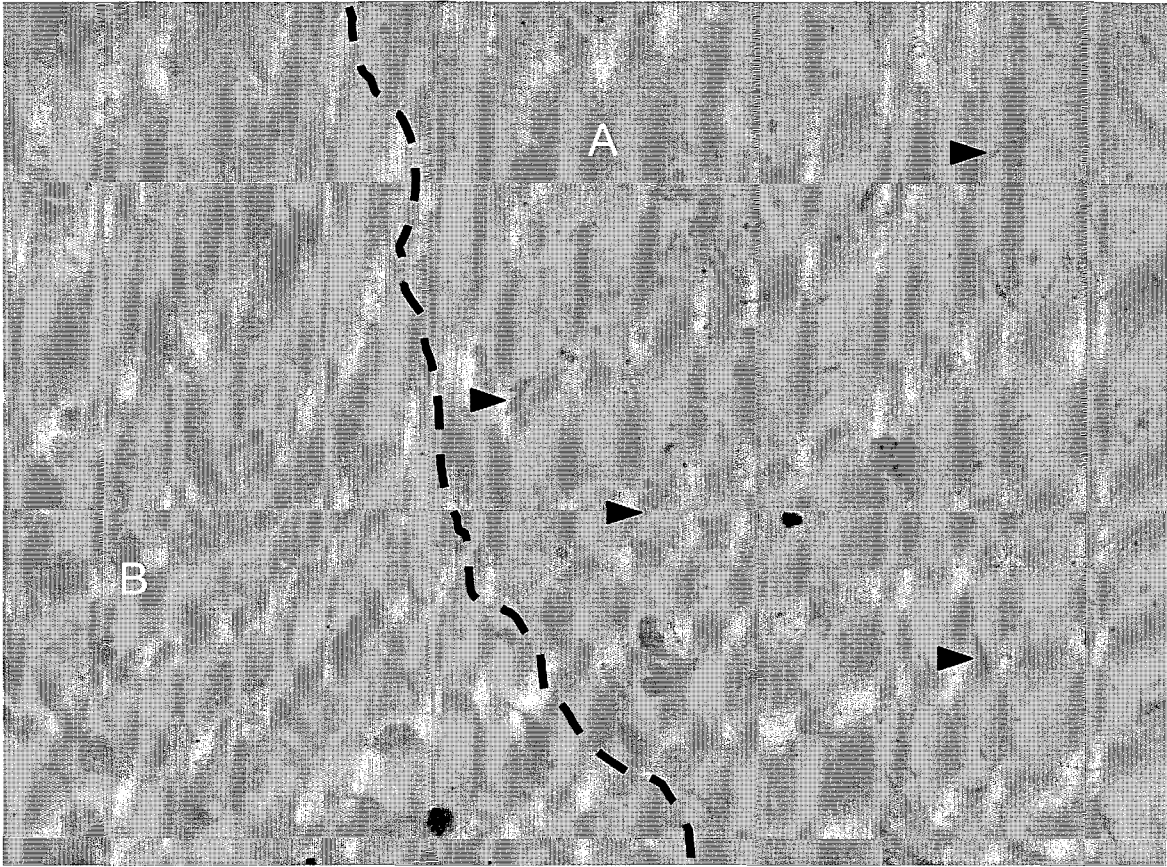


Figure 5: Histologic section through the coelomic cavity of an experimentally infected zebrafish showing two mesenteric granulomas. This pathology is commonly associated with *M. marinum* infection. The top granuloma (A) is well organized with a necrotic core. The lower granuloma (B) is more diffuse and not as well organized. Inflammatory cells can be seen infiltrating the area of infection. 400x



Figure 6: Ziehl Neelsen stain for acid fast bacteria of *M. marinum* infected zebrafish. The dotted line separates healthy tissue (B) from granuloma (A). Bacteria are all located within the granuloma and are recognized as thin strand like objects, in some cases having a beaded appearance (arrow heads). 600x



Appendix A: Immunohistochemical analysis for CYP1A using the C10-7 1° antibody

Introduction

Immunohistochemistry is the process by which monoclonal antibodies are applied to histological sections allowing - detection of specific molecules in or around cells. This method is advantageous over other laboratory methods, such as western blotting, in that whole fish sections can be analyzed allowing all tissues to be observed. Of particular importance to this dissertation was observing differences in CYP1A expression between the treatment groups. This method had been used previously to examine CYP1A expression in *Fundulus heteroclitus* after exposure to the PAH benzo[a]pyrene (Van Veld et al. 1997). These researchers found high levels of CYP1A expression in gill pillar cells, heart endothelium, hepatocytes and renal tubules. After observing the dramatic alteration in phenanthrene metabolism when zebrafish were challenged with both phenanthrene and *M. marinum*, an attempt was made to gain a better understanding of the mechanisms behind the observed suppression. Phenanthrene is not thought to induce the cytochrome P450 pathway (Barron et al. 2004), and that endogenous CYP1A activity is responsible for metabolism. It was anticipated that fish exposed only to the high dose of phenanthrene would appear similar to our control fish, and that dually challenged fish would exhibit CYP1A suppression below the control fish and our phenanthrene only treatment groups.

Material and Methods

For this study the C10-7 monoclonal antibody was used. This antibody was originally designed by Dr. Charles Rice (Clemson University) for the detection of CYP1A protein in the fathead minnow. Prior to conduction the IHC assay, reactivity with zebrafish CYP1A was confirmed using a western blotting assay (See Chapter 3 section 2.6 for methods). Fish used for IHC had been previously sampled for histological analysis. Additional sections of these samples were then prepared for IHC analysis.

Tissues were fixed as previously mentioned (Section 2.8) prior to immunohistochemical analysis. Slides were deparafinized using 2x changes in xylene (5 minutes each), 2x 100% EtOH (3 minutes each) and 1x 95% EtOH (3 minutes). Endogenous peroxidase activity was quenched using 3% H₂O₂ for 10 min in a moist incubation chamber, followed by a 2x rinse in phosphate buffer solution (PBS) (Ph 7.4) (5 minutes each). Slides were then steamed in 95° citrate buffer for 30 minutes in order to unmask epitopes. Slides were not submerged, but suspended above the citrate buffer solution. Slides were rinsed 2x in PBS followed by 20 minute incubation in antibody diluent (BD Biosciences). Without rinsing, slides were incubated in the primary antibody (C10-7) overnight at 4°C. Two to three minute rinses in PBS were followed by a 30 minute incubation in dilute biotinylated anti-mouse Ig secondary Ab (BD Biosciences Anti-Ig HRP detection kit) 1:50 with Ab diluent at room temperature. This was followed by 2, 3minute rinses in PBS. Streptavidin-HRP (BD Biosciences Anti-mouse Ig HRP detection kit) was then applied to each slide for 30 minutes at room temperature. Diaminobenzidine (DAB) substrate was used for color development. One drop DAB chromagen was added to 1mL DAB buffer to make a working solution (BD Biosciences Anti-Ig HRP detection kit). Slides were then incubated in the DAB substrate for 5

minutes at room temperature, followed immediately by 3x deionized water rinses for 3 minutes each. Slides were then dehydrated using 2-5 minute changes in 95% ETOH followed by 2, 5 minute changes in 100% EtOH. Finally slides went through 3x xylene changes for 5 minutes each. Slides were coverslipped and read using standard light microscopy.

Results

A large degree of variability was observed in our samples. However livers of control fish consistently stained very lightly. Of the “High” treatment group, one of the three samples showed a marked increase in staining and was darker than controls by day 9 (Figure 1). The heaviest staining was seen in hepatocytes and renal tubules. Of the other two samples, one showed a slight increase above controls, although this was not quantified, and the third showed no difference from controls. The same phenomenon was observed in the dually challenged fish, although not as heavily stained as the “High” treatment. One of the three samples showed a dramatic increase in staining above the controls by day 9 (Figure 1); however, the other two samples were indistinguishable from controls.

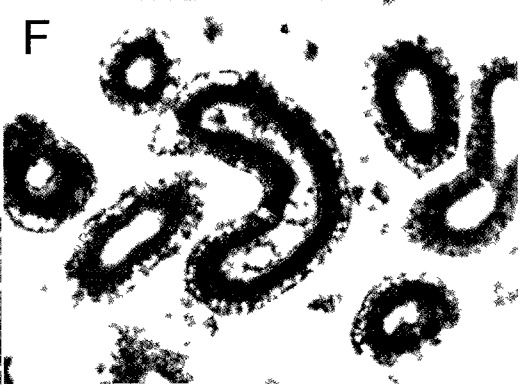
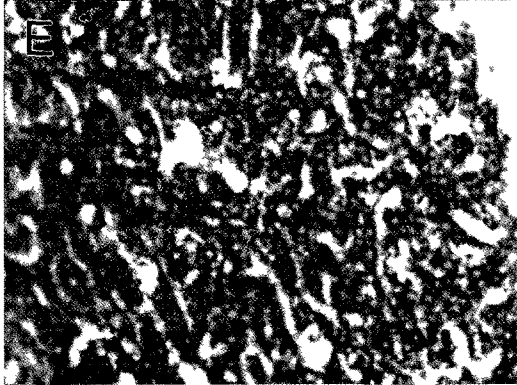
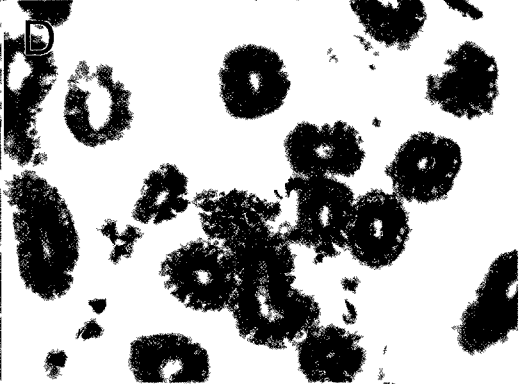
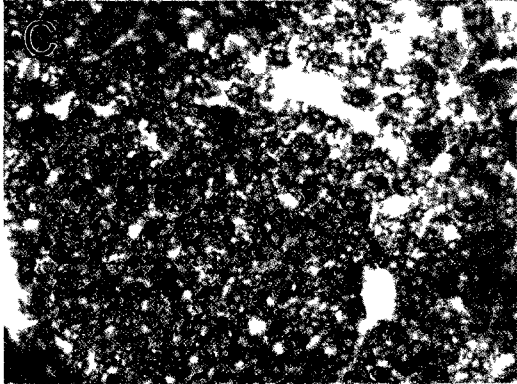
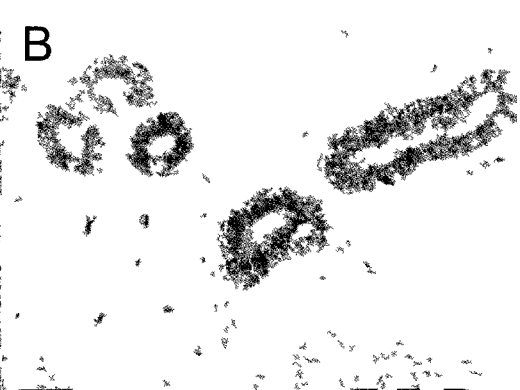
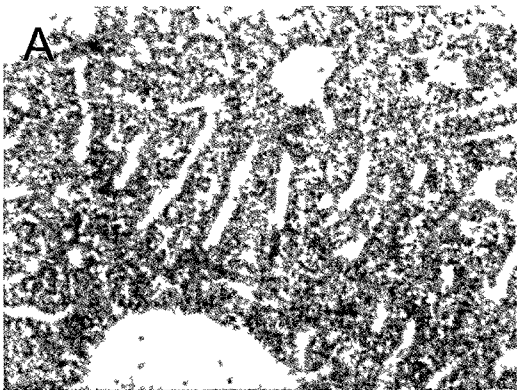
Discussion

The small sample size used in this assay makes definitive conclusions difficult; however, in samples where increased staining was observed, the tissue locations and cell types stained for CYP1A activity were consistent with previous research (Van Veld et al. 1997). The increased staining was highly surprising, as phenanthrene is thought to act as a Type I narcotic and is not known to bind the Ah receptor, a crucial step for initiating CYP1A production. Although the increase in CYP1A levels was unexpected, I did predict the dually challenged fish to have levels depressed below that of the “High” treatment. The results from this assay do support that hypothesis. Despite the high variability between samples, the results are suggestive that phenanthrene may be able to induce the CYP1A pathway, and supports our hypothesis of reduced metabolism in dually challenged fish. This warranted further investigation and therefore in each subsequent study larger sub-samples of fish were analyzed specifically to examine CYP1A activity. Due to difficulties in quantifying IHC results however, subsequent analyses used western blotting to quantify CYP1A activity.

References

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- Van Veld, P., W. Vogelbein, M. Cochran, A. Goksoyr and J. Stegman. 1997. Route-specific cellular expression of cytochrome P4501A (CYP1A) in fish (*Fundulus heteroclitus*) following exposure to aqueous and dietary benzo [a] pyrene. *Toxicol. Appl. Pharmacol.* 142:348-359.

Figure 1 (A-F): Histologic sections of liver (A, C, E) and kidney (B, D, F) from zebrafish following immunohistochemical detection of CYP1A protein (400x). A,B: Control fish show basal levels of CYP1A in both hepatocytes and renal tubules. C, D: “High” treatment group. Both hepatocytes and renal tubules of exposed fish are stained darker than those of control fish. E, F: “Myco+High” treatment. Both hepatocytes and renal tubules of exposed fish are stained darker than those of control fish, but not as dark as in the “High” treatment suggesting a reduction in CYP1A expression as a function of mycobacterial infection and the associated inflammation



CHAPTER 3

Phenanthrene and *Mycobacterium marinum* exposure in the zebrafish (*Danio rerio*): additive mortality from dual stressors

1 Introduction

Laboratory exposure studies are an effective approach to examine the mechanisms by which environmental stressors can adversely impact aquatic species. To a large extent, these types of studies have been conducted under optimal environmental conditions (temperature, salinity, nutrition, pH, etc.) and have typically focused on the impacts of a single specific stressor (Review by Walker et al. 2001). Because of this limited focus, these studies generally do not accurately reflect the complex interactions among multiple simultaneous stressors typically encountered by aquatic animals in nature. At the organismal level, simultaneous exposure to multiple stressors may negatively affect immune function, reproduction, growth, survival and/or predator-prey interactions, thereby posing a serious threat to population health (Adams 2005). This underscores the need for concerted effort to better understand the effects of multi-stressor exposures in aquatic organisms.

With the ever-advancing urbanization of coastal environments, the potential for adverse chemical impacts on aquatic animals will steadily increase (Lee et al. 2006). Of increasing concern in teleost fishes are the interactive effects between chemical toxicants and environmental stressors including low dissolved oxygen and temperature extremes have been described. For example, Landman et al. (2006) observed lowered hematocrits in trout (*Oncorhynchus mykiss*) simultaneously exposed to low dissolved oxygen levels and pulp mill effluent for 4 weeks. Osterauer and Köhler (2008) observed elevated mortality in zebrafish (*Danio rerio*) embryos exposed to the insecticide diazinon in conjunction with temperatures elevated 2 degrees above control.

Infectious diseases are widely seen as important environmental stressors simultaneously impacting organisms through multiple physiological systems and ultimately affecting population health (Holmstrup et al. 2010). Decreased resistance to infectious disease agents has been reported in aquatic organisms, and immunosuppression is now widely perceived to be a negative consequence of contaminant exposure. Grouper (*Epinephelus* sp.) exposed concurrently to heavy metal pollution (zinc, cadmium and copper) and infectious pancreatic necrosis virus exhibited higher mortality than fish exposed individually to either stressor (Chou et al. 1999). Salmon (*Oncorhynchus tshawytscha*) inhabiting a polluted estuary in Puget Sound, Washington displayed significantly higher mortality (e.g., lower disease resistance) following challenge by *Vibrio anguillarum*, than compared to salmon inhabiting non-polluted estuaries (Arkoosh et al. 1998). Carlson et al. (2002) reported decreased resistance (as expressed by increased mortality) to the bacterial pathogen *Yersinia ruckeri* in Japanese medaka (*Oryzias latipes*) challenged in the laboratory by IP injection of the polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene. Laboratory studies have also shown decreased B lymphocyte function following exposure of Chinook salmon to the PCB mixture Aroclor 1254 (Jacobson et al. 2003). However, studies examining toxicant induced immunosuppression have not fully elucidated the mechanisms resulting in decreased disease resistance (reviewed in Bols et al. 2001; Reynaud et al. 2006).

In this study I investigated the interactions between the toxicant phenanthrene and the bacterial pathogen, *Mycobacterium marinum*, in a model aquatic organism, the zebrafish, *Danio rerio*. The zebrafish is widely accepted by the National Institutes of Health as a model for human disease (Henken et al. 2004) and is rapidly becoming a

model for toxicological studies as well (Spitsbergen and Kent, 2003). The small size of the zebrafish allows for robust statistical analysis and ease of maintenance. In addition, it is a natural host for *M. marinum* (Watrall and Kent, 2007). I chose *M. marinum* because it causes a chronic infection characterized histologically by a prominent granulomatous inflammatory response (Gauthier and Rhodes, 2009). I chose phenanthrene, a 3 ring PAH, because it is ubiquitous in coastal environments, has a relatively high water solubility ($1280 \mu\text{g L}^{-1}$) and is toxic to vertebrates, causing both Type I narcosis (Sikkema et al. 1994) and dose dependent oxidative stress (Sun et al. 2006; Yin et al. 2007).

Earlier work (Prosser et al. 2011) has shown an antagonistic relationship when zebrafish were infected with *M. marinum* followed by phenanthrene exposure 24 hours later. In that study fish exposed to phenanthrene alone exhibited significantly higher mortality than fish exposed to phenanthrene and challenged with *M. marinum* infection simultaneously. Additionally, dually challenged fish showed a reduction in phenanthrene metabolism which I hypothesized was due to pro-inflammatory cytokines produced during the early inflammatory response to mycobacterial infection. This resulted in decreased production of toxic metabolites (e.g., hydroxy-phenanthrene), and lower mortality in the dually stressed fish than in fish exposed only to phenanthrene. I believed these surprising observations to be influenced by the short duration of the study (14 days), and relatively high target concentration of phenanthrene ($450\mu\text{g L}^{-1}$) used. The goal of this study was to more critically examine the interactions (additive, synergistic, or antagonistic) between multiple stressors using a more environmentally relevant phenanthrene target concentration ($250\mu\text{g L}^{-1}$) and longer study duration (21 days). For

the current study, I hypothesized that at lower phenanthrene concentrations we would not see the acute mortalities observed in the first study. Additionally, I believed a longer study duration (21 vs. 14 days) would permit synergistic effects to manifest.

2 Materials and Methods

2.1 *Experimental Animals*

Adult fish (wild type AB strain) were originally obtained from the Zebrafish International Resource Center (ZIRC) (Eugene, OR). Fish were maintained in an Aquatic Habitats © (Apopka, FL) recirculating system. Reverse osmosis (RO) water was adjusted to a conductivity of approximately 1000 $\mu\text{S}/\text{cm}$ and a pH of approximately 7.5 using Instant Ocean ® (Madison, WI) salts. Adult animals were actively bred according to standard procedures (Westerfield 2000). Additional animals were purchased from Segrest Farms (Gibsonton, FL). Experimental animals not bred in house were quarantined for one month. Prior to introduction into the colony, ten individuals were randomly selected, euthanized, necropsied and evaluated histologically to prevent the introduction of infectious agents (e.g., *Mycobacterium* infection or other parasites) into the breeding colony.

2.2 *Experimental Design*

This study was designed to examine the interactive effects of dual stressors using an environmentally relevant toxicant over a three week study duration. Zebrafish (n=800) were maintained in one of sixteen 30L glass aquaria at 28° C receiving aeration. Fish were randomized and distributed into four experimental treatments, each comprised of four replicates. Treatments were as follows: 1) fish sham-injected with phosphate

buffered saline (PBS) (“Control”), 2) *M. marinum* infected (“Myco”), 3) phenanthrene exposed (“Phen”) and 4) concurrently phenanthrene exposed and *M. marinum* infected (“Phen+Myco”). The target phenanthrene concentration was $250\mu\text{gL}^{-1}$, based on previously conducted experiments in our laboratory (Prosser et al. 2011) and shown to cause narcosis, but not inhibit feeding behavior. All fish received an intra-coelomic (IC) injection (20 μl) of either PBS (pH 7.4) (“Control” and “Phen” treatments) or *M. marinum* suspended in PBS (2.3×10^3 bacteria/fish) (“Myco” and “Phen+Myco” treatments).

Toxicant exposure was initiated first on day 0 of the experiment for the “Phen” and “Phen+Myco” fish, with the “Control” and “Myco” treatments held in clean water. IC injections, both *M. marinum* inoculum and sham, took place on day two. The experiment was a static renewal design, with approximately 95% of exposure solutions renewed every 24 hours. An additional 5 mL spike of phenanthrene ($900\ \mu\text{g}\cdot\text{mL}^{-1}$) in 100% acetone was added to the “Phen” and “Phen+Myco” groups at 12.1 hours post renewal to make up for losses of phenanthrene through volatilization, sorption and metabolism. Tanks housing “Control” and “Myco” treatment groups received 5 mL of acetone only. Phenanthrene exposures were continued for 21 days.

Fish were subdivided within each tank using a glass partition. One group (n=15) was used for survivorship analysis and monitored throughout the day for mortalities. The second group (n=35) was sampled temporally for histology, phenanthrene body burden analyses, plasma glucose levels, and western *blotting* analyses (CYP1A).

2.3 Preparation of Bacterial Cultures

Mycobacterium marinum (strain M30) was originally isolated from Chesapeake Bay striped bass (*Morone saxatilis*) using standard aseptic necropsy protocols in a BSL2 cabinet and cultured by standard bacteriological methods (Rhodes et al. 2004). This strain is pathogenic, causing high mortality in zebrafish (Ostland et al. 2008, Prosser et al. 2011). Archived samples were inoculated aseptically into Middlebrook 7H9 broth with OADC enrichment and incubated at 30°C for two weeks. The culture in log growth phase was centrifuged at 10,000 x g for 15 minutes to obtain a cell pellet. The supernatant was aspirated and the pellet was resuspended in 2 mL Butterfield's phosphate buffer (PB) (pH 7.4) with 0.05% Tween 80 to reduce adhesion and clumping of bacteria. To further reduce bacterial clumping, the culture was vortexed for approximately 30 seconds with sterile glass beads (~50µm diameter). An additional 5 mL of PBS were added and the optical density was measured at 590 nm. The desired OD of 0.1, representing approximately 5×10^4 bacteria/µl (as previously determined through serial dilutions and plate counting in the laboratory of Dr. Howard Kator) was achieved by adjusting with additional PBS. Serial ten fold dilutions were prepared to obtain a nominal bacterial density of 75 bacteria µL⁻¹. Plate counting was used to quantify actual bacterial densities.

2.4 Generation of Phenanthrene Solutions

Aqueous phenanthrene dosing solutions were made fresh every 24 hours during the experiment using a generator column. The generator column design and procedure to produce a saturated aqueous phenanthrene solution has been described previously (Unger et al. 2007, Prosser et al. 2011). Briefly, sand coated with phenanthrene (97%; Acros Organics © New Jersey, USA) was dry packed into a 7.5cm x 59.2cm aluminum

column. Reverse osmosis (RO) water was passed through the column against gravity, at a rate of 2.0 L min^{-1} . This flow rate was reported to be sufficient to achieve a saturated aqueous solution (Unger et al. 2007). RO water was conditioned with 0.3 g/L artificial sea salt (Instant Ocean ®) and used to dilute the saturated phenanthrene solution to the desired dosing concentration of $250 \mu\text{g L}^{-1}$.

2.5 Phenanthrene Analysis of Water Samples

Water was analyzed for phenanthrene using high performance liquid chromatography (HPLC) with a fluorescence detector (SpectraSYSTEM® P400 Controller, SpectraSYSTEM® AS3000 Autosampler [San Jose, CA, USA], Waters 474 Fluorescence Detector [Milford, MA, USA] and Restek® Allure C18 column [Bellefonte, PA, USA]) by methods used previously (Unger et al. 2007). A solvent gradient of water/acetonitrile was used with the gradient shifting from 100% water to 80% acetonitrile and back to water over 35 minutes at a flow rate of 1 mL min^{-1} . The fluorescence detector was set to an excitation wavelength of 265nm and an emission wavelength of 370nm. Data were collected using ChemStation (Hewlett Packard Santa Clara, Ca, USA) software. Calibration using a 9 point calibration curve and an internal standard of 1-methylnaphthalene (MN) ($0.40 \mu\text{g/mL}$) was done prior to analysis of experimental samples.

Water samples (1.0 mL) were removed from each tank and spiked with 0.030 mL MN in acetonitrile. Samples were collected at time 0 hours, 12 hours, 12.1 hours and 24 hours each day. Samples were analyzed immediately after collection, or refrigerated to minimize phenanthrene loss. Blanks of acetonitrile were run at the start of every new sample set, and replicates were taken every ten samples and analyzed to document

sampling and analytical precision. In addition, a calibration standard was run at the start of each day to ensure analytical accuracy.

2.6 CYP1A Analysis

Livers from three fish were pooled. Microsomal CYP1A content was quantified by Western Blot analysis using the C10-7 monoclonal antibody kindly provided by Dr. Charles Rice (Clemson University). C10-7 was originally produced to CYP1A protein of the mummichog (*Fundulus heteroclitus*); however, affinity of this monoclonal antibody to the zebrafish CYP1A protein has been previously verified in our laboratory using a western blotting assay. Zebrafish were sampled on days 1, 3 and 5 of the study, with six fish (representing two samples of three pooled liver each) removed from each replicate tank per sampling day resulting in eight data points per treatment group per sampling day. Fish were immediately euthanized by overdose with MS-222 followed by dissection of the liver. Each sample (3 pooled livers) was homogenized and immediately frozen in liquid nitrogen. Total microsomal proteins were quantified using the Bradford method (Bradford, 1976). Approximately 25 μ g total protein from each sample was separated on 12% polyacrylamide gels at 200V for 45 minutes. Proteins were then transferred to 0.20 μ m nitrocellulose membranes (Biorad) at 130V for 1hr. Nitrocellulose membranes were blocked overnight in 5% nonfat dry milk in 1X Tris buffered saline (TBS) (pH 7.5). Nitrocellulose membranes were then incubated for 1 hr in the primary antibody (C10-7: no dilution) at room temperature followed by washing 3x for 10 minutes in 1X TBS (pH 7.5). Nitrocellulose membranes were then incubated in a secondary antibody (goat anti-mouse: BioRad) for 1 hr at room temperature. Nitrocellulose membranes were washed 3x with 1X TBS (pH 7.5) and then developed using nitroblue tetrazolium chloride

(Biorad) and 5-bromo 4-chloro 3-indolyl phosphate (Biorad) for 2 minutes. Membranes were placed in deionized water to stop color development and held until imaging.

Membranes were imaged using an Alpha Innotech Flourchem system (Santa Clara, CA) and subsequently quantified using AlphaEase FC software for windows. Samples were quantified to previously purified *Fundulus heteroclitus* CYP1A standards provided by Dr. Van Veld.

2.7 Glucose Measurements

Induction of the integrated stress response in fishes is accompanied by increased levels of plasma catecholamines and corticosteroids. These rises are mirrored by an elevation of plasma glucose (Bonga, 1997). Because only small volumes (~ 2 μ l) of blood can be obtained from zebrafish, we obtained a simple measurement of plasma glucose to get an indication of the integrated stress response. Two fish were sampled from each replicate (n=8 per treatment) tank at days 1, 3, 5, 7 and 14. Fish were individually euthanized using MS-222 (150 μ g L⁻¹). The caudal peduncle was then severed with a single edged razor blade and a small drop of blood was permitted to accumulate on the cut tissue surface. Blood was then applied to an AGA Matrix Wavesense Presto glucose monitor (Salem, NH) which provided a read-out of plasma glucose levels in mg/dL.

2.8 Histology

Standard paraffin histological techniques were used (Prophet et al. 1992). Briefly, moribund fish were euthanized by overdose with MS-222 (500mgL⁻¹). A small mid ventral incision was made to allow the fixative to penetrate into the coelomic cavity and the entire fish were placed into Z-fix, a buffered zinc formalin fixative (Anatech LTD)

for at least 48 hours. Fish were subsequently decalcified overnight in a 50:50 formic acid: sodium citrate solution and rinsed in running tap water for 3 hours. They were then bisected sagittally using a single-edged razor blade and placed into uniquely labeled tissue cassettes. Tissue specimens were then dehydrated through a graded ethanol series, cleared and infiltrated in hot paraffin wax (TissuePrep; Fisher Scientific) in a Shandon Excelsior Tissue Processor (Miami FL, USA), after which they were embedded on a Microm EC350-1 tissue embedding center (Microm Inc. [Mound MN, USA]). Sections (5µm) were cut on an Olympus rotary microtome 4055, and stained with hematoxylin and eosin, in a Varistain Gemini ES automatic slide stainer [Thermo Scientific Miami, FL, USA].

Two fish per replicate (n=8 per treatment group) were sampled on days 3, 5, 7, 14 and 21 for histological examination. Total number of granulomas was quantified for a sagittal section of each fish. In addition, histologic tissue sections were scored semi-quantitatively for diffuse leukocytic infiltration (none, low, medium and high), stage of granuloma organization (none, diffuse inflammatory loci, epithelioid without a necrotic core, and epithelioid with necrotic core). Each category was assigned a rank number from 0-3 with 0 representing no evidence of a diseased state, and 3 having the most severe indications of disease. To avoid bias towards experimental groups, samples were randomly selected and scored blind. Values were then averaged for each experimental group (8 fish) and assessed for statistical significance (See below).

2.9 Phenanthrene Tissue Concentrations (Body Burdens)

Fish (2 per replicate tank) were sampled on day 1, 3 and 5 (n=8 per treatment per day). Analysis of phenanthrene in fish tissues followed previously described protocols for

tissue PAH concentrations in grass shrimp (*Palaemonetes pugio*) and zebrafish (Unger et al. 2007, Prosser et al. 2011). Briefly, individual fish were thawed, rinsed in deionized water, weighed and homogenized in 1 ml deionized water and 500 ng deuterated (d10) phenanthrene surrogate standard using Kontor® glass tissue homogenizers (Vineland, NJ, USA). Homogenates were then transferred to 50 ml Teflon centrifuges tubes, mixed with 2 ml of concentrated hydrochloric acid and 2 ml hexane, sonicated for ten minutes, and centrifuged (9000 rpm, 15 min). Hexane/HCl supernatant was transferred to a 15-ml glass tube. Homogenates were re-extracted, using the same sequence, with two additional ml of hexane. Extracts (~4 ml) were gently evaporated under a stream of nitrogen until the desired volume of 0.1 ml was attained. An internal standard of p-terphenyl was added (0.8 µg) before analysis on a 4D ion trap mass spectrometer (Varian, Walnut Creek, Ca, USA). Analytes and ions measured were p-terphenyl internal standard [152+230], phenanthrene D10 [187-189], phenanthrene [176-179], 1-hydroxyphenanthrene [165+194] and 3-hydroxyphenanthrene [165+194]. A seven point calibration curve was generated for all target analytes. Identification was based on matching retention time and spectra to standards (1-hydroxyphenanthrene: EQ Laboratories Inc. Augsburg, Germany; and 3-hydroxyphenanthrene: Cambridge Isotope Laboratories, Andover MA, USA) or to those in the NIST 05 mass spectral library.

As a way to quantify how efficiently phenanthrene was metabolized, we created a simple equation to measure metabolic efficiency (ME). ME, as defined here, is the ability of zebrafish to convert parent phenanthrene (p) to hydroxylated metabolites (m). This calculation used the measured tissue concentrations obtained from GC/MS analysis and was performed on individual animals. ME is the proportion of parent phenanthrene

in the total quantified sample (i.e. parent + metabolites). Thus, $ME=p/(p+m)$. As ME approaches 1, the parent compound is closer to constituting the entirety of the sample. This calculation allows us to normalize to individual fish regardless of age, sex, weight or dose and facilitated comparisons between treatment groups.

2.10 Water Quality

Exposure tanks were monitored daily for temperature, pH, dissolved oxygen (DO) and total ammonia. DO and temperature were monitored using an Oxyguard® Handy Polaris (Birkerød, Denmark). Salifort® box kits were used for analysis of ammonia and pH was measured with an Orion 250A pH meter with Thermo pH electrode (Waltham, Ma, USA).

2.11 Statistical Analysis

The dual exposure study was analyzed for differences in time to death using the Kaplan Meier method. This method (Newman, 1995) estimates survival function of an exposed group of individuals and allows comparison of two or more survival curves and evaluation of treatment effects. The log rank test was used to establish if there were significant deviations between survivorship curves. Bioaccumulation data, phenanthrene/metabolite ratios and semi quantitative histological data (e.g., severity of granuloma formation) were analyzed by the non-parametric Mann-Whitney U test. ANOVA was used to evaluate if aqueous phenanthrene concentrations differed significantly between replicates and treatments. All statistical analyses were performed using SAS software [Cary NC, USA].

3 Results

3.1 Water Quality

Water quality data are presented in Table 1. The mean values (+/- S.D.) for dissolved oxygen, pH, temperature and total ammonia were $6.9 \pm 0.9 \text{ mgL}^{-1}$, 6.5 ± 0.27 , 27.8 ± 1.4 , and <0.25 respectively. All water quality parameters remained within the physiological range for zebrafish for the study duration and were not considered to be stressful (Lawrence 2007).

3.2 Phenanthrene Water Concentration

Mean daily concentrations (+/- S.D.) were $179 \pm 100 \mu\text{g L}^{-1}$ for the “Phen” treatments and $157 \pm 110 \mu\text{g L}^{-1}$ for the “Phen+Myco” treatments (Figure 1). Average 12 hour loss of aqueous phenanthrene from all treatment groups was 60%. There were no significant differences in phenanthrene concentrations between replicate tanks for any treatment allowing replicates to be combined for survival analysis. Additionally, there were no significant differences between “Phen” and “Phen+Myco” treatments. Phenanthrene concentrations in all control and “Myco” tanks were repeatedly below detection limits ($1 \mu\text{g L}^{-1}$).

3.3 Fish Mortality

Narcotic effects from phenanthrene exposure were evident in fish exposed to phenanthrene regardless of mycobacterial infection. Clinical signs included erratic swimming and disorientation. Two days post phenanthrene exposure, all exposure groups returned to normal swimming patterns. This rapid recovery is consistent with animals which had been exposed to a Type I narcotic.

Twenty one day survivorship showed significant differences in mortality between treatment groups (Figure 2). Lowest survivorship occurred in the “Phen+Myco” groups with only 25% alive at the end of the study. This was significantly lower than the “Myco”

groups which exhibited 41% survival ($p < 0.01$) by the end of the study. Both of these treatments also differed significantly from the “Phen” groups which exhibited 88% survival ($p < 0.001$). There was 100% survivorship in the “Control” groups.

3.4 Tissue Concentrations

Mass spectrometry identified the presence and concentrations of parent compound (phenanthrene) as well as two phenanthrene metabolites, 3-hydroxyphenanthrene and 1-hydroxyphenanthrene in the fish tissues. Metabolic efficiency, a proportion of the measured phenanthrene tissue concentrations to total phenanthrene products (parent plus metabolites) was calculated as described in section 2.9. No significant differences were observed in ME between the “Phen” group and “Phen+Myco” group on any day, or over the course of the experiment (Figure 3). Within the “Phen” samples, the ME on day 1 was significantly higher than it was on day 5 ($p < 0.05$). The ME’s for the day 5 and 7 “Phen” samples were depressed but not significantly different from other treatment groups. No statistically significant differences were observed for ME between any of the “Phen+Myco” samples.

3.5 Histology

Fish were examined histologically to evaluate mycobacterial disease severity (granuloma number, degree of leukocyte infiltration) and phenanthrene associated pathology. Only fish infected with *M. marinum* displayed inflammation and granulomas. Over the course of the study, the inflammatory response to mycobacterial exposure progressed from an initial loosely organized leukocytic infiltration of the coelomic cavity at the early time points, to a more organized granulomatous inflammatory response

comprised of diffuse cellular infiltrates and discrete granulomas composed of epithelioid cell layers surrounding a zone of central necrosis (Figure 4).

Granulomas were evident initially associated with mesenteric exocrine pancreas; however, as the disease progressed temporally, granulomas also became evident within the liver, spleen and kidney. The earliest signs of granuloma formation occurred on day three post-infection, with one fish exhibiting early granuloma organization. However, it was not until day 14 that the disease was widespread in affected fish. By day 21 the coelomic cavity in both the “Myco” and “Phen+Myco” treatments exhibited severe granulomatous inflammation with breakdown of organ boundaries and tissue necrosis. Advanced granulomatous inflammation and tissue necrosis also made it difficult to distinguish between individual granulomas. No significant differences in the number of granulomas or their degree of organization were evident between any groups exposed to *M. marinum* over the first 14 days (Table 2). Because of the severe pathology by day 21, precise quantification of granulomas was difficult and so is an estimate in Table 2. However, this was consistent between *M. marinum* infected treatments. No significant differences were observed in total granuloma number, degree of inflammation or granuloma organization between any of the groups infected with *M. marinum*. No fish displayed any pathology that could be attributed to toxicant exposure (e.g., liver cell necrosis, megalocytosis; for review of toxicant induced pathologies see DiGiulio and Hinton 2008), an observation consistent with previous findings (Prosser et al. 2011)

3.6 CYP1A Analysis

Constitutive CYP1A levels in control fish (as pmol protein +/-S.D.) were 0.08 +/- 0.05, and were only examined on day 1. CYP1A concentrations for the “Phen”

treatments (pmol \pm S.D.) were 0.149 \pm 0.099, 0.054 \pm 0.007 and 0.168 \pm 0.193 for days 1, 3 and 5 respectively. Values for the “Phen+Myco” group were 0.059 \pm 0.004 and 0.230 \pm 0.228 for days 3 and 5 respectively. There were no significant differences observed between any of the treatment groups on any day, or over the five days sampled (Figure 5).

3.7 Plasma Glucose Analysis

On day 1, a significant difference in plasma glucose concentrations was observed between “Control”, and “Phen” groups ($p < 0.05$) (Figure 6). A significant difference was also observed between the “Phen” and “Control” treatments on day 5 ($p < 0.05$). Also on day 5, plasma glucose was significantly higher in “Phen” fish than in “Myco” fish ($p < 0.05$). No significant differences were observed between the “Phen+Myco” and “Phen” groups, or between the “Phen+Myco” and “Myco” groups on any day.

On day 3 there was a general elevation in plasma glucose levels above other days. For the “Myco” treatment, levels were significantly elevated beyond what was observed on days 5 and 7. The “Phen+Myco” group was significantly elevated on day 3 vs. day 14. Additionally, the day 3 “Control” levels, while not significantly above day one levels, were significantly higher than measured levels on days 5, 7 and 14.

4 Discussion

4.1 Survivorship

Previous research (Prosser et al. 2011) demonstrated an antagonistic relationship between the toxicant phenanthrene and bacterial disease challenge in dually-challenged zebrafish, resulting in significantly higher mortality in fish exposed to phenanthrene only. However, the duration of that study was short (14 days) and phenanthrene concentrations

were high (nominal: 450 μgL^{-1} , measured mean daily concentration: 284 μgL^{-1}). To further explore the role of dose, stressor order, and temporal scale in the current study additional dual-stressor challenges were conducted over a longer time scale (21 days) and with a more environmentally relevant target toxicant concentration (Target: 250 μgL^{-1} , mean daily concentration: 168 μgL^{-1})

In direct contrast to the prior study (Prosser et al., 2011), survival analysis showed significantly higher mortality in dually-challenged fish (“Phen+Myco”) than in all other treatments ($p < 0.001$). These findings are more consistent with previous research that has documented elevated mortalities in fishes simultaneously exposed to PAHs and infectious disease agents. Carlson et al. (2002) showed decreased survivorship in the Japanese medaka (*Oryzias latipes*) to the bacterium, *Yersinia ruckeri*, following IP injection of benzo[a]pyrene (BaP: 20 $\mu\text{g/g}$ body weight). In a followup study (Carlson et al., 2004), toxicant exposure alone (200 $\mu\text{g/g}$ body weight) was insufficient to kill fish over a 7 day period. Fish challenged only with *Yersinia ruckeri* exhibited <20% mortality. However, fish challenged concurrently with BaP and *Y. ruckeri* exhibited 70% mortality. Similarly, juvenile chinook salmon (*Oncorhynchus tshawytscha*), exposed to sub-lethal concentrations of the toxicants esfenvalerate or chlorpyrifos and challenged concurrently with infectious hematopoietic necrosis virus (IHNV) over a 21 day period (Clifford et al., 2005), displayed 68.5% mortality. Salmon exposed to IHNV or toxicant alone exhibited no mortalities. Arkoosh et al. (2001) showed higher mortalities in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) challenged with *Vibrio anguillarum* one week after IP injection of a sub-lethal mixture of ten high molecular weight PAHs than in salmon receiving only *V. anguillarum* challenge. These studies show clear synergistic interactive

effects between stressors in which dually-challenged fish exhibit significantly higher mortality than observed in individual exposures alone.

In contrast, with respect to zebrafish mortality, dual stressor interactions between phenanthrene and *M. marinum* occur in an additive, and not synergistic manner. This is supported by the survivorship and histological data. Additivity, in the case of multiple stressors, is defined as a measured effect (e.g., survivorship) that is roughly equivalent to the sum of the individual stressor effects. Conversely, synergy, also referred to as a multiplicative effect, occurs when the impacts of multiple simultaneous stressors significantly exceed the sum of the impacts of the individual stressors (Newman and Unger 2003). I have clearly demonstrated that the concentrations of *M. marinum* and phenanthrene used in this study are sufficient to cause mortality alone. By summing the mortalities for the individual “Phen” and “Myco” treatments, we can predict what survivorship for the dual challenge should be assuming an additive mode of action. Over the last five days of the experiment, summation of mortalities in the “Phen” and “Myco” groups yielded predicted values of 41%, 49%, 61%, 70% and 80% for the dual challenge. Measured mortalities for the “Phen+Myco” group were 52%, 61%, 67%, 71% and 75%. The predicted values are equivalent with measured values, and therefore suggest an additive effect due to combined phenanthrene toxicity and *M. marinum* mortality.

Additional support for an additive mode of action between stressors comes from the histological data. Histological examination for both the “Phen+Myco” and “Myco” groups shows no significant difference in the number of granulomas/fish, or severity of inflammation for any sampling day. As *M. marinum* disease severity is correlated with numbers of granulomas, (Watrall and Kent, 2006) this suggests that disease progression

and severity were similar in the experimental groups and not influenced by toxicant exposure. Although phenanthrene exposure did not influence disease severity or degree of inflammatory response, I did not directly measure individual immune parameters (either innate or acquired) and therefore cannot rule out other subtle immuno-modulatory influences of phenanthrene not expressed at the morphologic level.

4.2 Body burden/CYP1A analysis

To investigate if phenanthrene biotransformation was modulated by the infectious disease process under the current experimental conditions, contaminant metabolism and CYP1A levels were measured and compared to previous results (Prosser et al., 2011), which demonstrated a clear modulation of phenanthrene metabolism in *M. marinum* exposed fish. By examining the metabolic efficiency, I was able to determine what proportion of total phenanthrene was in parent form. This gave insight into how efficiently animals were able to metabolize the toxicant, and allowed for a quantitative comparison between treatments. In the current study, biotransformation of phenanthrene was not altered during the course of the experiment, regardless of *M. marinum* infection. Additionally, no significant differences were observed in CYP1A levels between any of the treatments. Although this study shows no significant differences in phenanthrene metabolism between any of the treatments, previous researchers have shown the ability of inflammatory cytokines to suppress toxicant metabolism in fishes *in vivo* (Chambras et al., 1999; Marionnet et al., 2006; Reynaud et al., 2005; Reynaud et al., 2008). In a previous study (Prosser et al. 2011) the results showed significantly lower phenanthrene metabolism in zebrafish exposed concurrently to phenanthrene and *M. marinum* infection

than in fish exposed to phenanthrene alone. The specific mechanisms responsible for the observed differences between these two studies are not known, but may be the result of different experimental protocols (e.g., lower phenanthrene concentration, order of challenge). These differences highlight the complexity of stressor pathway interactions, and underscore the need to gain better insights into the mechanisms involved.

4.3 Stress Response

The effects of the integrated stress response on fishes are multiple and complex, with both immunosuppressive and immunostimulatory effects having been reported (Bonga 1997). Although cortisol is the dominant stress hormone traditionally measured in vertebrates, the small size of zebrafish leaves this measurement prone to extraction error. Plasma glucose has been suggested as a secondary indicator of the stress response in fishes (Bonga 1997), and I therefore chose this as a surrogate for assessing the integrated stress response. Had I observed excessively high, or consistently high glucose levels, it could have suggested the integrated stress response was active and potentially skewing our results. However, I did not observe significant differences in plasma glucose levels between the “Phen+Myco” treatment and either the “Myco” or “Phen” treatments on any day measured. Therefore I suggest the stress response did not influence the observed differences in survivorship between those treatments. On day 3, all four groups were elevated above other sampling days. This was most likely due to the physical manipulations of the fish associated with IC injections which had been conducted the day prior. This elevation in plasma glucose supports the view that plasma glucose is a possible surrogate for measuring the integrated stress response (for review see Bonga 1997).

Results of this study differ significantly from the previous work showing the highest mortality in fish exposed to phenanthrene alone relative to dually challenged fish (Prosser et al. 2011). I believe these differences may be the result of a lower phenanthrene dose. The mean daily water concentration was $157 \pm 110 \mu\text{g L}^{-1}$ (\pm S.D.) compared to $284 \pm 187 \mu\text{g L}^{-1}$ in the previous experiment (Prosser et al. 2011). The original work showed a depression in phenanthrene metabolism (based on ME ratios) for dually challenged fish relative to the “Phen” group suggesting that the phenanthrene metabolites were more toxic (Hawkins et al, 2001) and were significant contributors to increased mortality (Prosser et al, 2011). Therefore, the reduced mortality observed in the current study for the “Phen” exposure group is most likely due to a reduction in toxic metabolites produced under the lower exposure concentrations used.

As previously stated, in the original study (Prosser et al. 2011) dually challenged fish demonstrated a suppression of the biotransformational pathway which I hypothesize was mediated by pro-inflammatory cytokines. Here I postulate as to why this phenomenon was not observed in the current study. Most CYP reactions follow hyperbolic saturation kinetics and can be modeled using Michaelis-Menton analysis (Korzekwa et al. 1998). At concentrations below cell saturation, the amount of CYP1A enzyme actively involved with biotransformation is dependent on the amount of phenanthrene present within a cell (i.e. as the number of toxicant molecules decreases, there are fewer substrates on which the enzymes can act). In the original study, an excessively high concentration of phenanthrene (measured $285 \mu\text{g L}^{-1}$) likely resulted in near saturation with reaction rate occurring close to its maximum velocity (V_{max}). Due to the hyperbolic nature of saturation kinetics, any disruption of this process would have

dramatic effects on metabolite production, and be readily calculated using ME.

However, in the current study, the concentration of phenanthrene was considerably lower (measured $157 \mu\text{g L}^{-1}$), and most likely well below saturation. Thus there would be less enzyme actively involved with metabolism, and more free, unbound protein available. In this sense, a disruption of metabolism would not be as readily seen because below saturation enzyme is in excess and available to compensate for any perturbations to the pathway.

The duration of experimental exposures can also influence the observed importance of stressor interaction on mortality. In the original study, a deviation in survivorship between the dually challenged fish, and the "Myco" group was observed towards the end of the study (14 days). Although not statistically significant, I felt that a longer study duration would allow for chronic effects to be elucidated. The current work therefore was extended for an additional 7 days. During this extension, I observed a significant difference in survivorship between the "Myco" and "Phen+Myco" groups ($p < 0.01$). This suggests that exposure duration is a significant factor in dealing with dual-stressor exposure.

The purpose of exposure studies is to gain a better understanding of how organisms respond to perturbations within their environment. However, single stressor laboratory exposures fail to account for the complexity of the natural environment where organisms are exposed to multiple stressors simultaneously. My goal, in using multiple stressor laboratory experiments, was to more closely mimic adverse impacts fishes may encounter in natural settings. It is evident from this work and prior investigations that multiple stressors can result in complex interactions leading to antagonistic, synergistic or

additive effects. Furthermore the studies have demonstrated that changes in toxicant concentration or exposure duration can alter these interactions (i.e. antagonistic → additive) resulting in dramatically different survivorship and conclusions. These studies suggest that stressor interactions are complex and difficult to predict *a priori*.

Currently, the majority of ecotoxicological risk assessments are based on simplistic, single stressor exposure studies that are used primarily because they are quick, easy and cheap (Calow and Forbes 2003). The majority of these studies are short term and do not take into account chronic exposures or additional stressors. To compensate for this, safety factors are often employed to account for uncertainties related to assessing chemical risk; however, safety factors are an arbitrary empirical method having no corroborative evidence (Chapman et al. 1998). This represents a major shortcoming in the standard practices of regulatory ecotoxicology. In reality, different pathogens, toxicant, dose, and duration of exposure all significantly influence stressor interaction and survivorship. My studies have shown that simple changes in the dose and/or exposure duration of phenanthrene can greatly influence stressor interaction with either an antagonistic or additive relationship being the ultimate outcome. Similar dose dependent synergism/antagonism has been reported with toxicant mixtures (Jonker et al. 2005). Moreover, pathogen type may greatly influence stressor interactions. *M. marinum* elicits a cell-mediated immune response characterized by granulomatous inflammation, a response that is very different from the humoral immune response elicited by many other pathogens (e.g. gram⁻ bacteria such as *Vibrio* spp.). The assumption that ecological risks can be compensated for by the use of safety factors promotes uncertainty and possible errors for risk assessment. With better elucidation of stressor interactions and how they

influence the “true” risk to organisms, more accurate regulatory guidelines may be developed. Multiple stressor studies, although still nascent, represent an ever growing and important field of study. This work demonstrates the continued need for detailed, controlled laboratory studies designed to further elucidate the complex interactions that can be better compared to the natural environment.

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Table 1: Water quality data for treatment group tanks

	Mean	+/-
DO	6.9	0.9
pH	6.5	0.27
C°	27.8	1.4
Ammonia	<0.25	0

Table 2: Granuloma counts and semi quantitative histological data for Day 3, 5, 7, 14 and 21. Granulomas were counted as the number of visible granulomas per fish. Other categories were ranked from 0-3 with 0 indicating no pathology observed and 3 indicating a high degree of pathology observed. Table values are the mean rank for each experimental group (St. Dev). No significant differences were calculated between any of the experimental groups for a given day

Number of Granulomas					
Day	d3	d5	d7	d14	D21
Control	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Phenanthrene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>M. marinum</i>	0,13(0 35)	0(0)	2(5 3)	4 1(2 8)	11(10 9)
<i>Phenanthrene+M. marinum</i>	0(0)	2 7(6 3)	0 38(0 7)	5 8(4 4)	11(6 5)
Granuloma Organization					
Day	d3	d5	d7	d14	D21
Control	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Phenanthrene	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>M. Marinum</i>	0 13(0 35)	0(0)	0 5(1 1)	1 4(0 9)	2(1 4)
<i>Phenanthrene+M. marinum</i>	0(0)	0 7(1 2)	0 4(0 7)	1 3(0 8)	2 8(0 5)
Degree of Inflammation					
Day	d3	d5	d7	d14	D21
Control	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)
Phenanthrene	0 (0)	0 (0)	0 2(2)	0(0)	1 3 (1 1)
<i>M. marinum</i>	0 25(0 7)	0(0)	0 6(0 7)	2 4(0 5)	2 1(0 9)
<i>Phenanthrene+M. marinum</i>	0(0)	0 57(1 0)	1 1(0 8)	2 1(0 7)	2 6(5 5)

Figure 1: Daily fluctuations in phenanthrene concentration (“Phen”-♦-, “Phen+Myco” - ■-). Phenanthrene solutions were made fresh every 24 h with additional phenanthrene spikes added at time 12 h each day. Error bars represent standard deviations. No significant differences were observed between experimental groups.

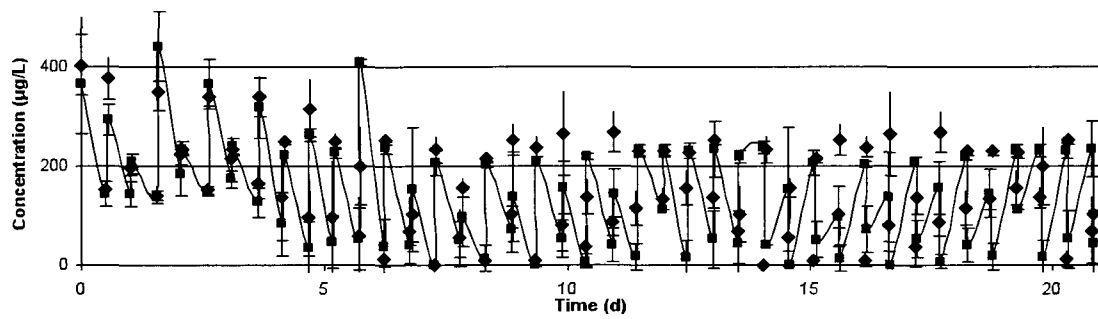


Figure 2: Kaplan–Meier survivorship curves for zebrafish, *D. rerio*, exposed to phenanthrene and *M. marinum*. Survival in fish exposed to phenanthrene + *M. marinum* was significantly lower than all other treatments. Error bars: standard error of the mean. Key: “Phen+Myco” (-◆-), “Phen” (-■-), “Myco” (-▲-), Control (-x-). Letters denote significant statistical difference (Kaplain Meier $p < 0.05$).

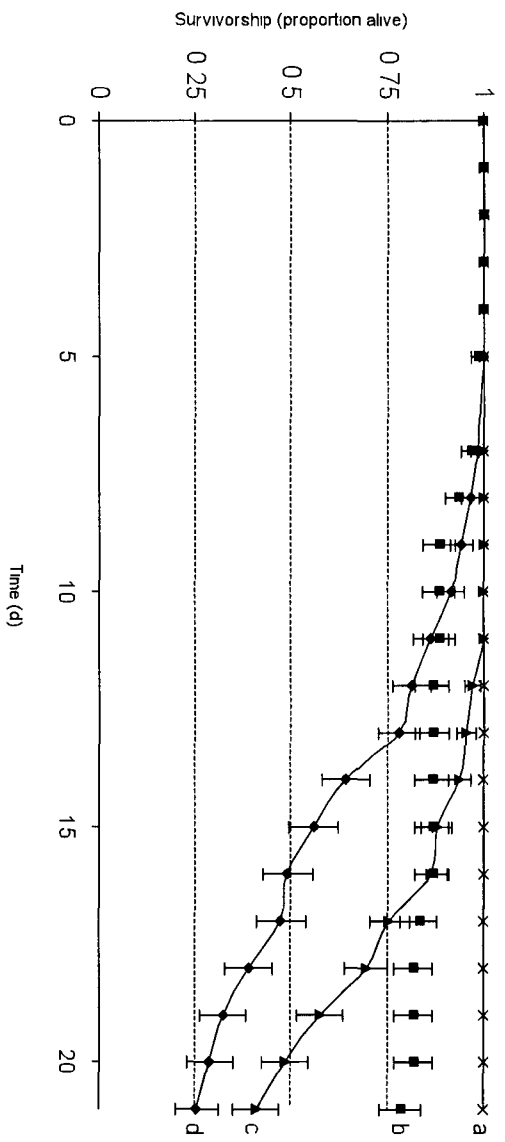


Figure 3: Metabolic efficiency (ME) in zebrafish, *D. rerio*, exposed to phenanthrene and *M. marinum* over 21 days. Error bars represent 95% confidence limits. Key: horizontal patterning represents the “Phen” group. Vertical patterning represents the “Phen+Myco” group.

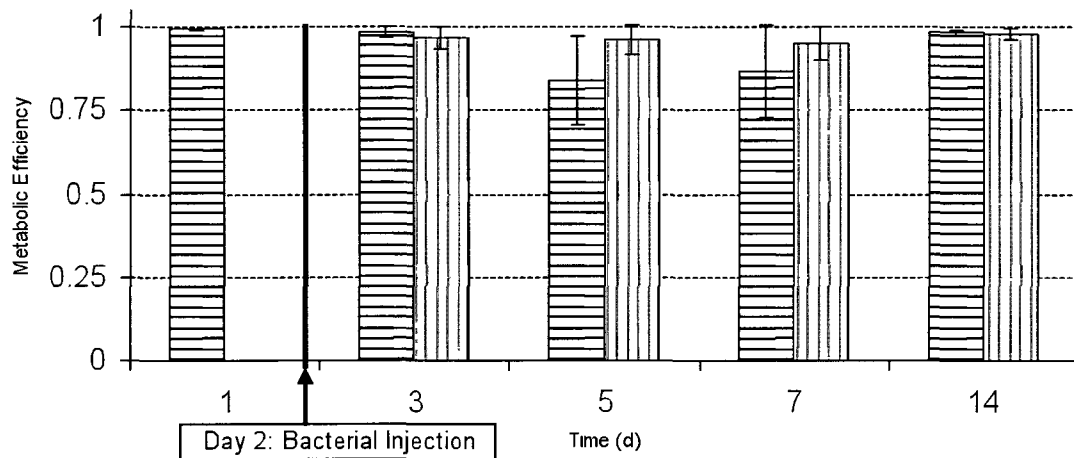


Figure 4: Progression of *Mycobacterium marinum* granulomatous inflammation A) loose association of inflammatory cells with no defined organization, but often with areas of inflammatory cell foci (Arrows). B) well organized granulomas comprised of epithelioid cells (arrowhead) and exhibiting central necrosis. 200x

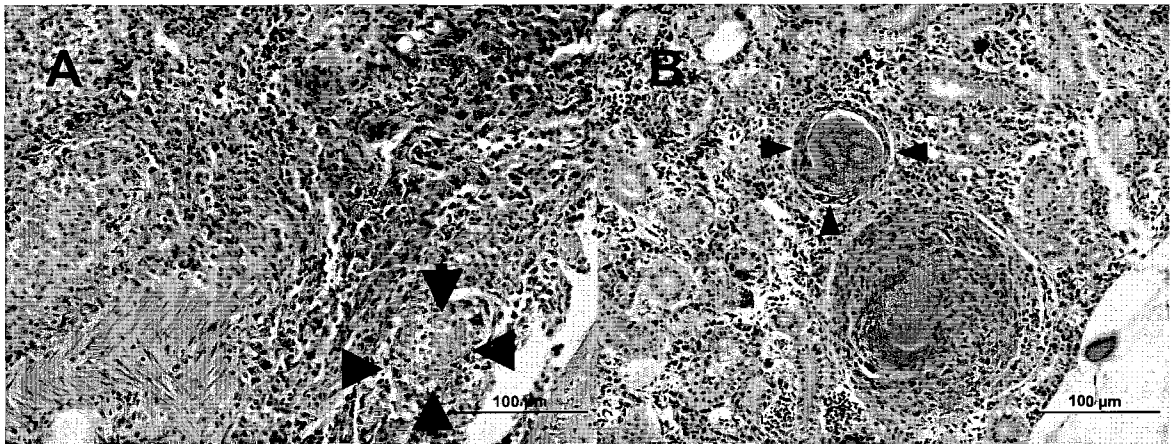


Figure 5: Hepatic CYP1A levels for zebrafish in various treatments over the first five days of the study. No significant differences were observed between treatments on any day. No significant differences were observed between treatments and control on any day. Error bars represent 95% confidence limits. Key: slanted lines represent the control treatment; vertical lines represent phenanthrene only exposed fish; checkered pattern represents the fish exposed to phenanthrene + *M. marinum*.

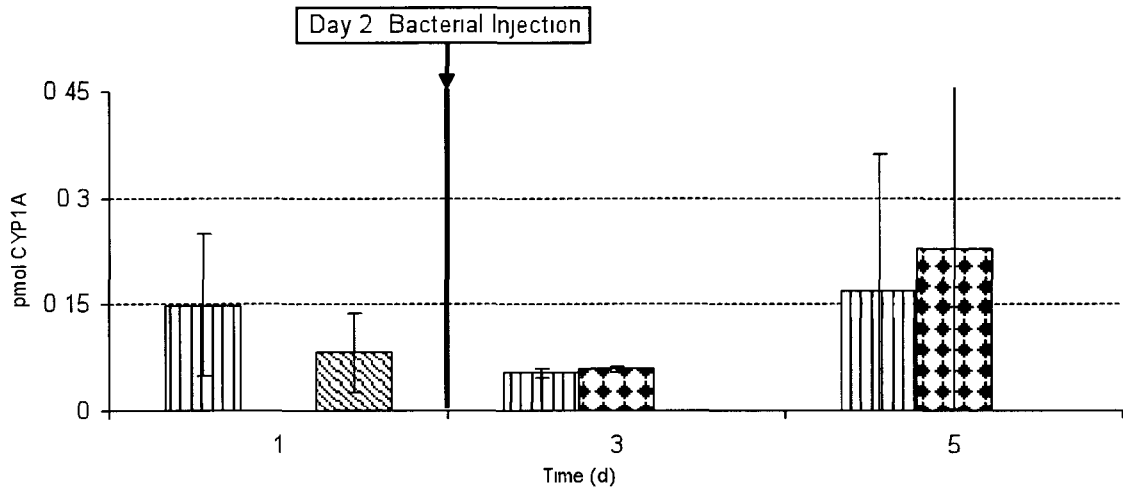
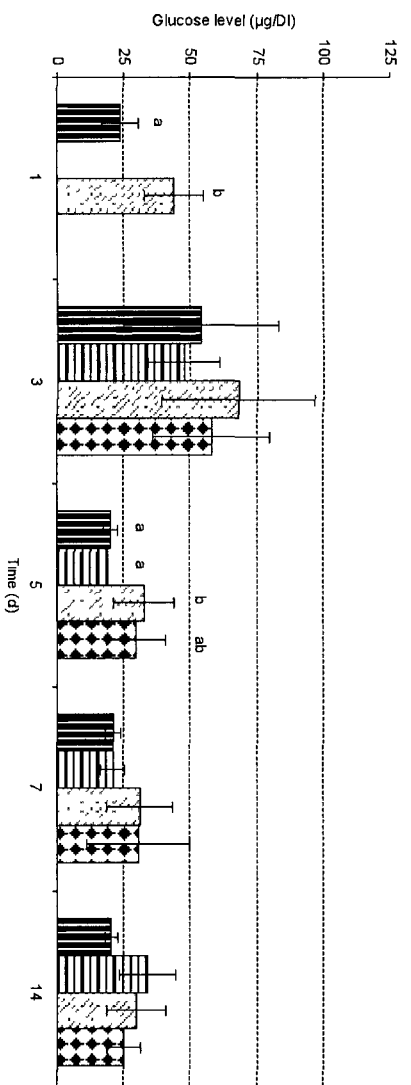


Figure 6: Plasma glucose levels for zebrafish over the first 14 days of the study. Different letters represent significant differences for that day ($p < 0.05$). Error bars represent 95% confidence limits. Key: horizontal bars represent controls; vertical bars represent *M. marinum* only treatment; slanted bars represent the phenanthrene only treatment and checkered pattern represents the phenanthrene + *M. marinum* treatment.



CHAPTER 4

Comparative pathogenicity of *Mycobacterium pseudoshottsii* and *Mycobacterium marinum* in the zebrafish (*Danio rerio*)

Introduction

Mycobacteriosis, a chronic bacterial disease caused by gram⁺ bacteria belonging to genus *Mycobacterium*, is common in both wild and aquacultured finfishes. This disease was first described in carp (*Cyprinus carpio*) (Bataillon et al 1897) and since that time it has been reported to occur in over 150 different fishes (Gauthier and Rhodes 2009). Until recently, the only recognized causative agents for the disease in fishes were three species, *Mycobacterium marinum*, *M. chelonae*, and *M. fortuitum* (Decostere et al 2004), however, recent findings indicate that a number of other mycobacterial species, including several that are new to science, also cause disease in fishes (See review in Gauthier and Rhodes 2009). Two species recently isolated from Chesapeake Bay striped bass (*Morone saxatilis*), *M. shottsii* and *M. pseudoshottsii*, are believed to be the predominant etiologic agents of the disease in this economically important finfish (Rhodes et al. 2005).

Mycobacteriosis in fishes can be characterized by non-specific gross clinical signs (emaciation, fin erosion, scale loss, dermal ulcerations and pale gray to tan nodular lesions in the visceral organs) (Gauthier and Rhodes, 2009), and by a granulomatous inflammatory response (Decostere et al. 2004), which can be observed using standard H&E histological techniques. Granulomas are spherical lesions ranging in structure from poorly organized inflammatory foci comprised of lymphocytes and macrophages with minimal epithelioid cell formation to lesions with highly eosinophilic, necrotic centers

surrounded by a layer of tightly apposed epithelioid macrophages (Gauthier and Rhodes, 2009). Ziehl-Neelsen staining often reveals the presence of acid-fast bacilli residing within granulomas.

Mycobacterium marinum is one of the most commonly reported and most widely studied mycobacterial pathogens of fishes worldwide (Hedrick et al. 1987, Kent et al. 2004, Watral and Kent 2007, Gauthier and Rhodes, 2009). It is a ubiquitous pathogen and has been found in both fresh and salt water environments (Ostland et al. 2008). However, its pathogenicity is dependent on both strain (van der Sar et al. 2004), and host factors (Ostland et al. 2008). Ostland et al. (2008), using eight *M. marinum* isolates observed differences in disease severity between zebrafish (*Danio rerio*) and hybrid striped bass (*Morone chrysops x Morone saxatilis*). In addition, Broussard and Ennis (2007) observed differential survivorship in zebrafish and medaka (*Oryzias latipes*) exposed to the same strain and bacterial density. *M. marinum* has also been widely established as a model organism for studying mycobacteriosis. Broussard and Ennis (2007) showed that *M. marinum* infection in the Japanese medaka (*Oryzias latipes*) forms chronic granulomatous inflammation and may act as a surrogate for studying tuberculosis in humans. Similarly, the zebrafish-*M. marinum* model has been used compare aspect of the host immune response in the fish to that of humans with tuberculosis (Swaim et al. 2006).

During the late 1990's, a major epizootic of mycobacteriosis was identified in Chesapeake Bay striped bass (Vogelbein et al. 1998). This outbreak currently poses a serious threat to stock health with >70% of the resident schooling fish showing clinical signs of disease (Gauthier et al. 2008). Recent work has identified a new species of

Mycobacterium, *M. pseudoshottsii*, as an important etiologic agent of the disease in the striped bass (Rhodes et al. 2001). *M. pseudoshottsii* is closely related to *M. marinum*, sharing >99% of the 16S rRNA gene sequence (Rhodes et al. 2005). *M. pseudoshottsii* has also been found to possess the insertion sequences IS2404 and IS2606, previously thought to be specific to only *M. ulcerans*, the causative agent of Buruli ulcer in humans (Ranger et al. 2006). Additionally, *M. pseudoshottsii* has been shown to produce mycolactone F, a unique secretory toxin (Ranger et al 2006). This mycolactone has a molecular structure similar to that produced by *M. ulcerans* but has been shown to be less toxic to mouse fibroblasts *in vivo* (Ranger et al. 2006). *M. pseudoshottsii* is the only known mycobacterial species from Chesapeake Bay fishes capable of producing mycolactone. Despite its high prevalence in Chesapeake Bay striped bass, little is known about the pathogenicity of *M. pseudoshottsii*, or how its pathogenicity compares to that of the better characterized *M. marinum*.

The goal of this study was to describe the comparative pathogenicity of *M. pseudoshottsii* and *M. marinum* in the zebrafish (*Danio rerio*). Zebrafish were selected as our model species because they have been shown to be a natural host for *Mycobacterium spp.* Outbreaks of mycobacteriosis in zebrafish colonies at various research facilities have been caused by *M. marinum* as well as other species, however, *M. pseudoshottsii* infections have not been reported (Watrall and Kent, 2006; Ostland et al., 2008). Additionally, the zebrafish has been suggested as a model for the study of piscine mycobacteriosis in general (Prouty et al. 2003), and human tuberculosis specifically (Swaim et al. 2006). This was suggested for several reasons including the ability to induce both chronic and acute infections, the similarity in granuloma structure to human

subjects with tuberculosis, and the extensive knowledge of the zebrafish genome. In our study, we used the M30 (isolated from Chesapeake Bay striped bass) and ATCC#BAA-535 (Human isolate) of *M. marinum*, and the L15 strain of *M. pseudoshottsii* (Chesapeake Bay striped bass isolate) to evaluate their relative virulence and pathogenicity. M30 has been shown in our laboratory to be extremely virulent in zebrafish with a 14 day LD₅₀ of 2x10³ bacteria/fish by IC injection (Prosser et al., 2011). In contrast, nothing is known about the virulence of either ATCC#BAA-535 or L15 in zebrafish.

2 Materials and Methods

2.1 *Experimental Animals*

Adult zebrafish were purchased from a local pet store “Luv-a-pet” (Virginia Beach, VA). Newly acquired fish were quarantined for one month prior to introduction into the colony. Fish were maintained in an Aquatic Habitats © (Apopka, FL) recirculating system. Reverse osmosis (RO) water was used that had been adjusted to a conductivity of approximately 1000 µS/cm and a pH of approximately 7.5 using Instant Ocean ® (Madison, WI) salts. Ten individuals were randomly selected, euthanized by overdose with MS-222 (Tricaine methanesulphonate), necropsied and evaluated histologically for the presence of infectious diseases including mycobacteriosis to prevent their introduction into the experimental colony.

2.2 *Experimental Design*

Fish were placed into 30L glass aquaria and allowed to acclimate for one week. Water was maintained at 28°C with individual 200W aquarium heaters (Aquatic Inc. Moorpark, CA), and total ammonia, pH, and dissolved oxygen were monitored daily to ensure they were within physiological limits for zebrafish as established by Lawrence

(2007). Hydrosponge filters (10 gal) (Aquarium Technology Inc. Decatur, GA) were used for biological filtration. Partial water changes were conducted at 3 day intervals, or as needed by syphoning of detritus that accumulated on the bottoms of the tanks. Fish were randomly divided into four treatments, each comprised of three replicates: control (3x n=20), *M. marinum* M30 strain (3x n=20), *M. marinum* ATCC #BAA-535 strain (3x n=30) and *M. pseudoshottsii* (3x n=30). Prior to injections, zebrafish were lightly anesthetized using MS-222 (150mg/L). Fish were injected intracoelomically (IC) with 18µl of bacterial inoculum (~1500 CFU/fish) using a 1cc syringe attached to an Eppendorf Repeater plus pipetter (New York, NY), with the controls receiving sterile phosphate buffer (PB) (pH 7.4). Fish were monitored for 24 hours post injection with dead fish removed at the time they were observed. Fish were maintained for 28 days with dead animals removed and placed into Z-Fix histological fixative (Anatech LTD) after making a ventral incision into the coelomic cavity. At 28 days, the remaining live fish were euthanized and placed into fixative for histological examination.

2.3 Bacterial preparation

Bacterial cultures of *M. pseudoshottsii* (L15 strain) and *M. marinum* (M30 strain) used in this study were originally isolated from the spleens of diseased Chesapeake Bay striped bass, *Morone saxatilis*, as described previously (Rhodes et al., 2004). The ATCC #BAA-535 strain of *M. marinum* was originally isolated from humans (Clinical Lab, Moffett Hospital, University of California, San Francisco) and obtained from ATCC (Manassas, VA). Archived samples of this culture were inoculated aseptically into Middlebrook 7H9 broth with OADC enrichment and incubated at 30°C for two weeks. The culture in log growth phase was centrifuged at 10,000 x g for 15 minutes to obtain a

cell pellet. The supernatant was aspirated and the pellet was re-suspended in 2 mL Butterfield's phosphate buffer (PB) (pH 7.4) with 0.05% Tween 80. To reduce adhesion and clumping of bacteria, the culture was vortexed for approximately 30 seconds with sterile glass beads (~50µm diameter). An additional 5 mL of PB was added and the optical density was measured at 590 nm. The desired OD of 0.1, representing approximately 5×10^4 bacteria/µl, was achieved by adjusting with additional PB. Serial ten-fold dilutions were prepared to obtain the desired bacterial density (75 bacteria/µL). Plate counting on MDA was used to quantify actual bacterial densities.

2.4 Histology

Standard histological techniques were used (Prophet et al. 1992). Briefly, fish allocated for histology were euthanized by overdose with MS-222 (500mg/L). A small mid-ventral incision was made into the abdomen to allow for fixative penetration and the entire fish were then placed into Z-fix, a buffered zinc formalin fixative (Anatech LTD, Battle Creek, MI) for at least 48 hours. Fish were subsequently decalcified overnight in a 50:50 formic acid: sodium citrate solution, rinsed in running tap water for 3 hours, bisected sagittally using a single-edged razor and placed into uniquely labeled tissue cassettes. Tissues were dehydrated through a graded ethanol series, cleared and infiltrated in hot paraffin wax in a Shandon Excelsior Tissue Processor (Miami FL, USA), after which they were embedded on a Microm EC350-1 tissue embedding center (Microm Inc. [Mound MN, USA]). Sections (5 µm) were cut on an Olympus rotary microtome 4055, and stained with hematoxylin and eosin, in a Varistain Gemini ES automatic slide stainer [Thermo Scientific Miami, FL, USA]. Additional slides were hand stained using the Ziehl-Neelsen technique for acid-fast bacteria.

Histologic tissue sections were scored semi-quantitatively for disease severity (0-3: none, low, medium and high) and degree to which bacilli were contained within granulomas (0-4: no containment, low containment, moderately contained, highly contained and completely contained). Disease severity was estimated based on the number of granulomas present and quantity of bacilli observed with Ziehl-Neelsen staining. In addition, samples were analyzed for how often bacilli were found in various tissue types. To avoid bias towards experimental groups, histologic slides were randomly selected and scored blind. Values were then averaged for each experimental group and assessed for statistical significance (See below).

2.5 Statistical analysis

Exposure groups were analyzed for differences in time-to-death by the Kaplan-Meier method. This method estimates survival function of an exposed group of individuals (Newman, 1995) and allows comparison of two or more survival curves and evaluation of treatment effects. Log-rank test was used to determine significant deviations between survivorship curves. Semi-quantitative data were compared using the nonparametric Mann-Whitney U test. Statistical analyses were performed using SAS software [Cary NC, USA].

3 Results

At 28 days post-infection, the *M. marinum*-infected fish (Both ATCC and M30 strains) exhibited significantly higher mortality than the controls and *M. pseudoshottsii*-infected groups ($p < 0.0001$) (Figure 1). Only one fish infected with ATCC #BAA-535 *M. marinum* (1.1%) and eight fish (13%) infected with M30 *M. marinum* survived to the end of the study. Little mortality was seen in control and *M. pseudoshottsii* exposure groups,

with 93.3% and 96.5% survival respectively, by 28 days post-infection. The M30 and ATCC groups did not significantly differ from each other, nor did the control and *M. pseudoshottsii* groups.

The high mortality observed in the *M. marinum* infected fish resulted in a low sample size for histological examination (ATCC n=1; M30 n=8); however, both *M. marinum* strains elicited severe visceral disease with extensive granulomatous inflammation in 8/9 surviving fish examined at 28 days post-infection (Table 1); however, one *M. marinum* infected fish did not display any histological signs of disease. Granulomas were observed in liver, spleen, kidney, pancreas, and gonads (Table 2). Granulomas in these fish ranged in morphology from the occasional loosely organized, early inflammatory cell foci with irregular undefined boundaries accompanied by diffuse prominent inflammatory infiltrates in the surrounding mesenteric tissues (Figure 2), to the more common well organized, typical granulomas comprised of multi-layered, tightly apposed epithelioid cells with necrotic centers (Figure 3). In 66.6% (6/9) of these fish granulomatous inflammation was severe enough to result in peripheral organ damage with extensive fibrosis and fusion of visceral organs and the affected mesenteric tissues (Figure 4). *M. marinum* bacilli were located predominantly within granulomas, within the more peripheral epithelioid cells or in the immediate proximity to the granuloma within free macrophages (Figure 5). In some instances, areas of extensive inflammation were observed (Figure 6A, B). These areas often had small leukocytic foci (perhaps precursors to granulomas), but no overt granulomatous inflammation was observed (6A). Ziehl-Neelsen staining revealed no acid fast bacilli in the loose inflammatory infiltrate, but individual bacteria were occasionally observed within the leukocytic foci (6B). Acid-

fast bacilli were not observed extra-cellularly in any of the fish infected with *M. marinum*.

Based on observable acid-fast bacilli, zebrafish response to *Mycobacterium pseudoshottsii* was varied. One fish displayed no disease signs and 15 fish (50%) showed only minor pathology. Another 11 fish (36.3%) displayed moderate infection and 3 fish (10%) exhibited severe pathology and infection (Table 1). Additionally, the degree of containment of the bacteria within granulomas was limited, and significantly less than that observed in *M. marinum* ($p < 0.05$) (Table 1). *M. pseudoshottsii* was systemic, with bacilli observed in the heart, pancreas, liver, gut wall, kidney, spleen, with large bacterial colonies also observed free in the gut lumen (Table 2). The proportion of fish with bacteria found in the pancreas was similar to that of *M. marinum*; however fewer fish were found to have bacteria in the liver, kidney and gonads. Conversely, more fish were observed to have bacteria in the heart, gut wall, and gut lumen, with free bacteria also being observed.

Fish infected with *M. pseudoshottsii* exhibited significantly lower mortality (Fig 1) and distinctly different pathology from what was observed in the *M. marinum* infected fish. Granulomatous inflammation was significantly reduced in these fish, with well-formed epithelioid granulomas being rarely observed. Ziehl-Neelsen staining confirmed the presence of acid-fast bacilli in 96.7% (29/30) of fish examined, in many cases extremely high numbers of bacilli were present. However, only 43% of the fish (13/30) exhibited organization of the leukocytic infiltrate into clearly defined epithelioid granulomas, and even in these fish the number of granulomas was significantly lower than in the *M. marinum*-infected fish. When granulomas were present, they were

generally much more poorly organized, smaller in size, and mostly lacking in epithelioid differentiation. Most notable however was the inability of the granulomatous response to effectively contain bacteria (Figure 7). This ineffectual containment often resulted in large areas of diffuse leukocytic infiltration and cellular necrosis containing large numbers of acid-fast bacteria in absence of any granulomatous inflammation (Figures 6C, D). Although similar areas of more diffuse inflammation were occasionally observed in *M. marinum* infected fish, acid-fast bacilli were rarely observed (Figures 6A, B). The lack of an organized granulomatous response to infection stands in sharp contrast to what was observed in *M. marinum*-infected fish.

In contrast to *M. marinum*, *M. pseudoshottsii* was commonly observed within isolated free macrophages. Individual isolated macrophages often contained large numbers of acid-fast bacteria within single large phagolysosomes (Figure 8). However, large numbers of extracellular bacteria were observed in 26.6% (8/30) of fish in areas of intense inflammation (Figure 9). Leukocyte lysis could be observed, with acid-fast bacteria being shed into the intercellular space (Figure 10). A very striking observation was what appeared to be the trafficking of acid-fast bacteria from the coelomic space across the alimentary tract serosa, muscularis and mucosa cavity ultimately to be shed into the gut lumen. About 57% (17/30) of the fish exhibited large colonies of acid-fast bacteria within the gut lumen. (These animals are not included in the percentage of animals previously mentioned to have “free [extracellular] bacteria”). These large colonies were usually observed in close proximity to acid-fast bacteria breaching the alimentary tract wall from the coelomic side (Figure 11). Acid-fast bacilli within the gut lumen were often smaller than those located within the visceral tissues and coelomic

cavity. This suggests rapid proliferation by these organisms. *M. pseudoshottsii* also appeared to employ both an intra- and extracellular lifestyle with the ability to replicate within leukocytes and within the gut.

4 Discussion

The results show significant differences in both survivorship and pathology of zebrafish experimentally infected with *Mycobacterium marinum* and *M. pseudoshottsii*. This was a highly surprising result as these two species have been shown to be very closely related, sharing >99% of the 16S rRNA gene sequence (Rhodes et al., 2005). Both strains of *M. marinum* caused a predictable, intense granulomatous inflammation and extensive tissue damage, mostly as a result of necrosis associated with fibrosis and fusion of the visceral organs. In contrast, the host cellular response to *M. pseudoshottsii* was poorly organized and with only minor granuloma formation and organization. Instead, bacteria were proliferating actively within isolated macrophages and widely disseminated throughout a poorly organized leukocytic infiltrate. Extensive and severe cellular necrosis was a common observation in these fish. The host inflammatory response to *M. pseudoshottsii* appeared to be largely ineffectual with bacteria appearing to be capable of extensive replication within macrophages, ultimately causing their lysis, and release into the coelomic cavity. These characteristics of the infection were not observed in *M. marinum*-infected zebrafish. Surprisingly, despite the extensive bacterial proliferation and associated tissue damage, mortality in *M. pseudoshottssi*-infected fish was not significantly different from the control fish over the 28 day study period. The extensive inflammation and tissue necrosis observed in *M. pseudoshottsii* infected fish suggests these animals would be experiencing mortality udy been lengthened. The

results of this study contrast with previous research in which the lowest mortality was observed for those *Mycobacterium* spp. inducing the most vigorous and well-formed granulomatous inflammatory response and highest mortality (virulence) in those species that caused a chronic, diffuse peritonitis (Ostland et al. 2008; Watral and Kent, 2006). Watral and Kent (2006) examined several species of mycobacteria infecting zebrafish research facilities and reported the most robust granulomatous response in the least pathogenic species (*M. marinum* ATCC-927) of the ones investigated. Similarly, Ostland et al. (2008) observed the most pathogenic strains of *M. marinum* infecting zebrafish to have fewer well organized granulomas as compared to the less pathogenic strains.

The underlying mechanisms responsible for the lower virulence of *M. pseudoshottsii* in this study are not as yet known, but may be related to the ability of *M. pseudoshottsii* to secrete a unique plasmid-encoded macrolide toxin, mycolactone F. The better studied *Mycobacterium ulcerans* also produces a mycolactone toxin. The mycolactone secreted by *M. ulcerans* is an immunosuppressive toxin and the causative agent of an extracellular cutaneous infection, Buruli ulcer, in humans (Adusumilli et al. 2005). Buruli ulcers are characterized by severe cellular necrosis at the site of infection (van der Werf et al. 1999). The mycolactone secreted by *M. ulcerans* is structurally very similar to that secreted by *M. pseudoshottsii*, and thus it is tempting to speculate that their functions may be similar (Ranger et al. 2006). Mycolactone F has an identical core lactone structure to that secreted by *M. ulcerans*, but has a unique side chain. The side chain differs in that it is only 14 carbons long (vs. 16 for that secreted by *M. ulcerans*), possesses an extra methyl group on C-8, and lacks a methyl group on C-10 and hydroxyl group on C-12 (Ranger et al. 2006). The production of this macrolide toxin by *M.*

pseudoshottsii may explain the differences in the host inflammatory response and bacterial proliferation and dissemination observed between *M. marinum* and *M. pseudoshottsii*-infected zebrafish. As noted previously one of the most striking differences in pathology was the absence granulomatous inflammation in the majority of *M. pseudoshottsii*-infected fish. The *M. ulcerans* mycolactone is a known immunosuppressant in mammals (van der Werf et al. 1999) and the production of mycolactone F by *M. pseudoshottsii* may similarly suppress immune function in the zebrafish, ultimately resulting in the inability of zebrafish to mount an effective, well-organized granulomatous inflammatory response. Similarly, immunosuppression has been postulated as the reason behind increased dissemination of *M. marinum* infections in zebrafish (Ramsay et al. 2009). Although immunosuppression in Ramsay et al. (2009) was due to increased stress, the hypothesis that reduced immune function resulted in the inability of host organisms to sequester the bacteria within granulomas is still relevant to the current work.

This study also clearly demonstrated a large degree of leukocyte death and lysis in *M. pseudoshottsii*-infected fish. George et al. (1999) demonstrated cell death via apoptosis in mouse macrophages exposed *in vitro* to mycolactone. Additionally, human neutrophils and fibroblasts exposed to mycolactone for as little as 24 hours exhibited leaky membranes and dose-dependent cell death (Adusumilli et al. 2005). Thus it is reasonable to hypothesize that zebrafish macrophages are capable of phagocytizing *M. pseudoshottsii*, but are subsequently unable to kill them. It is tempting to speculate that production of mycolactone F plays a role in the ability of *M. pseudoshottsii* to evade the intra-cellular killing mechanisms of zebrafish macrophages allowing them to proliferate

and actively disseminate throughout the tissues of this piscine model species. The production of mycolactone F may be responsible for the lack of organized immune response and cellular necrosis observed within the *M. pseudoshottsii*-infected zebrafish.

M. pseudoshottsii-infected zebrafish exhibited extensive dissemination of viable acid-fast bacilli throughout the visceral organs and mesenteric tissues. A striking observation in this study was the dissemination of viable bacilli across the alimentary tract wall, with bacterial cells actively breaching the serosal lining and disseminating through the muscularis and mucosal membrane of the gut. Many of these fish exhibited extensive colonies of acid-fast bacilli in the gut lumen in close proximity to the site of gut wall involvement. This was not observed in zebrafish infected with *M. marinum*. However, a similar phenomenon has been documented in zebrafish infected with *Mycobacterium haemophilum* (Whipps et al. 2007) suggesting migration of the bacteria and subsequent shedding. It is possible that bacteria were delivered into the alimentary tract of some fish when given the original inocula at the initiation of the study. However, if this were the case we would expect to see a similar phenomenon in our *M. marinum*-infected animals. Clearly, active dissemination of *M. pseudoshottsii* occurs in the zebrafish although the exact mechanisms for this movement and the potential involvement of mycolactone F requires further investigation. The lack of granulomatous inflammation suggests that *M. pseudoshottsii* infection in zebrafish is not an appropriate model for *M. tuberculosis*. However, the similarity in pathologies between zebrafish/*M. pseudoshottsii* and humans/*M. ulcerans* suggests our system as a viable model for investigation of Buruli ulcer.

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Table 1: Semi-quantitative values for overall disease severity and ability of host to contain bacteria within granulomas (** denotes statistically significant differences ($p < 0.01$))

		Disease severity	Containment	Relative number of bacteria	Immune response
<i>M. pseudoshottsii</i>	Mean	1.25	0.56**	High	Diffuse/ unorganized
	SD	0.84	0.69		
<i>M. Marinum</i>	Mean	2.56	3.75	Low	Organized
	SD	1.01	0.46		

Table 2: Shows the proportion of experimental animals in each tissue type. Large differences are observed in the kidney, gut lining, gut lumen between *M. marinum* and *M. pseudoshottsi*. Also note that free, extracellular bacteria are observed in *M. pseudoshottsi* infected zebrafish, but not *M. marinum* infected fish.

	Pancreas	Liver	Gut lining	Gut lumen	Kidney	Gonads	Spleen	Extracellular
<i>M. pseudoshottsii</i>	0.75	0.29	0.64	0.57	0.27	0.14	0.10	0.25
<i>M. marinum</i>	0.89	0.78	0.00	0.00	0.67	0.56	0.11	0.00

Figure 1: Percent survival for zebrafish with mycobacteriosis over 28 days. Control (-◆-), *M. pseudoshottsii* (--■--), *M. marinum* M30 (-●-), *M. marinum* ATCC (--x--).

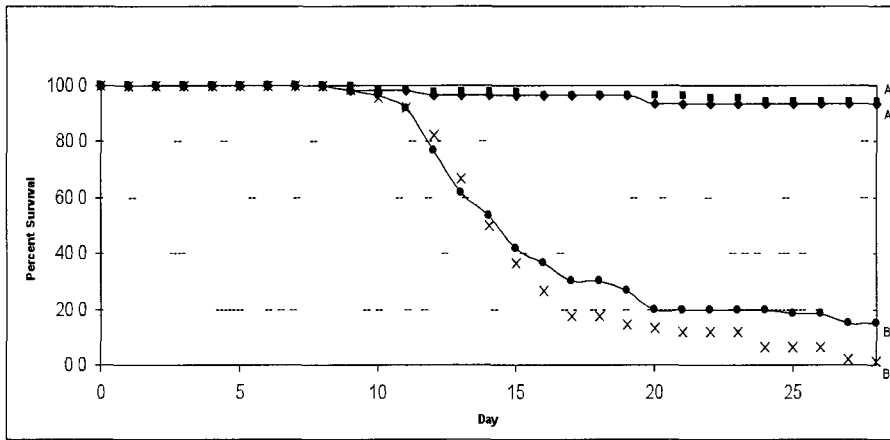


Figure 2: Early stage *M marinum* granuloma. No well-defined boundaries can be seen, however a general outline is shown around the inflammatory focus (arrow heads). Early stage granulomas lack epithelioid cells, and do not possess a highly eosinophilic, necrotic core. 400x

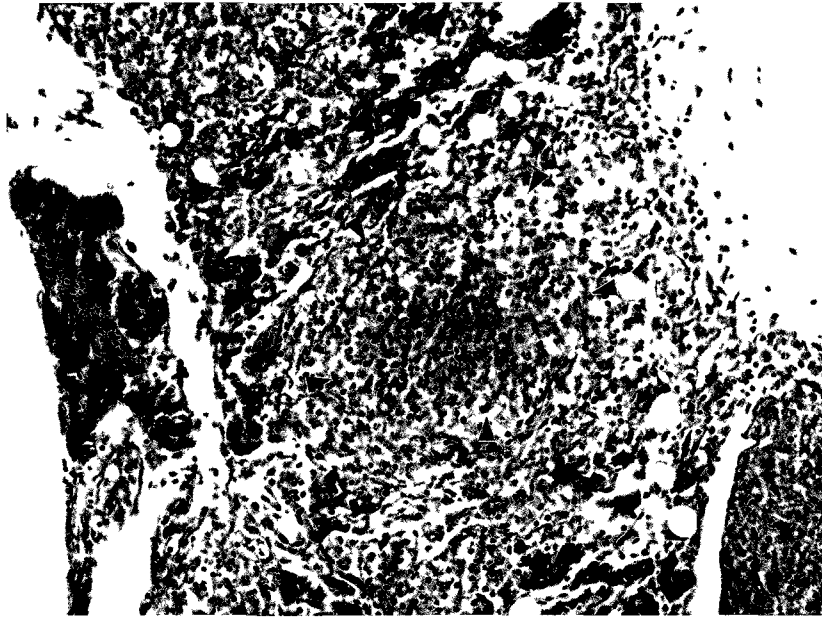


Figure 3: A well organized epithelioid granuloma from a fish experimentally infected with *M. marinum*. The center of the granuloma is heavily eosinophilic and necrotic with well defined epithelioid cells (arrow heads). Inflammatory cells (arrows) can be seen infiltrating the area. 600x

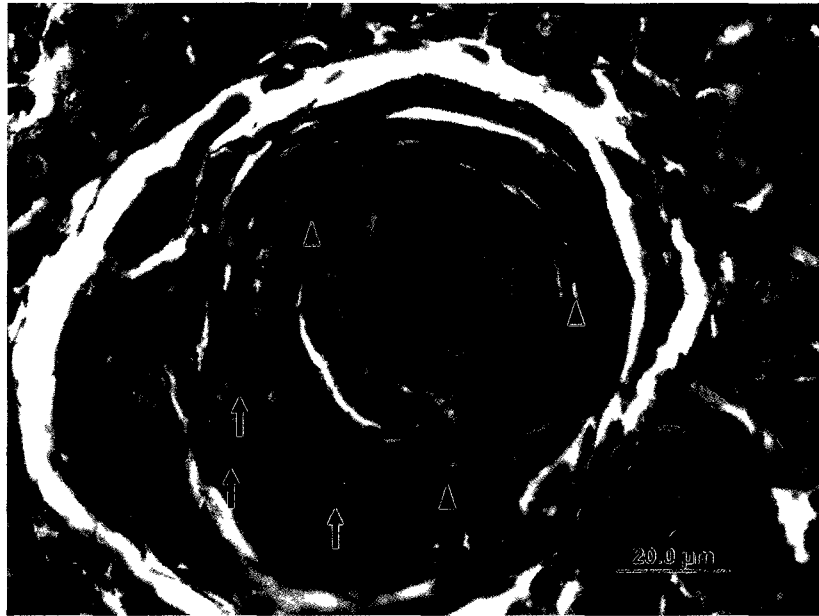


Figure 4: Extensive granulomatous inflammation throughout the mesenteries of a zebrafish infected with *M. marinum*. Multiple, well organized epithelioid granulomas (arrows) can be seen throughout the spleen (A), pancreas (B) and liver (C). 40x

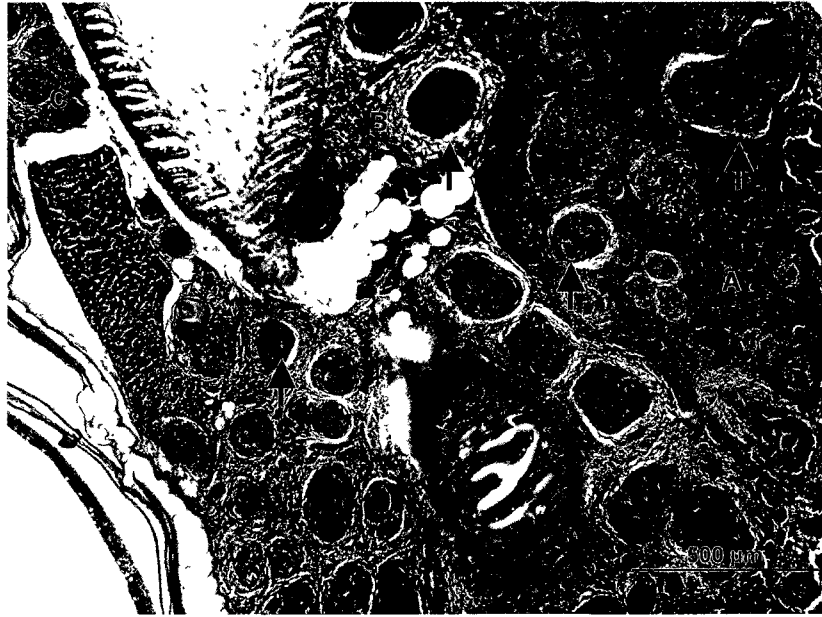


Figure 5: Ziehl-Neelsen staining of two well-defined granulomas in a *M. marinum* infected zebrafish showing the vast majority of bacilli (arrows) located within the granuloma, largely within the necrotic center. Small numbers of bacteria (arrow heads) can be seen within epithelioid cells of the granulomas. 200x

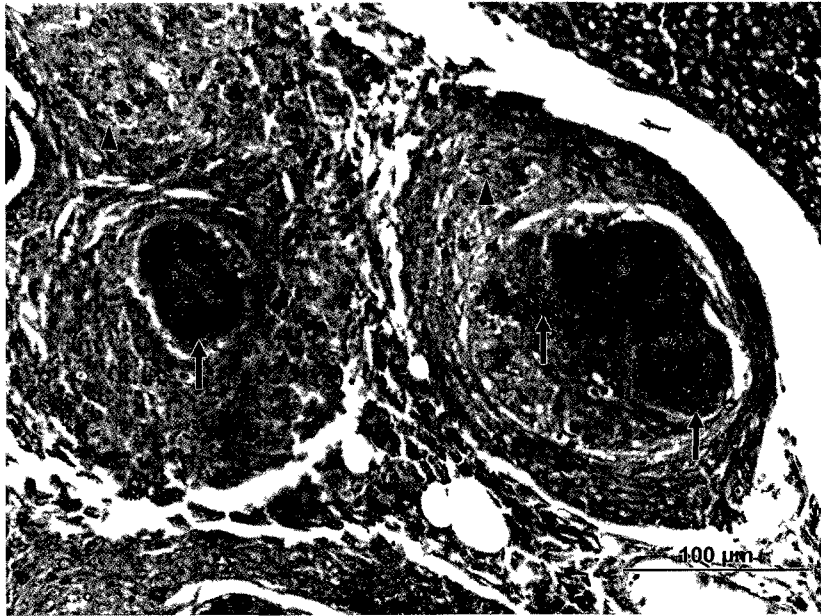


Figure 6 A-D: Chronic diffuse mesenteric inflammation in zebrafish infected with *M. marinum* (A, B) or *M. pseudoshottsii* (C, D). H&E staining reveals a similar loose leukocytic infiltrate in both *M. marinum* and *M. pseudoshottsii* infected zebrafish (A and C); however Ziehl-Neelsen staining reveals very few bacteria present in the *M. marinum* infected fish (B), which is in sharp contrast to the widely disseminated infection in the *M. pseudoshottsii* fish (D). Arrows (A) show a loose aggregate of leukocytic cells in which visible bacilli (Arrow head) from 6B are located. Arrow heads (6D) indicate clusters of bacteria. 400x

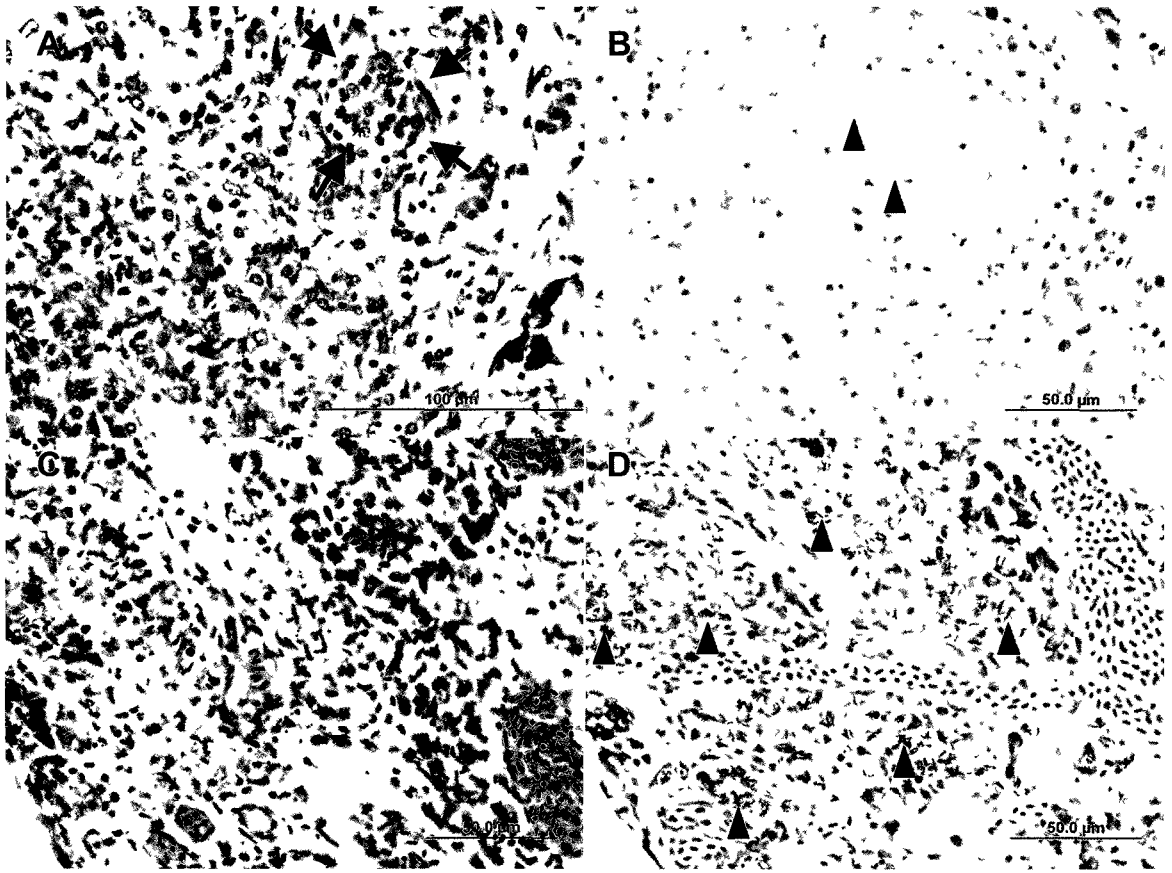


Figure 7: H&E (A) and Ziehl-Neelsen (B) sections of a *M. pseudoshottsii* infected zebrafish. H&E staining reveals a large area of diffuse inflammation and cellular necrosis with the presence of one well formed granuloma (arrow). In contrast to what is seen in *M. marinum* infections, Ziehl-Neelsen staining reveals a fast number of acid fast bacilli dispersed throughout the area of inflammation. 400x

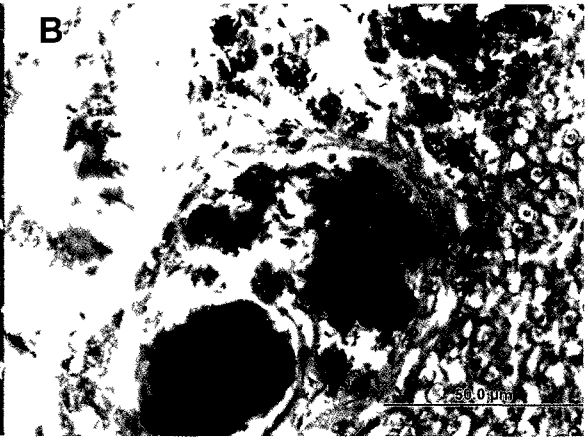
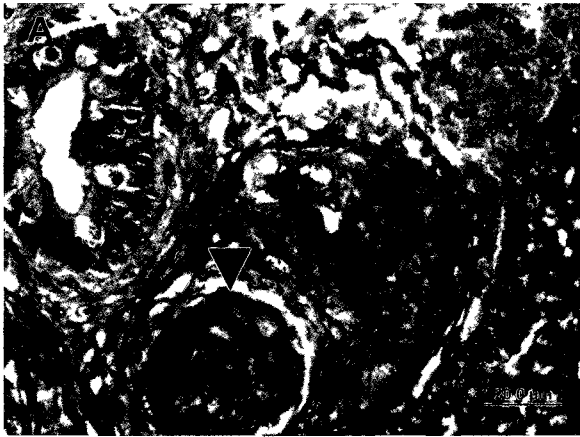


Figure 8: Ziehl-Neelsen image of a *M. pseudoshottsii* infected zebrafish. Multiple acid fast rods can be seen within a phagolysosome of multiple macrophages (arrows). I hypothesize the bacteria are able to persist and replicate intracellularly. 600x

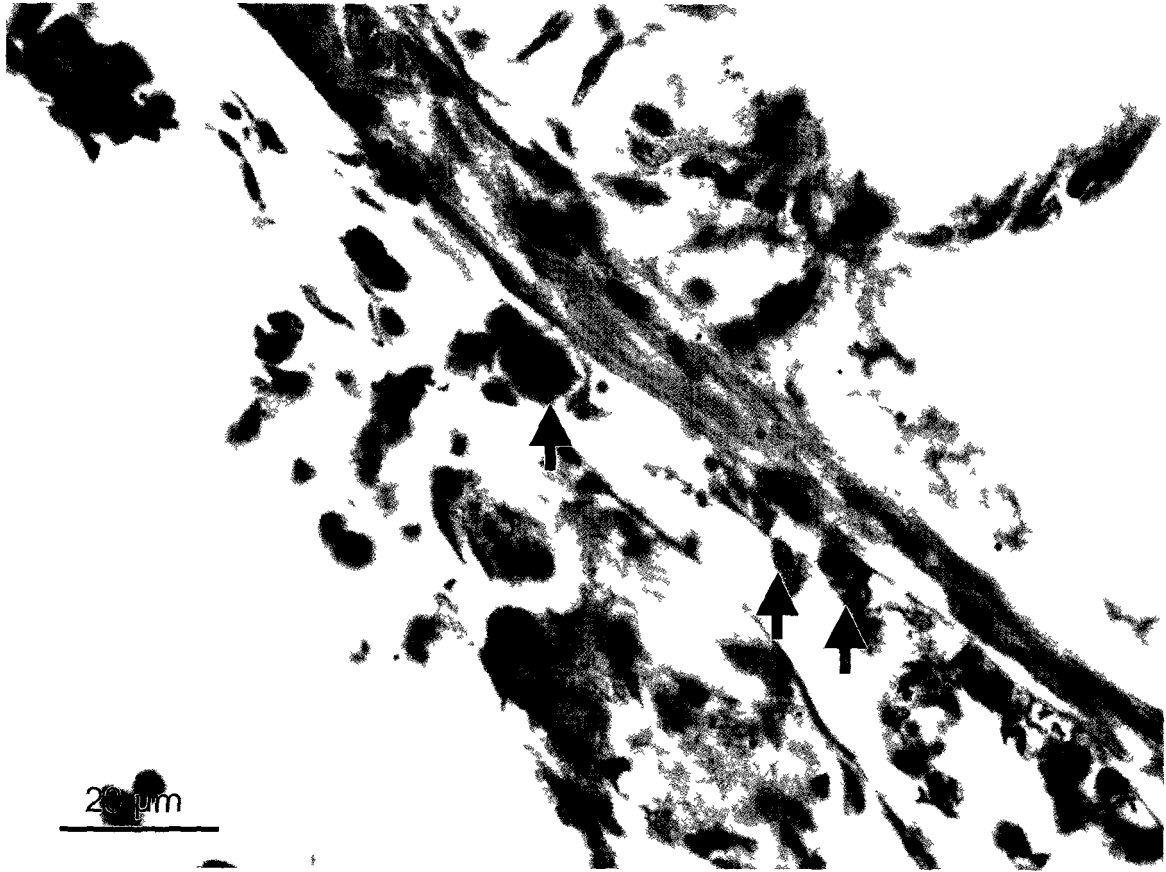


Figure 9: Ziehl-Neelsen images of a *M. pseudoshottsii* infected zebrafish revealing free bacilli. A, Extra-cellular bacilli (arrows) observed in areas of intense inflammation. B, free bacilli located along the swim bladder. 600x

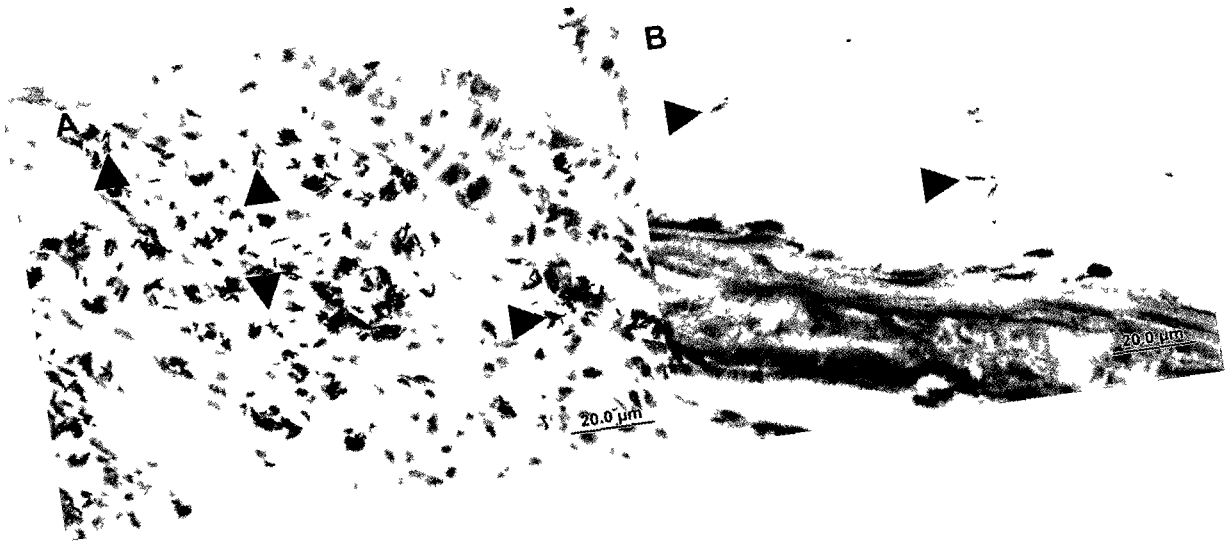
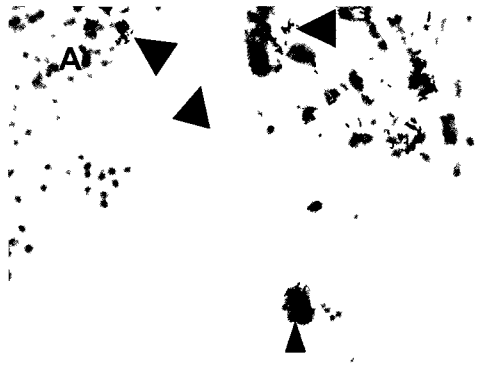


Figure 10: Ziehl-Neelsen images of a *M. pseudoshottsii* infected zebrafish showing macrophage lysis (arrow heads). This phenomenon was often accompanied by the presence of free bacteria (arrows). 600x

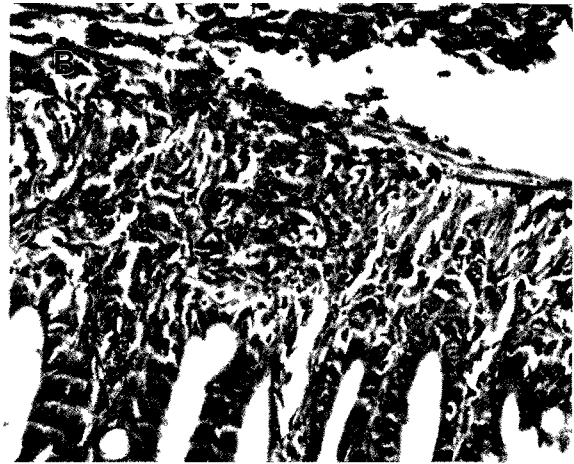
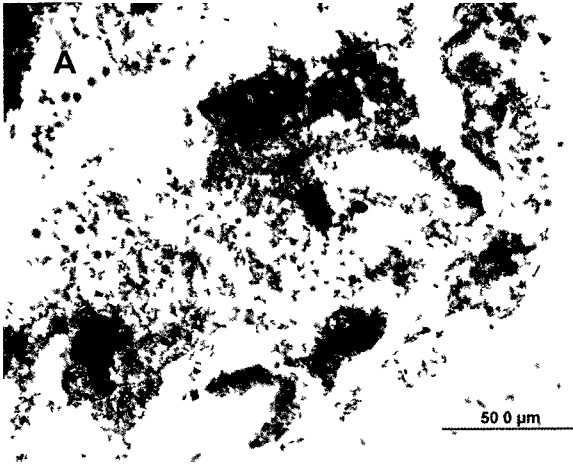


20.0 μ m



20.0 μ m

Figure 11: A, Ziehl-Neelsen images of a *M. pseudoshottsii* infected zebrafish showing extensive bacterial aggregates in the gut lumen. This was observed in 57% of examined fish. These bacteria were often slightly smaller than those seen throughout the mesenteric tissues, but also in much more dense colonies suggesting rapid proliferation of the bacteria within the gut. B, aggregates of bacteria throughout the muscularis of the gut. 64% of fish examined had bacteria located in the gut muscularis. 400x



Concurrent phenanthrene exposure and *M. marinum* infection induces a dose-dependent, additive effect in the zebrafish *Danio rerio*

1 Introduction

Laboratory studies investigating the interactions of multiple stressors are central to gaining a better understanding of the adaptive capabilities of aquatic species. Stressors are extrinsic or intrinsic stimuli that threaten the homeostasis of an organism (Chrousos and Gold 1992). Stressors include a variety of physical, chemical or biological stimuli, however laboratory exposure studies have traditionally focused on individual stressors, with other environmental parameters (temperature, water quality, pH etc.) kept within optimal ranges (Review by Walker et al. 2001). In the natural environment however, animal populations are intermittently exposed to sub-optimal conditions. Thus laboratory exposures employing single stressors cannot predict the potential for additive, synergistic or antagonistic effects associated with multiple stressors commonly encountered in the natural environment and therefore, may not accurately estimate the “true” risk to a species or population.

Multiple stressor studies represent an increasingly growing field of study within environmental science. A large degree of multiple-stressor work has been field oriented, having focused on population-level impacts including changes in growth, reproduction or mortality (Power 1997). However, the inherent variability within the natural environment makes establishing causation extremely difficult (Adams et al. 2005). Additionally, changes in reproduction or mortality may be cryptic in that their effects may not be immediately evident. Further complicating elucidation of dual-stressor affects is the potential for individual stressors to affect multiple physiological systems

concurrently. Therefore, dual-stressor exposures can result in complex interactions with multiple biological pathways impacted simultaneously (Schulte 2007).

Biological stressors, especially disease causing agents, are becoming of increasing concern to environmental researchers. Infectious disease can be a significant threat to biodiversity by causing population declines in both aquatic and terrestrial systems, significantly altering population structure (Harvell et al. 2002; Holmstrup et al. 2010). Of increasing concern is the interaction between chemical toxicants and potential disease causing agents, with immunosuppression now widely perceived to be a consequence of contaminant exposure. This is true of both individual and dual-stressor challenges of aquatic organisms. Salmon (*Oncorhynchus tshawytscha*) inhabiting a polluted estuary in Puget Sound, Washington displayed increased mortality (e.g., lower disease resistance) following challenge by *Vibrio anguillarum*, than salmon inhabiting nonpolluted estuaries Arkoosh et al. (1998). Carlson et al. (2002) reported that infection with the bacterial pathogen *Yersinia ruckeri* in Japanese medaka (*Oryzias latipes*) simultaneously exposed by IP injection to the polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene resulted in lower survivorship than in fish exposed to BaP or *Y. ruckeri* alone. Chinook salmon exposed to the PCB mixture Aroclor 1254 exhibited a decrease in the number of plaque forming cells, a measure of B cell function (Jacobson et al. 2003). However, a large degree of variability in toxicant immunosuppression (with some studies showing immunostimulation) has been demonstrated with overall results dependent on the toxicant, species, dose, and study duration (See reviews by: Bols et al. 2001; Reynaud et al. 2006).

The vertebrate immune system and the xenobiotic metabolizing enzyme systems such as the cytochrome P450 (CYP) pathway have both evolved as highly effective mechanisms to detect and eliminate exogenous and endogenous molecules. Additionally, these two systems have been shown to be able to interact (Reynaud et al. 2008). Samaras and Dietz (1953) first demonstrated that heightened immune function influenced drug metabolism by suppressing the CYP pathway (Reviewed in Renton, 2001). Perturbations of the CYP enzyme cascade have been shown to interfere with the ability of an organism to adequately metabolize and excrete toxicants, leading to their increased bioaccumulation and subsequent toxicity and impacts on organism health (Wessel et al. 2010; Varanasi et al. 1986). Several immune system mediated mechanisms of CYP down regulation have been proposed with most involving proinflammatory cytokines, including interleukins 1 and 6 (IL1, IL6) and tumor necrosis factor alpha (TNF α) (Bayne and Gerwick, 2001). Nicholson and Renton (1999) demonstrated that CYP activity was depressed in rat astrocytes when cells *in vitro* when exposed to lipopolysaccharide, a highly toxic cell wall component of gram negative bacterial pathogens. The authors speculate that the reduction in CYP activity was due to the production of IL1 and TNF α by microglial cells. Similarly, carp, *Cyprinus carpio*, exposed to 3-methylcholanthrene, a known CYP inducer, showed a marked increase in the phase II enzyme, glutathione S transferase (GST); however, subsequent injection of IL1 and TNF α significantly reduced GST activity (Reynaud et al. 2005). Marionnet et al. (2006) hypothesized that macrophages, activated by dextran sulfate or LPS, may also down regulate some CYP isoforms through generation of reactive oxygen species. They suggested that because

IL1 and TNF α are potent macrophage activators, they may therefore also act indirectly in the modulation of the cytochrome P450 pathway.

1.1 Previous work

In a previous dual-stressor study, (Prosser et al. 2011) zebrafish (*Danio rerio*) were individually exposed to *Mycobacterium marinum* and phenanthrene (nominal concentration: 450 $\mu\text{g L}^{-1}$, mean daily concentration: 284 μgL^{-1}), or concurrently to a combination of the two. In dually challenged treatments, animals were initially given intracoelomic (IC) injections of *M. marinum*, followed by continuous phenanthrene exposure starting 24 hours later. In that study fish exposed to phenanthrene alone exhibited significantly higher mortality than fish exposed simultaneously to phenanthrene plus *M. marinum* infection suggesting an antagonistic relationship between stressors. Additionally, we were able to demonstrate *M. marinum* infected zebrafish had a significantly depressed phenanthrene metabolism. We hypothesized that pro-inflammatory cytokines, produced during the early inflammatory response to mycobacterial infection inhibited metabolism of phenanthrene. This resulted in decreased production of toxic metabolites (e.g., hydroxy-phenanthrene), and lower mortality in the dually stressed fish than in fish exposed only to phenanthrene.

A subsequent study (Chapter 3 unpublished) was designed to examine how a more environmentally relevant concentration of phenanthrene (nominal concentrations: 250 $\mu\text{g L}^{-1}$, mean daily concentration: 168 μgL^{-1}) and longer exposure duration (21 vs. 14 days) would influence dual-stressor interactions. We also reversed the order of stressor exposure, starting first with phenanthrene followed 48 hours later with *M. marinum* infection. This was suggested by Dr. Charles Rice (Personal Communication) as a means

to determine if an already active biotransformational pathway was susceptible to suppression via inflammatory cytokines. In contrast to our original study, we found the highest mortality in dually challenged fish with an additive interaction between stressors. We also did not observe any difference in phenanthrene metabolism between treatments. This contrasting result to our original study indicates that slight alterations in experimental design can result in significant changes to organism health and demonstrates the continued need for detailed, controlled laboratory studies designed to further elucidate the complex interactions that can be better compared to the natural environment.

Due to the differences in phenanthrene concentration, study duration and a reversal of exposure order, our previous experiments cannot be directly compared thus the underlying mechanisms behind the contrasting results could not be discerned. Because of this, additional work was required to fully elucidate the influence of dose, and/or exposure order on organism health. In the current study we exposed zebrafish to *M. marinum* plus two different concentrations of phenanthrene. Groups were staggered so that half of the fish were challenged first by mycobacterial infection followed by initiation of toxicant exposure 48hrs later, whereas the other half were first exposed to toxicant followed by bacterial infection 48hrs later. Our goal was to gain a better understanding of multiple stressor interactions and how toxicant concentration and order of exposure affect host health.

2 Materials and Methods

2.1 *Experimental Animals*

Additional animals were purchased from Segrest Farms (Gibsonton, FL), and quarantined for one month. Prior to introduction into the colony, ten individuals were randomly selected, euthanized, necropsied and evaluated histologically to prevent the introduction of infectious agents (e.g., *Mycobacterium* infection or other parasites) into the colony. Fish were maintained in an Aquatic Habitats © (Apopka, FL) recirculating system. Reverse osmosis (RO) water was adjusted to a conductivity of approximately 1000 $\mu\text{S}/\text{cm}$ and a pH of approximately 7.5 using Instant Ocean ® (Madison, WI) salts.

2.2 Experimental Design

To examine the effects of combined stressors, zebrafish were simultaneously exposed to *Mycobacterium marinum* and the toxicant phenanthrene. Seven hundred (100 control animals, 600 experimental) zebrafish were randomized and distributed into fifteen 30 L glass aquaria comprising five experimental treatments, with each treatment consisting of three replicates receiving aeration and maintained at 28° C. Treatments consisted of either a “High” or “Low” dose of phenanthrene (Targeted concentrations: 250 $\mu\text{g}/\text{L}$ or 150 $\mu\text{g}/\text{L}$) and then divided further by the order of exposure (Figure 1). For each phenanthrene dose, half were exposed on day 0 and then injected with *M. marinum* on day 2. The other half received *M. marinum* on day 0, and were exposed to phenanthrene on day 2. The five treatments were therefore 1) control, 2) *M. marinum* 1st, “High” Phenanthrene 2nd (Myco+High), 3) *M. marinum* 1st, “Low” phenanthrene 2nd (Myco+Low), 4) “High” phenanthrene 1st, *M. marinum* 2nd (High+Myco) and 5) “Low” phenanthrene 1st, *M. marinum* 2nd (Low+Myco). Phenanthrene doses were chosen because they were sufficient to induce narcosis, but did not inhibit fish feeding behavior. All fish received an intracoelomic (IC) injection (18 μl) of phosphate buffer (pH 7.4)

(Controls) or *M. marinum* suspended in phosphate buffer (2.3×10^3 bacteria/fish) (treatments).

The experiment was conducted as an aqueous static renewal, with approximately 95% of exposure solutions renewed every 24 hours. An additional 5 mL spike of phenanthrene in acetone ($900 \mu\text{g mL}^{-1}$ and $540 \mu\text{g mL}^{-1}$ for “High” and “Low” treatments respectively) was added at 12.1 hours post renewal to make up for losses due to volatilization, sorption and metabolism. Control fish received a spike of acetone only. Toxicant dosing was continuous for 21 days.

Fish were subdivided within each tank using a glass partition. One group (n=15) was used for survivorship analysis and monitored throughout the day for mortalities. The second group (n=35) was sampled temporally for histology and phenanthrene body burden analyses, plasma glucose levels, and western *blotting* analysis (CYP1A).

2.3 Preparation of Bacterial Cultures

Mycobacterium marinum (strain M30) was originally isolated from Chesapeake Bay striped bass (*Morone saxatilis*) using standard aseptic necropsy protocols in a BSL2 cabinet and cultured by standard bacteriological methods (Rhodes et al. 2004). This strain is pathogenic, causing high mortality in zebrafish (Ostland et al. 2008, Prosser et al. 2011). Archived samples were inoculated aseptically into Middlebrook 7H9 broth with OADC enrichment and incubated at 30°C for two weeks. The culture in log growth phase was centrifuged at $10,000 \times g$ for 15 minutes to obtain a cell pellet. The supernatant was aspirated and the pellet was resuspended in 2 mL Butterfield’s phosphate buffer (PB) (pH 7.4) with 0.05% Tween 80 to reduce adhesion and clumping of bacteria. To further reduce bacterial clumping, the culture was vortexed for approximately 30

seconds with sterile glass beads (~50µm diameter). An additional 5 mL of PBS were added and the optical density was measured at 590 nm. A desired OD of 0.1, representing approximately 5×10^4 bacteria/µl was achieved by adjusting with additional PBS. Serial ten fold dilutions were prepared to obtain a nominal bacterial density of 75 bacteria µL⁻¹. Plate counting was used to quantify actual bacterial densities.

2.4 Generation of Phenanthrene Solutions

Aqueous phenanthrene dosing solutions were made fresh every 24 hours during the experiment using a generator column. The generator column design and procedure to produce a saturated aqueous phenanthrene solution has been described previously (Unger et al. 2007, Prosser et al. 2011). Briefly, sand coated with phenanthrene (97%; Acros Organics © New Jersey, USA) was dry packed into a 7.5cm x 59.2cm aluminum column. Reverse osmosis (RO) water was passed through the column against gravity, at a rate of 2.0 L min⁻¹. This flow rate was reported to be sufficient to achieve a saturated aqueous solution (Unger et al. 2007). RO water was conditioned with 0.3 g/L artificial sea salt (Instant Ocean ®) and used to dilute the saturated phenanthrene solution to the desired dosing concentration of 250µg L⁻¹.

2.5 Phenanthrene Analysis of Water Samples

Water was analyzed for phenanthrene using high performance liquid chromatography (HPLC) with a fluorescence detector (SpectraSYSTEM® P400 Controller, SpectraSYSTEM® AS3000 Autosampler [San Jose, CA, USA], Waters 474 Fluorescence Detector [Milford, MA, USA] and Restek® Allure C18 column [Bellefonte, PA, USA]) (Unger et al. 2007). A solvent gradient of water/acetonitrile was used with the gradient shifting from 100% water to 80% acetonitrile and back to water

over 35 minutes at a flow rate of 1 mL min^{-1} . The fluorescence detector was set to an excitation wavelength of 265nm and an emission wavelength of 370nm. Data were collected using ChemStation (Hewlett Packard Santa Clara, Ca, USA) software. Calibration using a 9 point calibration curve and an internal standard of 1-methylnaphthalene (MN) ($0.40\mu\text{g/mL}$) was done prior to analysis of experimental samples.

Water samples (1.0 mL) were removed from each tank and spiked with 0.030 mL MN in acetonitrile. Samples were collected at time 0 hours, 12 hours, 12.1 hours and 24 hours each day. Samples were analyzed immediately after collection, or refrigerated to minimize phenanthrene loss. Blanks of acetonitrile were run at the start of every new sample set, and replicates were taken every ten samples and analyzed to document sampling and analytical precision. In addition, a calibration standard was run at the start of each day to ensure analytical accuracy.

2.6 CYP1A Analysis

Livers from three fish were pooled. Microsomal CYP1A content was quantified by Western Blot analysis using the C10-7 monoclonal antibody kindly provided by Dr. Charles Rice (Clemson University). C10-7 was originally produced to CYP1A protein of the mummichog (*Fundulus heteroclitus*); however, affinity of this monoclonal antibody to the zebrafish CYP1A protein has been previously verified in our laboratory using a western blotting assay (Chapter 3). Zebrafish were sampled on days 1, 3 and 5 of the study, with six fish (representing two samples of three pooled livers each) removed from each replicate tank per sampling day resulting in 6 data points per treatment group per sampling day. Fish were immediately euthanized by overdose with MS-222 followed by dissection of the liver. Each sample (3 pooled livers) was homogenized and immediately

frozen in liquid nitrogen. Total microsomal proteins were quantified using the Bradford method (Bradford, 1976). Approximately 25 µg total protein from each sample was separated on 12% polyacrylamide gels at 200V for 45 minutes. Proteins were then transferred to 0.20µm nitrocellulose membranes (Biorad) at 130V for 1hr. Nitrocellulose membranes were blocked overnight in 5% nonfat dry milk in 1X Tris buffered saline (TBS) (pH 7.5). Nitrocellulose membranes were then incubated for 1 hr in the primary antibody (C10-7: no dilution) at room temperature followed by washing 3x for 10 minutes in 1X TBS (pH 7.5). Nitrocellulose membranes were then incubated in a secondary antibody (goat anti-mouse: BioRad) for 1 hr at room temperature. They were then washed 3x with 1X TBS (pH 7.5) and then developed using nitroblue tetrazolium chloride (Biorad) and 5-bromo 4-chloro 3-indolyl phosphate (Biorad) for 2 minutes. Membranes were placed in deionized water to stop color development and held until imaging. Membranes were imaged using an Alpha Innotech Flourchem system (Santa Clara, CA) and subsequently quantified using AlphaEase FC software for windows. Samples were quantified to previously purified *F. heteroclitus* CYP1A standards.

2.7 Histology

Standard paraffin histological techniques were used (Prophet et al. 1992). Briefly, moribund fish were euthanized by overdose with MS-222 (500 mgL⁻¹). A small mid ventral incision was made to allow the fixative to penetrate into the coelomic cavity and the entire fish were placed into Z-fix, a buffered zinc formalin fixative (Anatech LTD) for at least 48 hours. Fish were subsequently decalcified overnight in a 50:50 formic acid: sodium citrate solution and rinsed in running tap water for 3 hours. They were then bisected sagittally using a single-edged razor blade and placed into uniquely labeled tissue

cassettes. Tissue specimens were then dehydrated through a graded ethanol series, cleared and infiltrated in hot paraffin wax (TissuePrep; Fisher Scientific) in a Shandon Excelsior Tissue Processor (Miami FL, USA), after which they were embedded on a Microm EC350-1 tissue embedding center (Microm Inc. [Mound MN, USA]). Sections (5 µm) were cut on an Olympus rotary microtome 4055, and stained with hematoxylin and eosin, in a Varistain Gemini ES automatic slide stainer [Thermo Scientific Miami, FL, USA].

Two fish per replicate (n=6 per treatment group) were sampled on days 3, 5, 7, 14 and 21 for histological examination. Total number of granulomas was quantified for a sagittal section of each fish. In addition, histologic tissue sections were scored semi-quantitatively for diffuse leukocytic infiltration (none, low, medium and high), stage of granuloma organization (none, diffuse inflammatory loci, epithelioid without a necrotic core, and epithelioid with necrotic core). Each category was assigned a rank number from 0-3 with 0 representing no signs of a diseased state, and 3 having the most severe indications of disease. To avoid bias towards experimental groups, samples were randomly selected and scored blind. Values were then averaged for each experimental group (8 fish) and assessed for statistical significance (See below).

2.8 Phenanthrene Tissue Concentrations (Body Burdens)

Fish (2 per replicate tank) were sampled on day 1, 3, 5 and 7 (n=6 per treatment per day). Analysis of phenanthrene in fish tissues followed previously described protocols for tissue PAH concentrations in grass shrimp (*Palaemonetes pugio*) and zebrafish (Unger et al. 2007, Prosser et al. 2011). Briefly, individual fish were thawed, rinsed in deionized water, weighed and homogenized in 1mL deionized water and 500 ng

deuterated (d10) phenanthrene surrogate standard using Kontes® glass tissue homogenizers (Vineland, NJ, USA). Homogenates were then transferred to 50 mL Teflon centrifuge tubes, mixed with 2 mL of concentrated hydrochloric acid and 2 mL hexane, sonicated for ten minutes, and centrifuged (9000 rpm, 15 min). Hexane/HCl supernatant was transferred to a 15-mL glass tube. Homogenates were re-extracted, using the same sequence, with two additional mL of hexane. Extracts (~4 mL) were gently evaporated under a stream of nitrogen until the desired volume of 0.1 mL was attained. An internal standard of p-terphenyl was added (0.8 µg) before analysis on a 4D ion trap mass spectrometer (Varian, Walnut Creek, Ca, USA). Analytes and ions measured were p-terphenyl internal standard [152+230], phenanthrene D10 [187-189], phenanthrene [176-179], 1-hydroxyphenanthrene [165+194] and 3-hydroxyphenanthrene [165+194]. A seven point calibration curve was generated for all target analytes. Identification was based on matching retention time and spectra to standards (1-hydroxyphenanthrene: EQ Laboratories Inc. Augsburg, Germany; and 3-hydroxyphenanthrene: Cambridge Isotope Laboratories, Andover MA, USA) or to those in the NIST 05 mass spectral library.

As a way to quantify how efficiently phenanthrene was metabolized, we created a simple equation to measure metabolic efficiency (ME). ME, as defined here, is the ability of zebrafish to convert parent phenanthrene (p) to measured hydroxylated metabolites (m). This calculation used the measured tissue concentrations obtained from GC/MS analysis and was performed on individual animals. ME is the proportion of parent phenanthrene in the total quantified sample (i.e. parent + metabolites). Thus, $ME = p / (p + m)$. As ME approaches 1, the parent compound is closer to constituting the

entirety of the sample. This calculation allows us to normalize to individual fish regardless of age, sex, weight or dose and facilitated comparisons between treatment groups.

2.9 Water Quality

Exposure tanks were monitored daily for temperature, pH, dissolved oxygen (DO) and total ammonia. DO and temperature were monitored using an Oxyguard® Handy Polaris (Birkerød, Denmark). Salifort® box kits were used for analysis of ammonia and pH was measured with an Orion 250A pH meter with Thermo pH electrode (Waltham, Ma, USA).

2.10 Statistical Analysis

The dual exposure study was analyzed for differences in time to death using the Kaplan Meier method. This method (Newman, 1995) estimates survival function of an exposed group of individuals and allows comparison of two or more survival curves and evaluation of treatment effects. The log rank test was used to establish if there were significant deviations between survivorship curves. Bioaccumulation data, ME and semi quantitative histological data (e.g., severity of granuloma formation) were analyzed by the non-parametric Mann-Whitney U test. ANOVA was used to evaluate if aqueous phenanthrene concentrations differed significantly between replicates and treatments. All statistical analyses were performed using SAS software [Cary NC, USA].

3 Results

3.1 Water Quality

Water quality data are presented in Table 1. The mean values (+/- standard deviation) for dissolved oxygen, pH, temperature and total ammonia were 6.7 +/- 0.85

mgL⁻¹, 6.5 +/- 0.31, 27.5 +/- 1.0 °C, and <0.25 mg/L respectively. All water quality parameters remained within the physiological range of zebrafish and not considered to be stressful (Lawrence 2007).

3.2 Phenanthrene Water Concentration

Mean daily concentrations (+/- standard deviation) were 146 +/- 140 µg L⁻¹ and 167 +/- 141 µg L⁻¹ for the “Myco+High” and “High+Myco” groups respectively (Figure 2A). Value for the “Myco+Low” and “Low+Myco” groups were 81 +/- 78 µg L⁻¹ and 91 +/- 70 µg L⁻¹, respectively (Figure 2B). The 12 hour loss of aqueous phenanthrene was as high as 100%; this high degree of loss, as compared to previous aqueous exposure experiments, is suggestive of bacterial degradation. However, phenanthrene reductions were consistent across all treatments and replicates. There were no significant differences in phenanthrene concentrations between replicate tanks for any treatment allowing replicates to be combined for survival analysis. Additionally, there were no significant differences between “Myco+High” and “High+Myco” treatments, or between “Myco+Low” and “Low+Myco” treatments. Phenanthrene concentrations in all control tanks were repeatedly below detection limits (1 µg L⁻¹).

3.3 Fish Mortality

Narcotic effects from phenanthrene exposure were evident in fish exposed to both concentrations of phenanthrene. Narcosis was more severe in fish exposed to the “High” concentration, but no differences in degree of narcosis were evident between groups based on exposure order. Clinical signs included erratic swimming and disorientation. Two days after completion of the experiment, all exposure groups returned to normal swimming behaviors consistent with Type I narcosis.

Twenty-one day survivorship showed significant differences ($p < 0.05$) between the groups exposed to the “High” phenanthrene concentration and the “Low” phenanthrene concentration regardless of exposure order (Figure 3). No significant differences however were observed between the “Myco+High” and “High+Myco” groups (43% and 42% survival respectively). Similarly, no significant differences were seen between the “Myco+Low” and “Low+Myco” groups (60% and 69% survival respectively). All four treatments were significantly different from the controls, which exhibited 100% survival.

3.4 Tissue Concentrations

Mass spectrometry confirmed the presence of two phenanthrene metabolites, 3-hydroxy phenanthrene and 1-hydroxy phenanthrene in whole body samples from phenanthrene exposed treatments. No significant differences in ME were observed between any of the experimental groups on any day, or over the course of the experiment (Figure 4). Although day three displayed the lowest mean values for all treatments, error between groups results in no significant differences being observed. By Day 7 of the experiment, ratios were almost 1 for every group suggesting the vast majority of toxicant was in the parent phenanthrene form. No parent or metabolites were found in any of the control animals.

3.5 CYP1A Analysis

CYP1A levels (pmol protein +/-SD) for control fish were 0.059 +/- 0.021, 0.033 +/- 0.020 and 0.062 +/- 0.047 for days 1, 3 and 5 respectively (Figure 5). Concentration for the “High+Myco” and “Low+Myco” treatments on day 1 were 0.083 +/- 0.075 and 0.097 +/- 0.075 respectively. Although these levels were elevated above the

“Myco+Low” and “Myco+High” treatments, which had not yet been exposed to phenanthrene, they were not significant. At day 3 there was no significant increase from day 1 in any treatment, or between any experimental groups. However, at day 5, all treatment groups receiving phenanthrene were significantly elevated above day 1 levels ($p < 0.01$). On no day did order of exposure have significant impacts on CYP1A levels, and there was no difference in CYP1A levels between “High” and “Low” concentration groups.

3.6 Histology

Fish were examined histologically to evaluate for potential differences in infectious disease severity (granuloma number, and degree of leukocyte infiltration) between treatments. Control fish showed no signs of disease whereas all fish infected with *M. marinum* displayed granulomatous inflammation (Figure 6). Granulomas were evident initially in exocrine pancreas; however, as the disease progressed, granulomas were also evident in the liver, spleen, gonad and kidney. The earliest signs of granulomatous inflammation occurred on day five, with one fish examined showing an early stage granuloma. However, it was not until day 14 that the disease was wide spread. Over the course of the study, the inflammatory response ranged from a loose association of leukocytic cells with no defined organization, to highly structured granulomas with epithelioid cells surrounding a highly eosinophilic necrotic core. No significant differences in number of granulomas or degree of organization were evident between any treatment groups regardless of phenanthrene dose, or exposure order (Table 2). By day 21 mortalities had greatly reduced the number of available fish and so sample sizes at the later time periods was greatly reduced. Both the “Myco+High” and

“Myco+Low” groups had no fish alive for sampling. The “High+Myco” and “Low+Myco” groups were also greatly reduced, with 2 and 3 fish sampled, respectively. Therefore, no values are reported for day 21 due to no fish being alive for sampling, or because the sample size was too small to draw significant conclusions.

4 Discussion

4.1 Survivorship

Our results demonstrate that mortalities in zebrafish simultaneously exposed to phenanthrene and *Mycobacterium marinum* occur in a dose-dependent manner. Additionally, we show phenanthrene does not influence the *M. marinum* disease state or exacerbate the disease process. Dose effects are evident by the significantly higher mortalities for the “Myco+High” and “High+Myco” treatments as compared to “Low+Myco” and “Myco+Low” groups. Actual mean daily aqueous phenanthrene concentrations ranged from 81 $\mu\text{g L}^{-1}$ for the “Myco+Low” group to 167 $\mu\text{g L}^{-1}$ for the “High +Myco” group showing that a doubling in measured concentrations is sufficient to degrade host health. However, no significant difference was observed in either treatment receiving the “High” dose, nor was there any significant difference between treatments receiving the “Low” dose, regardless of exposure order. This suggests that the order of exposure is not a significant factor in predicting survivorship for dual-stressor experiments.

It should additionally be noted that the amount of time post-bacterial infection appears to be a significant factor in predicting survivorship. Despite the “High+Myco” group having been exposed to phenanthrene for 48 hours longer than the “Myco+High” group, significant mortalities began approximately the same time post-infection, 11 days

and 12 days for the “Myco+High” and “High+Myco” groups respectively. Similar results were seen in the treatments receiving the “Low” dose for which mortalities began 16 days post infection in both groups. This suggests that disease progression is not affected by an increase in exposure duration to phenanthrene. This is also supported by histological data in which there was no significant difference in total number of granulomas or degree of inflammatory response between any treatments. A dose response to phenanthrene, no perceived impact on the disease process and no modulation of phenanthrene metabolism, suggests these two stressors act independent of the other. Although not definitive in this study, the data presented here adds additional lines of evidence supporting an additive relationship between stressors as supported in my previous study (Prosser et al. 2011; Unpublished Chapter 3)

Although the results of this study support our previous work in which phenanthrene did not appear to modulate disease progression (Prosser et al. 2011; Unpublished Chapter 3), other researchers have shown reduced immune function after PAH exposure in controlled laboratory studies. Carlson et al. (2002) using IP injections of benzo[a]pyrene (a five-ring PAH) with the Japanese medaka (*Oryzias latipes*) followed by exposure to *Yersinia ruckeri* showed decreased immune function as measured by reduced lymphocyte proliferation and antibody forming cells. In the same study, a synergistic relationship was observed between stressors with significantly decreased survival in dually challenged fish. However, in a review of this topic, Reynaud and Deschaux (2006) point out significant many of the discrepancies regarding immunomodulation by PAH exposure, highlighting the fact that immune suppression can vary with host species, type and concentration of the PAH used and the specific immune

parameters being measured. Therefore, as demonstrated in the present study, it cannot be assumed that all PAHs universally act as immunosuppressors.

The intracellular lifestyle of *M. marinum* may also explain why phenanthrene does not appear to influence disease progression. *M. marinum* has evolved a strategy whereby it can survive within phagocytic cells *in vivo* (Stamm and Brown 2004). This results in a chronic granulomatous disease in which bacilli are sequestered within epithelioid granulomas (Gauthier and Rhodes 2009). Although the exact mechanisms whereby mycobacteria evade the immune response are not well understood, the pathogens are thought to be able to arrest phagosomal acidification (Sturgill-Koszycki et al. 1994). Therefore, for example, if a process such as lysosomal fusion, or phagosomal acidification is modulated due to toxicant exposure, no effect may be evident since the bacteria are already capable of evading that mechanism. Similarly if toxicant exposure influences humoral immunity, which is generally not thought to be involved in piscine mycobacteriosis, no differences in disease progression would be expected. However, because immune parameters were not directly measured in this study we cannot definitively determine if, or to what extent phenanthrene may act to modulate the immune system of zebrafish and so future work may aim to directly examine immune function. Additionally, the outcome of this study may be dependent on the bacteria used. Future studies may examine the use of *Mycobacterium pseudoshottsii*. Although closely related to *M. marinum*, *M. pseudoshottsii* elicits a significantly different immune response with greatly reduced granulomatous inflammation (Prosser unpublished, Chapter 4). However, based on our current and previous results, we can conclude that phenanthrene does not influence the progression of *M. marinum* in the zebrafish.

4.2 CYP1A induction

One of the most striking results we have shown is the ability for phenanthrene exposure to increase CYP1A levels through activation of the cytochrome P450 pathway. Previously it was believed that phenanthrene lacked the ability to bind the Ah receptor, a necessary step in activating the CYP pathway, thereby limiting metabolism to endogenous CYP1A (Barrone et al. 2004). However, we clearly demonstrated a significant elevation in CYP1A above basal levels over a five day period for all treatments exposed to phenanthrene regardless of dose or exposure order. Preliminary immunohistochemical data from our laboratory (unpublished) suggested that both hepatocytes and renal tubules of zebrafish are actively involved with CYP1A production. The location of activity is consistent with previous researchers using immunohistochemical analysis to analyze CYP1A induction after exposure of *Fundulus heteroclitus* to the 5-ring PAH Benzo[a]pyrene (Van Veld et al. 1997).

Although the general perception has been that phenanthrene is a nonspecific membrane disrupter primarily acting as a Type I narcotic (Sikkema et al. 1994; Schirmer et al. 1998), there is mounting evidence of dose dependent oxidative stress associated with increased cytochrome P450 activity. Using *Carassius auratus* Sun et al. (2006) demonstrated significant increases in hepatic hydroxyl radicals following phenanthrene exposure. Using the same species of fish, Yin et al. (2007) demonstrated a direct correlation ($R^2=0.97$) between hydroxyl radical production and increasing phenanthrene concentrations and suggested the possibility of dose dependent oxidative stress. Oliveira et al. (2007) measured CYP1A induction through EROD analysis after exposure to phenanthrene. They found that a range in phenanthrene concentrations from $54\mu\text{g L}^{-1}$ -

482 $\mu\text{g L}^{-1}$ was sufficient to significantly elevate EROD levels above controls. Using tilapia (*Oreochromis niloticus* x *O. aureus*), Wenju et al. (2009) also demonstrated elevated EROD levels within 4 days at phenanthrene concentration as low as 50 $\mu\text{g L}^{-1}$. Each of the aforementioned studies used an indirect method for ascertaining the extent to which CYP is activated by phenanthrene. In our study however, we were able to directly measure the CYP1A protein and definitively measure induction of the CYP pathway above basal levels as a function of phenanthrene exposure. However, neither in previous research, nor in our study was the exact mechanism for induction ascertained. As previously stated, phenanthrene has been reported to be unable to bind the Ah receptor (Barron et al. 2004), so alternate modes of activation should be explored. One area that deserves attention is the ability of glycine-N-methyl transferase (GNMT), to act as a Ah receptor independent method for CYP induction. As a homotetramer, GNMT is responsible for the transfer of a methyl group from S-adenosylmethionine (SAM) to glycine forming S-adenosylhomocysteine (SAH) and sarcosine (Bhat et al. 1997). However, in its homodimeric form, GNMT has been found to bind PAHs and stimulate CYP1A1 production in rats (Bhat et al. 1997). This same phenomenon was recently documented in *Fundulus heteroclitis* exposed to benzo[a]pyrene (Fang et al. 2010), illustrating the same Ah receptor independent mechanism for inducing CYP in fish. Although not demonstrated with phenanthrene, this alternate pathway to CYP induction may indicate on additional mechanisms for CYP activation in phenanthrene exposed fishes.

We have however, previously reported that phenanthrene does not appear to induce CYP1A production (Prosser unpublished, Chapter 3). We believe differences in

CYP1A induction between two studies may be related to animal age as this factor has been reported to influence CYP activity (see review by Andersson and Forlin 1992). In our previous work (Prosser unpublished, Chapter 3), histological analysis revealed that 47% of females were juvenile, as evident by undeveloped oocytes. Although the fish sampled for CYP1A were not sexed, we feel that the histological sample size (n=160; 20% of the total population) was sufficiently large to be representative of the whole which suggests that a large proportion of our fish were juveniles. By comparison, 100% of females in our current study were histologically determined to be adults as evident by fully developed oocytes. Again we feel that this sample size (n=150; 21.4% of the total population) is a sufficient reflection of our entire group. Research has shown that CYP1A levels are elevated in adult fishes as compared to juveniles exposed under similar circumstances. In adult flounder (*Pleuronectes americanus*) CYP1A levels were significantly higher as compared to juveniles sampled from a polluted site (Kahn and Payne 2002). Similar elevations in CYP1A have also been reported for adult turbot (*Scophthalmus maximus*) as compared to juveniles exposed to organic contaminants (Peters and Livingstone 1995). Therefore, in Prosser (Unpublished, Chapter 3) the large degree of inconsistency in CYP1A levels observed between treatments may be due to ~50% of our population being immature. By measuring CYP1A levels between different age classes we may have inadvertently added a large degree of variability thus resulting in no significant difference between treatments.

Fish size, and related lipid content, may also play a significant role in CYP1A expression. As juvenile fish mature lipid stores are minimal as energy is required for growth; however, as adults, growth has ceased, and lipid stores increase (Shearer et al.

1994). Although we did not directly measure lipid content of our fish, the weight of fish from our current study was over 2X that of our previous work (Weight \pm S.D.: 0.41g \pm 0.16 vs. 0.17g \pm 0.06), suggesting adult animals with the potential for high lipid content. Additionally, as previously stated, all histologically examined females in the current study were sexually mature adults having large stores of lipid rich eggs. Higher lipid content in our adult fish therefore poses a greater potential for the bioaccumulation of phenanthrene. A review of the literature supports increased cytochrome P450 activity due to toxicant bioaccumulation. Using three fresh water fish species (*Rutilus rutilus* (Roach), *Anguilla anguilla* (eel), and *Esox lucius* (pike)) collected from Nieuwe Meer, Amsterdam, Van der Oost et al. (1991) demonstrated an increase in the level and activity of cytochrome P450 enzymes with an increase in bioaccumulation. This correlation was evident using micropollutants (PAHs, PCBs and organochloride pesticides) that were below detection limit in the surrounding waters highlighting the significant role of bioaccumulation. Cheikyula et al. (2008) examined the effects of bioaccumulation of PAHs and EROD activity in three fish species: java medaka (*Oryzias javanicus*), Japanese flounder (*Paralichthys olivaceus*) and red sea bream (*Pagrus major*). These authors also found correlations between the bioaccumulation of PAHs and increased cytochrome P450 activity. Therefore, we believe the differences in CYP1A expression between our current and previous studies are due to the age of the animals used, and their differing lipid stores.

Conclusions

The goal of our study was to definitively determine how phenanthrene dose and/or order of stressor exposure affect zebrafish health when dually challenged with

phenanthrene and *Mycobacterium marinum*. This work was a continuation of two previous studies that gave insight into interactions between the inflammatory and biotransformational pathways, but that were not directly comparable (Prosser et al. 2011; Unpublished Chapter 3). Based on our results we conclude that the order of exposure is not a significant factor in determining organism health. However, we have strong support for a dose response to phenanthrene. Additionally, similar to our previous findings (Prosser Unpublished Chapter 3) we feel that increased mortality due to an additive effect between stressors with no modulation of metabolism or influence on disease progression. We also acknowledge discrepancies within the literature regarding toxicant induced immunomodulation and how differences in pathogen lifestyle may influence observed results. Finally, we have shown that phenanthrene exposure can induce the production of the cytochrome P450 protein, CYP1A. Our studies highlight the complexities in performing controlled dual-stressor experiments and underscore the importance of host, pathogen, toxicant, dose and duration in organism health. Future experiments should be conducted to examine the relationship between new toxicants and pathogens across several concentrations and temporal scales.

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Table 1: Mean water quality values for treatment groups

	Mean	+/-
DO	6.7	0.85
pH	6.5	0.31
C°	27.5	1.0
Ammonia	<0.25	0

Table 2: Granuloma counts and semi-quantitative histological data for days 3, 5, 7, 14 and 21 for *M. marinum* infected fish. Granulomas were quantified as the number of visible granulomas in one sagittal histologic tissue section per fish. Degree of inflammation ranked from 0-3 with 0 indicating no pathology observed and 3 indicating a high degree of pathology observed. Table values are the mean rank for each experimental group (St. Dev). No significant differences were calculated between any of the experimental groups for a given day.

Day	Number of Granulomas					Degree of Inflammation				
	d3	d5	D7	d14	d21	d3	d5	d7	d14	d21
Control	0 (0)	0 (0)	0 (0)	0 (0)	N/A	0 (0)	0 33(0 5)	0 67(0 52)	2(0 89)	NA
High+Myco	0 (0)	0 (0)	0 (0)	5(5 1)	N/A	0 17(0 41)	0 17(0 4)	0 5(0 55)	2(0 63)	NA
Low+Myco	0(0)	0(0)	0(0)	3 67(1 63)	N/A	0 25(0 7)	0(0)	0 6(0 7)	2 4(0 5)	NA
Myco+High	0(0)	0(0)	0(0)	1 75(1 15)	N/A	0 33(0 52)	0 67(0 5)	1 33(0 52)	1 5(0 58)	NA
Myco+Low	0(0)	0(0)	0 25(0 5)	N/A	N/A	0 17(0 41)	0 5(0 5)	1 5(0 58)	NA	NA

Figure 1: Schematic of experimental design illustrating fish assignments for the experimental challenge study. Seven hundred fish were divided into the control or one of four treatments. Two treatments were first continuously exposed to either a “High” or “Low” dose of phenanthrene followed 48 hours later by injection of *M. marinum*, or fish were first injected with the bacteria followed by a “High” or “Low” dose phenanthrene exposure starting 48 hours later.

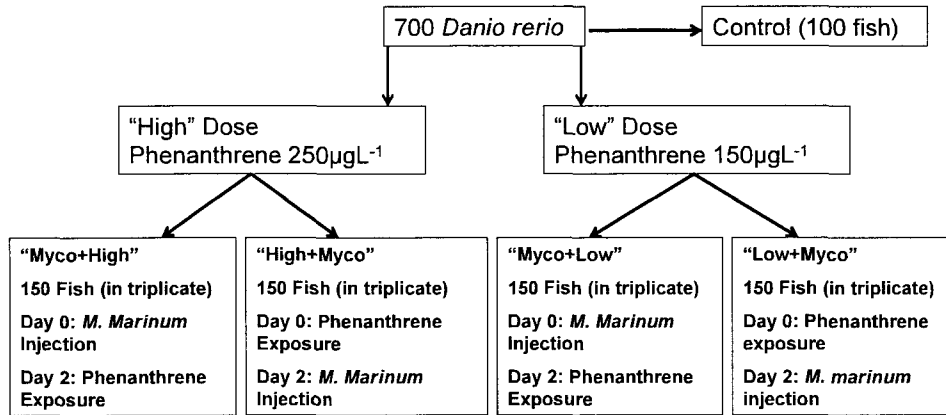


Figure 2: Daily fluctuations in phenanthrene concentration for A) high dose groups (“High+Myco”-x-, “Myco+High” --◆--) and B) low dose groups (“Low+Myco” --x--, “Myco+Low” -◆-). Fresh phenanthrene solutions were made every 24hours with additional phenanthrene spikes added at time 12.1 hours each day. Error bars represent standard deviations.

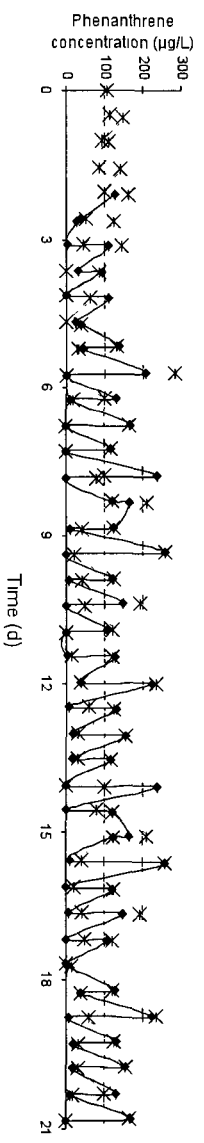
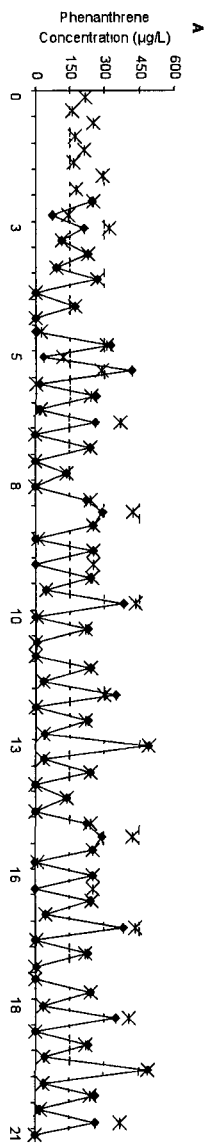


Figure 3: Kaplan-Meier survivorship curves for zebrafish, *Danio rerio*, exposed to phenanthrene and *M. marinum*. Survival in fish exposed to high dose phenanthrene was significantly lower than in fish exposed to high dose phenanthrene+*M. marinum*. Error bars: standard error of the mean. Key: “High+Myco” (-♦-), “Low+Myco” (--■--), “Myco+High” (--X--), “Myco+Low” (--▲--) Control (-●-). Different letters denote statistical significance ($p < 0.05$)

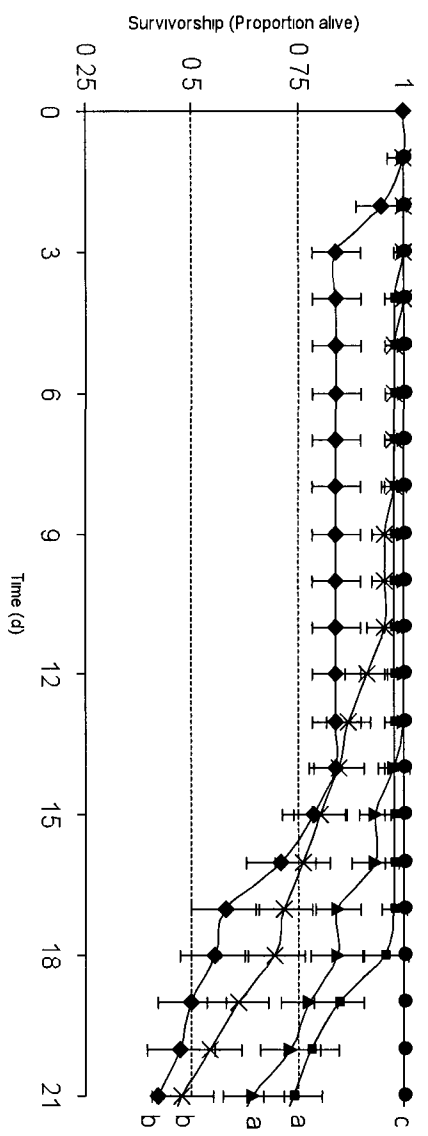


Figure 4: Mean phenanthrene:(Phenanthrene+hydroxyl-phenanthrene) ratios in zebrafish, *D. rerio*, exposed to phenanthrene and *M. marinum* over 21 days. Error bars represent 95% confidence limits. Key: horizontal pattern represents the “High+Myco” group, vertical patterning represents the “Myco+High” group, slanted tile pattern represents “Myco+Low” and checkered represents “Low+Myco”.

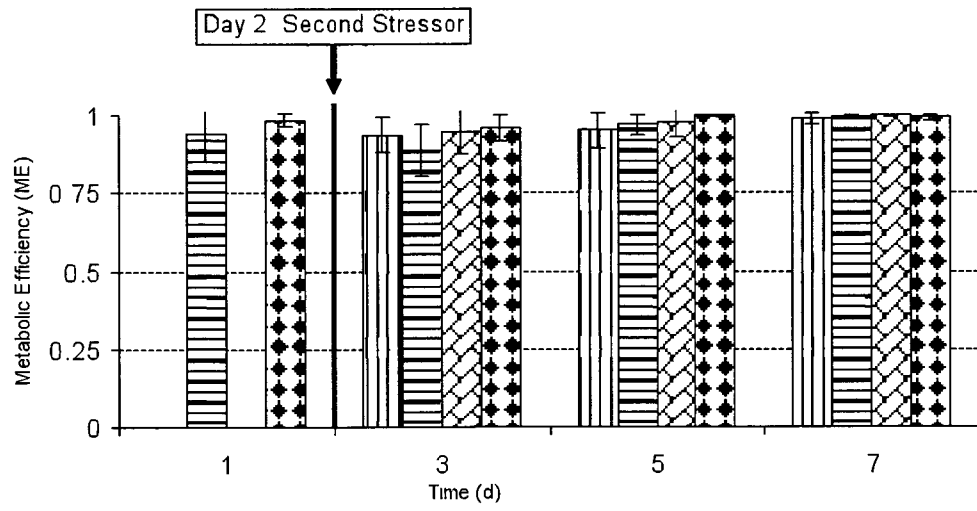


Figure 5: Hepatic CYP1A levels for zebrafish in various treatments over the first five days of the study. Letters denote significant differences between individual treatments from day 1 to day 5 ($p < 0.05$). Error bars represent 95% confidence limits. Key: Vertical lines represent the “Myco+High” group; horizontal lines represent the “High+Myco” group; slanted tiles represent the “Myco+Low” treatment; checkered pattern represents the “Low+Myco” group; spotted pattern represent controls.

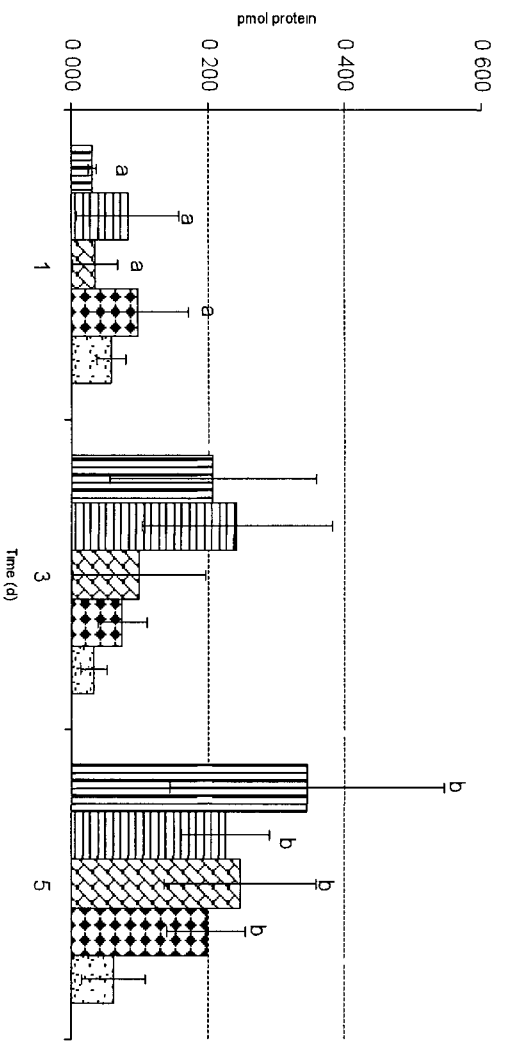
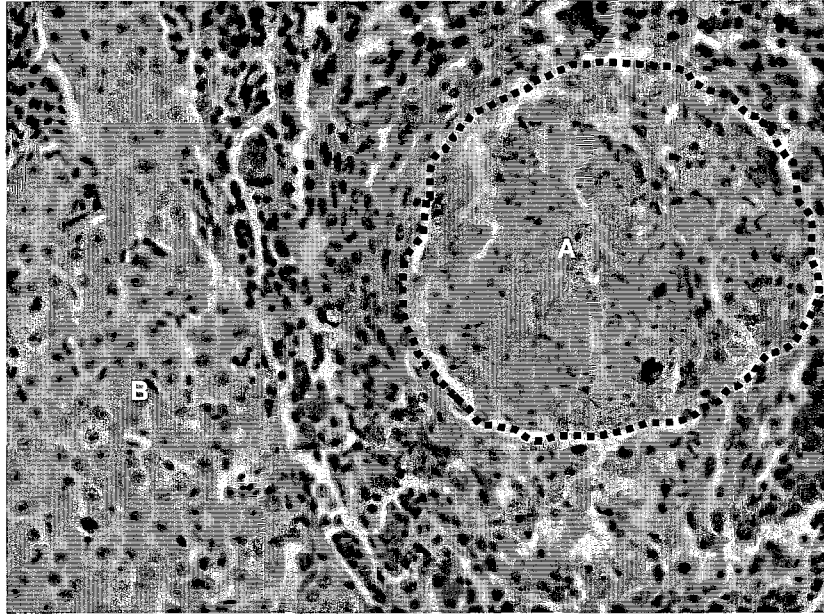


Figure 6: Histologic section through the coelomic cavity of an experimentally infected zebrafish showing a hepatic granuloma. This pathology is commonly associated with *M. marinum* infection. Granuloma is well organized with a necrotic core (A) and clearly differentiated from healthy hepatic tissue (B). 400×.



SUMMARY

The goal of this dissertation was to examine how multiple stressor interactions affect the health of the zebrafish, *Danio rerio*. Based on the first experiments I can draw several conclusions. Primarily I was able to demonstrate an antagonistic relationship between phenanthrene and *M. marinum* at a dose of $284\mu\text{g L}^{-1}$. This was evident by a significantly lower survivorship for fish exposed only to the high dose of phenanthrene as compared to the dually challenged fish. By examining body burden data I was also able to show a difference in metabolism between fish only exposed to the high dose of phenanthrene and the dually challenged fish. I believe that this disruption is responsible for the observed differences in mortality and demonstrates that phenanthrene metabolism, either through the generation of toxic intermediates or reactive oxygen species, plays a significant role in toxicity. I hypothesize that the disruption in metabolism for the dually challenged fish was due to inflammatory cytokines released as a result of bacterial infection. The results from this study were highly surprising as we had originally expected to observe immunosuppression and a subsequent increase in disease prevalence, or increases in phenanthrene body burden leading to increased mortality.

My second dual challenge study was designed to further examine the effects of dual-stressors using a more environmentally relevant phenanthrene concentration (Target: $250\mu\text{g L}^{-1}$, measured daily mean: $157\mu\text{g L}^{-1}$) and longer study duration (21 days). This study showed dramatically different results from the first. Dually challenged fish showed a significantly higher mortality than did all other treatments. In addition phenanthrene metabolism was consistent between treatments. I did however demonstrate the dual-stressor interaction to behave in an additive manner. In this sense we can predict

the multiple stressor effects by summation of our singlet exposed animals. These results are significant because they demonstrate that subtle changes in experimental design (toxicant concentration, exposure duration, and/or exposure order) can have dramatic impacts on stressor interaction and ultimately organismal health.

My third study set up a matrix of phenanthrene dose and exposure order. Fish were exposed to either $86 \mu\text{g L}^{-1}$ or $157 \mu\text{g L}^{-1}$ (Mean daily concentrations) of phenanthrene and infected with *M. marinum*. There were therefore four different treatment groups: 1) high phenanthrene first, bacteria second, 2) low phenanthrene first, bacteria second, 3) bacteria first, high phenanthrene second, and 4) bacteria first, low phenanthrene second. The results from this study demonstrated that the order of exposure did not influence organism health. This was evident by no significant differences in mortality between treatments within the “High” phenanthrene dose, regardless of exposure order. The same result was seen for both treatments within the “Low” phenanthrene dose. However, I did demonstrate phenanthrene dose as a significant factor in survivorship based on both treatments receiving the “High” dose of phenanthrene having a significantly lower survivorship than both treatments receiving the “Low” dose. I did not show significant differences in phenanthrene metabolism between any treatment groups. A surprising result from this study was the induction of the cytochrome P450 pathway as evident by increased CYP1A levels. To my knowledge this is the first study to directly show increased CYP1A protein after phenanthrene exposure in teleosts.

The ultimate conclusions that we can draw for my dissertation research are: 1) Phenanthrene does not only act as a non-specific membrane disruptor (Type I narcosis),

but at elevated concentrations phenanthrene metabolism significantly contributes to toxicity either directly from metabolite toxicity or indirectly from the generation of reactive oxygen species; 2) At elevated concentrations of phenanthrene ($284 \mu\text{g L}^{-1}$), an antagonistic relationship is observed with *M. marinum*; 3) at doses of $157 \mu\text{g L}^{-1}$ phenanthrene, an additive effect is seen with *M. marinum* 4) Phenanthrene can stimulate the cytochrome P450 pathway causing an elevation in CYP1A levels; 5) Phenanthrene does not influence the disease progression of *M. marinum*; 6) dual-stressor effects observed here were ultimately due to toxicant dose, and not influenced by order of exposure, and 7) the toxicant, its dose and the pathogen selected may all influence how dual-stressor interactions affect organism health.

Vita

Christopher Michael Prosser was born in Pensacola Fl July 26, 1979, but grew up in O'Fallon Il. After high school he attended Coastal Carolina University where he received his B.S. in biology and marine science in 2002. In 2004 he received his Master's in Environmental Management from Duke University. Entered the doctoral program at the College of William and Mary, Virginia Institute of Marine Science in Fall 2006.