AN ABSTRACT OF THE DISSERTATION OF

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Michael L. Kent

Since its inception as a laboratory animal in the early 1970s, the zebrafish has proven itself a rising star in the world of comparative biomedical sciences due to its short generation time, ease of care, external fertilization, and transparent larvae. In a very few decades, the zebrafish has been utilized as a model organism for as many experimental topics and modalities as its rodent counterparts, which have been in use for centuries. Whereas the rodent world has had time to develop biosecurity protocols and specific pathogen free lines, the zebrafish community has had little time to cleanse the animals of contaminating infectious organisms from their tenure as ornamental fish in the pet trade. Initially, because they were used primarily for developmental genetics studies, the need for biosecurity and elimination of infectious disease was not viewed as particularly important. However, as zebrafish have been used as model organisms in sensitive studies of biomedical and neurobehavioral phenomena, the scientific community must evaluate the potential for subclinical infections to alter experimental data. *Pseudoloma neurophilia*, a microsporidian endoparasite, is currently one of the most commonly diagnosed infections in zebrafish facilities worldwide. Because of its tropism for the central nervous system, and because of the opportunistic nature of microsporidia, it is vital to understand the potential effects that infection can have on behavior and studies involving immunosuppression. Armed with this knowledge, researchers will be able to make informed decisions regarding biosecurity and husbandry protocols in order to mitigate the effects of infection-associated, non-protocol induced variation. The hypothesis of this dissertation is that infection of zebrafish by *Pseudoloma neurophilia* causes non-protocol induced variation in

neurobehavioral and biomedical experiments. In order to explore experimental protocols most likely to be influenced by *P. neurophilia*, I first performed a retrospective study with the intention of identifying the most common features of neuronal and muscular infections. Five hundred fifty-nine zebrafish infected with *P. neurophilia* submitted to ZIRC (Zebrafish International Resource Center, Eugene, OR) from 86 laboratories between the years 2000 and 2013 were examined via histopathology. Parasite clusters (PCs) occurred in distinct axonal swellings, frequently with no associated inflammation. Inflammation was observed in viable cell bodies distant from PCs. Multiple PCs occasionally occurred within a single axon, suggesting axonal transport. PCs occurred most frequently in the spinal cord ventral white matter (40.3% of all PCs) and the spinal nerve roots (25.6%). Within the rhombencephalon, PCs were most common in the primary descending white matter tracts. Within the rhombencephalon gray matter, PCs occurred most frequently in the reticular formation and the griseum centrale (61% and 39%, respectively). High numbers of PCs within brain and spinal cord structures mediating startle responses and anxiety suggest that related behaviors could be altered by neural microsporidiosis. Infection could, therefore, introduce unacceptable variation in studies utilizing these behaviors.

I chose a commonly utilized neurobehavioral testing protocol that involved motor activity and anxiety-associated responses since it appeared to be the most likely experimental protocol to be influenced by *P. neurophilia* infection: The progressive tap test for startle response habituation. Fish infected via cohabitation were tested for startle response habituation in parallel with controls in a device that administered ten taps over 10 min along with taps at 18 and 60 min to evaluate habituation extinction. After testing, fish were euthanized and evaluated for infection via histopathology. Infected fish had a significantly smaller reduction in startle velocity during habituation compared to uninfected tankmates and controls. Habituation was eliminated in infected and control fish at 18 min, whereas exposed negative fish retained partial habituation at 18 min. Infection was also associated with enhanced capture evasion: Despite the absence of external symptoms, infected fish tended to be caught later than uninfected fish netted from the same tank. The combination of decreased overall habituation, early extinction of habituation compared to uninfected cohorts, and enhanced netting evasion indicates that *P. neurophilia* infection is associated with a behavioral phenotype distinct from that of controls and uninfected cohorts.

In order to demonstrate a causative link between infection and behavior change, and to evaluate another common neurobehavioral experimental protocol, we performed a shoaling test, which examines social behavior, before and after infection. Tanks containing 10 fish each were divided into 6 control and 6 experimental shoals and recorded prior to exposure. Over 123 days, control fish were exposed to water housing uninfected fish and experimental fish were exposed to water housing infected fish. Shoals were re-recorded following exposure and infection status was determined via histopathology. There were no significant differences in mean interfish distance and percent of top-dwelling fish between control and experimental shoals prior to exposure. Following the exposure period, shoals exposed to and infected by P. neurophilia showed a significantly reduced mean interfish distance compared to controls. The percentage of top-dwelling fish was also reduced in infected shoals, although this difference was not statistically significant. This study supports the fact that *P. neurophilia* infection causes altered behavior in zebrafish and it should act as a warning to neurobehavioral researchers to use parasite-free fish in their research.

Because of the opportunistic nature of microsporidial infections as demonstrated by fatal *Enterocytozoon bieneusii* and *Encephalitozoon intestinalis* infections in human AIDS patients, we decided to evaluate the effects of immunosuppressive gamma irradiation protocols on zebrafish infected with *P. neurophilia*. In this study we exposed zebrafish to combinations of *P. neurophilia* infection and gamma irradiation in order to explore the interaction between this immunosuppressive experimental modality and a normally subclinical infection. Zebrafish infected with *P. neurophilia* and exposed to gamma irradiation exhibited higher mortality, increased parasite loads, and increased incidences of myositis and extraneural parasite infections than fish exposed either to *P. neurophilia* or gamma irradiation alone.

Pseudoloma neurophilia in the Zebrafish *(Danio rerio)*: Consequences of Infection on Neurobehavioral and Biomedical Research using a Burgeoning Model Organism

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Sean Spagnoli, Author

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Their sacrifice was not in vain.

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Brandon Crews, you make me want to be a better man.

CONTRIBUTION OF AUTHORS

For chapter 2, Dr. Lan Xue performed statistical analyses. Dr. Katrina N. Murray provided archived glass slides from the ZIRC diagnostic service. Fidelis Chow performed the datamining necessary to identify appropriate cases. For chapter 3, Dr. Lan Xue performed statistical analyses. For chapter 4, Justin Sanders performed statistical analyses. For chapter 5, Justin Sanders performed statistical analyses and Virginia Watral provided support for technical aspects of experimentation.

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Chapter 1

INTRODUCTION

1.1 The Zebrafish: *Danio rerio*

Since its inception as a model organism for developmental genetics studies in the 1970s the zebrafish (*Danio rerio*) has skyrocketed in terms of its popularity and breadth of use, surpassing *C. elegans* and *Drosophilia* in Pubmed keyword searches every year from 2011 to the present (Gunwald and Eisen 2002; Kent et al. 2012). As vertebrates, zebrafish can be used in nearly any experiment that utilizes rodents with the distinct advantages of space efficiency, shorter generation times, large numbers of offspring, and external fertilization which considerably reduces the difficulties of precise breeding practices and the genetic manipulation of embryos (Gunwald and Eisen 2002; Phillips and Westerfield 2014). That is not to say that zebrafish are a "better" model organism than mice—as mammals, mice share more conserved traits with humans than zebrafish at immunological and anatomical levels—rather, the zebrafish should be viewed as a complementary laboratory organism possibly better suited to initial high-throughput screenings requiring large numbers of subjects in order to fine-tune initial protocols before moving on to more complex, expensive, and labor-intensive mammalian species.

One of the greatest challenges facing the research community regarding zebrafish compared to mice is their relative novelty as a laboratory organism. Rodents, having been utilized in comparative biomedical research for over a century, have had sufficient time to be relatively cleansed of infectious organisms that produce overt disease or cause more insidious subclinical infections which cause non-protocol induced variation in experiments. Hence, tight biosecurity measures and specific pathogen free (SPF) mouse lines are commonplace in many laboratories (Compton and Macy 2015). That is not to say, however, that all of the bugs have been worked out of rodent models. Until very recently, pneumonia in laboratory rats caused by the fungus *Pneumocystis carinii* was thought to be caused by an undiscovered "rat respiratory virus" (Livingston et al. 2011) and the infamous supposed carcinogenic nature of the artificial

sweetener Saccharine was brought into question in the 1980s when rodents suffering from transitional cell carcinomas also tended to be infected with the bladder threadworm *Trichosomoides crassicauda* (Weihrauch and Diehl 2004). It is important for the research community to learn from the rodent world's history so that the zebrafish community does not repeat similar mistakes. It is with this in mind that this thesis was dedicated to evaluating the potential effects of the highly prevalent but usually subclinical, infectious organism *Pseudoloma neurophilia* on the following research endpoints: neurobehavior and the pathogenesis of the parasite in severely immune compromised zebrafish.

1.2 *Pseudoloma neurophilia*

Pseudoloma neurophilia is a microsporidium, an obligate intracellular microparasite that forms environmentally resistant spores and is most closely related to fungi (Kent et al. 2014; Sanders 2012). Microsporidia are well recognized to cause disease in fishes. However, *P. neurophilia* is similar to *Encephalitozoon cuniculi* of rabbits and dogs (Percy and Barthold 2007; Kent et al. 2014), and *Enterocytozoon bieneusii* both of which generally have no effect on healthy humans, but can produce fatal disease in the immunosuppressed hosts such as AIDS patients (Didier and Weiss 2006; Carr et al. 1998). The origins of *P. neurophilia* infections in zebrafish are unclear. Although the zebrafish is native to tributaries of the Ganges river, the first experimental animals were purchased from a commercial supplier, which is a common practice to this day (Gunwald and Eisen 2002; Spagnoli et al. 2015). Interestingly, although I have observed intramuscular microsporidia similar in appearance to *Pleistophora* species in wild-caught zebrafish from India, no organisms resembling *P. neurophilia* have been observed outside of laboratory or pet fish stocks (anecdotal). This conspicuous absence, along with the ability of *P. neurophilia* to infect fathead minnows (*Pimephales promeras*) and medaka (*Oryzias latipes*) (Sanders et al. 2016), which are commonly housed alongside zebrafish, suggests that *P. neurophilia* infections may have originated in commercial supplier housing rather than from the wild.

P. neurophilia can infect nearly any cell in the zebrafish body, however, it has a powerful tropism for the nervous system, most commonly forming intra-axonal parasite clusters

containing all stages of development (Spagnoli et al. 2015). Because these infections tend to be deep within the animal's tissues, a common route of infection is thought to be carcass consumption as zebrafish tend to eat their dead soon after expiration. Unlike *E. cuniculi* and *E. bieneusii*, which infect renal and intestinal epithelial cells and are shed in urine and feces, respectively, fecal and urine shedding make minimal contributions to *P. neurophilia* transmission (Percy and Barthold 2007; Carr et al. 1998). Rather, *P. neurophilia* commonly infects the zebrafish ovary and spores are frequently observed either within ova or in the adjacent ovarian stroma (Sanders et al. 2013). Since female zebrafish are frequent spawners, spores consumed in shed eggs and egg associated debris are considered to be a constant source of environmental contamination throughout an individual's lifetime. Vertical transmission between mothers and larvae also occurs which, along with the presence of spores adhered to egg surfaces, is a particular problem for zebrafish facilities since most genetic lines are shared between laboratories through embryonated eggs (Sanders et al. 2013). Not only do spores resist bleaching up to 100 ppm (Ferguson et al. 2007), which is higher than the concentration used by most facilities to wash eggs, but spores within the egg itself are never exposed to bleach at all (Sanders et al. 2013). This ease of transmission between facilities, along with the subclinical and therefore occult nature of most infections, resulted in up to 75% of facilities having the infection, based on samples to the Zebrafish International Resource Center (ZIRC, Eugene, OR) diagnostic service between 1999 and 2013 (Murray 2015).

The fact that most *P. neurophilia* infections are subclinical has traditionally resulted in a relatively laissez-faire attitude on the part of the research community regarding screening and biosecurity, and not without cause. Traditionally, zebrafish were developed as a model primarily for developmental genetics, an area of research that likely would not be impacted by a chronic, subclinical infection (Gunwald and Eisen 2002). However, over the relatively few decades that zebrafish have been used in comparative biomedical studies, their utility has blossomed to encompass nearly every conceivable area of research from toxicology to immunology to infectious disease and neurobehavior (Gunwald and Eisen 2002; Kalueff and Stewart 2012; Phillips and Westerfield 2015). Due to their ease of use and care, however, the zebrafish's myriad applications as a model organism have far outstripped the scrutiny of potential complications inherent in its own pathology and ecology.

1.3 Zebrafish and neurobehavioral research.

Based on *P. neurophilia*'s prominent tropism for the zebrafish nervous system, one such area of research that will be highlighted in this thesis is comparative neurobehavior, which benefits largely from the zebrafish's robust and consistent behavioral responses, their high fecundity, and the transparency of their larvae (Kalueff and Stewart 2012). While there are obvious differences between zebrafish and mammalian/human neuroanatomy, there are just as many conserved features of development, physiology, and behavioral integration among vertebrates. Various basic behaviors are conserved across all vertebrates, including the auditory startle response, habituation to stimuli, and exploratory behavior (Kalueff and Stewart 2012). Similarly, specific neuroleptic drug trials have allowed researchers to identify various facets of behavior including, but not limited to, fear and anxiety. Furthermore, zebrafish tend to exhibit behavioral syndromes, a form of "personality" similar to mammals in which individuals respond in a consistent, syndrome-dependent manner to a variety of stimuli (Kalueff et al. 2012).

In addition to behavioral similarities between zebrafish, mice, and humans, zebrafish have the further advantage of producing transparent larvae. This transparency coupled with a thorough knowledge of cell fates during embryonic development, has allowed for the creation of the "brainbow" strain in which specific neurons are fluorescently labelled (Richier and Salecker 2015). Taken together, these features of zebrafish neurobehavior and physiology have led researchers to utilize them for a panoply of research topics including neurotoxin screening, pharmaceutical screening of psychoactive drugs, and the evaluation of long-term behavioral effects of larval exposure to various toxins (fetal alcohol syndrome in particular) (Kalueff et al. 2012). Zebrafish are currently being used to model a wide range of human neurobehavioral disorders including autism, Parkinson's Disease, schizophrenia, alcoholism, addiction, social anxiety, and depression (Kalueff et al. 2012; Maximinio et al. 2010; Morris 2009; Pittman and Lott 2014; Stewart et al. 2014; Willemsen et al. 2008). Inherent to behavioral studies is a large amount of variation between fish and between tanks (Spagnoli et al. 2015). The addition of a neurotropic parasite in research animals could result in altered data based on infection. It is for this reason that this thesis will carefully explore the potential effects of *P. neurophilia* infection on various neurobehavioral testing modalities used by zebrafish researchers.

1.4 Zebrafish and biomedical research

Shifting from neurobehavioral research to another realm of biomedical research, this thesis also also explores the interactions between immunosuppression and *P. neurophilia* infection because microsporidia are notoriously opportunistic organisms that produce severe infections in immune suppressed individuals, particularly in AIDS patients (Kent et al 2014; Didier and Weiss 2006; Carr et al. 1998). Furthermore, Ramsay et al. (2012) demonstrated that stress and cortisol, both of which are consummately immunosuppressive, are associated with increased mortality and infection severity due to *P. neurophilia*. Appropriate to this discussion is the use of gamma irradiation in various studies of the immune system. Some laboratories use gamma irradiation to knock down the immune system of zebrafish for tissue transplant studies (Trede et al. 2004; Traver et al. 2004; Paik and Zon 2010) and it is possible that underlying *P. neurophilia* infections in these fish may become clinical. Gamma radiation is an ionizing radiation usually emitted by the isotope Cobalt 60. The high frequency electromagnetic waves excite atoms and molecules within the target tissue to the extent that electrons are released from their orbits, producing free radicals. Free radicals, with their incomplete outer electron shells, are highly reactive, and their interactions with complex molecules such as DNA are highly damaging. In the case of DNA, free radicals result in either single- or double-strand breaks. The cell's DNA repair and monitoring processes respond to DNA damage either by inducing apoptosis or by attempting to repair the damage. Generally, repair attempts result in deletions since double strand breaks may lack appropriate template DNA for replication and repair. These deletions can result in cellular dysfunction or death, or apoptosis induction by DNA proofreading mechanisms. While gamma irradiation has some effect on all exposed tissues, rapidly dividing cells are most susceptible to destruction since the "checkpoint" stages of mitosis are under close scrutiny for DNA damage and the resulting damage-induced apoptosis. (Hosoya and Miyagawa 2014; Lord and Ashworth 2012).

Gamma irradiation is commonly used in hematopoietic and immunological research to eliminate rapidly dividing host hematopoietic cells as a precursor to replacement with foreign cells in a "rescue" scenario (Trede et al. 2004; Traver et al. 2004; Paik and Zon 2010). Because of the immunosuppressive effects of gamma irradiation, opportunistic infections are a particular risk. This is well-illustrated by incidences of severe opportunistic infections following the

Chernobyl incident, leading to the rather colorful term “Chernobyl AIDS” (Yablokov 2009). There is also precedence for increased infection severity in zebrafish secondary to gamma irradiation: Parrikka et al. (2012) found that gamma irradiation of fish with latent *Mycobacteria marinum* infections resulted in severe, active infections. It is for these reasons that the interactions between gamma irradiation and *P. neurophilia* infection will be investigated in this thesis.

1.5 Conclusion

In this dissertation, we will evaluate, for the first time, the influence of *Pseudoloma neurophilia* infection on various research methodologies in the adult zebrafish, *Danio rerio*. Chapter 2 describes a wide-ranging retrospective study to provide anatomical insight into the infection patterns and pathogenesis of *P. neurophilia*. Chapter 3 concerns the effects of *P. neurophilia* infection on startle response habituation in adult zebrafish, the test most likely to be sensitive to influence by infection based on the parasite’s most common neuranatomic distribution. Chapter 4 further explores the effects of infection on zebrafish behavior by evaluating social interactions via the shoaling test. Chapter 5 details the interactions between *P. neurophilia* infection and gamma irradiation of adult fish. I also include a book chapter reviewing stress and its impacts in on research in laboratory fishes in the Appendix. By demonstrating the influence of infection on experimental results, we can alert the research community to the importance of screening and biosecurity protocols in order to improve the zebrafish as a model organism.

1.6 References

Grunwald D and Eisen J. Headwaters of the zebrafish—emergence of a new model vertebrate. Nat Rev: Genetics 3. 2002;717-724.

Phillips JB and Westerfield M. Zebrafish models in translational research: tipping the scales toward advancements in human health. Dis Models Mech. 2014;7(7):739-743.

Kent ML, Harper C, and Wolf JC. Documented and potential research impacts of subclinical diseases in zebrafish. ILAR J. 2012:53;126-134.

Compton SR and Macy JD. Effect of cage-wash temperature on the removal of infectious agents from caging and the detection of infectious agents on the filters of animal bedding-disposal cabinets by PCR analysis. J Am Assc Lab Anim Sci. 2015;54(6): 745-755.

Livingston RS, Besch-Williford CL, Myles MH, Franklin CL, Crim MJ, and Riley LK. *Pneumocystis carinii* infection causes lung lesions historically attributed to rat respiratory virus. Comp Med. 2011;61(1):45-59.

Weihrauch MR and Diehl V. Artificial sweeteners—do they bear a carcinogenic risk? Ann Oncol. 2004; 15(10):1460-1465.

Kent ML, Shaw RW, and Sanders J. Fish Microsporidia. Chapter 20, in: LM Weiss, JJ Becnel (Eds), Microsporidia: Pathogens of Opportunity, Wiley Enterprise, 2014, pp. 493-520.

Sanders GE. Zebrafish housing, husbandry, health and care: IACUC considerations. ILAR J 2012;53:205–207.

Percy DH, and Barthold SW, eds. Pathology of laboratory rodents and rabbits, third edition. Blackwell, Iowa. 2007.

Didier ES and Weiss LM. Microsporidiosis: current status. Curr Opin Infect Dis 2006; 19: 485-492.

Carr A, Marriott D, Field A, Vasak E, and Cooper DA. Treatment of HIV-1 associated microsporidiosis and cryptosporidiosis with combination antiretroviral therapy. Lancet. 1998; 351: 256-261.

Spagnoli S, Xue L, Murray KM, Chow F, and Kent ML. *Pseudoloma neurophilia*: a retrospective and descriptive study of nervous system and muscle infections with new implications for pathogenesis and behavioral phenotypes. Zebrafish. 2015;12:189-201.

Sanders J, Watral V, Clarkson K, and Kent ML. Verification of intraovum transmission of a microsporidium of vertebrates: *Pseudoloma neurophilia* infecting the zebrafish, *Danio Rerio*. PLoS One 2013;8(9).

Ferguson J, Watral V, Schwindt A, and Kent ML. Spores of two fish Microsporidia (*Pseudoloma neurophilia* and *Glugea anomola*) are highly resistant to chlorine. Dis Aquat Org. 2007;76:205-214.

Murray K. Summary of submissions to the ZIRC Diagnostic Service on The Zebrafish International Resource Center webpage. URL: http://zebrafish.org/health/diseaseManual.php#SUMMARY OF SUBMISSIONS TO THE ZIRC DIAGNOSTIC SERVICE. 2015.

Zebrafish Protocols for Neurobehavioral Research. Kalueff AV and Stewart AM (Eds). Springer, New York, 2012.

Richier B and Salecker I. Versatile genetic paintbrushes: Brainbow technologies. Wiley Interdiscip Rev Dev Biol. 2015;4(2):161-180.

Maximino C, de Brito TM, Silva Batista AW, Herculano AM, Morato S and Gouveia A Jr. Measuring anxiety in zebrafish: a critical review, Behav Brain Res. 2010;214:157–171.

Morris JA. Zebrafish a model system to examine the neurodevelopmental basis of schizophrenia. Prog Brain Res. 2009;179:97–106.

Pittman JT and Lott CS. Startle response memory and hippocampal changes in adult zebrafish pharmacologically-induced to exhibit anxiety/depression-like behaviors. Physiol Behav. 2014;123:174–179.

Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, and Kalueff AV. Modeling anxiety using adult zebrafish: a conceptual review, Neuropharmacology 2012;621:35–143.

Stewart A, Wong K, Cachat J, Gaikwad S, Kyzar E, Wu N, Hart P, Piet V, Utterback E, Elegante M, Tien D, and Kalueff AV. Zebrafish models to study drug abuse-related phenotypes, Rev. Neurosci. 2011; 22:95–105.

Stewart AM, Nguyen M, Wong K, Poudel KW, and Kalueff AV, Developing zebrafish models of autism spectrum disorder (asd). Prog Neuropsychopharmacol Biol Psychiatry. 2014;50:27–36.

Willemsen R, Hasselaar W, van der Linde H and Bonifati V. Zebrafish as a new model organism for Parkinson's disease. Proc Meas Behav. 2008;5:0–1.

Spagnoli S, Xue L, and Kent ML. The common neural parasite *Pseudoloma neurophilia* is associated with altered startle response habituation in adult zebrafish (*Danio rerio*): Implications for the zebrafish as a model organism. Behav Brain Res. 2015;291:351-360.

Ramsay JM, Watral V, Schreck CB, Kent ML. *Pseudoloma neurophilia* infections in zebrafish *Danio rerio*: effects of stress on survival, growth, and reproduction. Dis Aquat Organ. 2009;88:69–84.

Trede NS, Langenau DM, Traver D, Look AT, Zon LI. The use of zebrafish to understand immunity. Immunity. 2004;20(4):367-379.

Traver D, Winzeler A, Stern HM, Mayhall EA, Langenau DM, Kutok JL, Look AT, and Zon LI. Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. Blood. 2004;104(5): 1298-1305.

Paik EJ and Zon LI. Hematopoietic development in the zebrafish. Int J Dev Biol. 2010;54:1127-1137.

Hosoya N, Miyagawa K. Targeting DNA damage response in cancer therapy. Cancer Sci. 2014;105:370–388.

Lord CJ, Ashworth A. The DNA damage response and cancer therapy. Nature 2012;481:87–294.

Trede NS, Langenau DM, Traver D, Look AT, Zon LI. The use of zebrafish to understand immunity. Immunity. 2004;20(4):367-379.

Traver D, Winzeler A, Stern HM, Mayhall EA, Langenau DM, Kutok JL, Look AT, and Zon LI. Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. Blood. 2004;104(5): 1298-1305.

Paik EJ and Zon LI. Hematopoietic development in the zebrafish. Int J Dev Biol. 2010;54:1127-1137.

Yablokov AV. Nonmalignant Diseases after the Chernobyl Catastrophe, in Chernobyl: Consequences of the catastrophe for people and the environment. Yablokov AV, Nesterenko VB, and Nesterenko AV, eds. Annals of the New York Academy of Sciences, vol. 1181. 2009, pp 58-161. Blackwell Publishing. Boston, Mass.

Parikka M, Hammare´n MM, Harjula S-KE, Halfpenny NJA, Oksanen KE, et al. Mycobacterium marinum Causes a Latent Infection that Can Be Reactivated by Gamma Irradiation in Adult Zebrafish. PLoS Patho (2012) g 8(9): e1002944.doi:10.1371/journal.ppat.1002944

Chapter 2

Pseudoloma neurophilia: A Retrospective and Descriptive Study of Nervous System and Muscle Infections, with New Implications for Pathogenesis and Behavioral Phenotypes.

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2.1 Abstract

Pseudoloma neurophilia is a microsporidium of zebrafish (*Danio rerio*) that preferentially infects neural tissue. It is one of the most common pathogens of zebrafish in research laboratories based on diagnostic data from the Zebrafish International Resource Center diagnostic service (Eugene, OR). Five hundred fifty-nine zebrafish infected with *P. neurophilia* submitted to ZIRC from 86 laboratories between the years 2000 and 2013 were examined via histopathology to develop a retrospective study of the features of neural microsporidiosis. Parasite clusters (PCs) occurred in distinct axonal swellings, frequently with no associated inflammation. Inflammation was observed in viable cell bodies distant from PCs. Multiple PCs occasionally occurred within a single axon, suggesting axonal transport. PCs occurred most frequently in the spinal cord ventral white matter (40.3% of all PCs) and the spinal nerve roots (25.6%). Within the rhombencephalon, PCs were most common in the primary descending white matter tracts. Within the rhombencephalon gray matter, PCs occurred most frequently in the reticular formation and the griseum centrale (61% and 39%, respectively). High numbers of PCs within brain and spinal cord structures mediating startle responses and anxiety suggest that related behaviors could be altered by neural microsporidiosis. Infection could, therefore, introduce unacceptable variation in studies utilizing these behaviors.

2.2 Introduction

Pseudoloma neurophilia is a common microsporidian parasite of zebrafish (*Danio rerio*) that can infect nearly every tissue in the body (Kent et al. 2011; Sanders 2012). It has an overwhelming preference for neural tissue, particularly the nerve roots, spinal cord, and hindbrain (Sanders et al. 2014). First described in 2001, *P. neurophilia* has since been identified as one of the most common infectious organisms diagnosed in laboratory zebrafish. Clinical infection is most frequently characterized by emaciation and spinal deformities (Matthews et al. 2001; Murray et al. 2011). Transmission occurs through the consumption of environmentally resistant spores either free in the water or embedded in fish carcasses. Maternal transmission occurs, either by shedding spores at spawning or by parasites within the egg (Sanders et al. 2013). Spores are resistant to bleaching at a concentration of 25–50 ppm for 10 min, which makes transmission prevention difficult even when spores are adhered to the exterior egg surface

(Murray et al. 2011). The Zebrafish International Resource Center (ZIRC) located in Eugene, OR, provides a diagnostic service to the zebrafish community by performing histopathologic, molecular, and bacteriologic analyses of samples from both clinically ill and routine sentinel fish. From the years 2006 to 2013, this service diagnosed *P. neurophilia* in an average of 50% of submitting facilities (range 19%–74%) (Murray et al. 2011; Kent et al. 2012; Chow et al. 2015). Recently, a survey of the ZIRC diagnostic service database revealed that only 26% of fish submitted between the years 1999 and 2013 diagnosed with *P. neurophilia* were submitted due to clinical disease, while the rest were submitted as routine sentinel cases with no reported clinical disease (Kent et al. 2012; Chow et al. 2015). Taken together, these facts indicate that *P. neurophilia* is prevalent throughout zebrafish facilities and that most cases *of P. neurophilia* infection are subclinical, making this disease both widespread and insidious (Murray et al. 2011).

Diagnosis of *P. neurophilia* infection is usually made via histopathologic examination of fixed tissues or by polymerase chain reaction analysis. Microscopic lesions in fish include myositis, meningitis/meninxitis, myelitis, encephalitis, granulomatous inflammation, and, commonly, the presence of parasite clusters (PCs) in nervous and other tissues without inflammation (Matthews et al. 2001; Murray et al. 2011). PCs were first described as xenomas (Matthews et al. 2001), but more extensive ultrastructural analysis by Cali et al (2012) showed that they are not xenomas as defined by Lom and Dykova (2005). Although a particular name has not been officially suggested for these structures created by *P. neurophilia*, we will refer to them simply as ‘‘parasite clusters’’ in this article, because they contain all stages of the life cycle but are not surrounded by a distinct membrane.

Because most *P. neurophilia* infections are often subclinical and because the zebrafish is still a relatively new addition to the laboratory animal stable, the drive to screen for and to eliminate *P. neurophilia* within zebrafish facilities has been relatively low compared with the efforts made with pathogens that cause acute mortality, such as *Mycobacterium marinum*, *Mycobacterium haemophilum*, and *Edwardsiella ictulari* (Murray et al. 2011).

P. neurophilia has been extensively studied with regard to life cycle, transmission, microscopic and ultrastructural features, as well as the effects of stress on infection (Sanders et al. 2014; Matthews et al. 2001; Murray et al. 2011; Sanders et al. 2013; Cali et al. 2012; Ramsay et al. 2009). Zebrafish are now extensively used in behavior research (Morris 2009; Pittman and Lott 2014; Stewart et al. 2011; Stewart et al. 2012; Stewart et al. 2014; Willemsen et al. 2008),

and it is possible that these neural infections *by P. neurophilia* may introduce nonprotocol-induced variation to these experiments.

Based on a relatively small sample size from one facility, Matthews et al. (2001) first associated severity of infection and concurrent myositis with clinical disease (i.e., emaciation). Ramsay et al. (2009) later showed that infected fish that were subjected to crowding stress had an increased incidence of myositis. We took advantage of the large collection of histologic slides, including both clinical and apparently normal infected zebrafish at ZIRC, to conduct a more comprehensive retrospective study evaluating the overarching trends of *P. neurophilia* infection with respect to grossly observable clinical disease, inflammation, anatomic location, and the simultaneous presence of other potentially complicating diseases. The purpose of our detailed study was to further characterize the features of *P. neurophilia* infections with an eye toward developing a hypothesis as to which specific behaviors or other research endpoints are most likely to be influenced by this common infection.

2.3 Materials and Methods

Fish were selected based on data reviewed from the ZIRC diagnostic service database. These archival samples ranged from the years 1999 to 2014 (Chow et al. 2015). To determine whether any microscopic features of infection were correlated with grossly visible clinical disease, infected fish that were submitted as ‘‘clinical’’ were examined (n = 175). To compare the features of cases submitted as ‘‘routine’’ or nonclinical, a set of 175 fish from this group was arbitrarily selected. As the study progressed, we decided to match the number of clinical and routine cases for each year to help reduce potential effects of submission date as a confounding factor. This resulted in the examination of a total of 559 fish, with 175 noted as clinical and 384 noted as routine.

Samples examined were glass slides made from paraffin embedded, fixed tissues. The vast majority were stained with hematoxylin and eosin (H&E), although a small number of slides were stained by utilizing the Giemsa, Gram, acid fast, and Luna techniques utilizing standard procedures. All specimens examined were sectioned sagitally. Also included in our analysis were the year of submission, the submitting laboratory, the sex of the fish, and the presence of other potential causes of primary disease besides *P. neurophilia*.

A negative binomial regression was used to determine whether the total PC numbers were significantly different between male and female fish, and between routine and clinical fish while controlling for other factors: year, whether or not fish displayed myositis, and whether or not the fish had other diseases. A logistic regression was used to assess the effects of sex, clinical disease, the presence of other diseases, meninxitis, and encephalitis on the prevalence of myositis, while controlling for the effect of the year in which each case was submitted. An ordered logistic regression was used to determine the effects of sex, the presence of other diseases, and encephalitis on the severity of meninxitis. At each of the anatomic locations under study (brain and spinal cord), a negative binomial regression was used to determine whether the total PC numbers observed in white matter versus gray matter were significantly different while controlling for other factors: year of submission, sex, clinical disease, myositis, and the presence of other diseases.

All analyses were conducted using the statistical software R (version 3.1). The R function glm.nb() was used for negative binomial regression analysis, polr() was used for ordered logistic regression analysis, and glm() with a logit link was used for the logistic regression analysis.

2.4 Results

Although extraneuromuscular lesions were noted during the study (spores or associated granulomas in the viscera), their observation was rare. Furthermore, Sanders et al. (2014) and Peterson et al. (2001) showed that light *P. neurophilia* infections, particularly those in the viscera, frequently require special staining to highlight small numbers of spores. Because this was a retrospective study ranging from 1999 to 2013 and because the Luna stain was not used for zebrafish diagnotics until ~2008, we focused on neural and muscular infections observable by H&E staining alone.

To provide an objective analysis of the data to complement the descriptive aspects of the study, the number of PCs in various anatomic locations throughout the nervous system were counted (Fig. 1). Similarly, to provide an objective assessment of the extent of inflammation (meninxitis and encephalitis/ myelitis), a rubric was developed based on the character and extent of inflammation within the central nervous system (Fig. 2).

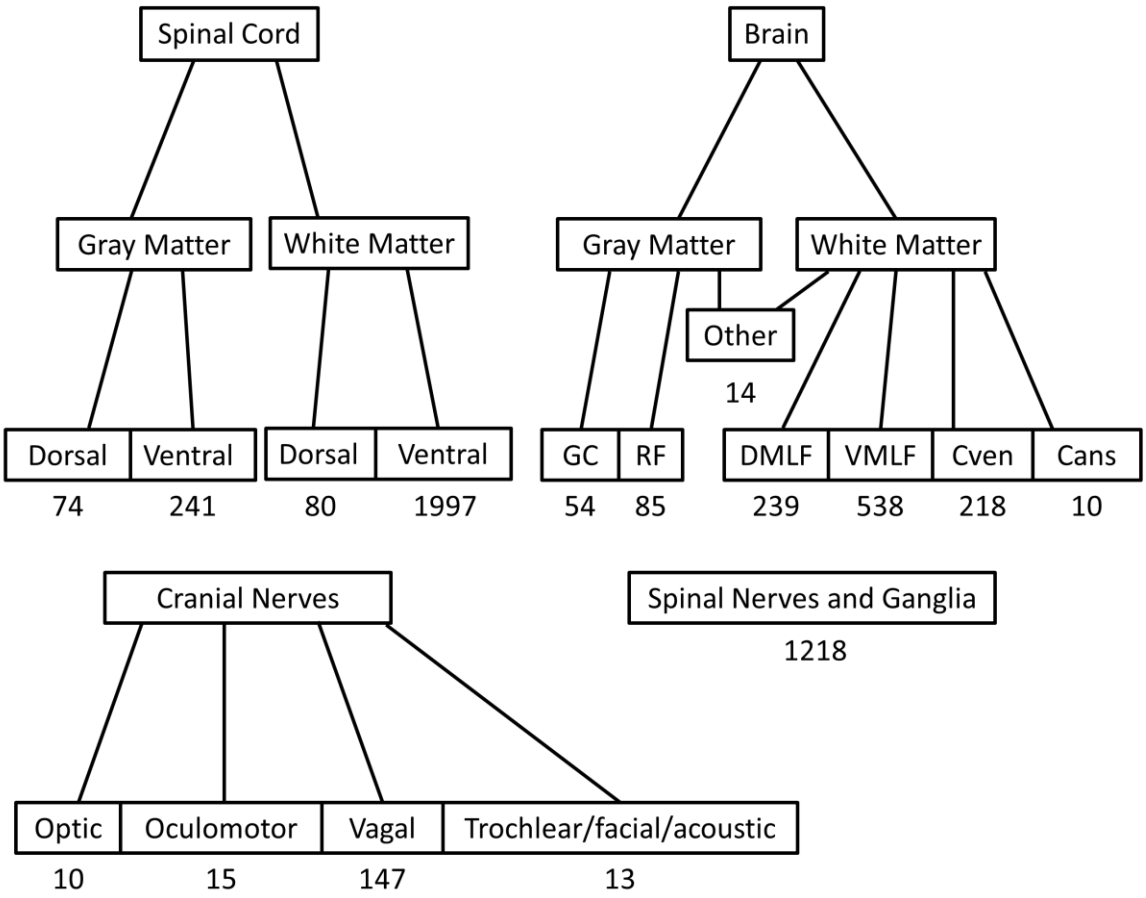

FIG. 1. Anatomic locations and spore cluster numbers in the nervous system. PCs were most commonly observed in the ventral spinal cord white matter. Spinal nerves and ganglia were a close second. Spores observed in the brain were most common in the VMLF. For both the spinal cord and the brain, PCs were overwhelmingly more commonly observed in the white matter than in the gray matter. Within gray matter in the hindbrain, spores were most commonly observed in the RF and the GC. A strikingly large number of PCs were observed in the vagal nerve. Because of the sectioning of archival samples, it was difficult to distinguish between the trochlear, facial, and acoustic nerves and so these are grouped in single category. The ‘‘Other’’ category applies to all locations in the brain in which less than 10 PCs were observed out of all fish examined. This category includes both white and gray matter structures. It is important to note that no PCs were observed in any gray matter structure farther rostral than the hypothalamus. PCs were never encountered in the telencephalon. The vast majority of the PCs were observed in the rhombencephalon. DMLF, dorsal medial longitudinal fascicle; Cven, commisura ventralis rhombencephali; Cans, commisura ansulata; PCs, parasite clusters; VMLF, ventral medial longitudinal fascicle; RF, reticular formation; GC, griseum centrale.

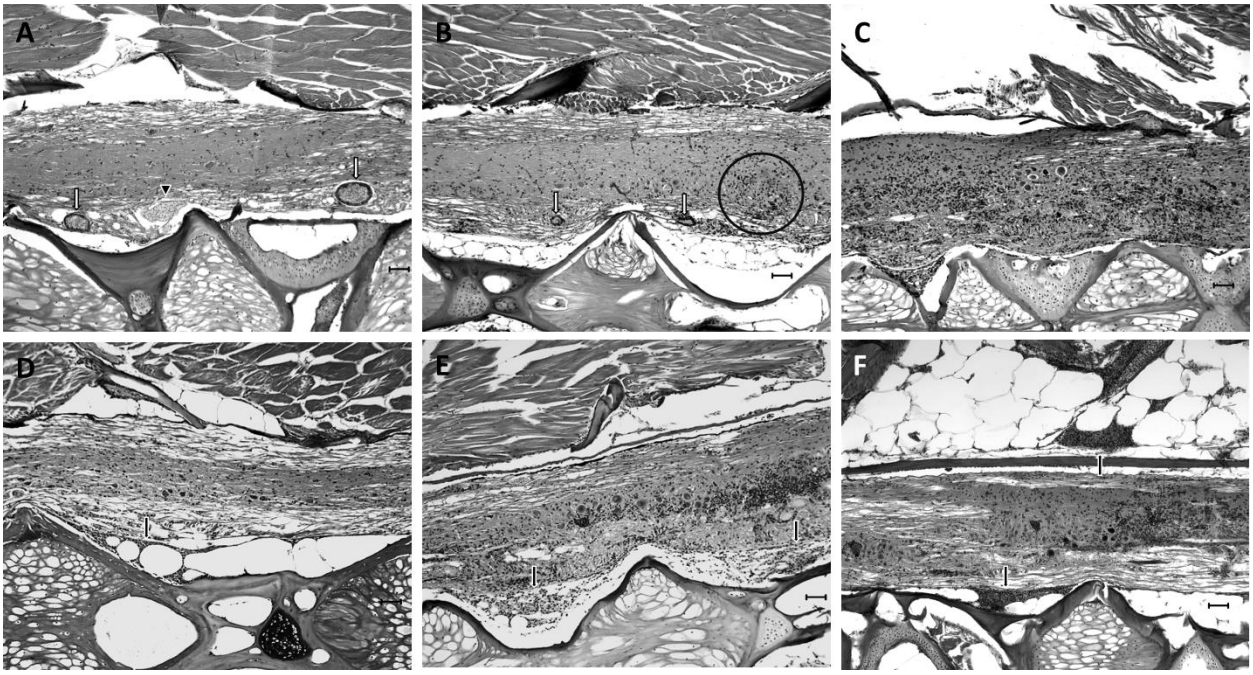

FIG. 2. Visual grading scheme for encephalitis/myelitis and meninxitis. H&E. Scale bars = 50 µM. (A) Grade 1 myelitis: commonly, encephalitis/myelitis in P. neurophilia-infected fish were characterized by localized inflammation surrounding discrete PC in the neuropil. These PC were encapsulated by a layer of flattened, epithelioid cells that are most likely macrophages or microglial cells (white arrows). If an animal's encephalitis or myelitis was confined entirely to these inflammation-encapsulated PC, it was given a grade of 1. These could be present alongside uninflamed PCs (arrowhead). (B) Grade 2 myelitis: animals with grade 2 encephalitis/myelitis had multifocal to coalescing areas of gliosis within the neuropil that are commonly associated with scattered piecemeal neuronal necrosis and/or sattelitosis and/or neuronophagia (circled area). Inflammation-encapsulated PC may have been observed occasionally, as well as PC that appeared to have ruptured (white arrows). The latter PC were characterized by dense clusters of macrophages, presumptive microglial cells, granulocytes, and lymphocytes that surround and separate densely packed, but poorly organized clusters of spores and pre-sporogonic stages. (C) Grade 3 myelitis: animals with grade 3 encephalitis/myelitis had changes similar to those described in animals with grade 2 inflammation, but the encephalitis and myelitis was more severe and extensive, affecting more than 20% of the observable spinal cord and possibly involving granulocytes and amorphous cellular debris as well as large numbers of presumptive microglial cells. (D) Grade 1 meninxitis was characterized by occasional (up to three in a single animal) multifocal mats of granulocytic meninxitis (black arrow), confined to either the dorsal or ventral visible aspect of the spinal cord. (E) Grade 2 meninxitis was characterized by moderate numbers (up to one dozen in a single animal) of discrete inflammatory mats that are generally thicker than grade 1 mats (black arrow). Inflammation is limited to either the dorsal or ventral aspect of the visible cord in grade 2 meninxitis. (F) Grade 3 meninxitis was characterized by thick, widespread mats of inflammation that were frequently circumferential (observed both dorsal and ventral to the spinal cord). In extreme cases, the inflammation will extend past the ectomeninx to surround nerve roots (black arrows). H&E, hematoxylin and eosin.

2.4.1 Characteristics of neuronal infection

When PCs of P. neurophilia were present within the white matter or within nerve roots, they were always observed within the axon rather than within neuron cell bodies, glial cells, or capillary endothelial cells. In certain views, PCs of *P. neurophilia* were distinctly observed within the axonoplasm, producing gradual to abrupt swellings in the axon itself (Fig. 3).

Strikingly, in most cases, there was no directly observable evidence of axonal (Wallerian) degeneration (axonal swelling, vacuolation, or digestion chambers) either cranial or caudal to the intra-axonal PCs. Frequently, multiple PCs were observed along a single axon separated by segments of normal-appearing axon, forming a ‘‘string of pearls’’ configuration (Fig. 3). Also of note is the fact that PCs definitively observed within neurons were always present in the axons: No PCs or spores were ever identified in neuron cell bodies. When present in gray matter, as with the white matter, PCs were observed in the neuropil and not in glial cells, endothelial cells, or neuron cell bodies.

Despite the fact that intraneuronal PCs were only ever observed in axons, neuron cell bodies were not spared the consequences of infection. In cases with varying PC numbers throughout the spinal cord, nerve roots, and hind brain, multifocal individual necrotic neurons characterized by cell shrinkage, pyknosis or karyolysis, and cytoplasmic vacuolation were observed within either hindbrain or spinal cord gray matter distant from observable PC. This piecemeal neuronal necrosis was occasionally associated with encephalitis or myelitis of varying severity and was occasionally present when no other inflammation was observed proximal to the neuron cell bodies. PCs were usually not observed within the affected gray matter (Figs. 4 and 5).

Multifocal scattered neuron cell bodies surrounded by putative microglial cells that either lined the periphery of the cell (satellitosis) or were observed within the cytoplasm of these cell bodies (neuronophagia) were frequently observed in gray matter distal to observable PCs. This suggests that the PCs were the cause of these lesions (particularly in the absence of any other neuronal disease). To clarify, neuronophagia is defined as the invasion, destruction, and/or consumption of neurons by phagocytic cells (commonly microglial cells in mammals). These neuron cell bodies frequently had viable, non-pyknotic nuclei and no indication of necrosis or degeneration beyond the inflammatory changes of satellitosis and neuronophagia. Satellitosis

and/or neuronophagia of visibly necrotic neurons (shrinkage, hypereosinophilia, vacuoloation, pyknosis, karyolysis) was also common.

Overall, the spinal cord ventral white matter, nerve roots, and ganglia were the most common sites for PCs. Regarding the brain, nearly all PCs observed were found in the rhombencephalon. Within the hind brain, there was a consistently striking pattern of infection that appeared to closely follow the primary descending white matter tracts. PCs were frequently found in the dorsal and ventral medial longitudinal fascicle (MLF) as well as the commissura ventralis rhombencephali where fibers were associated with the dorsal and ventral MLF decussate (Fig. 6). When PCs were present in gray matter, they were most commonly found in the reticular formation and the griseum centrale (GC).

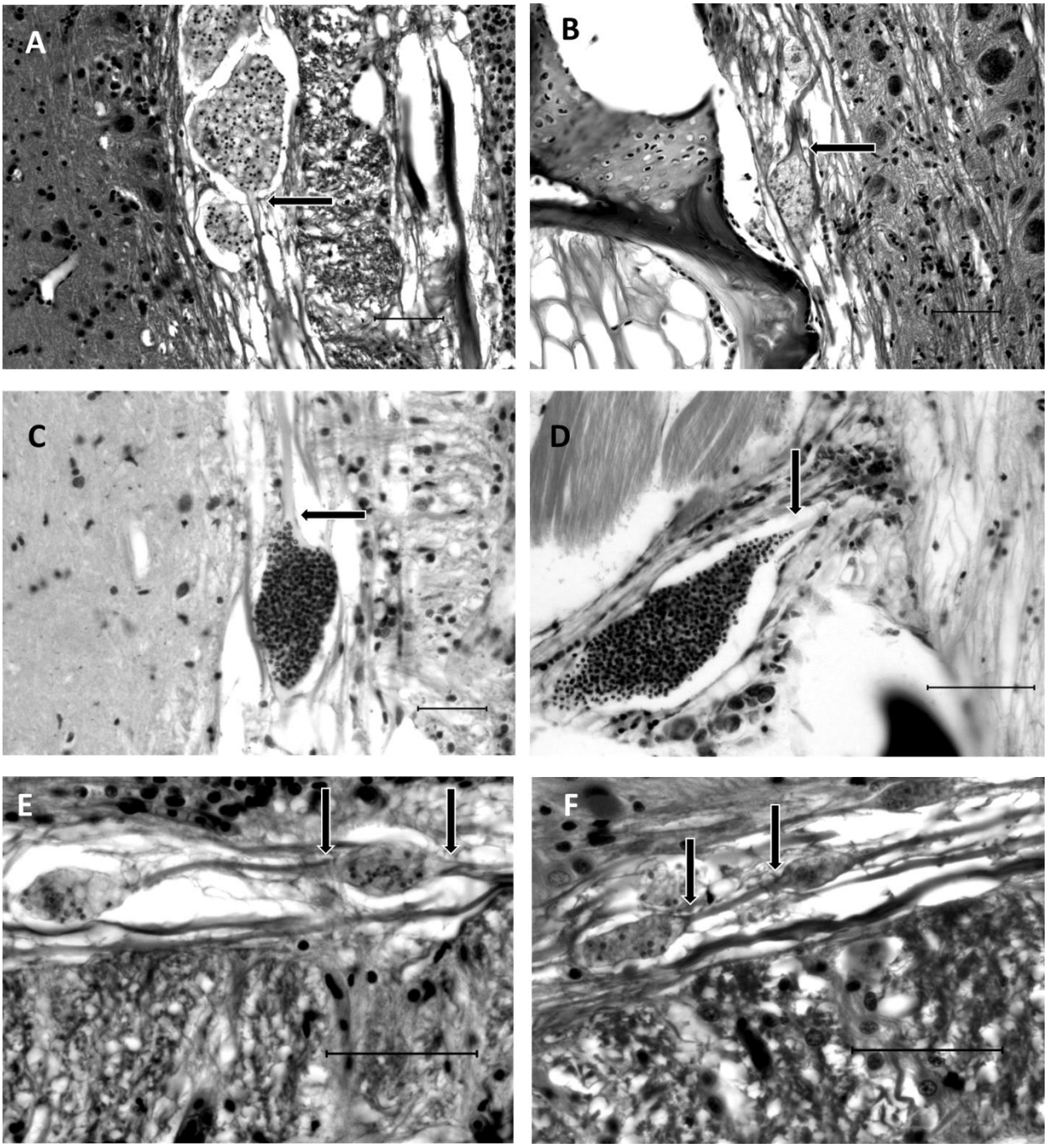

FIG. 3. Intra-axonal PCs. (A and B) Frequently, PCs are observed in distinct axonal swellings with no associated inflammation. There appears to be an abrupt swelling of the axonoplasm before the PC distends the axon. There is a distinct absence of axonal degeneration (Wallerian degeneration). H&E. Scale bar = 50 µM. (C) Occasionally, spores can be seen trailing off at the base of the axonal swelling. Luna. Scale bar = 50 µM. (D) Within a nerve root, a distinct line of spores can be seen trailing past the axonal swelling and up the axon. Giemsa. Scale bar = 50 µM. (E and F). Occasionally, multiple PC are observed in a single axon, forming a ‘‘string of pearls’’ arrangement. H&E. Scale bar = 50 µM. Bases of axonal swellings are indicated by arrows.

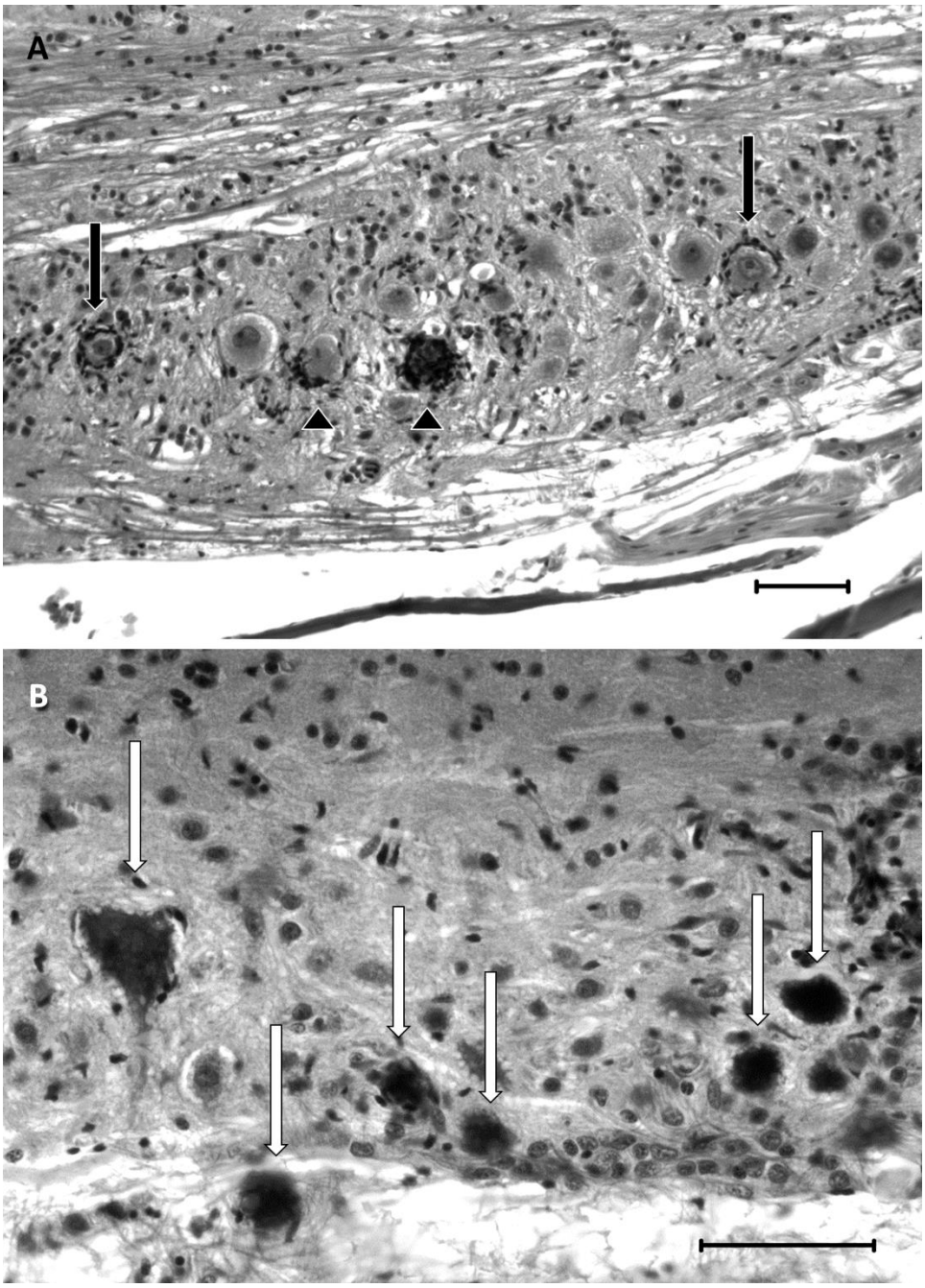

FIG. 4. Patterns of encephalitis and myelitis distal to visible PCs. H&E. Scale bars = 50 µlM. (A) Neuron cell bodies (in the reticular formation, here) appear to be targeted by inflammation, primarily composed of presumptive microglial cells. While some neurons undergoing neuronophagia appear degenerate (chromatolysis, pyknosis, shrunken, and hypereosinophilic; black arrowheads) many neurons that appear completely healthy are surrounded by presumptive microglial cells (black arrows). (B) Inflammation in the spinal cord (myelitis) is characterized primarily by presumptive microglial cells admixed with occasional lymphocytes. Multifocally, there may be individual necrotic neurons (white arrows) characterized by a shrunken, angular profile, karyolysis, and severe hypereosinophilia.

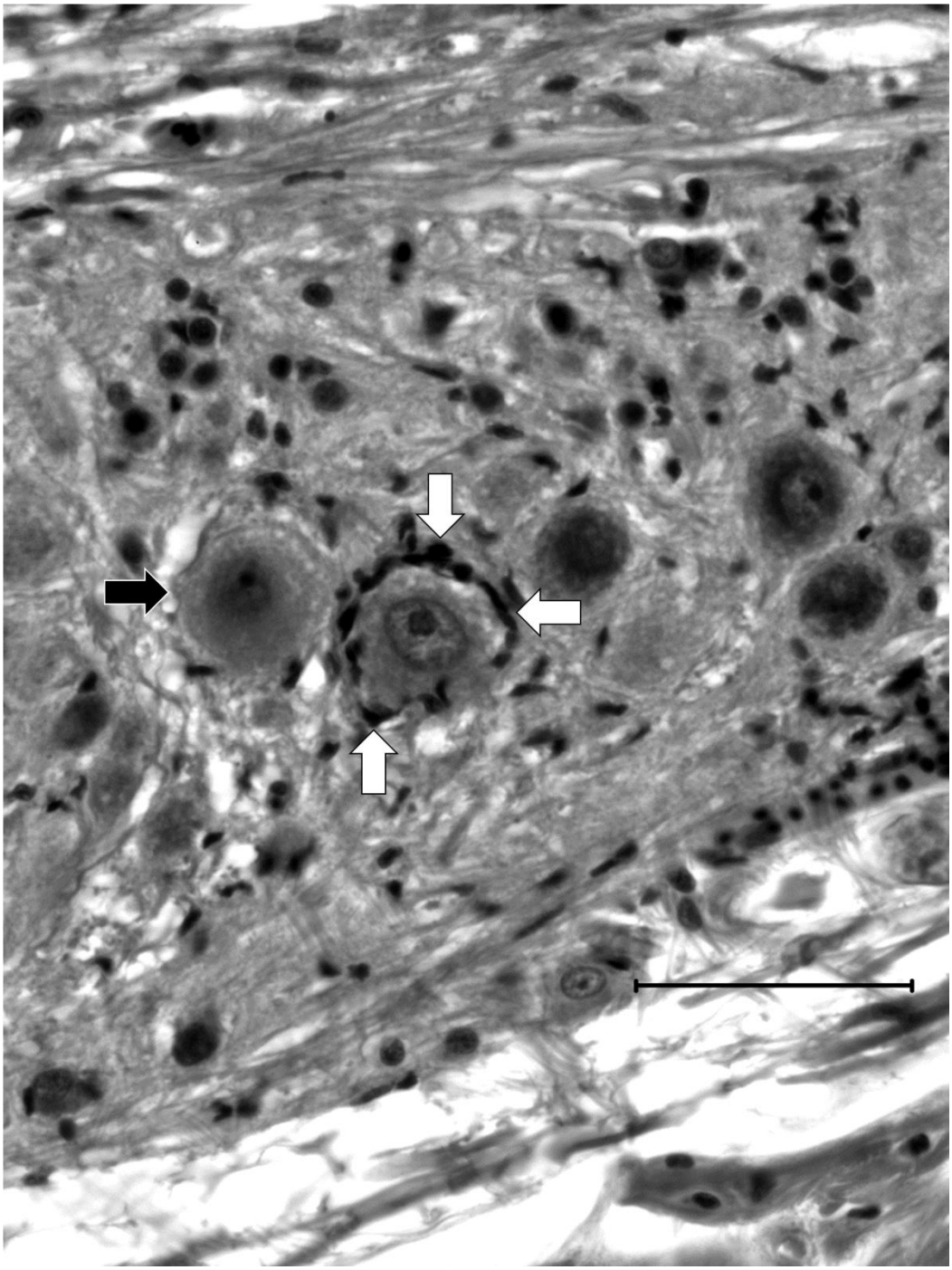

FIG. 5. Detailed features of perineuronal inflammation. Frequently, neuron cell bodies with no sign of degeneration including chromatolysis, vacuolation, or pyknotic nuclei, were surrounded by what appeared to be microglial cells (white arrows) when PCs were observed at sites distal to the inflammation. Frequently, affected neurons were directly adjacent to normal, healthy-looking neurons (black arrow).

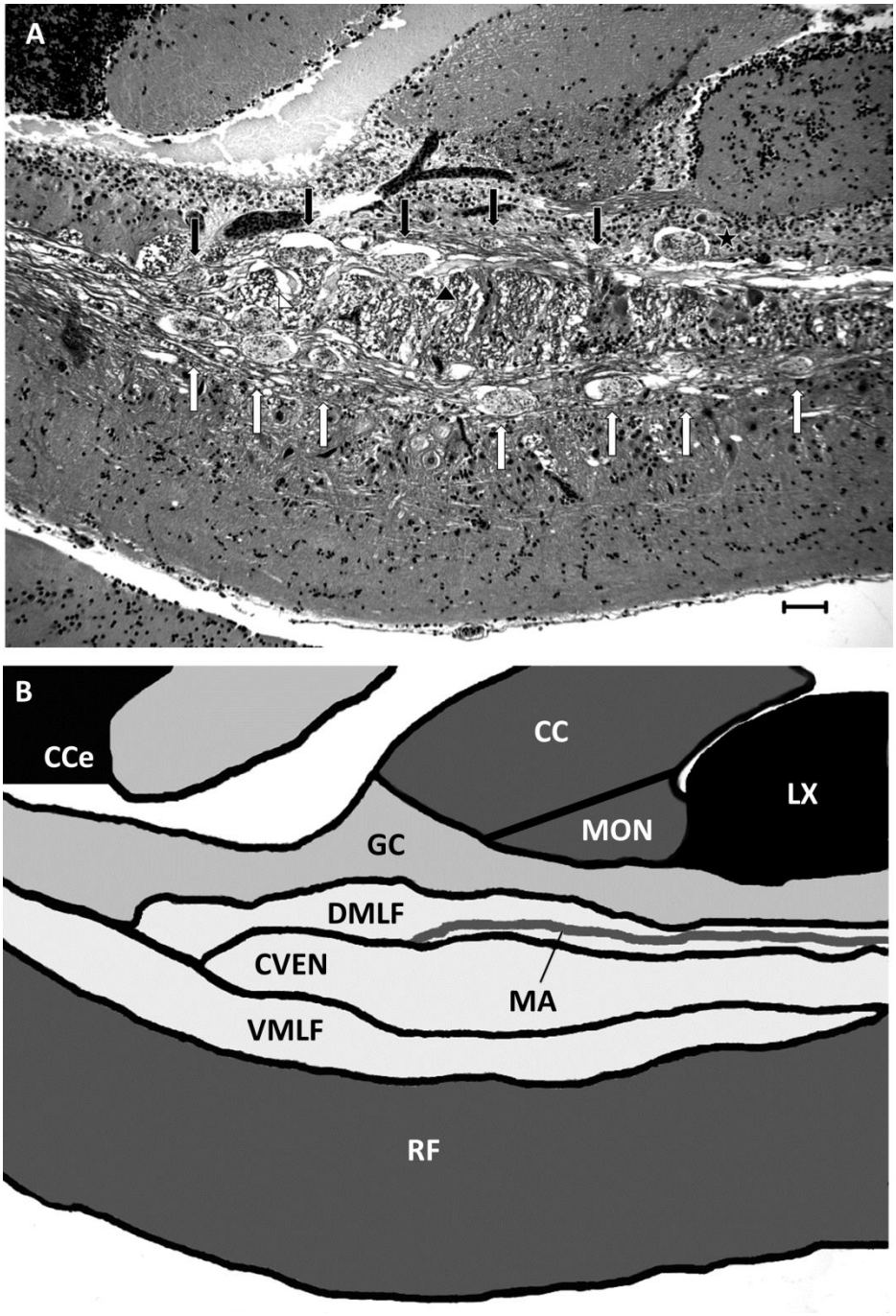

FIG. 6. PCs in the rhombencephalon. (A) PCs in therhombencephalon tended to follow a distinct anatomic distribution, being most commonly found in the major descending white matter tracts: The DMLF (black arrows) in which a PC can be seen impinging on (but not directly infecting) the MA (black arrowhead) and the VMLF (white arrows) along with the Cven (white arrowhead) in which fibers of the MLF decussate. PC are also observed in rhombencephalic gray matter, including the GC (black star). H&E. Scale bar = 50 µM. (B) Hindbrain anatomy schematic for comparison to Figure 3A. Cce, corpus cerebelli; CC, Crista cerebrallis; MON, medial octavolateralis nucleus; MA, Mauthner axon.

2.4.2 MLF and the ventral spinal white matter

Most PCs were observed in the ventral spinal cord white matter (40% of the total observed PCs) and in the spinal nerves and ganglia (25%). A total of 23% of all observed PCs were found in the hindbrain. Of these PCs, 12% were observed in the gray matter and 88% were observed in the white matter. Of PCs observed in the hindbrain gray matter, 61% were found in the reticular formation and the rest were observed in the GC. Of PCs observed in the hindbrain white matter, 53% were observed in the ventral medial longitudinal fascicle (VMLF). A total of 4% of all observed PCs were found in the cranial nerves. Of these, the vast majority (79%) were observed in the vagus (Cranial nerve X).

2.4.3 Inflammation

Inflammation associated with *P. neurophilia* infection was localized based on the anatomical structure in which it was present and categorized as encephalitis/myelitis when present in the neuropil of the brain or spinal cord, meninxitis when associated with the membranes surrounding the central nervous system, and myositis when present in the muscle.

2.4.4 Encephalitis/myelitis

Of the 561 infected fish, 59% had this lesion. Of these, 21% received a grade of 1, 55% received a grade of 2, and 24% received a grade of 3. Among fish submitted without clinical disease, 66% had encephalitis or myelitis. Of these, 24% were grade 1, 54% were grade 2, and 22% were grade 3. Among fish submitted with clinical disease, 54% had encephalitis/myelitis. Of these, 12% were grade 1, 58% were grade 2, and 30% were grade 3.

PCs were frequently observed in neuronal tissue with no visible inflammation either directly associated with the PCs or scattered throughout the neuropil (Figs. 2, 4, 5, and 7). Fish with these features were given an encephalitis/myelitis grade of 0. Inflammation was never associated with the vasculature, and there was no evidence of perivascular inflammation or vasculitis. Gliosis and inflammation in animals with grade 2 and 3 inflammation was randomly

scattered throughout the neuropil or closely associated with apparently ruptured PCs (Fig. 8). Distinct perivascular patterns were never identified.

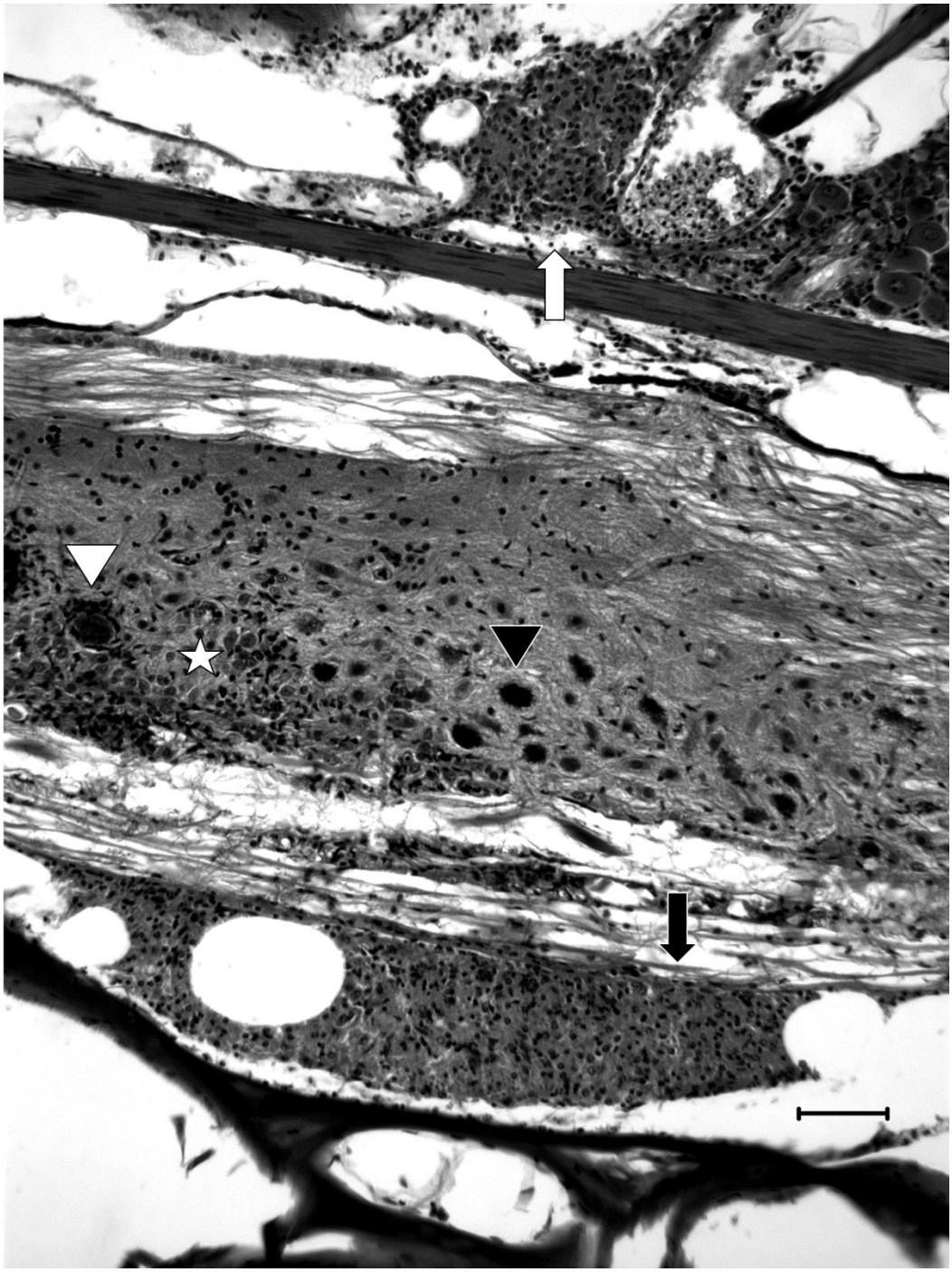

FIG. 7. General patterns of inflammation. Meninxitis was frequently composed of granulocytes associated with free spores (arrows). In grade 3 meninxitis, inflammation appeared both ventral (black arrow) and dorsal (white arrow) to the spinal cord. In severe cases, inflammation frequently extended through the ectomeninx to cause periradiculoneuritis (white arrow). In this image, grade 2 myelitis is observed with generalized gliosis (star), neuronophagia (white arrowhead) and piecemeal neuronal necrosis with peripheral vacuolation (black arrowhead). H&E. Scale bar = 50 µM.

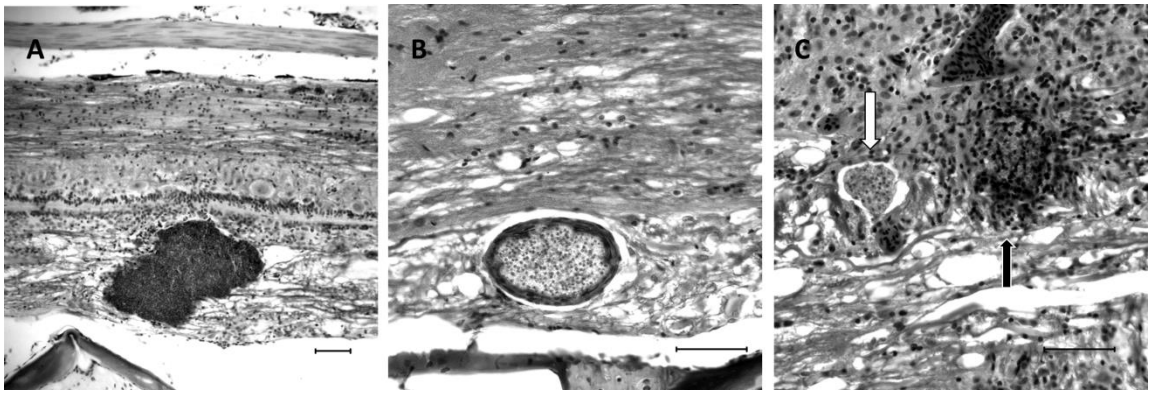

FIG. 8. Patterns of inflammation associated directly with PCs in the nervous system. (A) PC can grow to massive sizes, in this case, almost half the diameter of the spinal cord, while inducing only a minimal inflammatory response. Luna. Scale bar = 50 µM. (B) Even smaller PC may induce a localized inflammatory response wherein epithelioid macrophages (likely transformed microglial cells) form a layer around the PC presumably in an attempt to ‘‘wall it off.’’ H&E. Scale bars = 50 µM. (C) Inflammation associated with individual PC is highly variable, even when they are directly adjacent to each other. One can be completely intact and have no associated inflammation (white arrow), while another can be completely effaced by inflammation separating individual spores and presporogonic stages (black arrow). The latter cluster is presumed to have ruptured. H&E. Scale bar = 50 µM.

2.4.5 Meninxitis

The severity of these inflammatory changes was graded by extent of the lesions (1–3), as described in Figure 2. Of the 561 infected fish, 50% showed this lesion. Of these, 22% received a grade of 1, 53% received a grade of 2, and 25% received a grade of 3. Of the 384 fish submitted as routine cases, 52% had meninxitis. Of these, 20% were grade 1, 54% were grade 2, and 26% were grade 3. Of the 177 fish submitted as clinical cases, 45% had meninxitis. Of these, 26% were grade 1, 49% were grade 2, and 25% were grade 3.

About 50% of the fish exhibited inflammation that was associated with the membranes surrounding the brain and spinal cord. In the zebrafish studied here, granulocytic meninxitis frequently formed discrete, well-demarcated mats within the perimeninxial space that contained granulocytes, cellular debris, and, frequently, free *P. neurophilia* spores (but no observable presporogonic stages) (Fig. 7). This granulocytic meninxitis frequently followed nerve roots for a short distance outside of the vertebral canal and was frequently associated with periganglioneuritis and periradiculoneuritis (Fig. 7).

2.4.6 Myositis

The most commonly observed extraneural sequelae of microsporidiosis are detailed in Figures 9 and 10. Myositis was far rarer than expected, occurring in only 13% of all examined fish. This lesion was observed in 16% of fish submitted with clinical disease and 7% of the fish submitted without clinical disease. Relatively few fish had myositis, which was generally characterized by multifocal to coalescing areas of granulocytic to granulomatous inflammation. Because the locations of myositis were inconsistent between fish and relative severity was difficult to determine, we decided to simply note the presence or absence of myositis rather than producing an ordered grading system. The presence or absence of myodegeneration without inflammation was similarly treated.

2.4.7 Other diseases

This study focuses on *P. neurophilia* infection. However, potentially clinically significant (health-affecting) histopathologic lesions were frequently observed concurrent with neural microsporidiosis. These lesions included, but were not limited to, aerocystitis (inflammation of the swim bladder), oophoritis and ovarian granulomas, mycobacteriosis, nephrocalcinosis (mineral deposits in renal tubules), and neoplasias such as seminomas and ultimobranchial gland adenomas. For the purposes of this study, all histopathologic lesions (besides neural microsporidiosis) that could have produced clinical disease were categorized as ‘‘positive for other disease.’’

2.4.8 Statistical Analysis

There were significant differences among years of submission in terms of the PC number per fish ($p < 0.01$), total PCs in the brain ($p < 0.01$), in the spinal cord ($p < 0.01$), in the nerve roots ($p < 0.01$), in the brain white matter ($p < 0.01$), in the spinal cord white matter ($p < 0.01$), and in the spinal cord gray matter ($p < 0.01$). The submission year was then accounted for in the next analyses to correct for the effect of this variable. There was a significant difference between

females and males in terms of total number of PCs/fish ($p < 0.01$). The corresponding estimated coefficient was 0.35, which indicated that male fish had significantly more total PCs compared with the females. There was, however, no significant difference between clinical and normal fish ($p = 0.45$) in terms of total number of PCs/fish.

The presence of clinical disease was a significant predictor for the prevalence of myositis ($p < 0.01$), as the odds of having myositis for fish submitted as routine cases were only 27.51% of the odds for fish that were submitted as clinical cases. There was strong evidence that sex and encephalitis were significant predictors for meninxitis and males tended to have more severe meninxitis than females (estimated coefficient 0.41, $p < 0.01$), and fish with more severe encephalitis also tended to have more server meninxitis (estimated coefficient for encephalitis categories 1, 2, and 3 are 0.47, 1.51, and 2.41, respectively). However, clinical disease was not a significant predictor of either meninxitis or encephalitis/ myelitis ($p = 0.4$ and $p = 0.08$, respectively. There were significantly more PCs observed in the white matter than in the gray matter of both the brain and spinal cord ($p < 0.01$ for each). When combined, total PCs in the gray and white matter of the brain were significantly associated with the presence of other diseases ($p = 0.047$).

Surprisingly, there were no statistically significant associations between total PC number and clinical disease ($p = 0.45$); total parasite number in any specific anatomic location and clinical disease ($p > 0.05$); and either meninxitis or encephalitis/myelitis and clinical disease ($p > 0.05$ for each). We also explored whether or not the submitting laboratory had an effect on infection patterns. Because there were so many submitting laboratories, we decided that it would be best to focus on the four laboratories submitting the most samples. Using 163 samples from these laboratories, we found that certain correlations that were previously not statistically significant became so. When accounting for submitting laboratory: total PCs in the brain were significantly associated with the presence of other diseases ($p = 0.047$); total PCs in the nerve roots were significantly associated with myositis ($p = 0.01$); total PCs in brain white matter were significantly associated with the presence of other disease ($p = 0.019$); total PCs in spinal cord white matter was significantly associated with the presence of other diseases ($p = 0.041$); total PCs in the gray and white matter of the brain were highly correlated with the presence of other diseases ($p = 0.0091$); total PCs in the white matter of the spinal cord were correlated with the

presence of other diseases (p = 0.041); and parasites in the combined gray and white matter of the spinal cord were strongly correlated with the presence of other disease (p = 0.011).

Moreover, when submitting laboratory was taken into account, myositis was significantly correlated with the presence of other diseases (p = 0.0033 compared with p = 0.079 when laboratory was not taken into account). Following this trend, when laboratory was taken into account, myositis was no longer significantly correlated with clinical disease (p = 0.89 compared with 0.0089 when submitting laboratory was ignored).

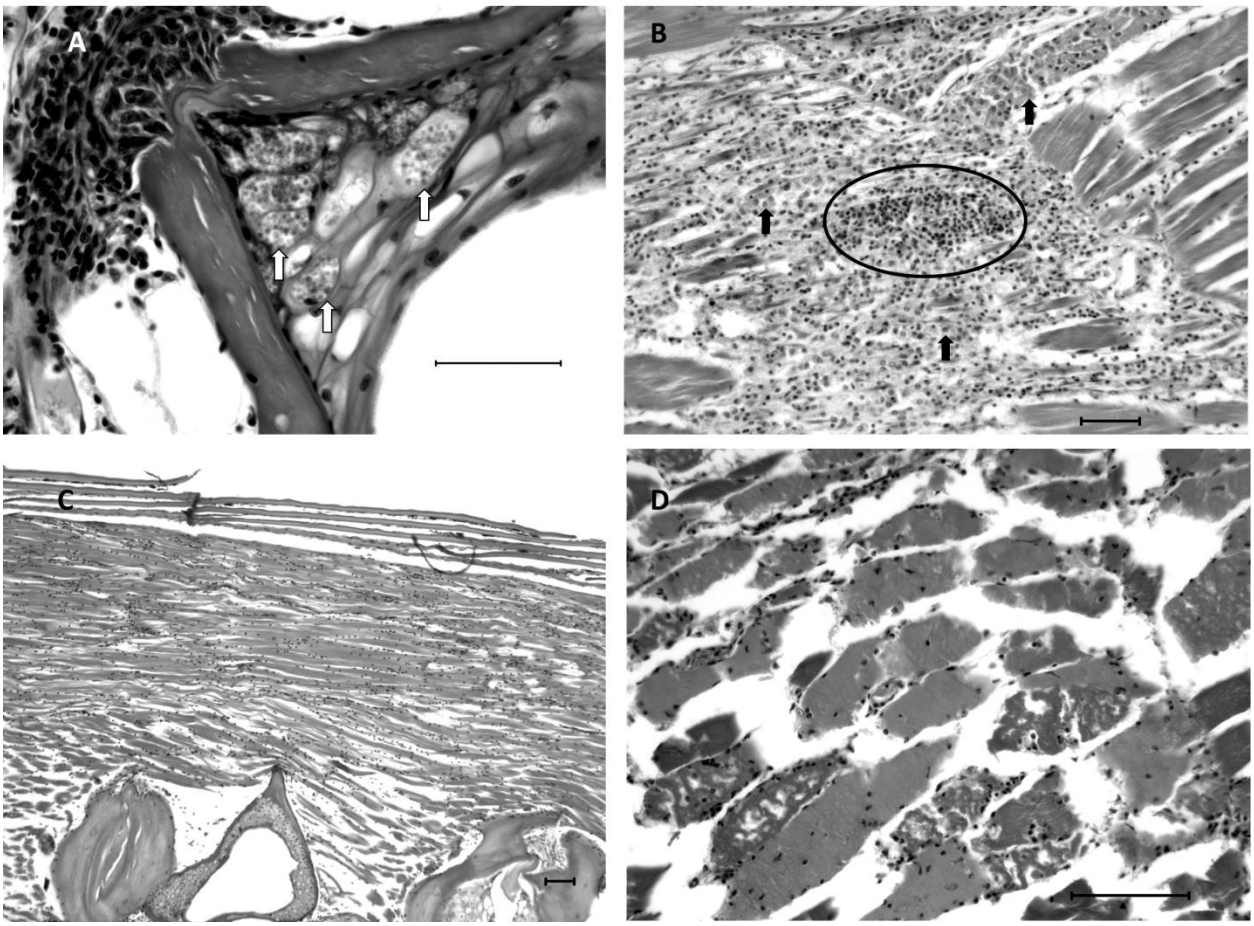

FIG. 9. Extraneural manifestations of P. neurophilia infection. H&E. Scale bar = 50 µM. (A) Notochord remnants in vertebrae contain large intracytoplasmic PC (white arrows). This is an uncommon, but not rare, presentation of P. neurophilia infection. (B) Granulocytic myositis occasionally contains free spores (circled cluster of black spots) and is commonly adjacent to nerve roots either containing PC or surrounded by granulocytic inflammation (black arrows) extending from a meninxitis. (C) Animals with P. neurophilia infection will frequently have noninflammatory myodegeneration.This manifestation is chronic, resulting in muscle atrophy and loss, with increased space between myocytes. (D) Myodegeneration without inflammation can also occur acutely or subacutely with the loss of cross-striation in myocytes, the formation of contraction bands, and cytoplasmic fragmentation.

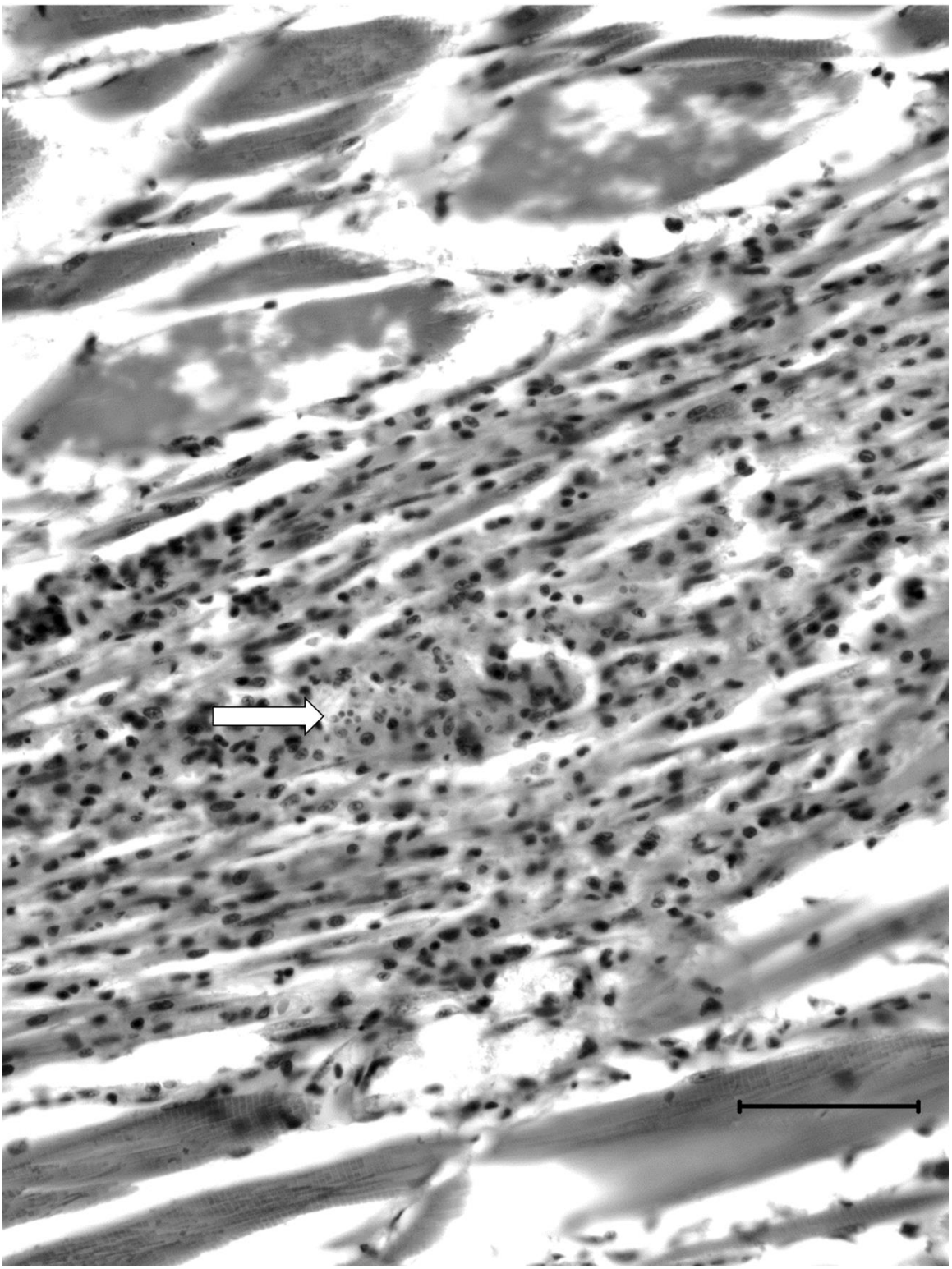

FIG. 10. Myositis with intralesional spores. Acute to subacute granulocytic myositis was occasionally associated with spores free in the inflammatory infiltrate (arrow). H&E. Scale bar = 50 µM.

2.5 Discussion

Sanders et al. (2014) described the entry of *P. neurophilia* spores into the gut and their sequential distribution to viscera surrounding the gut followed by infection of the central nervous system. The initial distribution into the viscera adjacent to the gut is likely due to the launching of the polar tubule through the gut wall and into the surrounding viscera. Because it appears that this must occur before the appearance of parasites in the CNS, there is an unidentified step between the first occurrence of parasites in the viscera and their transport to the CNS because *P. neurophilia* has no capacity for motility beyond the use of the polar tubule and, as seen in this study and others, there is a very specific tropism for the CNS. Hematogenous spread is a potential mechanism. However, the consistent presence of PCs in nerve roots and white matter along with the fact that PCs are present within axonal swellings could suggest a simultaneous or alternative method of transport to the CNS; retrograde axonal transport.

Retrograde axonal transport is the movement of viruses, toxins, organelles, and proteins along microtubules in the axon toward the cell body via the action of molecular motors (LaVail et al. 2003). Prions, the rabies virus, and herpesviruses utilize this method to spread from their points of entry in the peripheral tissues to the CNS. Currently, the only reported infectious moieties that move via axonal transport are viruses and prions; however, theoretically, the size of an object that can be transported via the axon is limited only by the diameter of the axon itself (LaVail et al. 2003). Vesicles and organelles as large as mitochondria are regularly transported along the axon (Salinas et al. 2010). Adult zebrafish axons (besides the Mauthner axon) are generally at least 7–8 μm in diameter and all developmental stages of *P. neurophilia* are considerably smaller than this (Cali et al. 2012), making axonal transport of at least certain stages of the parasite theoretically possible. The presence of PCs within the axoplasm rather than within the myelin sheath (Cali et al. 2012), along with the presence of multiple PCs in a single axon and the fairly specific tropism of the parasite for the commissura ventralis rhombencephali and MLF, both of which are continuous with the spinal cord ventral white matter, makes axonal transport highly plausible (Figs. 3 and 6). This provides an explanation of how an organism with no known method of motility besides its polar tubule would be able to infect such specific

structures as the MLF and commissura ventralis rhombencephali, which happen to be white matter tracts.

If this were found to be true in the case of *P. neurophilia*, it would be the first documented case of any organism more complex than a virus utilizing retrograde axonal transport to move through the nervous system. Axonal transport of mitochondria is well documented (Salinas et al. 2010) as is the close association between microsporidia and mitochondria. Microsporidia are obligate intracellular parasites and in certain species, including *Encephalitozoon cuniculi*, parasitophorous vacuoles of the meront stage bind to host mitochondria using a special transmembrane pore to siphon host ATP out of the organelle. Electron microscopy has revealed that the parasitophorous vacuoles of *E. cuniculi* are partially coated by host mitochondria (Hacker et al. 2014). Because axonal transport of mitochondria is well documented and because microsporidia tend to associate closely with host mitochondria, axonal transport of mitochondria could be a method by which *P. neurophilia* ‘‘hitches a ride’’ down the axon if it cannot hijack axonal molecular motors on its own.

Intracellular PCs were occasionally observed within multiple vertebrae distending the cytoplasm of notochord remnant cells (Fig. 9). This pattern of intravertebral infection could be explained if spores, consumed when the fish are larvae (or even as embryos in the case of vertical transmission), launch their polar tubules and hit the notochord (Sanders et al. 2014). This would allow for both the spread of the microsporidium throughout notochord cells and their retention within the notochord remnants of adjacent vertebrae in adult fish.

Satellitosis and neuronophagia of otherwise healthy-appearing neuron cell bodies could indicate that *P. neurophilia* antibodies are expressed in neuron cell bodies associated with infected axons. Observation of the parasite within the axonoplasm is corroborated by transmission electron microscopy of *P. neurophilia* that showed ultrastructurally that spores and pre-sporogonic stages were present within the axonoplasm (Cali et al. 2012). Inflammation of neuron cell bodies in the absence of directly observable pathogens in the cell body has some precedent in mammals: Human neurons express atypical MHC-I molecules in response to infection by rabies or herpesviruses, and rodent neurons in vitro can internalize and express foreign ovalbumin antigens via MHC-I complexes leading to cytotoxic T-cell-mediated killing (Cebrian et al. 2013; Megret et al. 2007). Histopathologically, most of the inflammatory cells surrounding cell bodies in zebrafish with *P. neurophilia* resembled microglial cells rather than T

cells (Figs. 4 and 5). Of course, it is impossible to definitively identify these cells without proper immunohistochemical labeling, which does not exist at this stage in the development of the zebrafish as a laboratory animal. It is also possible that neuron cell bodies in these fish may be damaged in ways that are not observable via light microscopy and that this, rather than antigen expression, is the reason for the presumptive microglial attack of neuron cell bodies. Regardless of the specific mechanism, this piecemeal neuronal necrosis, satellitosis, and neuronophagia suggest that *P. neurophilia* infection of axons may have retrograde effects on neuron cell bodies. The caveat to this suggestion is that, during this retrospective study, there was no clear identification of a single inflamed or necrotic neuron cell body connected directly to a PC-containing axon.

Perhaps the existence of PCs in the axon protects the organisms from an immune reaction, providing an explanation for why PCs were often indirectly associated with inflammation and why a subset of fish evaluated had encephalitis/myelitis had scores of zero. Similarly, *Loma salmonae*, a microsporidium of salmon, infects the pillar cells and endothelium of gills, and elicits essentially no tissue reaction when within intact xenomas. Severe chronic inflammation associated with free spores generally occurs only after xenoma rupture (Kent et al. 1999). A similar phenomenon appears to occur with *P. neurophilia*—that is, inflammation is enhanced when PCs rupture their host axon, exposing organisms to the immune system.

Interestingly, some of the largest PCs observed in this study were associated with minimal to no inflammation (Fig. 8). Perhaps PCs may be able distend the axon to astounding dimensions without rupturing them, as seen with xenomas formed by other microsporidian species. If axon rupture is indeed the cause of inflamed PCs, the cause of rupture is unclear, particularly because size itself does not seem to be a factor. A possible explanation for this could be the rate of PC growth within the axon: Slow growth may allow for progressive stretching of the axon, but if PCs expand too quickly, the axon may be unable to compensate and may rupture.

About 50% of the fish exhibited inflammation associated with the membranes surrounding the brain and spinal cord. Teleosts generally do not have a distinct arachnoid, pia, or dura mater (Caruncho and Pinto Da Silva 1994). Cyprinids have an ectomeninx, composed of mucinous tissue, collagen fibers, and blood vessels, that is most comparable to the mammalian dura mater. Directly apposed to the brain is the endomeninx, composed of an outer, intermediate, and inner layer, which is comparable to the leptomeninges in mammals (Caruncho and Pinto Da

Silva 1994). Between the ectomeninx and the endomeninx is a layer of fatty tissue called the perimenixial space (Ariens Kappers 1924). Meninxitis associated with *P. neurophilia* infection in this study was primarily granulocytic, and it was located largely within the perimeninxial space. The pattern of meninxitis in these fish was strikingly different from that generally observed in mammals with meningitis (Hatta and Korn 1998). In mammals, due the fact that the pia mater follows blood vessels deep into the neuropil, leptomeningitis usually results in a perivascular pattern of inflammation with the expansion of the leptomeninges by inflammatory cells (Maxie and Youssef 2007).

2.5.1 Myositis and extraneural infections

Once outside of the ectomeninx, inflammation associated with *P. neurophilia* infections was generally unrestricted. Frequently, it produced perivertebral myositis and, in some cases, interstitial nephritis. Myositis, however, is not pathognomic for *P. neurophilia*, as only 13% of infected fish had this lesion. As reported by Matthews et al. (2001) in the first histopathologic description of *P. neurophilia*, myositis was associated with clinical disease: Fish with clinical disease were twice more likely to have this lesion than apparently healthy fish in our study. It may be that the presence of myositis makes it more likely for fish to become visibly emaciated due to a decreased ability to find and acquire food. It may also predispose these fish to grossly visible vertebral abnormalities, due to early infection of the notochord or due to the chronic effects of differential muscle tension on vertebrae. Ramsay et al. (2009) showed that zebrafish subjected to stressors and with increased cortisol had an increased incidence of *P. neurophilia*-associated myositis. Interestingly, many cases of granulocytic myositis contained extracellular spores free within the inflammation. It is possible that these incidents of myositis are largely due to the rupture of peripheral nerves and release of spores outside of the plane of section with spread into the surrounding muscle (Fig. 10).

When the submitting laboratory was ignored, we found a positive correlation between the presence of diseases other than microsporidiosis and myositis. The presence of these other diseases could increase the severity of *P. neurophilia* infection due to stress and subsequent immunosuppression (Ramsay et al. 2009). However, other infections may cause these lesions in the absence of *P. neurophilia*. Infections with *Mycobacterium* spp. are the second most common

disease diagnosed in laboratory zebrafish (Whipps et al. 2012) and mycobacteriosis may cause myositis. Conversely, taking into account the submitting laboratory, it appeared that multiple factors, including the parasite number in several anatomic locations, became significantly correlated with the presence of other diseases. Myositis was significantly correlated with the presence of other diseases, but not with clinical disease when submitting laboratory was taken into account. One explanation for this could be the following: If one of the laboratories under study submitted a large number of fish with mycobacteriosis that were coinfected with *P. neurophilia*, we would likely see a strong association between myositis and the presence of other diseases (mycobacteriosis).

The fact that myositis was no longer correlated with the presence of clinical disease caused by *P. neurophilia* when the submitting laboratory was taken into account illustrates one of the most difficult aspects of working with a large number of facilities when constructing a retrospective study: Not all labs are created equal, and even when they are, variations are bound to occur over time. Most zebrafish facilities are similar in that they maintain their fish at about 28°C in large, recirculating systems and use similar breeding methods (Lawrence et al. 2012). The zebrafish as a laboratory animal is still in its infancy relative to mice, and although standards of care exist, there is marked variation between individual labs in terms of stocking densities and individual system dynamics. With the infinite variations in genetic knockouts and the wide variety of strains available, the interaction between host genetics and the parasite may be very different between individual laboratories. In addition, pathogen profiles vary dramatically between laboratories, as researchers use fish with a wide range of pathogen status and history, from pet store fish reared in ponds and exposed to other fishes, to fish from ZIRC with documented disease histories, to SPF fish from Oregon State University (Kent et al. 2011). Compounding this issue is the fact that only a small fraction of zebrafish research facilities submit moribund fish for pathogen diagnoses (Lawrence et al. 2012) and thus their pathogen profiles are unknown. As we have seen in this study, the interaction between microsporidiosis and other coinfecting pathogens can dramatically influence the health of the animal and the incidence of other diseases varies between submitting laboratories.

Perhaps even *P. neurophilia* strains may differ between laboratories. The rapid reproductive cycle of both zebrafish and the parasite, along with different modes of transmission of the parasite (e.g., vertical and horizontal), could drive the rapid evolution of *P. neurophilia*

toward more or less virulent strains. Each laboratory is its own ecosystem and each tank is its own microenvironment, both of which could nudge the parasite into various evolutionary directions. These same conditions and the development of individual laboratories along with intermittent changes in housing conditions could also provide an explanation for the statistically significant effects of submission year on infection patterns.

2.5.2 Inflammation and clinical disease

The driving forces behind inflammation severity, parasite load, and easily observable clinical disease are as of yet unclear. While we may suggest that variability in these factors may be due to differences in stress and subsequent immunosuppression in individual fish, there may be more to the story such as differential behavioral predelictions to increased parasite intake (increased carcass consumption) or heritable differences in resistance or susceptibility. Further complicating the issue is that, while increased cortisol levels and increased parasite load can be experimentally correlated with weight loss and decreased fecundity (Ramsay et al. 2009), in this particular study, there was no statistical correlation between particular features of infection and the reporting of clinical disease by submitting personnel. This discrepancy highlights the need for further study of specific study of subtle changes produced by a chronic, seemingly subclinical parasite.

2.5.3 Binomial pattern

A well-recognized paradigm in parasitology is that macroparasites, such as nematodes, in wild animals generally exhibit a negative binomial distribution, with relatively few hosts harboring most parasites (Crofton 1971; Poulin 2013). This type of distribution extends to fish as well as to domestic terrestrial hosts (Lester 1984), and we actually recently demonstrated this phenomenon with *Pseudocapillaria tomentosa* in zebrafish that were experimentally infected in the laboratory (Collymore et al. 2014) Interestingly, *P. neurophilia*, which would be considered a ‘‘microparasite’’ as it replicates in its host, also showed a binomial distribution of infection in our study. This resulted in an overdispersion of infection severity, in which relatively few fish had very large numbers of PCs and most fish had relatively low numbers.

2.5.4 Sex

Sex may also influence infection patterns. Chow et al. (2015) using essentially the same data set as was used in this study, reported that males had a higher prevalence of infection by *P. neurophilia* compared with females, in both clinical and routinely submitted fish. Here, we expand these observations, showing that males also tend to have more total PCs than females. Males also tended to have more severe encephalitis/myelitis and meninxitis than females. Zebrafish in aquaria establish hierarchies, which are more pronounced in males. Filby et al. (2010) and Spence and Smith (2006) showed that subordinate males exhibited a greater rise in cortisol than subordinate females. Thus, Chow et al. (2015) suggested that a possible reason for the increased likelihood of clinical disease in males could be increased stress due to hierarchical inter-fish competition correlating with elevated cortisol levels resulting in more severe infections. Another possible explanation is that sexual differences in hormones may differentially influence the immune system and responses to stress.

2.5.5 Behavior

Over the past three decades, the zebrafish has become an important animal model for behavioral research (Hatta and Korn 1998; Eaton et al. 1977; Liu and Fetcho 1999; Liu and Westerfield 1988; Zottoli et al. 1999). The potential influence of *P. neurophilia* on these studies should be considered, given that it infects the CNS and is widespread in research facilities. Both the Mauthner axon and reticular formation are important for the functioning of the startle response in zebrafish (Hatta and Korn 1998; Eaton et al. 1977; Liu and Fetcho 1999; Liu and Westerfield 1988; Zottoli et al. 1999). It is possible that the infection may adversely affect behavioral studies that rely on the startle response, including the tap test, due to the frequent presence of *P. neurophilia* spores in the MLF proximal to and compressing the Mauthner axon, and the frequent infection and associated inflammation of reticular formation cell bodies. This is particularly plausible, because most experiments studying anxiety or the startle response use motor reflexes and movement as an experimental endpoint (Hatta and Korn 1998; Eaton et al. 1977; Liu and Fetcho 1999; Liu and Westerfield 1988; Zottoli et al. 1999). Because *P.*

neurophilia infects nerve roots and the ventral spinal cord (both containing motor neurons) and because infections can be associated with myositis and muscle atrophy, there could be impairments at every step of the startle response (Zottoli et al. 1999).

The GC receives input from the medial habenula, which has been implicated in anxiety and habituation in relation to stress (Mathuru and Jesuthusasan 2013). The frequent presence of PCs in this structure could, therefore, have effects on experiments that interrogate anxiety and habituation, which are frequently examined in zebrafish behavioral studies (Pittman and Lott 2014; Stewart et al. 2012; Mathuru and Jesuthusasan 2013). In fact, even studies that attempt to evaluate higher functions, such as learning and comprehension, utilize noxious stimuli and habituation in response to stress, both of which could be affected by neural microsporidiosis (Pittman and Lott 2014; Stewart et al. 2012; Mathuru and Jesuthusasan 2013).

Myxobolus arcticus infections in the hindbrain have been associated with decreased swimming speed of naturally infected salmon (Moles and Heifetz 1998). Because this myxozoan parasite exhibits a similar pattern of infection as *P. neurophilia* (mostly in the hind brain, often with no inflammation), this suggests that the latter infection could affect experiments with zebrafish that involve swimming or other motor functions. Another *Myxobolus* species, *M. balantiocheili* of tricolor sharks (*Balantiocheilos melanopterus*), causes prominent CNS infections that extend into the meninxes. Although it was not reported to cause significant inflammation, it was associated with uncoordinated darting, rolling and pitching (Levsen et al. 2004).

Infections of the central nervous system by various pathogens that do not elicit significant histopathologic lesions probably have effects beyond their presence as mere space occupying lesions. These parasites are living organisms, and there is active chemical ‘‘cross talk’’ between the host and parasite. For example, chronic infections with *Toxoplasma gondii* cysts in the brain have been associated with unusual and profound behavior changes infected rats (Berdoy et al. 2000; Dubey 1998). Although *T. gondii* is an apicomplexan and *P. neurophilia* is a microsporidium, the parasites share some similarities: Both are spread via the fecal-oral route with the eventual spread of the parasite from the gut to the central nervous system. Similar to *P. neurophilia, T. gondii* has a noninflammatory stage in tissues (encysted bradyzoites).

In conclusion, our retrospective study confirmed that many apparently healthy zebrafish from research facilities have significant *P. neurophilia* infections at a histological level,

particularly in the CNS. Close examination of numerous histological slides revealed evidence for axonal transport of the parasite throughout the nervous system. The anatomic locations of parasite-associated lesions indicates that the use of such infected fish may adversely affect in vivo experiments with zebrafish beyond merely inducing morbidity, emaciation, and reducing fecundity.

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2.6 References

Kent ML, Buchner C, Watral VG, Sanders JL, LaDu J, Peterson TS, et al. Development and maintenance of a specific pathogen free (SPF) zebrafish research facility for *Pseudoloma neurophilia*. Dis Aquat Org. 2011;95:73–79.

Sanders GE. Zebrafish housing, husbandry, health and care: IACUC considerations. ILAR J. 2012;53:205–207.

Sanders J, Peterson T, and Kent ML. Early development and tissue distribution of *Pseudoloma neurophilia* in the zebrafish, *Danio rerio*. J Eukaryot Microbiol. 2014;61:238–246.

Matthews JL, Brown AM, Larison K, Bishop-Steward JK, Rogers P, and Kent ML. *Pseudoloma neurophilia* n.g., n. sp., a new microsporidium from the central nervous system in the zebrafish (*Danio rerio*). J Eukaryot Microbiol. 2001;48: 227–233.

Murray KN, Dreska M, Nasiadka A, Rinne M, Matthews JL, Carmichael C, et al. Transmission, diagnosis, and recommendations for control of *Pseudoloma neurophilia* infections in laboratory zebrafish (*Danio rerio*) facilities. Comp Med. 2011;61:322–329.

Sanders J, Watral V, Clarkson K, and Kent ML. Verification of intraovum transmission of a microsporidium of vertebrates: *Pseudoloma neurophilia* infecting the zebrafish, Danio rerio. PLoS One. 2013;8.

Kent ML, Spitsbergen JM, Matthews JM, Fournie JW, and Murray KN, Westerfield M. Diseases of Zebrafish in Research facilities. ZIRC Health Services Zebrafish Disease Manual. 2012. Available at http://zebrafish.org/zirc/health/diseaseManual.php.

Chow FW, Xue L, Kent ML. Retrospective study of the prevalence of *Pseudoloma neurophilia* shows male gender bias in zebrafish *Danio rerio* (Hamilton-Buchanan). J Fish Dis. 2015. [Epub ahead of print]; DOI: 10.1111/jfd.12328.

Cali A, Kent M, Sanders J, Pau C, and Takvorian PM. Development, ultrastructural pathology, and taxonomic revision of the Microsporidial genus, *Pseudoloma* and its type species *Pseudoloma neurophilia*, in skeletal muscle and nervous tissue of experimentally infected zebrafish *Danio rerio*. J Eukaryot Microbiol. 2012;59:40–48.

Lom J, and Dykova I. Microsporidian xenomas in fish seen in wider perspective. Folia Parasitol (Praha). 2005;69–81.

Ramsay JM, Watral V, Schreck CB, and Kent ML. *Pseudoloma neurophilia* infections in zebrafish *Danio rerio*: effects of stress on survival, growth, and reproduction. Dis Aquat Organ. 2009;88:69–84.

Morris JA. Zebrafish: a model system to examine the neurodevelopmental basis of schizophrenia. Prog Brain Res. 2009;179:97–106.

Pittman JT, and Lott CS. Startle response memory and hippocampal changes in adult zebrafish pharmacologically induced to exhibit anxiety/depression-like behaviors. Physiol Behav. 2014;123:174–179.

Stewart A, Wong K, Cachat J, Gaikwad S, Kyzar E, Wu N, et al. Zebrafish models to study drug abuse-related phenotypes. Rev Neurosci. 2011;22:95–105.

Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, and Kalueff AV. Modeling anxiety using adult zebrafish: a conceptual review. Neuropharmacol. 2012;62:135–143.

Stewart AM, Nguyen M, Wong K, Poudel KW, and Kalueff AV. Developing zebrafish models of autism spectrum disorder (ASD). Prog Neuropsychopharmacol Biol Psychiatry. 2014;50:27–36.

Willemsen R, Hasselaar W, van der Linde H, and Bonifati V. Zebrafish as a new model organism for Parkinson's disease. Proc Measuring Behav. 2008;50–51.

Peterson TS, Spitsbergen JM, Feist SW, and Kent ML. Luna stain, an improved selective stain for detection of microsporidian spores in histologic sections. Dis Aquat Organ. 2001;95:175–180.

LaVail JH, Tauscher AN, Aghaian E, Harrabi O, and Sidhu SS. Axonal transport and sorting of herpes simplex virus components in a mature mouse visual system. J Virol. 2003;77:6117–6126.

Salinas S, Schiavo G, and Kremer EJ. A hitchiker's guide to the nervous system: the complex journey of viruses and toxins. Nat Rev Microbiol. 2010;9:645–655.

Hacker C, Howell M, Bhella D, and Lucocq J. Strategies for maximizing ATP supply in the microsporidian *Encephalitozoon cuniculi*: direct binding of mitochondria to the parasitophorous vacuole and clustering of the mitochondrial porin VDAC. Cell Microbiol. 2014;16: 565–579.

Cebrian C, Zucca FA, Mauri P, Steinbeck JA, Studer L, Scherzer CR, et al. MHC-I expression renders catecholaminergic neurons susceptible to T-cell mediated degeneration. Nat Commun. 2013;5.

Megret F, Prehaud C, Lafage M, Moreau P, Rouas-Freiss N, Carosella ED, et al. Modulation of HLA-G and HLA-E expression in human neuronal cells after rabies virus or herpes virus simplex type 1 infections. Hum Immunol. 2007;68:294–302.

Kent ML, Dawe SC, and Speare DJ. Resistance to reinfection in chinook salmon *Oncorhynchus tshawytscha* to *Loma salmonae* (*Microporidia*). 1999;37:205–208.

Caruncho HJ and Pinto Da Silva P. Alterations in the intermediate layer of goldfish meninges during adaptation to darkness. J Anat. 1994;184:355–362.

Ariens Kappers CU. The meninges in Cyclostomes, Selachians, and Teleosts, compared with those in man. Proc R Acad Amst. 1924;28:71–80.

Hatta K, and Korn H. Physiological properties of the Mauthner System in the adult zebrafish. J Comp Neurol. 1998;395:493–509.

Maxie MG, and Youssef S: Nervous system. In: Jubb, Kennedy, and Palmer's Pathology of Domestic Animals Volume 1. Maxie MG (ed), pp. 281–298, Elsevier Limited, Philadelphia, PA, 2007.

Whipps CM, Lieggie C, and Wagner R. Mycobacteriosis in zebrafish colonies. ILAR J. 2012;53:95–105.

Lawrence C, Ennis DG, Harper C, Kent ML, Murray K, and Sanders GE. The challenges of implementing pathogen control strategies for fishes used in biomedical research. Comp Biochem Physiol C Toxicol Pharmacol. 2012;155:160–166.

Crofton HD. A model of host-parasite relationships. Parasitology. 1971;63:343–364.

Poulin R. Explaining variability in parasite aggregation levels among host samples. Parasitology. 2013;140:541–546.

Lester R. A review of methods for estimating mortality due to parasites in wild fish populations. Helgol Mar Res 1984; 37:53–64.

Collymore C, Watral V, White JR, Colvin ME, Rasmussen S, Tolwani RJ, et al. Tolerance and efficacy of Emamectin benzoate and Ivermectin for the treatment of *Pseudocapillaria tomentosa* in laboratory
zebrafish (*Danio rerio*). Zebrafish. 2014;11:490–497.

Filby AL, Paull GC, Bartlet EJ, Van Look KJ, and Tyler CR. Physiological and health consequences of social status in zebrafish (*Danio rerio*). Physiol Behav. 2010;101:576–587.

Spence R, Smith C. Mating preference of female zebrafish, *Danio rerio*, in relation to male dominance. Behav Ecol. 2006;17:779–783.

Eaton RC, Bombardieri RA, and Meyer DL. The Mauthner-initiated startle response in teleost fish. J Exp Biol. 1977; 66:65–81.

Liu KS, and Fetcho JR. Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. Neuron. 1999;23:325–335.

Liu DW, and Westerfield M. Function of identified motoneurones and coordination of primary and secondary motor systems during zebrafish swimming. J Physiol. 1988;403: 73–89.

Zottoli SJ, Newma BC, Rieff HI, and Winters DC. Decrease in occurrence of fast startle responses after selective Mauthner cell ablation in goldfish (*Carassius auratus*). J Comp Physiol A. 1999;184:207–218.

Mathuru AS, and Jesuthusasan S. The medial habenula as a regulator of anxiety in adult zebrafish. Front Neural Circuits. 2013;7:1–3.

Moles A, and Heifetz J. Effects of the brain parasite *Myxobolus arcticus* on sockeye salmon. J Fish Biol. 1998;52:146–151.

Levsen A, Alvik T, and Grotmol S. Neurological symptoms in tricolor shark minnow *Balantiocheilos melanopterus* associated with *Myxobolus balantiocheili* n. sp. infecting the central nervous system. Dis Aquat Org. 2004;59:135–140.

Berdoy M, Webster JP, Macdonald DW. Fatal attraction in rats infected with *Toxoplasma gondii*. Proc Biol Sci. 2000; 267:1591–1594.

Dubey JP. Advances in the life cycle of *Toxoplasma gondii*. Int J Parasitol. 1998;28:1019–1024.

Chapter 3

The common neural parasite *Pseudoloma neurophilia* is associated with altered startle response habituation in adult zebrafish (*Danio rerio*): Implications for the zebrafish as a model organism.

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3.1 Abstract

The zebrafish's potential as a model for human neurobehavioral research appears nearly limitless despite its relatively recent emergence as an experimental organism. Since the zebrafish has only been part of the research community for a handful of decades, pathogens from its commercial origins continue to plague laboratory stocks. One such pathogen is *Pseudoloma neurophilia*, a common microparasite in zebrafish laboratories world-wide that generally produces subclinical infections. Given its high prevalence, its predilection for the host's brain and spinal cord, and the delicate nature of neurobehavioral research, the behavioral consequences of subclinical *P. neurophilia* infection must be explored. Fish infected via cohabitation were tested for startle response habituation in parallel with controls in a device that administered ten taps over 10 min along with taps at 18 and 60 min to evaluate habituation extinction. After testing, fish were euthanized and evaluated for infection via histopathology. Infected fish had a significantly smaller reduction in startle velocity during habituation compared to uninfected tankmates and controls. Habituation was eliminated in infected and control fish at 18 min, whereas exposed negative fish retained partial habituation at 18 min. Infection was also associated with enhanced capture evasion: Despite the absence of external symptoms, infected fish tended to be caught later than uninfected fish netted from the same tank. The combination of decreased overall habituation, early extinction of habituation compared to uninfected cohorts, and enhanced netting evasion indicates that *P. neurophilia* infection is associated with a behavioral phenotype distinct from that of controls and uninfected cohorts. Because of its prevalence in zebrafish facilities, *P. neurophilia* has the potential to insidiously influence a wide range of neurobehavioral studies if these associations are causative. Rigorous health screening is therefore vital to the improvement of the zebrafish as a translational model for human behavior.

3.2 Introduction

3.2.1 The Zebrafish: a burgeoning model organism

The use of zebrafish in neurobehavioral studies has increased exponentially since their inception as an experimental animal in the 1970s (Grunwald and Eisen 2002). In this short time,

these studies have come to utilize a variety of stimuli including, but not limited to, genetic manipulations, pharmaceutical products, and environmental toxins. As the reliance on zebrafish in neurobehavioral studies has increased, so too has their utility as a model organism for such diverse human behavioral traits as stress, memory, and learning. Zebrafish are also being developed as models for such complex human neurological diseases as schizophrenia, autism, and Parkinson's disease (Caramillo et al. 2015; Chanin et al. 2012; Eddins et al. 2010; Kalueff and Stewart 2012; Maximino 2010; Morris 2009; Pittman and Lott 2014; Stewart et al. 2012; Stewart et al. 2011; Stewart et al. 2014; Willemsen et al. 2008).

Like any relatively new laboratory animal, the zebrafish comes with its own menagerie of infectious diseases that range in severity from the merely problematic to the completely devastating. Paradoxically, the most severe can be the easiest to monitor: laboratories maintain intense vigilance against highly virulent organisms such as *Edwardsiella ictaluri*, *Mycobacterium marinum* and *Mycobacterium haemophilum*, which can decimate entire stocks (Kent et al. 2012). Because of their pathogenicity, these diseases generally produce visible symptoms and sick fish can be excluded from behavioral experiments. More sinister are the subclinical diseases of zebrafish (Kent et al. 2012). From a pure husbandry standpoint, a low virulence 'background' disease may not seem like an important concern. However, as zebrafish are increasingly used in sensitive experiments across the scientific spectrum, infection-associated, non-protocol induced variation is a rising threat.

Pseudoloma neurophilia is one of the most common pathogens identified in many zebrafish facilities with infections present in up to 74% of all facilities submitting zebrafish to the ZIRC diagnostic service between 2006 and 2010 (Zebrafish International Resource Center, Eugene, Oregon) (Kent et al. 2012). Currently, the only identified symptoms of infection are fairly nonspecific and include weight loss, decreased fecundity, and increased mortality (Ramsay et al. 2009; Sanders 2012; Sanders et al. 2012). *P. neurophilia*'s lack of pathogenicity compared to more florid organisms makes it nearly invisible to researchers without intensive monitoring, and subtle behavioral consequences of infection have not yet been identified.

3.2.2 *Pseudoloma neurophilia*

Members of the phylum Microsporidia are intracellular, fungus-like parasites that infect a wide range of host phyla (Cali et al. 2012). They are particularly prevalent and pathogenic in fishes (Kent et al. 2014). *P. neurophilia* is spread primarily through the consumption of environmentally resistant spores, either through scavenging of infected carcasses or through consumption of free spores released with eggs during spawning. Alternatively, spores can be transmitted vertically, as infections have been observed in both eggs and newly-hatched larvae (Sanders et al. 2013; Sanders et al. 2014). *P. neurophilia* spores are a particular problem for zebrafish facilities because they can survive bleaching at a concentration of 25–50 ppm, which is the standard concentration used in most facilities for embryo sterilization (Ferguson et al. 2007). This makes transmission of spores between facilities via embryonated eggs a risk (Murray et al. 2011).

Many zebrafish researchers are either unconcerned or uninformed about underlying *P. neurophilia* in their animals, because most infections are subclinical and many facilities lack screening protocols (Chow et al. 2015). There is currently only one zebrafish facility that is specific pathogen free (SPF) for *P. neurophilia* (Kent et al. 2011). Hence, zebrafish are usually obtained from non-SPF facilities. Simultaneously, there is almost universally no mention of pathogen screening in zebrafish-based neurobehavioral studies. Even laboratories maintaining in-house zebrafish populations tend to have a fairly high incidence of *P. neurophilia* infection (Murray et al. 2011; Chow et al. 2015; Kent et al. 2011; Spagnoli et al. 2015).

3.2.3 Zebrafish neuroanatomy and potential consequences of infection

In order to explore the potential consequences of subclinical infection, our group performed a retrospective study of *P. neurophilia* cases submitted to the ZIRC zebrafish diagnostic service between the years 1999 and 2013 (Spagnoli et al. 2015). In most chronic neural infections, *P. neurophilia* forms non-membrane-bound, intra-axonal aggregates of spores and pre-sporogonic stages termed parasite clusters (PCs) (Spagnoli et al. 2015; Kent and Bishop-Stewart 2003). *P. neurophilia* has a strikingly specific tropism for certain neural structures and by studying the most common anatomic locations of infection, we can hypothesize which behaviors might be altered by the parasite.

PCs were found most frequently in spinal nerve roots and spinal white matter (Spagnoli et al. 2015). PCs in these locations would most likely affect motor function (Wulliman et al. 1996). In the hindbrain, PCs were most frequently located in descending white matter tracts, (the dorsal and ventral medial longitudinal fasciculi) which transmit signals from the brain to the spinal cord and then to the rest of the body (Spagnoli et al. 2015). Lesions in these areas could also affect motor function (Wulliman et al. 1996). The dorsal medial longitudinal fasciculus contains the Mauthner axon, which runs the entire length of the spinal cord and plays a major role in coordinating the startle response. Since we found that PCs in the hindbrain and the spinal cord frequently impinge upon the Mauthner axon, it is likely that the startle response would be altered by infection (Spagnoli et al. 2015; Wulliman et al. 1996; Eaton et al. 1977; Hatta and Korn 1998; Liu and Westerfield 1988; Liu and Fetcho 1999).

PCs located in rhombencephalic gray matter were frequently observed in the griseum centrale and the reticular system (Spagnoli et al. 2015). Anxiety and fear-learning in mammals are generally associated with the amygdala (Maximinio et al. 2010; Mathuru and Jesuthusasan 2013). Although cyprinids lack an amygdala, the medial habenula of the telencephalon has been implicated in anxiety and aversion learning and it has descending connections that associate with the griseum centrale. Because many zebrafish-based behavioral experiments utilize avoidance learning and the memory of noxious stimuli (either directly or indirectly), it is possible that griseum centrale lesions could influence these experiments (Maximinio et al. 2010; Mathuru and Jesuthusasan 2013). The reticular formation contains arousal circuitry and acts with the Mauthner neurons to integrate the startle response (Liu and Fetcho 1999; Zottoli et al. 1999; Mathuru and Jesuthusasan 2013; O'Malley et al. 1996).

Based on the frequent presence of PCs in anatomic structures involved with motor function, anxiety, fear-learning, and the startle response, any experimental protocol that evaluates or involves one or more of these features could be unduly influenced by *P. neurophilia* infection. Startle response habituation tests, therefore, should be highly sensitive to alteration by neural microsporidiosis.

3.2.4 Startle response habituation

In this study, we explored the potential of *P. neurophilia* infection to influence a common neurobehavioral assay. We accomplished this by comparing the performances of infected and uninfected adult zebrafish to a progressive tap test for startle response habituation and habituation extinction. We evaluated the effects of *P. neurophilia* infection using the progressive tap test because we felt that this assay had the highest potential to be affected by neural microsporidiosis based on the parasite's anatomic tropisms as explained in Section 1.3. The progressive tap test for adult fish was used with protocols based largely on those described in Eddins et al. (2010).

3.3 Materials and methods

3.3.1 Fish

Zebrafish of the 5D strain were used and reared to an age of two months. These fish were obtained from the Sinnheuber Aquatic Research Laboratory (SARL, Corvallis, OR). The 5D strain is an outbred strain derived from zebrafish reared for the ornamental fish industry and the SARL is SPF for *P. neurophilia* (Kent et al. 2011). At 70 days (d) of age, approximately 250 fish from the SARL facility were transported to our laboratory where they were separated into two equally numbered groups. Fish were reared in the same room on the same lighting schedule and were exposed to the same system water at the same temperature (27 ◦C). Both groups of fish were housed in 28 L tanks on a flow-through system and fed twice daily with the same artificial commercial diet.

Test fish were exposed to *P. neurophilia* in the following manner: To optimize infection rates, and to allow enough time for the development of chronic infections, naïve fish were placed into water that had previously housed infected fish. The contaminated water containing naïve fish was then supplemented with effluent water from a tank containing infected adult 5D fish for 24 d beginning upon their arrival from the SARL facility (70 d old). The feeder tank containing adult infected fish was placed on a shelf above the exposure tank and an outflow tube was fed from the upper tank into the exposure tank. Effluent water was allowed to flow into the exposure tank by gravity. In order to maintain appropriate water quality levels, the exposure tank also received supplementary system water at a rate approximately twice that of the effluent flow rate.

Water quality values were comparable between the two tanks and considered within acceptable limits. At the end of the 24 d exposure period, neural infection of test fish (and, simultaneously, the non-infected status of control fish) was confirmed via histopathology. At this time, there was no histopathologic evidence of chronic exposure to poor quality water (proliferative branchitis, etc.). Exposure to effluent was chosen as the method of infection in this experiment in order to mimic conditions in an actual zebrafish facility where infections would be transmitted by exposure of naïve fish to spore-laden water.

Approximately half of the fish in the test tank died by 24 d post exposure, most likely due to the stress of transfer of young fish combined with exposure to parasite-laden water. This mortality rate far exceeded that of the control fish, and so at the end of the 24 d period (fish aged 94 d) the tank of control fish was divided in half, leaving both tanks at a stocking density of approximately 40–60 fish per liter. This was done in order to eliminate differences in survival rates and stocking densities as potential complicating variables.

3.3.2 Tap test

The startle response is a fast start response in which a loud noise or a sudden, frightening stimulus causes a fish to turn and swim rapidly away from the source (Eaton et al. 1977; Hatta and Korn 1998; Liu and Westerfield 1988; Liu and Fetcho 1999). The intensity of the startle response can be quantified in adult zebrafish by measuring swimming velocity following a stimulus (e.g., tapping on the aquarium wall). Habituation occurs when the post-startle response decreases over serial stimuli (Eddins et al. 2010; Maximino et al. 2010; Valsamis and Schmid 2011) and the degree of habituation can be measured by evaluating the overall reduction in startle velocity during the habituation period (Valsamis and Schmid 2011). The progressive tap test for startle response habituation combines multiple neural structures and behavioral circuits, and the results are best interpreted as an integration of multiple fear and anxiety responses resulting in a quantifiable motor response (Maximino et al. 2010).

We used a testing apparatus similar to that described by Eddins et al. (2010). It consisted of an aluminum frame measuring 52×42×220cm that held the arenas on top of a flat plastic board. Located beneath the board were four solenoid coil-driven pistons (Guardian brand Model TP6×12 Push-Type DC Tubular Solenoid). These were placed along the longitudinal midline of

the board at regular spaces directly between each pair of arenas so that the vibrations from each strike would be distributed evenly throughout the arenas (Fig. 1). The solenoid pistons were connected by wire to a push-button for activation. Video was captured using a Sony Handycam model HDR-CX240. The camera was attached to the frame at a height of 180cm above the upper rims of the arenas and was positioned to record all eight simultaneously from the top-down.

Opaque cylindrical white plastic arenas were used, each 12cm tall and 8 cmin diameter at the base (Fig. 1). These were filled with 250mL of water from the laboratory aquaculture system (27 ◦C). These containers and the water volume were chosen so individual fish could not see each other and to prevent fish from leaping out of their containers during the experiment. Water in the arenas was replaced and the arenas themselves were rinsed with system water between testing sessions in order to minimize the potential effect of alarm substance released by fish during testing. Tests were performed over the course of 4 d, beginning when the fish were aged 171 d (101 d after initial experimental exposure). Testing occurred between 0800 and 1700 h; during standard daylight activity times for tested fish. Three to five tests were performed per day, beginning at 0800, 1000, 1200, 1400, and/or 1600 h where applicable. Each testing session utilized eight fish. While fish from both control and exposed groups were available, four fish from each group were used during each testing session in order to both maintain similar stocking densities and to minimize the effect of individual session variation. The location of each fish (control or exposed) within rig arenas was randomized via coin toss to reduce locational variation. For each test, four fish were captured from the two tanks (exposure and control) using similar square nylon mesh nets measuring 10×25 cm. The same investigator performed each netting session.

The investigator was located in another room and separated from the testing apparatus by a closed door. The moment of door closing was used as the start of the ten minute acclimation period during which no taps were given. Using a stopwatch, the investigator manually pressed a button to activate tapping solenoids on the rig. Taps occurred once every sixty seconds over 10 min after the acclimation period. These 10 taps over 10 min were used to assess the fish's habituation to the startle response. After these stimuli, two more taps were administered at 8min and 50 min following the completion of the first set of taps. This was conducted to evaluate the extinction of the adaptation response. Eddins et al. (2010) utilized taps at 8min and 50 min following the last tap of the 10 min habituation series to evaluate the extinction of the habituation

response. This can be measured by comparing the post-tap startle velocities between tap 1 and taps at 18 min and 60 min. Retention of habituation is indicated by later post-tap velocities being reduced compared to the starting post-tap velocity: a larger difference indicates a greater degree of retention. If there is no difference in post-tap velocity between tap 1 and the later taps, then the habituation response developed during the initial ten minute series is said to be extinct (Eddins et al. 2010).

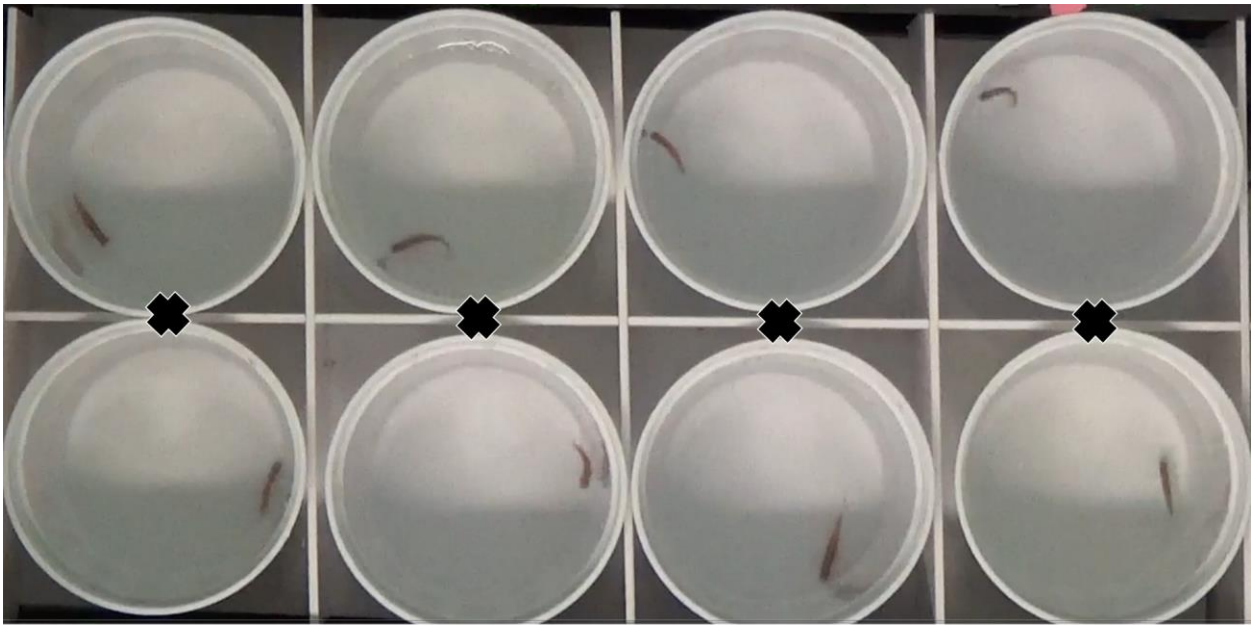

FIG 1. Testing Apparatus. Video still showing a top-down view of eight testing arenas containing fish. X = location of solenoid piston beneath the arenas.

3.3.3 Histopathology

Fish were euthanized by exposure to ice water, an approved protocol in the guidelines for the use of zebrafish in the NIH intramural research program (NIH 2013). The abdomen of the each fish was opened with a longitudinal cut, and fish were preserved whole, fixed in Dietrich's fixative, and decalcified using Cal-Ex II. Fish were then processed for histology. Fish were sectioned longitudinally in midline sections so that slides contained the brain and spinal cord, and sections were with hematoxylin and eosin. Fish with granulomas were further stained with Kinyoun's Acid Fast, Luna, and Hall's bile stains using standard methods. Severity of infection

and associated inflammatory changes were scored as described by Spagnoli et al. (2013)(meninxitis and encephalitis both on a scale of 0–3).

3.3.4 Video analysis

Video was analyzed using Ethovision XT 10.1 software (Noldus, The Netherlands). The average swimming velocity of each fish over five seconds post-tap was used as an indicator of startle responsestrength. The length of each fish was measured using still images from video recordings and 'length' was defined as the longitudinal measurement of the fish from the midpoint between the eyes to the thinnest visible portion of the caudal peduncle.

3.3.5 Statistical analysis

We applied the logarithm transformation to startle velocity to stabilize the variance and transform the positively defined variable to the entire real line. We modeled the logarithm of startle velocity as a linear function of tap number, but allowed different interceptors and slopes for three different exposure types (control, exposed negative, and infected), and accounted for the effects of bile duct hyperplasia, hepatic granulomas, fish length, presence of PCs in notochord remnants, total PC number, encephalitis score, and meninxitis score. Both session number and fish identification were considered as random effects to incorporate possible correlation of measurements taken on the same fish and in the same session. Along with the slope of the logarithmic linear function, the difference in startle velocities between tap1 and tap10 was used to quantify the degree of habituation over ten taps. A linear mixed model was used to quantify the effects of sex and exposure/infection status on fish habituation while controlling for bile duct hyperplasia, hepatic granulomas, fish length, presence of PCs in notochord remnants, total PC number, encephalitis score, and meninxitis score. The session was considered as a random effect to incorporate potential correlations within the same sessions into the model. Two similar linear mixed models were also used, where with logarithms of the 18 and 50 min time points were treated as response variables, respectively. These models evaluate the effects of exposure/infection and sex on startle velocity at these time points, while controlling for the effects of other variables. Differences in startle velocities between tap1 and tap18, and

betweentap1 and tap50 were used to quantify the degree of extinction of the habituation. A similar linear mixed model as above was used to assess the effects of exposure/infection and sex on the degree of extinction of the habituation, while controlling for the effects of other variables. At each tap, the sample variances of the startle velocity were calculated for fish of the same sex and exposure type. A linear regression model was then used to compare the variability of startle velocity for fish with different sex and exposure types. A one-way analysis of variance (ANOVA) with session as the response variable and exposure type as the explanatory variable was used to investigate the difference of capture times of fish with different exposure type. All analyses were conducted using PROC GLM, SAS Insititute 2015.

3.4 Results

Approximately half of the fish in the test tank died by 24 d postexposure, most likely due to the stress of transfer of young, small fish to a tank full of parasite spores. This mortality rate far exceeded that of the control fish.

3.4.1 Histopathology

Fish in the two separate tanks were divided into three categories based on exposure and infection status: Control (n = 60) in one tank and exposed positive/infected (n = 40) and exposed negative (n = 31) in the other tank. Infections were generally mild, with a median of 2 intraneural PCs per fish (standard deviation (SD) = 2.29). Central nervous system PCs in infected fish ranged in number from 0 to 11. The vast majority of PCs in infected fish were observed in the spinal cord white matter or in the nerve roots (Fig 2). Three of these fish had parasite clusters in notochord remnant cells and one of these had PCs only in notochord remnant cells and in no other anatomic structure. The fish with PCs only in the notochord remnant was counted as an infected fish with 0 intraneural PCs (Fig 2). Of the 40 infected fish, only 7 had PCs in the medial longitudinal fasciculus (descending white matter tract) in the hindbrain. No fish had PCs in any rhombencephalic gray matter structure. Myositis was not observed in any infected fish.

Inflammation was uncommon among the 40 infected fish, with encephalitis or myelitis observed in only 12 fish and meninxitis observed in 13. These were graded according to the

scheme outlined in Spagnoli et al. (2015). Scores for both encephalitis/myelitis and meninxitis ranged from 0 to 3 on a scale of 0–3 for both inflammation categories. Encephalitis scores had a mean of 0.5 and a standard deviation of 0.9. Meninxitis scores had a mean of 0.5 and a standard deviation of 0.8. Four fish in the exposed tanks had grossly visible skeletal deformities. Of these, three were infected and one was uninfected. One fish in the control tank had a skeletal deformity but it was confirmed negative for infection by histopathology. Over half of all fish in all exposure groups (control, exposed positive, and exposed negative) had some combination of biliary hyperplasia or hepatic granulomas (Fig 3). These lesions were negative for mycobacteria with Kinyoun's acid fast stain, negative for *P. neurophilia* spores with the Luna stain, and negative for bile via Hall's bile stain.

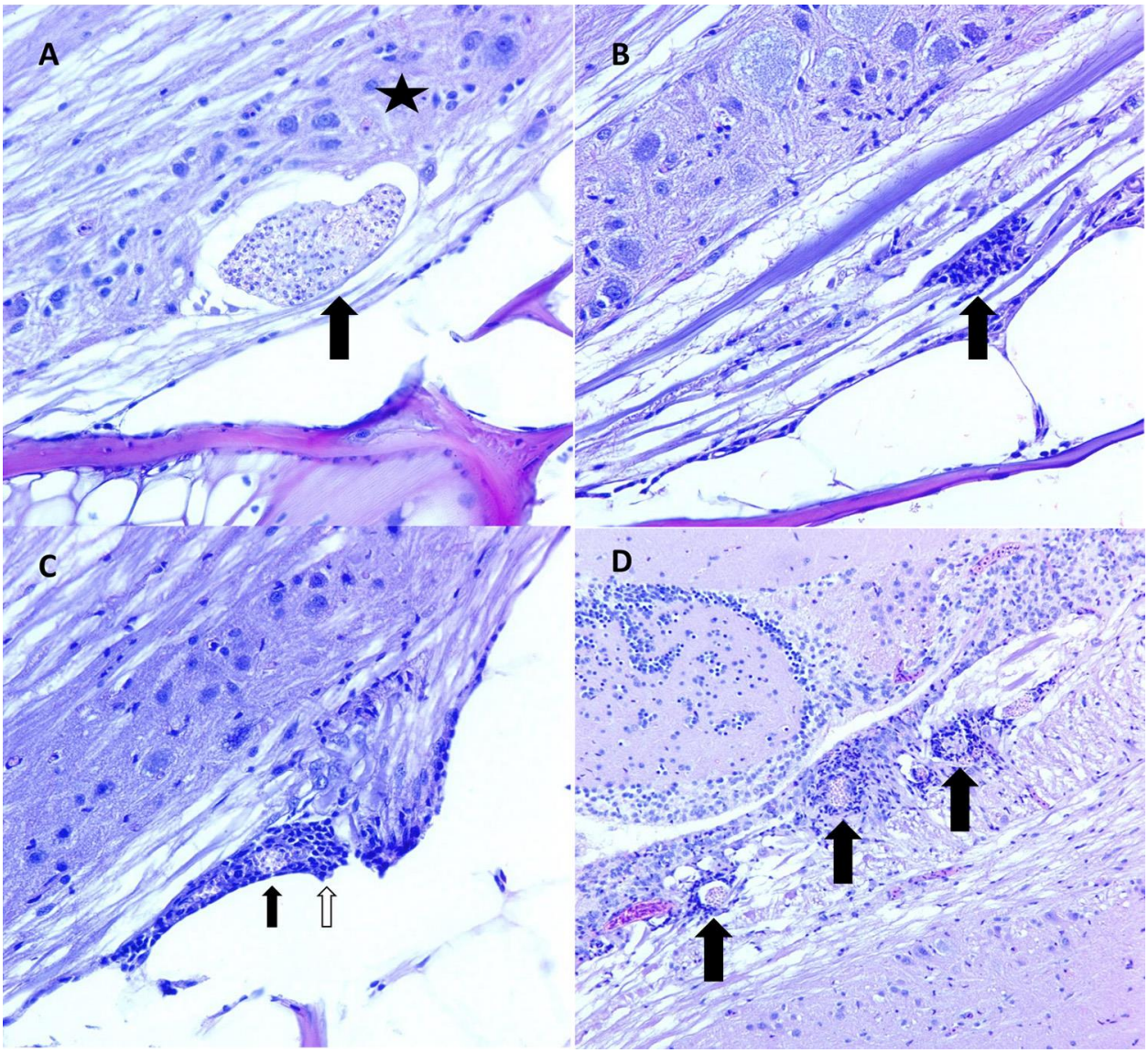

FIG 2. General features of infection. (A) Parasite clusters (PCs) were most commonly observed in the ventral white matter of the spinal cord. In this image, a PC in white matter (arrow) impinges of the gray matter (star). Photomicrograph. H&E. 400× magnification. (B) A common feature of infection was myelitis, or inflammation of the spinal cord neuropil. Myelitis in these fish was generally multifocal and composed of inflammatory cells that are most likely a combination of granulocytes and microglial cells (arrow). Photomicrograph. H&E. 400× magnification. (C) Meninxitis is inflammation of the perineural membranes of the teleost central nervous system, so called because they do not have a true set of meninges as in mammals. Here, granulocytes (white arrow) surround a ruptured PC (black arrow) at the base of a nerve root. (D) Encephalitis, inflammation of the brain, was associated with PCs (black arrows) in the medial longitudinal fasciculus, a descending white matter tract in the rhombencephalon Photomicrograph. H&E. 200× magnification.

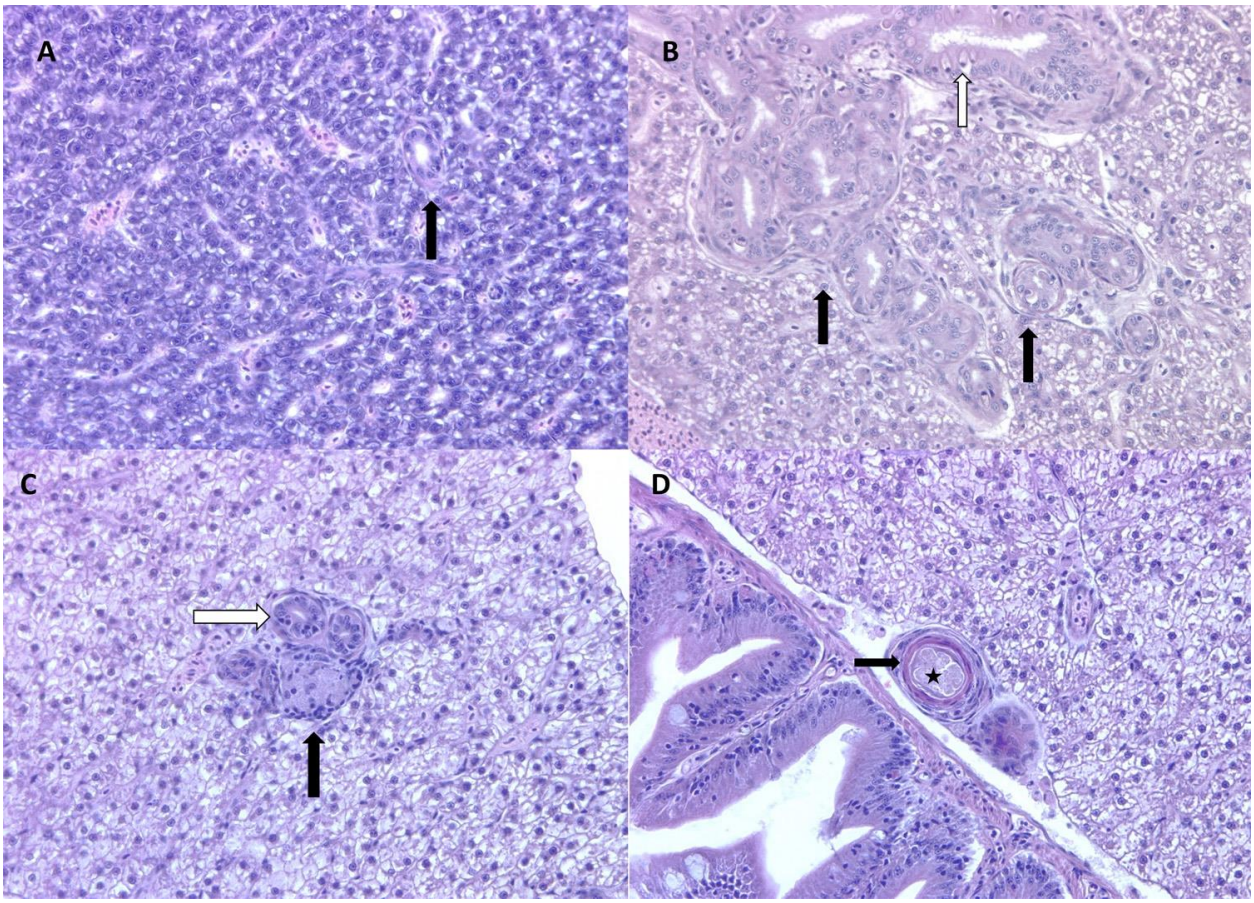

FIG 3. Biliary hyperplasia and granulomas. (A) Less than half of the fish in all groups examined had normal-appearing livers. Normal zebrafish bile ducts (arrow) are lined by a single layer of low cuboidal epithelium. Photomicrograph. H&E. 400× magnification. (B) In the most severe cases, biliary hyperplasia was characterized by well-differentiated, tortuous bile ducts of varying size with prominent basement membranes (black arrows). Ducts were lined by tall simple to pseudostratified columnar epithelial cells that were occasionally infiltrated by varying numbers of rodlet cells (white arrow). Photomicrograph. H&E. 400× magnification. (C) The vast majority of visceral granulomas in these animals were observed in the liver. They were commonly observed in association with hyperplastic bile ducts (white arrow). Some granulomas were relatively poorly organized with a central area of flocculent, acellar material admixed with and partially lined by lymphocytes and macrophages (arrow). Photomicrograph. H&E. 400× magnification. (D) Granulomas frequently had central acellular flocculent material (star) surrounded by what appeared to be lamellar keratin developed from stratified squamous epithelium (black arrow). Photomicrograph. H&E. 400× magnification.

3.4.2 Habituation testing

Log habituation slopes are listed in Table 1.When the log habituation slopes are compared to each other, statistical differences between the groups were as follows: Exposed positive versus control, significant ($p = 0.04$); exposed negative versus control, not significant ($p = 0.4$); exposed positive versus exposed negative, not significant ($p = 0.3$). This indicates that infected fish habituated significantly more slowly than control fish. There was no significant difference in log slope between infected and exposed negative fish. Slopes for control and exposed negative fish were quite similar and did not differ significantly. r^2 values were low, indicating that the data were a poor fit for the linear model, however, the negative slope of habituation velocity was sufficient to support the use of T1–T10 post-tap velocity difference as a quantitative measure of habituation.

Because all three exposure groups had negative velocity slopes on the log scale and because there was no significant difference between T1 post-tap velocities between groups, the reduction in post-tap velocity between tap1 and tap10 was used to quantify and compare the overall degree of habituation between groups. The reduction in post-tap velocity between tap1 and tap10 for the control and exposed negative groups did not differ significantly (8.193 and 7.45 cm/sec, respectively; $p = 0.9$). However, the reduction for exposed positive fish was 4.047 cm/sec, which was about half that of the exposed negative and control groups. This difference was statistically significant ($p = 0.03$).

Evaluation of post-tap velocities between the three groups at specific, individual time points, including the initial tap and taps given at 18 and 50 min showed no statistical differences (linear mixed model, $p > 0.05$). There were no significant effects attributable to parasite cluster number, severity of meningitis or encephalitis, hepatic lesions, or parasite cluster location (linear mixed model, $p > 0.05$ for all parameters).

Table 1
Slopes of log-transformed post-tap velocities.

Exposure group.	**Slope of log transformed post-tap velocity.**	**p-value.**	**r^2.**
Control	−0.105	<0.0001	0.096
Exposed negative	−0.0815	0.0005	0.081
Infected	−0.0502	0.0182	0.045

Note: Slopes of log transformed post-tap velocities for all three exposure groups. All three slopes were negative and statistically significant, indicating that habituation occurred in all three groups.

3.4.3 Extinction of the habituation response

The difference in mean post-tap velocity between the tap at one minute and the tap at 18 min (8min after the last tap of the 10 tap habituation series) was significantly different only for exposed negative fish (Table 2). There were no significant differences in mean post-tap velocity between the tap at one minute and the tap at 60 min (50 min after the last tap of the 10 tap habituation series) for any group.

Table 2
Habituation extinction

Group	**Velocity T1–T18**	**p-Value**	**Velocity T1–T60**	**p-Value**
Control	5.86	0.06	0.25	0.9
Exposed negative	8.63	0.01	2.37	0.4
Infected	2.93	0.3	−0.73	0.7

Note: Only exposed negative fish retained habituation at 18 min. No fish retained habituation at 60 min.

3.4.4 Variability

There were high levels of interfish variability within groups (Fig. 4). However, startle velocity variabilitywassignificantly different between control, exposed positive and exposed negative groups ($p = 0.01$). Controls (SD estimate = 5.633) were more variable than exposed positive fish (SD estimate = 5.148), and the latter were more variable than exposed negative fish (SD estimate = 5.0264). Whereas mean startle velocities did not differ based on sex, males had significantly greater variability than females (males SD estimate = 5.453, females SD estimate = 5.0854, $p = 0.03$)

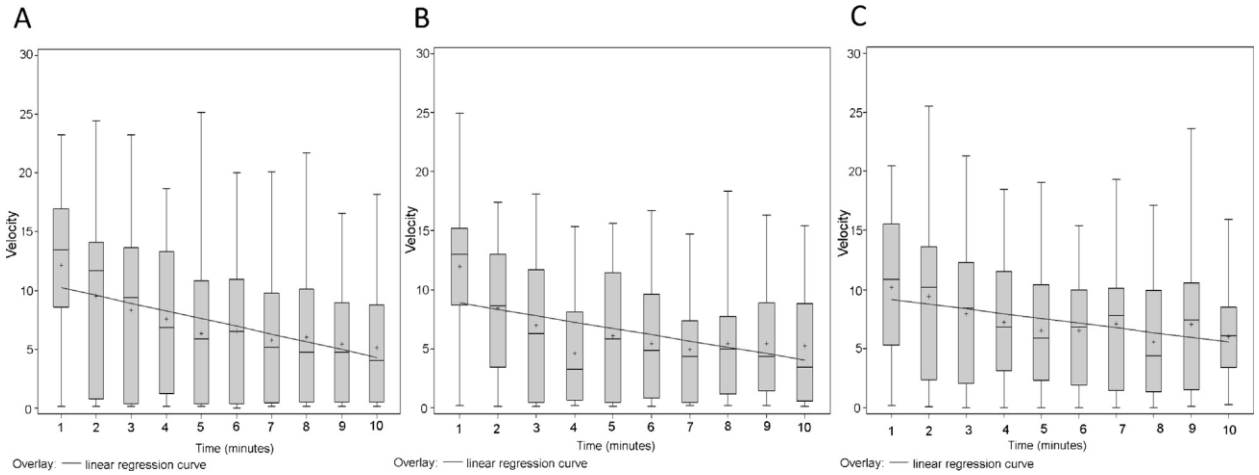

FIG 4. Mean post-tap velocity (cm/sec) for fish during habituation period (one tap per minute over ten minutes). Key: Box–Interquartile range, Whiskers–Range, Line–Median, Plus sign–Mean. Y-Axis: Tap/minute number. (A) Control, (B) exposed negative, (C) infected.

3.4.5 Capture avoidance

Within the exposure tank, infection was associated with the netting session in which a fish was caught (ANOVA, $p = 0.006$). Although it was not possible to visually differentiate between infected and uninfected fish, infected fish were caught, on average, approximately four sessions later than exposed negative fish captured from the same tank (ANOVA, $p < 0.001$).

3.5 Discussion

3.5.1 ***P. neurophilia*** infections in experimental animals

Approximately half of the larval fish in the exposure tank died during the exposure period. Because water quality values were similar to control tank values and because surviving exposed fish had no histologic lesions associated with poor water quality, the most likely explanation for the high mortality was transport stress combined with early exposure to parasites. Ramsay et al. established that elevated cortisol levels increase parasite load in stressed fish (Ramsay et al. 2009). Coupled with the age of the fish, this fact provides one possible explanation for the high mortality.

Of the several reports on *P. neurophilia*, only Ramsay et al. (2009) provided data on prevalence relating to time after exposure. The 56% infection rate in the exposure tank at 14 weeks post-exposure was lower than expected given that Ramsay et al., who observed greater than 80% infection at 13 weeks post-exposure. Left for a long enough time, 100% infection rates within a tank are achievable (Ramsay et al. 2009), however the rate of spread through a population varies from tank to tank. Anecdotally, infection rates observed on routine examination of tanks in contaminated facilities range from 40 to 60% and in these cases, the time of initial contamination is always unknown. The possibility also exists that different infection rates may be caused by different strains of *P. neurophilia* or different strains of the host. In this case, it is possible that the high early mortality rates selected for more resistant fish, resulting in an overall lower infection rate and relatively light infections among infected fish.

Our study resulted in two control groups, a separate tank of unexposed fish and negative fish within the exposed tank. The presence of uninfected fish along with infected fish in the exposure tank provided us with an internal control group, avoiding the confounding factor of tank conditions between control and exposed groups. Nevertheless, we believe that the while in a separate tank, the unexposed fish were also an appropriate control to use in our analyses as they were from the same stock of fish and held under otherwise identical conditions (Fig. 4).

3.5.2 Startle response habituation

The slope of the log habituation line for infected fish was significantly shallower than that of control fish. Although there was no statistically significant difference between habituation slopes for control and exposed negative fish, there was also no significant difference between exposed negative and exposed positive fish. This could have been due to the fact that exposed negative and infected fish had been reared in the same tank, making their behavioral phenotypes more similar than those of the fish in the control tank. Also, the r^2 value of all groups was low, likely due to the high interfish variability. Even though the linear model was likely not a good fit for the data, the negative value of the slopes proves that there was an overall reduction in startle velocity during the habituation period and supports the use of the T1–T10 startle velocity reduction as an overall measure of habituation.

There were no statistically significant differences between post-tap velocities at any individual time point, including the first tap, so any increased reduction in startle velocity between tap1 and tap10 corresponds to an increased degree of habituation. The use of the overall reduction in startle response to quantify the habituation response is similar to methods used in some rodent studies (Valsamis and Schmid 2011; Dulawa et al. 1997). The difference between velocities at tap1 and tap10 for infected fish was approximately half that of the control and exposed negative groups. This was statistically significant, indicating that infected fish had less of an overall habituation response than either control group. Moreover, differences for control and exposed negative groups did not differ significantly.

The overall reduced velocity for infected fish during the habituation period indicates that fish infected with *P. neurophilia* remain excitable despite repeated startling stimuli. A second possibility is that infected fish may simply have a higher baseline velocity than uninfected cohorts. Further research using an increased number of taps to discover a baseline velocity could determine whether the observation of overall reduced velocity was due to hyper-excitability or to an elevated baseline velocity in infected fish.

3.5.3 Extinction of the habituation response

Only exposed negative fish retained a degree of habituation at the 18 min tap. Habituation was extinct in control and infected fish at the 18 min tap, and in all groups at the 60

min tap (Table 2). Potential reasons for this finding include exposed negative fish having enhanced recall or reduced anxiety compared to control and infected fish. Alternatively, exposed negative fish could have simply had a lower baseline velocity than control or infected fish, meaning it might take longer for their post-tap velocity to return to starting levels. Regardless of the mechanism underlying this finding, it provides further evidence for a difference in behavioral phenotypes between infected fish and uninfected tankmates as well as potentially different phenotypes between exposed negative fish and control fish.

3.5.4 Variability

The greatest variation was observed among control fish, followed by infected fish, followed by exposed negative fish. While it is difficult (and impossible from this experiment alone) to determine why these differences occurred, some features of zebrafish behavior might lend themselves to a possible explanation. Individual variation within populations of zebrafish has been well-documented as has the formation of intrashoal hierarchies (Toms and Echevarria 2014), with more aggressive fish dominating and chasing less aggressive fish. Interestingly, the variation in response to the tap test was greater for infected fish than for exposed negative fish held in the same tank. Again, we see a difference between behavioral responses of infected and uninfected fish in the same tank as well as differences between infected fish, uninfected fish, and controls.

The formation of dominance hierarchies between fish of different sexes is unlikely to be the cause of the reduced variation of females compared to males. Spence et al. found that sex is not associated with the assumption of dominant or submissive roles (Spence et al. 2008). Also, even though size might contribute to the formation of dominance hierarchies, various studies have shown conflicting results with regards to whether size is positive or negatively associated with rank (Spence et al. 2008). Interestingly, the reduced variability of females compared to males may be an intrinsic facet of female zebrafish behavior. Tran and Gerlai (2013) demonstrated that females had more consistent behavior patterns than males during both passive observation and an open field task (Tran and Gerlai 2013)—this behavioral consistency could extend to habituation and the startle response.

3.5.5 Netting observations

The observation that infected fish tended to be caught in later sessions than uninfected fish from the same tank may be related to the results of the tap test. Fish that habituate poorly (have a smaller velocity reduction during habituation) may be particularly excitable and more apt to flee from a net. Alternatively, fish with a higher baseline velocity may escape netting for more sessions by virtue of their speed. While more research is needed to repeat the experiment using a pre-designed protocol, this particular experiment indicates that infected fish may be better at avoiding netting than uninfected fish. The difference in netting avoidance between infected and uninfected fish in the same tank provides more evidence for a difference in behavioral phenotypes between these groups.

3.5.6 Synthesis

The behavioral pattern of infected fish, highlighted by the reduction in startle velocity during habituation compared to controls and the enhanced netting avoidance, suggests that that infection is associated with a hyper-alert behavioral syndrome or an increased baseline swimming velocity. Further research is necessary to determine the factors underlying these observations, however, we have shown that *P. neurophilia* infection was associated with a behavioral phenotype distinct from those of control fish and uninfected fish reared in the same tank.

The most intriguing explanation for the distinct behavioral pattern is that *P. neurophilia* infection alters the behavior of infected fish. Because the observed behavioral differences involve the startle response, habituation, and motor function, the tropism of *P. neurophilia* for neuroanatomical sites connected to these behaviors makes a causative relationship eminently plausible (Spagnoli et al. 2015; Wulliman et al. 1996; Eaton et al. 1977; Hatta and Korn 1998; Liu and Westerfield 1988; Liu and Fetcho 1999; Zottoli et al. 1999; Mathuru and Jsuthusasan 2013; O'Malley et al. 1996). The complex behavioral consequences of neural parasitism are well documented in numerous species, particularly in trophic organisms (parasites that require the consumption of the intermediate host by a predator) (Barber et al. 2000; Thomas et al. 2005). A widely publicized example of a trophic parasite is the apicomplexan *Toxoplasma gondii*, which forms small lesions in its bradyzoite stage with almost no inflammation in chronic infections.

Pertinent to our study, these stages infect the central nervous system similar to *P. neurophilia*. Despite the noninflammatory nature of most chronic *T. gondii* infections, there is ample evidence from rodent experiments that the parasite suppresses predator avoidance behaviors, presumably as an evolutionary tactic to facilitate consumption by the final feline host (Gatkowska et al. 2012). There is also precedent for behavioral effects of parasitism on fish: metacercariae of *Diplostomum phoxini*, a trophic parasite, in Eurasian minnows (*Phoxinus phoxinus*) infects the brain and may induce subtle personality changes (measured by exploratory boldness) in the host (Kekalainen et al. 2014). Although it is unclear from an evolutionary standpoint how, or even if, *D. phoxini* facilitates predation by the final host, the fact that a neural parasite in fish produces behavioral changes provides an interesting precedent to our own findings.

Another possible explanation for our observations is that preexisting behavioral phenotypes may increase or decrease a fish's susceptibility to infection rather than infection itself causing behavioral changes. Different behavioral syndromes between animals can be associated with different immune responses and susceptibility to disease (Koolhaas 2008). Furthermore, behaviors such as increased egg and carcass consumption could increase the risk of infection, as stress and aggression have been suggested as the reason for higher infection rates among males compared to females (Chow et al. 2015).

If we further consider the idea of behavioral phenotypes influencing infection susceptibility, we may find a more subtle causative explanation for our observations. We observed high mortalities among naïve fish initially exposed to parasite-laden water. It is possible that the dead fish represented a third group of animals with a behavioral syndrome that made them particularly susceptible to fatal infections. If this was the case, then early infection could have selected for a population of adult fish with the behavioral patterns observed in our study. In this case, early *P. neurophilia* exposure could result in a population of adults with a particular set of behavioral traits even among uninfected fish. In this study, exposed negative fish had reduced variability and failed to abolish habituation at 18 min compared to controls. This distinct behavioral phenotype could be due to parasite-based selection or to interactions with their infected tankmates. Even if infection does not directly alter fish behavior, early infections present in contaminated facilities rearing their own fish could select for animals with altered behavioral phenotypes.

There were no statistically significant effects of PC number (a measure of parasite burden), PC location, encephalitis, or meninxitis on responses to the tap test. This could be explained by the relative lightness of infection patterns observed among fish in this study. Alternatively, the high mortality rate among young fish could have selected for a group of highly resistant adults, explaining the resulting combination of uninfected and mildly infected fish. A more intriguing explanation is that any P. neurophilia infection, no matter how mild, could produce similar effects across hosts due to molecular crosstalk with the parasite. Therefore, the parasite probably has more effects on the host than simply occupation of tissue spaces *T. gondii* is an excellent example of this, as mentioned previously (Gatkowska et al. 2012). The finding that disease severity was not significantly associated with any differences in behavioral responses coincides with the findings of our retrospective study: PC number, inflammation severity, and PC location were not associated with clinical disease (Spagnoli et al. 2015).

3.5.7 Startle response habituation in rodents: Using established models to improve zebrafish behavioral models

In order to improve the zebrafish as a powerful neurobehavioral model, we should look to established models as a guide for its development. The acoustic startle response has been well characterized in rodents for decades. Although similar to the fast start response in zebrafish, startling auditory stimuli elicit a whole body flinch in rodents that does not result in a ‘run’ stage as we have quantified in our study (Chanin et al. 2012; Eddins et al. 2010; Valsamis and Schmid 2011; Dulawa et al. 1997). Also, because rodents lack a lateral line, the perceptive aspect of their startle response is almost entirely auditory, whereas zebrafish also perceive pressure waves (Kalueff and Stewart 2012). As we described in our study, habituation is considered a decrease of response magnitude in the face of repeated stimuli (Kent and Bishop-Stewart 2003). Reduced habituation inhibition and pre-pulse inhibition (the reduction of a startle response following exposure to a non-startling stimulus) are observed in human schizophrenia along with a number of other psychological disorders (Valsamis and Schmid 2011; Dulawa et al. 1997; Dirks et al. 2002; Wang et al. 2003) and for this reason, habituation inhibition and pre-pulse inhibition have been thoroughly studied in rodent models. Auditory startle response habituation inhibition has been documented in adenosine A2A receptor knockout mice, seratonin1B receptor knockout

mice, and transgenic mice overexpressing corticotropin-releasing hormone among others (Valsamis and Schmid 2011; Dulawa et al. 1997; Dirks et al. 2002; Wang et al. 2003). Rodent facilities providing animals for such sensitive studies generally use extensive biosecurity protocols, and rightly so: The development of rodent models for human behavior, particularly those utilizing genetic knockouts, is both time-consuming and costly. It is nearly unthinkable that researchers would intentionally use rodents with viral encephalitis or chronic *T. gondii* infections in the experiments mentioned above, and zebrafish investigators should be similarly reluctant to use fish infected with *P. neurophilia* in their own studies (Percy and Barthold 2007).

3.5.8 Potential impacts of *P. neurophilia* on neurobehavioral Research

Whereas further research is needed to determine precisely how *P. neurophilia* induces behavioral changes or whether certain behavioral syndromes result in elevated infection susceptibility, our study shows that *P. neurophilia* is associated with a distinct behavioral phenotype in infected fish as well as in uninfected cohorts. If infection causes behavioral changes in a population, whether directly by molecular crosstalk with the animal's brain, or indirectly by mortality-based selection for particular behavioral syndromes, we can hypothesize what types of studies might be influenced by its presence.

The test most likely to be influenced by *P. neurophilia* is the one performed in this study: the serial tap test for startle response habituation, as well as any study that involves habituation to the startle response. This test is widely used as an indicator of anxiety, fear, stress, and psychomotor response in zebrafish (Chanin et al. 2012; Eddins et al. 2010; Kalueff and Edwards 2012; Maximino et al. 2010). Habituation inhibition may also be particularly useful in the study of human PTSD (Caramillo et al. 2015). If the zebrafish does become a model for PTSD, it will be vital for investigators to avoid *P. neurophilia*.

Although more research must be performed in order to determine precisely which facets of behavior may be influenced by *P. neurophilia*, the underlying traits potentially responsible for our findings could influence a wide range of neurobehavioral protocols if the association is causative. The high baseline velocity or hyper-alert behavioral phenotype observed in our infected fish may be an emergent property of several neurobehavioral components, the most likely of which are motor function, stress, fear, anxiety, and arousal (Chanin et al. 2012; Eddins

et al. 2010; Kalueff and Edwards 2012; Maximino et al. 2010). These traits are involved in the vast majority of zebrafish behavioral studies and they are the basis for innumerable protocols including the conditioned alarm reaction, shuttle box avoidance, inhibitory avoidance, open field testing, novel tank testing, novel object exploration, scototaxis (preference for dark over light areas), and Schrecksreaktion (place avoidance) (Kalueff and Edwards 2012; Maximino et al. 2010).

If *P. neurophilia* infection causes reliable behavioral changes in a population, its potential to confound research is augmented by its highly variable infection rates and uneven distribution of infection severity. Indeed, as with most other chronic parasite infections, *P. neurophilia* infections occur with a negative binomial distribution (Spagnoli et al. 2015) in which only a few fish have heavy infections and most have light or no infections.

Consider a theoretical neurobehavioral experiment in a contaminated facility: If control and experimental groups had equal numbers of infected animals, the influence might be equal between the two groups. However, it is unlikely that all tanks in an affected facility would be contaminated. While no surveys have been performed to directly measure the number of infected tanks within a contaminated facility (since an unintentionally contaminated facility would be unlikely to agree to such a survey), consider that in our own experiment, the control tank was maintained in a room full of contaminated tanks without becoming contaminated itself. While special precautions were taken to ensure that contamination did not occur, most facilities undertake similar precautions to reduce transmission of infectious diseases between tanks (Murray et al. 2011). Anecdotally, no facility contaminated with *P. neurophilia* has been observed with a 100% tank infection rate. If control and test fish in the theoretical neurobehavioral experiment are derived from two separate rearing tanks, which is especially likely in chronic exposure studies, it would be possible for one group to have infections but not the other, thus introducing an unintended bias. Furthermore, infection rates in contaminated tanks are rarely 100%. Even in this study, we achieved only a 56% infection rate after heavy exposure at an early age. Even if both tanks in the theoretical study were infected, it is unlikely that they would have the same rate of infection even if they were contaminated at exactly the same time with exactly the same dose of infectious material. If infection reliably produces a consistent behavioral phenotype, the varying infection rates between the two groups could adversely influence study data. Future research should focus on the interactions between *P.*

neurophilia infection and various experimental manipulations. Certain drugs, genetic knockouts, or toxicants could either enhance or reduce the effects of the parasite. In a similar vein, any alteration to a fish's immune system, stress included, could be associated with an increase or decrease in infection rates. Until this research is performed, this study should act as a red flag for investigators to screen their fish for this potentially damaging parasite.

3.5.9 Conclusion

P. neurophilia is a common contaminating parasite of laboratory zebrafish that preferentially infects the central nervous system. Based on this study, infection is associated with altered startle response habituation and potentially enhanced netting evasion. While further research is necessary to definitively prove a direct or indirect causal relationship between *P. neurophilia* infection and altered behavioral phenotypes, our observations indicate that this is a strong possibility. Because zebrafish are used in a wide variety of sensitive behavioral studies that may share fundamental traits affected by *P. neurophilia*, the potential influence of infection on fish behavior along with unpredictable intertank infection rates makes it vitally important to screen fish prior to experimentation.

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3.6 References

Maximino C, de Brito TM, da Silva Batista AW, Herculano AM, Morato S, and Gouveia A Jr. Measuring anxiety in zebrafish: a critical review. Behav Brain Res. 2010;214:157–171.

Morris JA. Zebrafish a model system to examine the neurodevelopmental basis of schizophrenia. Prog Brain Res. 2009;179:97–106.

Pittman JT and Lott CS. Startle response memory and hippocampal changes in adult zebrafish pharmacologically-induced to exhibit anxiety/depression-like behaviors. Physiol Behav. 2014:123;174- 179.

Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, and Kalueff AV. Modeling anxiety using adult zebrafish: a conceptual review. Neuropharmacol. 2012:62;135–143.

Stewart A, Wong K, Cachat J, Gaikwad S, Kyzar E, Wu N, Hart P, Piet V, Utterback E, Elegante M, Tien D, and Kalueff AV. Zebrafish models to study drug abuse-related phenotypes. Rev Neurosci. 2011;22:95–105.

Stewart AM, Nguyen M, Wong K, Poudel KW, and Kalueff AV. Developing zebrafish models of autism spectrum disorder (asd). Prog Neuropsychopharmacol Biol Psychiatry. 2014:50;27–36.

Willemsen R, Hasselaar W, van der Linde H, Bonifati V. Zebrafish as a new model organism for Parkinson's disease. Proc Meas Behav. 2008:5;0–1.

Kent ML, Spitsbergen JM, Matthews JM, Fournie JW, Murray KN, and Westerfield M. Diseases of Zebrafish in Research facilities. ZIRC Health Services Zebrafish Disease Manual. 2012; Online only: <http://zebrafish.org/zirc/health/diseaseManual.php>.

Kent ML, Harper C, and Wolf JC. Documented and potential research impacts of subclinical diseases in zebrafish. ILAR J. 2012:53;126–134.

Ramsay JM, Watral V, Schreck CB, and Kent ML. *Pseudoloma neurophilia* infections in zebrafish *Danio rerio*: effects of stress on survival, growth, and reproduction. Dis Aquat Organ. 2009;88:69–84.

Sanders GE. Zebrafish housing, husbandry, health and care: IACUC considerations. ILAR J. 2012;53: 205–207.

Sanders JS, Watral V, and Kent ML. Microsporidiosis in zebrafish research facilities colonies. ILAR J. 2012:53;106–113.

Cali A, Kent M, Sanders J, Pau C, and Takvorian PM. Development, ultrastructural pathology, and taxonomic revision of the microsporidial genus, pseudoloma and its type species *Pseudoloma neurophilia*, in skeletal muscle and nervous tissue of experimentally infected zebrafish *Danio rerio*. J Eukaryot Microbiol. 2012:59;40–48.

Kent ML, Shaw RW, and Sanders J. Fish Micorsporidia. Chapter 20, in: LM Weiss, JJ Becnel (Eds.), Microsporidia: Pathogens of Opportunity, Wiley Enterprise, 2014, pp. 493–520.

Sanders J, Watral V, Clarkson K, and Kent ML. Verification of intraovum transmission of a microsporidium of vertebrates: *Pseudoloma neurophilia* infecting the zebrafish, *Danio rerio*. PLoS One. 2013;8(9).

Sanders J, Peterson T, and Kent M, Early development and tissue distribution of *Pseudoloma neurophilia* in the zebrafish, *Danio rerio*. J Eukaryot Microbiol. 2014;61:238–246.

Ferguson J, Watral V, Schwindt A, and Kent ML. Spores of two fish microsporidia (*Pseudoloma neurophilia* and *Glugea anomola*) are highly resistant to chlorine. Dis Aquat Org. 2007;76:205–214.

Murray KN, Dreska M, Nasiadka A, Rinne M, Matthews JL, Carmichael C, Bauer J, Varga ZM, and Westerfield M. Transmission, diagnosis, and recommendations for control of *Pseudoloma neurophilia* infections in laboratory zebrafish (*Danio rerio*) facilities. Comp Med. 2011;61:322–329.

Chow FW, Xue L, and Kent ML. Retrospective study of the prevalence of *Pseudoloma neurophilia* shows male gender bias. Zebrafish. 2015;(in press) http://dx.doi.org/10.111/jfd.12328

Kent ML, Buchner C, Watral VG, Sanders JL, Lad J, Peterson TS, and Tanguay RL. Development and maintenance of a specific pathogen free (spf) zebrafish research facility for *Pseudoloma neurophilia*, Dis Aquat Organ. 2011;95:73–79.

Spagnoli S, Xue L, Murray KM, Chow F, and Kent ML. *Pseudoloma neurophilia*: a retrospective and descriptive study of nervous system and muscle infections, with new implications for pathogenesis and behavioral phenotypes. Zebrafish. 2015;12:189–201.

Kent ML and Bishop-Stewart JK. Transmission and tissue distribution of *Pseudoloma neurophilia* (Microsporidia) of zebrafish *Danio rerio*. J Fish Dis. 2003;26:1–4.

Wulliman MF, Barbara R, and Reichert H. Neuroanatomy of the Zebrafish Brain: A Topological Atlas, Birkhaüser, Boston, 1996.

Eaton RC, Bombardieri RA and Meyer DL. The Mauthner-initiated startle response in teleost fish. J Exp Biol. 1977;66:65–81.

Hatta K and Korn H. Physiological properties of the Mauthner system in the adult zebrafish, J Comp Neurol. 1998;395:493–509.

Liu DW and Westerfield M. Function of identified motoneurones and coordination of primary and secondary motor systems during zebrafish swimming. J Physiol. 1988;403:73–89.

Liu KS and Fetcho JR. Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. Neuron. 1999;23:325–335.

Zottoli SJ, Newma BC, Rieff HI and Winters DC. Decrease in occurrence of fast startle responses after selective Mauthner cell ablation in goldfish (*Carassius auratus*). J Comp Physiol A. 1999;184:207–218.

Mathuru AS and Jesuthusasan S. The medial habenula as a regulator of anxiety in adult zebrafish. Front Neural Circuits. 2013;7:1–3.

O'Malley DM, Kao YH, and Fetcho JR. Imaging the functional organization of zebrafish hindbrain segments during escape behaviors. Neuron. 1996;17:1145–1155.
Guidelines for use of Zebrafish in the N.I.H intramural research program. N.I.H. Online only 2013 <http://oacu.od.nih.gov/ARAC/documents/Zebrafish.pdf>.

Valsamis B and Schmid S. Habituation and prepulse inhibition of acoustic startle in rodents. J Vis Exp. 2011.

Dulawa SC, Hen R, Scaerce-Levie K, and Geyer MA. Serotonin 1b receptor modulation of startle reactivity, habituation, and prepulse inhibition in wild-type serotonin1b knockout mice, Psychopharmacol. 1997;132:125–134.

Toms CN and Echevarria DJ. Back to basics: searching for a comprehensive framework for exploring individual differences in zebrafish (*Danio rerio*) behavior. Zebrafish. 2014;11:325–340.

Spence R, Gerlach G, Lawrence C, and Smith C. The behavior and ecology of the zebrafish, *Danio rerio*. Biol Rev Camb Philos Soc. 2008;83:13–34.

Tran S and Gerlai R. Individual differences in activity levels in zebrafish (*Danio rerio*), Behav Brain Res. 2013;257:224–229.

Barber I, Hoare D, and Krause J. Effects of parasites on fish behavior: a review and evolutionary perspective. Rev Fish Biol Fish. 2000;10:131–165.

Thomas F, Adamo S, and Moore J. Parasitic manipulation: where are we and where should we go. Behav Process. 2005;68:185–199.

Gatkowska J, Wieczorek M, Dziadek B, Dzitko K, and Dlugonska H. Behavioral changes in mice caused by *Toxoplasma gondii* invasion of brain. Parasit Res. 2012;111:53–58.

Kekalainen J, Lai YT, Vainikka A, Sirkka I, and Kortet R. Do brain parasites alter host personality? Experimental study in minnows. Behav Ecol Sociobiol. 2014;68:197–204.

Koolhaas JM. Coping style and immunity in animals: making sense of individual variation. Brain Behav Immun. 2008;22:662–667.

Dirks A, Groenink L, Schipholt MI, van der Gugten J, Hijzen TH, Geyer MA, and Oliver B. Reduced startle reactivity and plasticity in transgenic mice overexpressing corticotrophin-releasing hormone. Soc Biol Psychiatry. 2002;51:583–590.

Wang JH, Short J, Ledent C, Lawrence AJ, and van den Buuse M. Reduced startle habituation and prepulse inhibition in mice lacking the adenosine A2A receptor. Behav Brain Res. 2003;143:201–207.

Percy DH. Pathology of Laboratory Rodents and Rabbits, in: D.H. Percy, S.W. Barthold (Eds.), Third edition, Blackwell, Iowa, 2007.

Chapter 4

The common neural parasite *Pseudoloma neurophilia* causes altered shoaling behavior in adult laboratory zebrafish (*Danio rerio*) and has wide-reaching consequences for neurobehavioral research.

Sean Spagnoli, Justin Sanders, Michael L. Kent

4.1 Abstract

Because of the zebrafish's recent rise in popularity as a model organism, fish may carry diseases that interfere with research. The microsporidium *Pseudoloma neurophilia* is one of the most commonly diagnosed infections in zebrafish facilities. Although primarily subclinical, infection has been associated with altered startle response habituation. Here, we performed a shoaling test before and after exposure to *P. neurophilia* - contaminated water to determine its effect on social behavior. 12 tanks containing 10 fish each were divided equally into control and experimental shoals and recorded prior to exposure. Over 123 days, controls were exposed to water housing uninfected fish and experimental shoals were exposed to water housing infected fish. Shoals were re-recorded following exposure and infection status was determined via histopathology. There were no significant differences in mean interfish distance and percent of top-dwelling fish between cohorts prior to exposure. Following exposure, shoals infected by *P. neurophilia* showed significantly reduced mean interfish distance compared to controls. The percentage of top-dwelling fish was also reduced in infected shoals, although this difference was not statistically significant. This study supports the fact that *P. neurophilia* infection causes altered behavior in zebrafish and it should warn neurobehavioral researchers to screen for infection.

4.2 Introduction

4.2.1 *Pseudoloma neurophilia*

Pseudoloma neurophilia is a microsporidian parasite that primarily causes subclinical infections in laboratory zebrafish (*Danio rerio*). It has a strong tropism for neural tissue, most commonly infecting spinal nerve roots, white matter of the ventral spinal cord, and descending white matter tracks in the rhombencephalon (Spagnoli et al. 2015). Transmission occurs vertically and via consumption of spores in carcasses, eggs, and contaminated water (Sanders et al. 2012; Sanders et al. 2013; Ferguson et al. 2007). Clinical signs, when present, are extremely nonspecific and include weight loss, decreased fecundity, and spinal deformities (Ramsay et al.

2009). Moreover, the vast majority of positive cases submitted to diagnostic laboratories exhibit no clinical signs, and so it is difficult to identify infections in laboratories or husbandry facilities without robust screening programs (Spagnoli et al. 2015). The subclinical nature of most *P. neurophilia* infections results in wide spread cryptic infections, making it so successful that, in 2006, 75% of all laboratories submitting fish to the Zebrafish International Resource Center (ZIRC, Eugene, Oregon) diagnostic service were contaminated (Murray 2015).

To make matters worse, *P. neurophilia* forms environmentally resistant spores, which makes both sterilization and transmission important issues when considering control (Sanders et al. 2013; Murray et al. 2011). Embryonated eggs are a particular risk because spores survive bleaching at 25-50ppm (Ferguson et al. 2007), which is the standard concentration used by most facilities for egg sterilization (Murray et al. 2011). Furthermore, Sanders et al. showed that spores can be transmitted vertically, so that even if the bleach concentration were high enough, it still would not reach the spore within the embryo (Sanders et al. 2013). Despite the fact that most curation facilities and laboratories ship fish in the form of embryonated eggs rather than as adult fish for biosecurity purposes, *P. neurophilia* can still slip through the cracks and spread between facilities (Murray et al. 2011).

4.2.2 The zebrafish as a neurobehavioral model organism

Early in their tenure as a laboratory animal, chronic infections in adult zebrafish were of minor concern as the vast majority of research involved developmental genetics, focusing almost exclusively on embryos and larvae (Grunwald and Eisen 2002). However, the explosion of the zebrafish as a model organism over the past forty or so years has seen this popular pet store staple being used in every field of study formerly reserved for rodents (Phillips and Westerfield 2014; Bugel et al. 2014). These fields include, but are not limited to, infectious disease, immunology, oncology, physiology, epigenetics, and, the focus of this paper, behavior and neurophysiology (Kalueff and Stewart 2012). Because of their relative ease of maintenance, rapid generation time, fecundity, and capacity for high throughput screening, the zebrafish is proving itself to be a powerful model organism for neurobehavioral studies. Zebrafish are used to study the behavioral effects of various pharmaceuticals, genetic manipulations, and situational

disturbances such as acute and chronic stressors (Maximino et al. 2010; Morris 2009; Pittman and Lott 2014; Stewart et al. 2012; Stewart et al. 2011; Stewart et al.2014; Willemsen et al. 2008). As their utility in this field has grown, zebrafish have been proposed as models for a wide range of human neurobehavioral disorders including autism, schizophrenia, epilepsy, anxiety, depression, and post-traumatic stress disorder (Maximino et al. 2010; Morris 2009; Pittman and Lott 2014; Stewart et al. 2012; Stewart et al. 2011; Stewart et al. 2014; Willemsen et al. 2008; Caramillo et al. 2015). Because of the sensitive nature of these studies, consistency among experimental subjects is critical. Behavioral studies in general are burdened by high variability between individual subject behavioral syndromes. The addition of an occult brain parasite poses an important risk of non-protocol induced variation (Kent et al. 2015).

4.2.3 *P. neurophilia* and zebrafish behavior

The question of whether or not *P. neurophilia* actually influences host behavior was partially answered by a prior study that used the progressive tap test for startle response habituation (Spagnoli et al. 2015). This study found that infected fish habituated significantly less overall than both exposed negative and control fish. Furthermore, infected fish tended to evade netting significantly longer than exposed negative fish from the same tank, even though the two were physically indistinguishable. We chose the tap test for startle response habituation because *P. neurophilia* tends to infect areas of the brain and spinal cord associated with stress, anxiety, the startle response, and motor function (Spagnoli et al. 2015). The primary disadvantage of this study, however, was that it was difficult to prove a direct causal relationship between behavioral changes and *P. neurophilia* because we could not test individual fish before and after infection.

In this experiment, we therefore conducted a shoaling assay to evaluate the effects of *P. neurophilia* infection on zebrafish behavior, which allowed us to track groups of fish, rather than individuals, before and after infection. To clarify, a "shoal" is defined as a group of fish that swim together in loosely organized manner, whereas a school is a group of fish that exhibits synchronized motion in specific directions (Miller and Gerlai 2011; Miller and Gerlai 2012). The social preferences of zebrafish, along with our extensive knowledge of their genome and

neuroanatomy have led them to be used as a robust model for human social behavior (Oliveira 2013). One of the great advantages of the zebrafish as a model for human neurobehavioral disorders is the fact that, along with similarities at the anatomical, physiological, and genetic levels, both are essentially social species (Miller and Gerlai 2011; Miller and Gerlai 2012). As with human beings, many fish species prefer the company of others: in experiments where fish are given the option to spend time next to a shoal of fish or next to an empty arena, adult zebrafish will generally spend more time near the group (Engeszer et al. 2007). Other studies have shown that chemical compounds can significantly influence shoaling: embryonic ethanol exposure, and acute LSD and ketamine exposure inhibit shoaling as measured by increased mean interfish distance (Buske and Gerlai 2012; Riehl et al. 2011; Grossman et al. 2010), whereas mescaline and alarm substance tend to improve shoaling as measured by decreased interfish distance (Kyzar et al. Kalueff 2012; Pham et al. 2012). Social behavior is considerably more complex than startle response habituation, as it involves the integration and emergent properties of circuitry throughout all areas of the brain (Oliveira 2013). This begs the question of whether or not we can expect to observe alterations in social interactions based on *P. neurophilia*'s neuroanatomic predilections. The "social behavior network" in mammals is a series of associated neuroanatomic structures that is thought to help regulate social behavior, however, because social behavior requires the simultaneous integration of multiple brain structures, pinpointing individual contributions is difficult. This is part of the reason why zebrafish are such an attractive model for social behavior research: With the development of powerful new imaging techniques and the "Brainbow" fish, researchers are getting closer to more specifically characterizing neurophysiological aspects of the social response (Oliveira 2013). With regards to our own study, this "social behavior network" includes the extended medial amygdala in mammals. While teleosts lack an amygdala, they do have a counterpart known as the medial habenula, which is involved in fear learning (Wulliman et al. 1996). In a retrospective study, we identified the griseum centrale as a structure common infected by *P. neurophilia* (Spagnoli et. al 2015). The griseum centrale receives and integrates signals from the medial habenula, which is involved in the social behavioral network in fish. The localization of parasites to the griseum centrale may indicate a direct neuroanatomic effect on social behavior, however, the fact that the social behavior network is so diffuse and integrative could mean that infections in any number of neuroanatomic locations could have an effect (Oliveira 2013; Wulliman et al. 1996).

4.2.4 *P. neurophilia* and shoaling behavior

Here we conducted an experiment on the effects of *P. neurophilia* on two established shoaling endpoints: Mean interfish distance and percentage of top-dwelling fish. The fish were maintained in groups during the long exposure and infection period to both reduce any potential undue stress resulting from extended isolation, and allow us to track the behavior of an entire shoal before and after infection. Comparing the shoaling behavior of the same groups of fish before and after infection allowed us to examine the causative relationship between *P. neurophilia* infection and behavioral changes.

4.3 Materials and Methods

4.3.1 Fish and pre-exposure testing

The procedures in this experiment are largely modeled after those described in Pham et al. (2012) and are modified, where appropriate, to accommodate the chronic exposure required by our study.

Zebrafish of the 5D strain were spawned and reared in-house to an age of approximately eight months. The source of the fish was the Sinnhubber Aquatic Resource Center, a SPF *Pseudoloma*-free facility (Kent et al. 2012). The experiments were conducted in our zebrafish laboratory, with a flow-through water supply. Incoming municipal water source was filtered, dechlorinated and heated to 27 ^{o}C, fish were subjected to a 14/10 h light/dark photoperiod, pH 7.0-7.4, alkalinity 80 ppm, hardness 75 ppm, conductivity 135 µS.

140 fish were netted from the stock tank and separated into 12 tanks containing 10 fish each. Fish were allowed to acclimate for seven days in their individual 2.8L tanks with green plastic lids and drain guards in order to make the experience between pre-and post- exposure assays as similar as possible (fish in the post-exposure portion of the experiment would be netted from their smaller tanks rather than a large stock tank). These tanks were housed on an

Aquaneering flow-through system (Aquaneering, inc. United States). Fish were fed once a day except weekends, with the original commercial diet "master mix" used at the SARL. Opaque dividers were placed between tanks to prevent viewing of fish in adjacent tanks. This was done to ensure consistency in rearing among all tanks since, without dividers, tanks in the middle of the rack would be able to view conspecifics on both sides while tanks at the ends of the racks would be able to view conspecifics on only one side. Husbandry and feeding were administered the same way throughout the exposure period.

On the day of pre-exposure testing, fish were netted from their holding tanks into a beaker. They were then transported to the staging area and poured into a novel container similar to those in which they had been previously housed. The novel container lacked the green lid, drain guard, and opaque dividers of the "home" tanks. The testing tank was also 2.8L capacity and measured 35x5X19cm. The tank was filled to a height of 16cm with system water. Fish were filmed using a Sony Handicam model HDR-CX for 10 minutes, beginning when the investigator left the room. After filming, fish were poured back into their home tanks from the testing tank. The order of testing was determined by random number generator and noted so that post-exposure testing could be performed in the same order to decrease the influence time-of-day effect might have on individual tanks. After testing, tanks with their shoals were individually identified and labeled for tracking purposes. All testing occurred during daylight activity hours between 0900 and 1600.

4.3.2 Exposure period

Following pre-exposure filming, tanks were divided into six control and six exposure tanks, with the addition of one sentinel tank holding 10 fish, which was used to evaluate the progression of infection. Two previously-established tanks containing about 70 four month old fish each of the 5D strain were used as water donor tanks. One tank was confirmed negative and one tank was confirmed positive for *P. neurophilia* via histopathology of resident fish. In order to mimic the exposure and transmission conditions present in most facilities (between-tank spread generally occurs through transfer of contaminated water or infected fish), 100mL of water from the contaminated donor tank was added to each tank in the exposure group once per day,

five days per week, for 123 days. Similarly, 100mL of water from the uncontaminated tank was added to each control tank once per day. This was done in order to increase similarities between the groups in terms of waste products and pheromones from donor fish. By exposing both control and "exposed" fish to "waste-water", we made sure that the only difference between the groups was the presence of *P. neurophilia* in the donor water. Water quality parameters (pH, ammonia, etc.) were within acceptable ranges and identical between donor tanks. 5 fish from the sentinel tank were sampled for histopathology at 4 months following the start of exposure. All of these sentinel fish were infected at this time, and hence and post-exposure testing was performed.

4.3.3 Post-exposure testing

Fish were netted from their home tanks into a beaker and transferred into a novel container—the same container that had been used in pre-exposure testing (section 2.1). Tanks were tested in the same order as they had been tested pre-exposure in order to maintain the most similarity between pre- and post- exposure testing in terms of time of day. Fish were recorded in a manner similar to that used in the pre-exposure test described in 2.1. Following filming, fish were euthanized via ice water in accordance with IACUC approval. Their coeloms were then opened and fish were fixed in Dietrich's fixative.

4.3.4 Histopathologic analysis

Fish were decalcified for 48 hours using Cal-Ex II solution, then placed in 70% ethanol. Decalcified fish were bisected sagitally to expose the spinal cord and placed *en face* into paraffin blocks for sectioning and staining with H&E in the standard manner. All fish from all exposed tanks were evaluated and five fish from each of the six control tanks were evaluated to confirm that controls were negative for infection. Slides were read and interpreted by a trained and boarded veterinary anatomic pathologist.

4.3.5 Image analysis

As is detailed in Pham et al. (2012), eight screen captures were taken per tank per recording session. Time 0 was defined as the beginning of recording. Following a three minute rest period, frames were analyzed at times 3:20, 3:40, 4:00, 4:20, 4:40, 5:00, 5:20, and 5:40 using Image J analysis software (Rasband 2015). Interfish distances were measured manually from the center of each fish—that is, the center of the fish's profile in the screen capture as was suggested by Pham et al. (2012)—by a trained investigator.

For percentage of fish at the surface, the tank was divided into a top half and a bottom half in the screen captures in order to measure the percent of fish at the top half of the tank. The height was based on the distance from the bottom to the water surface, which was 16 cm and the halfway line was drawn at 8cm.

4.3.6 Statistical analysis

"Mean interfish distance" was defined as the average of all distances between each fish and all tank mates over eight screen-captures (45 possible interfish measurements for a shoal of ten fish over eight screen captures or times equaling 360 interfish measurements per tank per recording session).

Analyses were performed using the statistical programming environment R version 3.1.2 (R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.).The average inter-fish distance and number of fish in the upper half of the tank was calculated from measurements between all fish in all 8 screenshots per tank as has been previously described (Pham et al. 2012) As the measurement data were not normally distributed, the nonparametric Wilcoxon-Mann-Whitney U- test was used to compare the average inter-fish distance measured between the two treatment groups at pretreatment and posttreatment time points and the average number of fish in the upper half of the tank.

4.4 Results.

4.4.1 *P. neurophilia* infection

Infection rates among exposure tanks were relatively high, ranging from 80-100% prevalence between tanks and a mean prevalence of 88%. Infections were generally mild with little to no meninxitis or encephalomyelitis and 1-3 parasite clusters were observed in each infected fish. None of the fish sampled from the control tanks were infected.

4.4.2 Mean interfish distance

There was no significant difference in average inter-fish distances between cohorts in pretreatment measurements ($p = 0.81$, U-test). After the four-month exposure period, the *P. neurophilia* exposure fish exhibited significantly less (about 15%) mean inter-fish distance than controls $p = 0.026$, U-test (Figure 1).

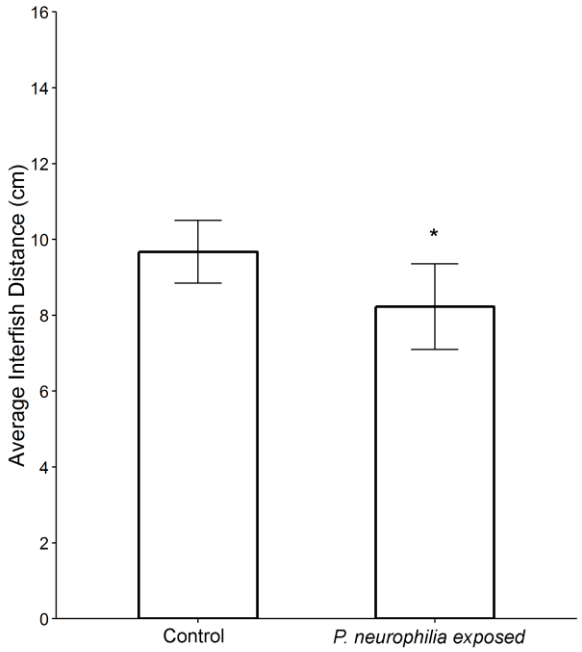

FIG 1. Effects of *Pseudoloma neurophilia* exposure on shoaling behavior of zebrafish. Exposure to *P. neurophilia* significantly decreased the average inter-fish distance with 12 shoals (tanks), 6 exposed and 6 controls. * p = 0.026, U-test.

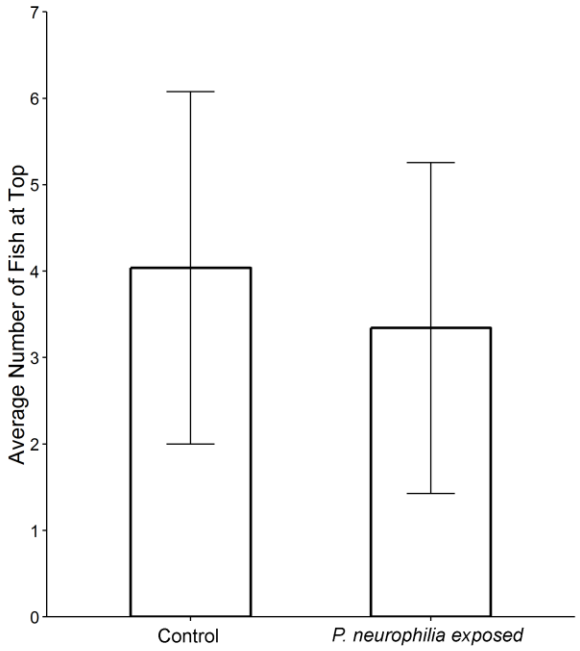

FIG 2. Effects of *Pseudoloma neurophlia* exposure on the average number of zebrafish localizing to upper half of tank in 6 exposed and 6 controls.

4.4.3 Percent of fish at surface

Exposed fish exhibited about 17% reduction in suface-dwelling fish compared to controls (Figure 2). There was a large variation between tanks within both the controls and treated fish, and the reduction observed in the latter was not statistically significant ($p = 0.70$, U-test). There was also no statistical difference between the average number of fish in the top half of the tank in the two treatment groups before exposure was initiated ($p = 0.69$, U-test).

4.5 Discussion

4.5.1 Potential interpretations of decreased mean interfish distance.

Our findings here were supported by those of our previous study in which the behavioral alterations associated with *P. neurophilia* infection were potentially suggestive of a hypervigilant or hyper-responsive behavioral phenotype (Spagnoli et al. 2015). This experiment shows that *Pseudoloma neurophilia* causes altered shoaling behavior in adult zebrafish as the infected fish formed tighter shoals than controls as measured by the reduced mean interfish distance. As with other animals, fish exposed to predators and other perceived threats tend to "pack tighter" (Brierly and Cox 2010). With zebrafish, Green *et al.* observed reduced inter-fish distance following exposure to alarm substance (Green et al. 2012). Conversely, zebrafish exposed to drugs that are recognized to reduce stress and awareness (e.g., high doses of alcohol and ketamine) can result in increased interfish distance (Kent et al. 2012). Whereas not statistically significant, the reduction in fish near the top of the tank seen in the exposed fish also was consistent with increased anxiety, and thus supported our interfish distance results (Kent et al. 2012). Indeed, the reduction in interfish distance and percent of fish in the top of tank showed very similar values; 15 or 17% reduction. The lack of statistically significance in the latter assay may have been caused by the considerable variation seen between tanks within each treatment regime.

4.5.2 *P. neurophilia* infection causes changes in shoaling behavior.

A unique aspect of the present study compared to the startle response habituation test is that here we provide a stronger causative link between the presence of *P. neurophilia* in a zebrafish shoal and altered shoaling behavior, which is supported by the fact that there were no significant differences in mean inter-fish distance between cohorts in the pre-exposure period. Following the exposure period, during which the only difference between cohort treatments was exposure to spore-laden fish water versus exposure to uninfected fish water, a significant difference in mean interfish distance developed, suggesting that infection caused this difference.

4.5.3 Parasites and host behavior

In both the habituation extinction experiment and the retrospective studies performed by our lab, we found that the severity of infection as measured by parasite numbers and the presence of neuronal inflammation had no effect on whether or not the disease had a clinical presentation. Hence, the presence of any *P. neurophilia* infection, regardless of severity or anatomic location, may influence fish behavior. This calls to mind subclinical infections of *Toxoplasma gondii*, which have widely reported effects on rodent behavior despite the fact that dormant infections produce minimal inflammation and nerve damage (Gatkowska et al. 2012; Thomas et al. 2005). Interestingly, *T. gondii* infections tend to very specifically influence behaviors associated with predation avoidance since the final vertebrate host is the cat. In contrast to our findings in *P. neurophilia*-infected zebrafish, *T. gondii* appears to reduce anxiety-associated behavior in rodents, particularly with regards to predator avoidance. *T. gondii* also seems to have a more specific effect on predator-associated anxiety like behaviors than *P. neurophilia* because since *T. gondii* appears to have no effect on rodent social behavior (Vyas et al. 2007). The divergent manifestations of these two infections is most likely due to the fact that *T. gondii* requires the rodent host to be consumed by a predator to complete its lifecycle, whereas *P. neurophilia* is a non-trophic parasite that can complete its lifecycle within a single species (Vyas et al. 2007). Moreover, *P. neurophilia* is maternally transmitted (Sanders et al. 2013; Sanders et al. 2014) and therefore it would be to the parasite's advantage to prolong the survival of its host, possibly through hypervigilance and predator avoidance.

4.5.4 Future avenues of inquiry

Whereas the tighter shoaling, reduced surface occupation, and reduced startle habituation all suggest a higher stress or higher anxiety behavioral phenotype, some goals of future research should be to dissect and identify the precise neurobehavioral syndromes that may be unique to *P. neurophilia* infection. This could then be taken even further in order to identify the precise neural pathways influenced by infection using various pharmaceutical compounds in an attempt to perform a "rescue" protocol. For example, our work suggests that neural microsporidiosis is associated with elevated anxiety compared to uninfected fish. Anxiety is associated with specific neural pathways and may be considered a distinct entity from stress. In order to specifically determine whether or not *P. neurophilia* infection truly influences anxiety rather than stress pathways, one could perform a behavioral testing array on infected fish and then administer an anxiolytic such as Paroxetene in order to see if the treated, infected fish then revert to the wild-type behavioral syndrome. If this was the case, one could then argue that *P. neurophilia* specifically increases anxiety in zebrafish rather than simply causing anxiety-associated behaviors (Maximino et al. 2010). By creating a specific protocol to identify specific effects of neural parasitism, one could extend these methodologies to study other neural parasites, such as *T. gondii* in rodents and humans.

4.5.5 Implications for neurobehavioral research.

Regardless of the specific mechanism of *P. neurophilia*-associated behavioral changes, the fact that infection causes changes in shoaling behavior has wide-ranging implications for neurobehavioral research. For example, the percent reduction in inter-fish distance associated with *P. neurophilia* was actually greater than that observed when fish are exposed to an alarm substance in another study (Green et al. 2012). Altered social interactions and social deficits are associated with a wide range of neurobehavioral disorders including schizophrenia, Asperger's disease, and most obviously, social anxiety (Maximino et al. 2010; Morris 2009; Pittman and Lott 2014; Stewart et al. 2012; Stewart et al. 2011; Stewart et al. 2014; Willemsen et al. 2008). Not only are zebrafish used to model a number of these disorders through the use of shoaling studies, they are used to study the effects of pharmaceuticals on social behavior (Maximino et al.

2010; Morris 2009; Pittman and Lott 2014; Stewart et al. 2012; Stewart et al. 2011; Stewart et al. 2014; Willemsen et al. 2008). A laboratory examining the treatment of social disorders using various pharmaceuticals could be adversely affected by the presence of *Pseudoloma*-contaminated fish, most obviously by parasite-associated reductions in mean interfish distance and top-dwelling behavior, but perhaps more subtly by anxiety-associated behaviors in infected fish.

4.5.6 Conclusion

In conclusion, this study further highlights the potential of subclinical *P. neurophilia* infections to cause non-protocol induced variation in neurobehavioral studies using adult zebrafish. This is particularly important for this area of research given the inherent high variability seen within treatment groups in behavioral assays. With *D. rerio* becoming a popular model in comparative neurobehavioral research, it is imperative that both curation facilities and laboratories utilizing zebrafish in their research introduce robust biosecurity and screening protocols to maintain parasite-free experimental populations.

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4.6 References

Spagnoli S, Xue L, Murray KM, Chow F, and Kent ML. *Pseudoloma neurophilia*: a retrospective and descriptive study of nervous system and muscle infections with new implications for pathogenesis and behavioral phenotypes. Zebrafish. 2015;12: 189-201.

Sanders JS, Watral V, and Kent ML. Microsporidiosis in zebrafish research facilities colonies. ILAR J. 2012;53:106–113.

Sanders J, Watral V, Clarkson K, and Kent ML. Verification of intraovum transmission of a microsporidium of vertebrates: *Pseudoloma neurophilia* infecting the zebrafish, *Danio rerio*. PLoS One. 2013;8.

Ferguson J, Watral V, Schwindt A, and Kent ML. Spores of two fish microsporidia (*Pseudoloma neurophilia* and *Glugea anomola*) are highly resistant to chlorine. Dis Aquat Org. 2007;76:205–214.

Ramsay JM, Watral V, Schreck CB, Kent ML. Pseudoloma neurophilia infections in zebrafish *Danio rerio*: effects of stress on survival, growth, and reproduction. Dis Aquat Organ 2009;88:69–84.

Murray K. Summary of submissions to the ZIRC Diagnostic Service on The Zebrafish International Resource Center webpage. 2015. URL: http://zebrafish.org/health/diseaseManual.php#SUMMARY OF SUBMISSIONS TO THE ZIRC DIAGNOSTIC SERVICE.

Murray KN, Dreska M, Nasiadka A, Rinne M, Matthews JL, Carmichael C, et al. Transmission, diagnosis, and recommendations for control of *Pseudoloma neurophilia* infections in laboratory zebrafish (*Danio rerio*) facilities. Comp Med. 2011;61:322–329.

Grunwald D and Eisen J. Headwaters of the zebrafish—emergence of a new model vertebrate. Nat Rev: Genetics 2002;3:717-724.

Phillips JB and Westerfield M. Zebrafish models in translational research: tipping the scales toward advancements in human health. Dis Models Mech. 2014;7:739-743.

Bugel SM, Tanguay RL, and Planchart A. Zebrafish: A marvel of high-throughput biology for 21st century toxicology. Curr Environ Health Rep. 2014;1:341-352.

Zebrafish Protocols for Neurobehavioral Research. Kalueff AV and Stewart AM (Eds). Springer, New York, 2012.

Maximino C, de Brito TM, Silva Batista AW, Herculano AM, Morato S and Gouveia A Jr. Measuring anxiety in zebrafish: a critical review. Behav Brain Res. 2010;214:157–171.

Morris JA. Zebrafish a model system to examine the neurodevelopmental basis of schizophrenia. Prog Brain Res. 2009;179:97–106.

Pittman JT and Lott CS. Startle response memory and hippocampal changes in adult zebrafish pharmacologically-induced to exhibit anxiety/depression-like behaviors. Physiol Behav. 2014;123:174–179.

Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, and Kalueff AV. Modeling anxiety using adult zebrafish: a conceptual review, Neuropharmacology. 2012;62:135–143.

Stewart A, Wong K, Cachat J, Gaikwad S, Kyzar E, Wu N, Hart P, Piet V, Utterback E, Elegante M, Tien D, and Kalueff AV. Zebrafish models to study drug abuse-related phenotypes. Rev Neurosci. 2011;22:95–105.

Stewart AM, Nguyen M, Wong K, Poudel KW, and Kalueff AV. Developing zebrafish models of autism spectrum disorder (asd). Prog Neuropsychopharmacol Biol Psychiatry. 2014;50:27–36.

Willemsen R, Hasselaar W, van der Linde H and Bonifati V. Zebrafish as a new model organism for Parkinson's disease. Proc Meas Behav. 2008;5:0–1.

Caramillo EM, Khan KM, Collier AD, and Echevarria DJ. P.T.S.D. Modeling In the zebrafish: are we there yet? Behav Brain Res. 2015;276:151–160.

Kent ML, Harpera C, and Wolf JC. Documented and potential research impacts of subclinical diseases in zebrafish. ILAR J. 2012;53:126–134.

Spagnoli S, Xue L, and Kent ML. The common neural parasite *Pseudoloma neurophilia* is associated with altered startle response habituation in adult zebrafish (*Danio rerio*): Implications for the zebrafish as a model organism. 2015. Behav Brain Res. 291: 351-360.

Sanders J, Peterson T, Kent M. Early development and tissue distribution of *Pseudoloma neurophilia* in the zebrafish, Danio rerio. J Eukaryot Microbiol. 2014;61:238–246.

Cheung E, Chatterjee D, and Gerlai D. Subcutaneous dye injection for marking and identification of individual adult zebrafish (*Danio rerio*) in behavioral studies. Behav Res Methods. 2014;46:619-624.

Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML and Schreck CB. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. Aquaculture. 2006;258:565-574.

Pham M, Raymond J, Hester J, Kyzar E, Gaikwad S, Bruce I, Fryar C, Chanin S, Enriquez J, Bagawandoss S, Zapolsky I, Green J, Stewart AM, Robison BD, and Kalueff AV. 2012. Assessing social behavior phenotypes in adult zebrafish: Shoaling, social preference, and mirror biting tests. In Zebrafish Protocols for Neurobehavioral Research. Kalueff AV and Stewart AM, eds. Springer, New York.

Miller NY and Gerlai R. Shoaling in zebrafish: what we don't know. Rev Neurosci. 2011;22:17-25.

Miller NY and Gerlai R. From schooling to shoaling: patterns of collective motion in zebrafish (*Danio rerio*). PLoS one. 2012.

Buske C and Gerlai R. Early embryonic ethanol exposure impairs shoaling and the dompaminergic and seratoninergic systems in adult zebrafish. Neurotox and Teratol. 2011;33:698-707.

Riehl R, Kyzar E, Allain A, Green J, Hook M, Monnig L, Rhymes K, Roth A, Pham M, Razavi R, DiLeo J, Gaikwad S, Hart P, and Kalueff AV. Neurotoxicol and Teratol. 2011;33:658-667.

Grossman L, Utterback E, Stewart A, Gaikwad S, Chung KM, Suciu C, Wong K, Elegante M, Elkhayat S, Tan J, Gilder T, Wu N, DiLeo J, Cachat J, and Kalueff AV. 2010;214:277-284.

Kyzar EJ, Collins C, Gaikwad S, Green J, Roth A, Monnig L, El-Ounsi M, Davis A, Freeman A, Capezio N, Stewart AM, and Kalueff AV. Effects of hallucinogenic agents mescaline and phencyclidine on zebrafish behavior and physiology. Prog Neuropsychopharmacol Biol Psychiatry. 2012;37:194-202.

Engeszer RE, Da Barbiano LA, Ryan MJ, Parichy DM. Timing and plasticity of shoaling behavior in the zebrafish, *Danio rerio*. Anim Behav. 2007;74.

Oliveira RF. Mind the fish: zebrafish as a model in cognitive social neuroscience. Front Neural Circuits. 2013;8:131.

Wulliman MF, Barbara R, and Reichert H. Neuroanatomy of the Zebrafish Brain: A Topological Atlas, Birkhaüser, Boston, 1996.

Brierly AS and Cox MJ. Shapes of krill swarms and fish schools emerge as aggregation members avoid predators and access oxygen. Curr Biol. 2010;20:1758-1762.

Green J, Collins C, Kyzar EJ, Pham M, Roth A, Gaikwad S, Cachat J, Stewart AM, Landsman S, Grieco F, Tegelenbosch R, Noldus LP, and Kalueff AV. Automated high-throughput neurophenotyping of zebrafish social behavior. J Neurosci Methods. 2012:210;266-271.

Gatkowska J, Wieczorek M, Dziadek B, Dzitko K, and Dlugonska H. Behavioral changes in mice caused by *Toxoplasma gondii* invasion of brain. Parasit Res. 2012;111:53–58.

Thomas F, Adamo S, and Moore J. Parasitic manipulation: where are we and where should we go. Behav Process. 2005;68:185–199.

Vyas A, Kim SK, Giacomini N, Boothroyd JC and Sapolsky RM. Behavioral changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. PNAS. 2007;104:6442-6447.

Rasband WS, ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2015.

Chapter 5

Pseudoloma neurophilia infection combined with gamma irradiation causes increased mortality in adult zebrafish (*Danio rerio*) compared to infection or irradiation alone: New implications for studies involving immunosuppression.

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5.1 Abstract

Gamma irradiation is commonly used as a bone marrow suppressant in studies of the immune system and hematopoiesis, most commonly in mammals. With the rising utility and popularity of the zebrafish (*Danio rerio*), gamma irradiation is being used for similar studies in this species. *Pseudoloma neurophilia*, a microparasite and common contaminant of zebrafish facilities, generally produces subclinical disease. However, like other microsporidia, *P. neurophilia* is a disease of opportunity and can produce florid infections with high morbidity and mortality secondary to stress or immune suppression. In this study we exposed zebrafish to combinations of *P. neurophilia* infection and gamma irradiation in order to explore the interaction between this immunosuppressive experimental modality and a normally subclinical infection. Zebrafish infected with *P. neurophilia* and exposed to gamma irradiation exhibited higher mortality, increased parasite loads, and increased incidences of myositis and extraneural parasite infections than fish exposed either to *P. neurophilia* or gamma irradiation alone. This experiment highlights the devastating effects of opportunistic diseases on immunosuppressed individuals and should caution researchers utilizing immunosuppressive modalities to carefully monitor their stocks in order to ensure that their experimental animals are not infected.

5.2 Introduction

Microsporidian parasites have long been known as pathogens of opportunity. While epizootic outbreaks of microsporidiosis have been documented in wild and cultured fishes (Kent et al. 2014), and fewer in mammals including *Encephalitozoon cuniculi* (Percy and Barthold 2007), microsporidiosis in man was virtually unheard-of until the AIDS epidemic of the 1980s and 1990s (Didier and Weiss 2006). During this time, organisms such as *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* rocketed from obscurity as virus-associated immune suppression allowed them to run rampant in the bodies of the afflicted (Carr et al. 2001). Similarly, *Pseudoloma neurophilia*, a microsporidium of zebrafish (*Danio rerio*) that generally produces subclinical infections, was largely ignored as an unimportant background contaminant in many zebrafish research facilities soon after its discovery (Matthew et al. 2001; Sanders et al. 2010). However, as the utility of zebrafish as a laboratory animal has grown, so too has the

potential for subclinical pathogens to produce clinical disease, with effects ranging from the addition of non-protocol induced variation in important experiments to the loss of valuable laboratory stocks (Kent et al. 2012).

Pseudoloma neurophilia is a particularly insidious microorganism in that it hides behind the veil of subclinical disease while propagating in the form of environmentally resistant spores (Spagnoli et al. 2015; Sanders et al. 2013). These spores resist bleaching at concentrations generally used by facilities that ship embryonated eggs (Ferguson et al. 2007). The parasite can also be transmitted vertically within developing embryos (Sanders et al. 2013), making the policy of bringing only embryonated eggs into a facility inadequate to avoid *P. neurophilia*. Between the years 1999 and 2013, up to 75% of facilities submitting fish to the Zebrafish International Resource Center (ZIRC) diagnostic service were contaminated with *P. neurophilia* (Murray 2015). While that percentage has decreased in recent years, contamination remains a problem for many laboratories, particularly those that acquire their fish from pet stores or whole sale suppliers without quarantine or sentinel programs to detect subclinical disease.

It has already been established that subclinical infections can produce measurable effects on fish behavior that can adversely influence neurobehavioral experiments (Spagnoli et al. 2015 [2]). If even subclinical infections interfere with relatively low-impact experimental manipulations, it is logical to assume that experimentation resulting in immune suppression would produce even more damaging effects. Ramsay *et al.* (2009) examined the effects of stress and cortisol on *P. neurophilia* infections in zebrafish and found that increased cortisol levels were correlated with more severe infections, reduced weight, reduced fecundity, and increased mortality (Ramsay et al. 2009). While cortisol is notoriously immunosuppressive, other experimental manipulations, such as exposure to gamma irradiation, are purposefully used to destroy hematopoietic tissue and immune function in the test subject. With the emergence and increased use of gamma irradiation protocols and the high prevalence of *P. neurophilia* in zebrafish research we must pay special attention to the interactions between this experimental modality and the contamination of facilities with *P. neurophilia* (Trede et al. 2004; Traver et al. 2004; Paik and Zon 2010).

Gamma irradiation exposure protocols are commonly used to study hematopoiesis by elimination of the host immune system followed by transplantation of new cell lines into the depleted tissue (Traver et al. 2004). Other laboratories utilize gamma irradiation to study the

immune system by analyzing suppression followed by recovery (Parikka et al. 2012). A facility utilizing gamma irradiation protocols has occasionally observed extensive mortalities following exposure. This facility also had endemic *Pleistopora hyphessobryconis* infections and it was discovered on post-mortem examination that these infections were far more severe, widespread, and inflammatory than is generally observed in non-irradiated animals (Sanders et al. 2010). *P. neurophilia* is also endemic in this facility. As this microsporidium is so widespread, it inspired us to investigate the influence of gamma irradiation on the severity of the infections and associated mortality.

5.3 Materials and Methods

Wild type zebrafish of the 5D strain were obtained from the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University. Fish were housed in a flow-through system. Incoming municipal water source was filtered, dechlorinated and heated to 27 ^{o}C, fish were subjected to a 14/10 h light/dark photoperiod, pH 7.0-7.4, alkalinity 80 ppm, hardness 75 ppm, conductivity 135 µS. Fish were fed twice daily, except weekends, with the original commercial diet "master mix" used at the SARL (Miller et al. 2014). This study was comprised of two experiments evaluating the interactions between *P. neurophilia* infections and gamma irradiation by either exposure to the infection and then irradiation (Task 1) or gamma irradiation then infection (Task 2). Both of these experiments aimed to mimic potential conditions of a laboratory contaminated with *P. neurophilia* utilizing gamma irradiation in its research. Hence, both experiments included four groups of fish; controls, *Pseudoloma* infected without irradiation (Pn+G-), *Pseudoloma* with irradiation (Pn+G+), and irradiation without *Pseudoloma* (Pn-G+). Irradiation was achieved using a Gammacell 220 60Co gamma irradiator. Fish were exposed for 250 sec to accomplish a dose of 16 Gray, twice, separated by 24h hours. Hence, each exposure gave 15-17 Gray (Gy), resulting in a total exposure of 30-34 Gy. Fish were first sedated with 100 ppm tricaine methane sulfonate (Aregent, Redmond, WA) and then exposed by being placed, approx. 20 at a time, in a square plastic dishes within the irradiator (100 by 100 mm). Appropriate groups of fish were experimentally infected with *P. neurophilia* by exposure to effluent from a tank containing about 100 infected fish.

5.3.1 Task 1: *P. neurophilia* followed by gamma irradiation

A total of 120 fish were exposed to *P. neurophilia* for 6 wk in a 10 gal aquarium. Sixty fish from this tank were then irradiated and 60 were not. They were then placed into 12 1L tanks (10 fish/tank). Likewise, 40 uninfected fish were irradiated, and 40 controls (exposed to neither gamma irradiation or *P. neurophilia* (10 fish/tank) were included. Fish were monitored daily, and moribund or dead fish were preserved for histology. Surviving fish were examined at 4 and 6 wk post-irradiation (10 wk and 12 wk post-exposure to *P. neurophilia*).

5.3.2 Task 2: Gamma irradiation followed by *P. neurophilia* infection.

A total of 50 fish were irradiated and 30 of these fish (3 tanks of 10 fish) were then exposed to *P. neurophilia*-effluent starting 3 d after the last gamma exposure. The other 2 tanks of 10 unexposed fish represented the Pn-G+ group. Likewise, 30 additional fish (3 tanks) were exposed to the parasite (Pn+G-) and 20 fish (2 tanks) represented the Pn-G- control group. Exposure to the parasite in Task 2 was accomplished by running the effluent of the identical group of infected donors in Task 1 into 6 3 L tanks (10fish/tank). This was done by diverting the out flow of the donor tank into 6 separate water lines. The water flow to each tank was regulated to be similar into each tank. In addition the tanks were rearranged every week to different out flow lines to minimize exposure variation.

Moribund and dead fish were collected and preserved for histology throughout the experiment. Many of the Pn+G+ fish had died or appeared moribund by 8 weeks (wk) post-exposure (PE) (Figure 1). Therefore, we collected all the remaining fish from this group and those in Pn-G- and Pn-G+ fish, and half of the Pn+G- fish. Only half of the latter were collected at this time point as the infection status was unknown and we decided to hold some of the fish from this group for an additional 4 wks to ascertain the development of the infection.

5.3.3 Fish sampling and histopathology

Sampled fish had their coeloms opened and then they were fixed in Dietrich's fixative and decalcified for 48h using Cal-Ex II. These fish were then sectioned longitudinally and

stained with hematoxylin and eosin (H&E) or the Luna stain using standard methods for histopathologic analysis (Sanders et al. 2013). Two en face longitudinal sections were examined per fish and the section with the most well-exposed spinal cord was chosen as the section to evaluate. Fish were evaluated via light microscopy to determine sex, percent of fish infected, percent of fish with nervous system infections, percent of fish with extraneural infections, percent of fish with myositis, and the abundance of parasite clusters within fish with nervous system infections.

5.3.4 Statistical Analyses

Survival curves for each experiment were generated using Kaplan-Meier estimate. Within-treatment heterogeneity for mortalities was analyzed to determine if there was a significant difference between mortality numbers between replicate tanks within treatments using the log rank test. If no significant difference was determined, data for the replicate treatment tanks were pooled, otherwise mortality data were analyzed separately for each tank within that treatment. Survival between treatments was compared for a difference from untreated controls using the log-rank test. Survival analyses were performed in GraphPad Prism version 5.1 (GraphPad Software). Significance was determined by a P value < 0.01 (Bonferroni correction to correct for five multiple comparisons) nonparametric Wilcoxon-Mann-Whitney U- test

The differences in the number of *P. neurophilia* exposed fish with CNS infections, myositis, and non-CNS infections were compared between gamma-irradiated and unirradiated groups using the chi-squared test. The difference in parasite abundance were compared between the two groups using the Wilcoxon-Mann-Whitney U-test. The effect of these histological endpoints on survival of *P. neurophilia* exposed fish was estimated using the Cox proportional hazards model to identify independent predictors of survival. Analyses were performed using the statistical programming environment R version 3.1.2 (R-project) and the survival package version 2.38 (Therneau 2015; Therneau and Grambsch 2000).

5.4 Results

Histological examination of control fish (Pn-G- fish) in all groups revealed no significant lesions in terms of myositis or *P. neurophilia* infections. Please note that in incidents of myositis within test groups, the most characteristic lesion is inflammation and muscle destruction with or without intralesional free spores. Free spores, rather than parasite clusters, are most commonly found in areas of myositis. Generally, when intact parasite clusters were visible in muscle, they were not associated with inflammation.

5.4.1 Task 1: *P. neurophilia* exposure followed by Total body irradiation

No significant difference between replicate tanks within treatments was found so fish within each treatment were pooled for survival analysis. At 4 wk post-irradition (PI), which was 10 wk post exposure to P. neurophilia (PE), there were no mortalities in untreated (Pn-G-) and *P. neurophilia*-exposed (Pn+G-) groups, and no significant difference was found in the survival of fish treated by TBI only (Pn-G+) compared to those exposed to *P. neurophilia* and subsequently treated with TBI (Pn+G+) (Fig 1. 90.5% survival versus 86.2%, respectively). At 6 wk PE (12 wk PI), there were no mortalities in the untreated control tanks and no significant difference between survival of the Pn+G- and Pn-G+ groups compared to the untreated controls (Pn-G- fish) (Fig 1. 93.3% and 95%, respectively). In contrast, survival within the Pn+G+ group declined to nearly half by 6 wk and differed significantly from controls (Fig. 1. 56.7%, $p < 0.0001$, log-rank test).

Histological examination revealed that the prevalence of infection increased from about 20% at 4 wk PI to about 60-70% at 6 wk PI in both the Pn+G- and Pn+G+ fish (Table 1; Fig. 2; Fig. 3)., At 6 wk PI, Pn+G+ fish exhibited over twice as many parasite clusters in the CNS as the Pn+G- fish. Moreover, the Pn+G+ fish had 5 times the prevalence of parasites in non-CNS tissues, and twice as many with myositis compared to the Pn+G- fish at this time.

	Treatment	Mortality (%)	CNS (%)	Abundance	Myositis (%)	Non-CNS (%)
Task 1,Time 1	No Gamma (n=34)	0	24	5.8	18	6
Task 1,Time 1	Gamma (n=29)	14	21	2.4	24	14
Task 1,Time 2	No gamma (n= 30)	07	62	5.4	25	7
Task 1, Time 2	Gamma (n=30)	43	70	13.7*	52**	37*
Task 2,Time 1	No Gamma (n=15)	7	53	2.8	47	13
Task 2,Time 1	Gamma (n=28)	50	62	8.9	58	42**
Task 2,Time 2	No Gamma (n=14)	0	71	8.1	7	21

Table 1. Histology results from Task 1 and Task 2 for zebrafish exposed to *P. neurophilia*, either with or without gamma irradiation. CNS=central nervous system *P. neurophilia* infections. Abundance = mean numbers of *P. neurophilia* clusters in CNS. Note that abundance includes uninfected fish with in the particular populations. Non-CNS = infections by *P. neurophilia* in other organs. Statistical differences at each time point between irradiated and non-irradiated fish are indicated by * ($p < 0.05$) using the Wilcoxon-Mann-Whitney U-test for abundance and the chi-squared test for numbers of CNS infections, myositis, and non-CNS infections. ** = marginally significant ($p=0.08$)

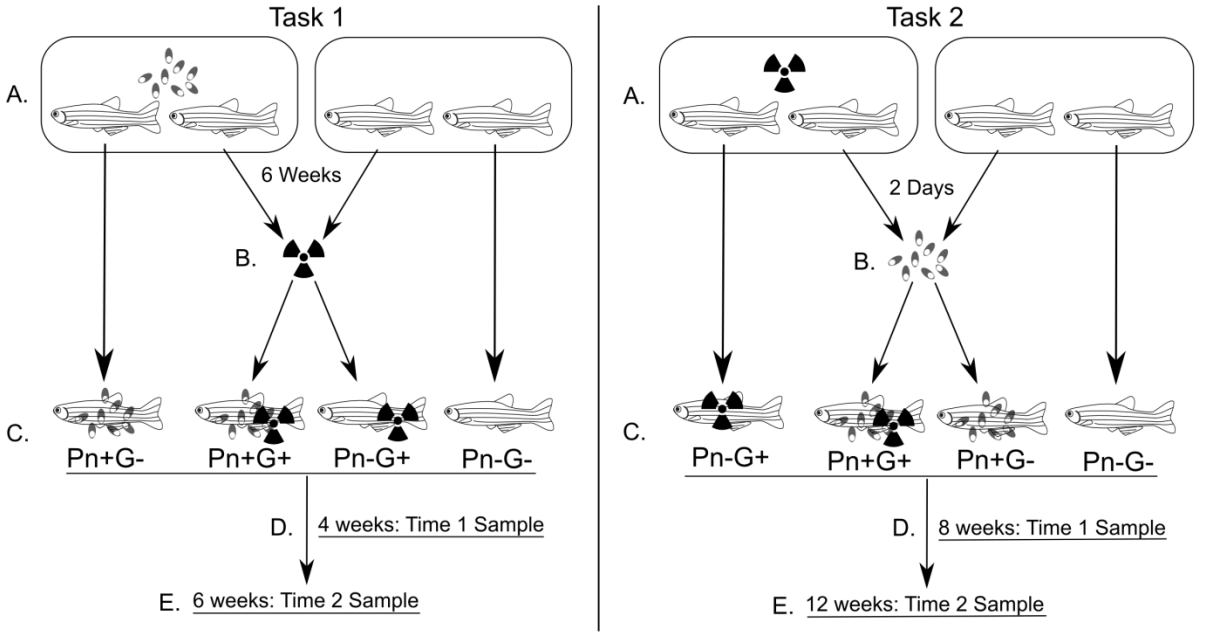

FIG 1. Overview of gamma irradiation/*Pseudoloma neurophilia* exposure of *Danio rerio.* Numbers of fish in each group and collection time are noted in Table 1 and in the text Task 1A. Zebrafish were exposed to *P. neurophilia* by running effluent from a tank of infected donor fish into the 10 gal aquarium they are housed in. An unexposed group was held in a separate 10 gal aquarium with filtered system water. 1B. After 6 weeks, one half of the fish from the exposed and the unexposed group (n = 40) were irradiated. 1C. Each treatment group was divided into 1L tanks (10 fish/tank): Pn+G- (6 tanks), Pn+G+ (6 tanks), Pn-G+ (4 tanks), Pn-G- (4 tanks). Fish were monitored daily and moribund fish were euthanized and preserved for histology. Fish that died prior to the time point collection were included in that time point if they were interpretable by histology (i.e. did not have significant post-mortem autolysis). 1D. Time 1 sampling: One half of the surviving fish from each group were sampled at 4 weeks post-irradiation by collecting all the fish in half of the tanks from each group 1E. Time 2 sampling: At 6 weeks post-irradiation, the remaining fish are euthanized and processed for histology, and any suitable moribund or dead fish after Time 1 where included in Time 2. Task 2A. A total of 50 fish were irradiated and separated into 1L tanks (10 fish/tank). An additional 50 fish were separated into 1L tanks (10 fish/tank) without gamma irradiation. 2B. At 3 days post irradiation, 3 tanks of irradiated and 3 tanks of non-irradiated fish were exposed to *P. neurophilia*. 2C. There were four treatment groups held in separate 1 L tanks: Pn-G+ (2 tanks), Pn+G+ (3 tanks), Pn+G- (3 tanks), Pn-G- (2 tanks). Fish were monitored daily and moribund fish were euthanized and preserved for histology. Fish that died prior to the time point collection were included in that time point if they were interpretable by histology. 2D. Time 1 sampling: At 8 weeks post-P. neurophilia exposure, half of the fish from each Pn+G- tank and all remaining Pn-G-, Pn-G+, and Pn+G+ fish were euthanized and fixed for histology. 2E. Time 2 sampling: At 12 weeks post-*P. neurophilia* exposure, the remaining Pn+G- fish were euthanized and fixed for histology.

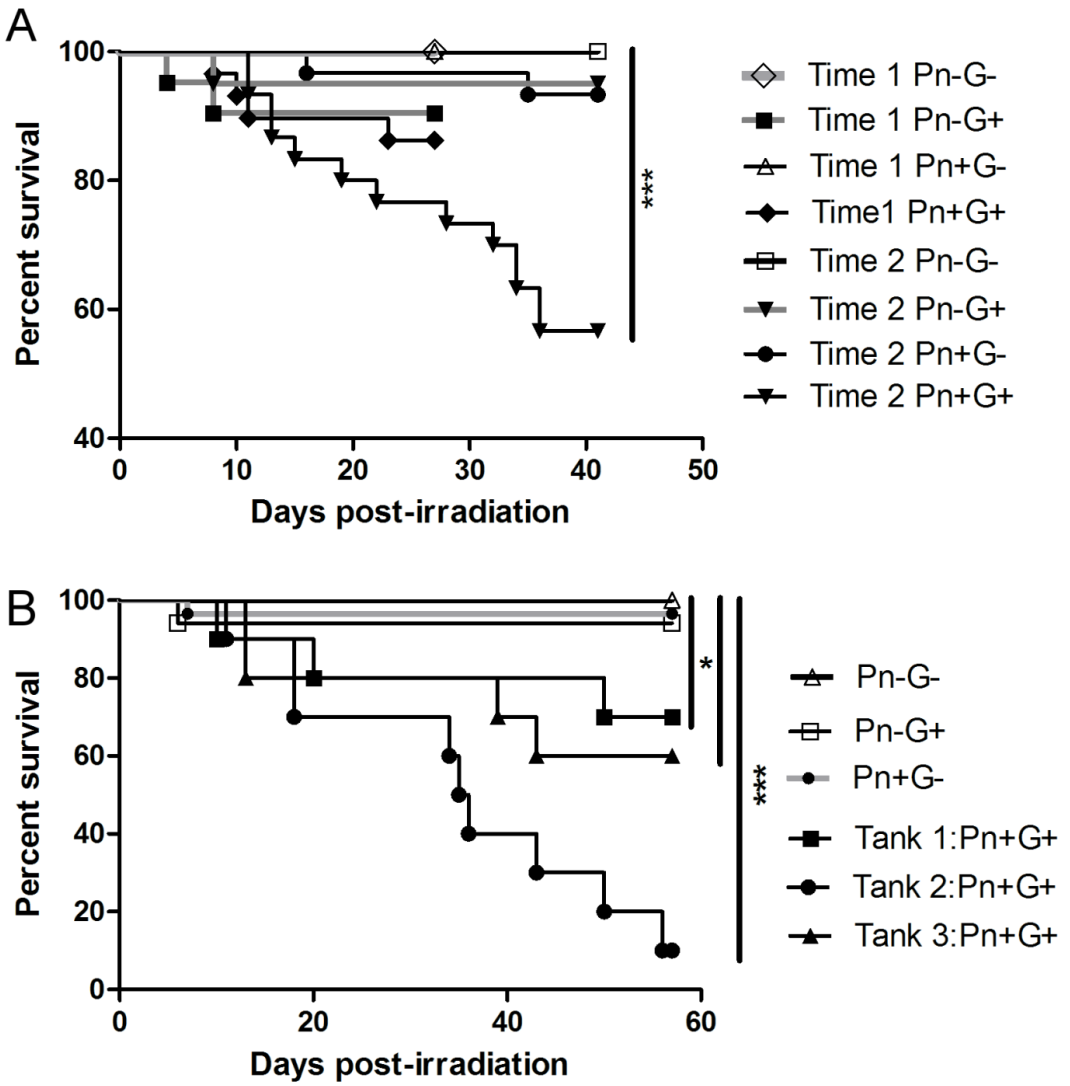

Figure 2. Survival of *Danio rerio* treated with total body irradiation (TBI) and exposed to *Pseudoloma neurophilia*. A. Percent survival of Task 1 in which fish were either untreated (Pn-G-), treated with TBI only (Pn-G+), exposed to *P. neurophilia* (Pn+G-), or exposed to *P. neurophilia* and then treated with TBI (Pn+G+). Fish were sacrificed and examined at two time points, 4 wk post-TBI (Time 1) and 6 wk post-TBI (Time 2). No significant difference in survival was observed between treatment groups and the untreated group at Time 1. At Time 2, the survival of the PN+G+ group differed significantly from the untreated group. B. Percent survival of Task 2 with similar groups as in Task 1, however Pn+G+ fish were exposed to *P. neurophilia* 3 d post-TBI. Survival between replicate tanks within the Pn+G+ group was compared separately to the untreated group. All three Pn+G+ tanks differed significantly from untreated controls. * $p < 0.01$, *** $p < 0.001$ log-rank test. n = number of fish/treatment group.

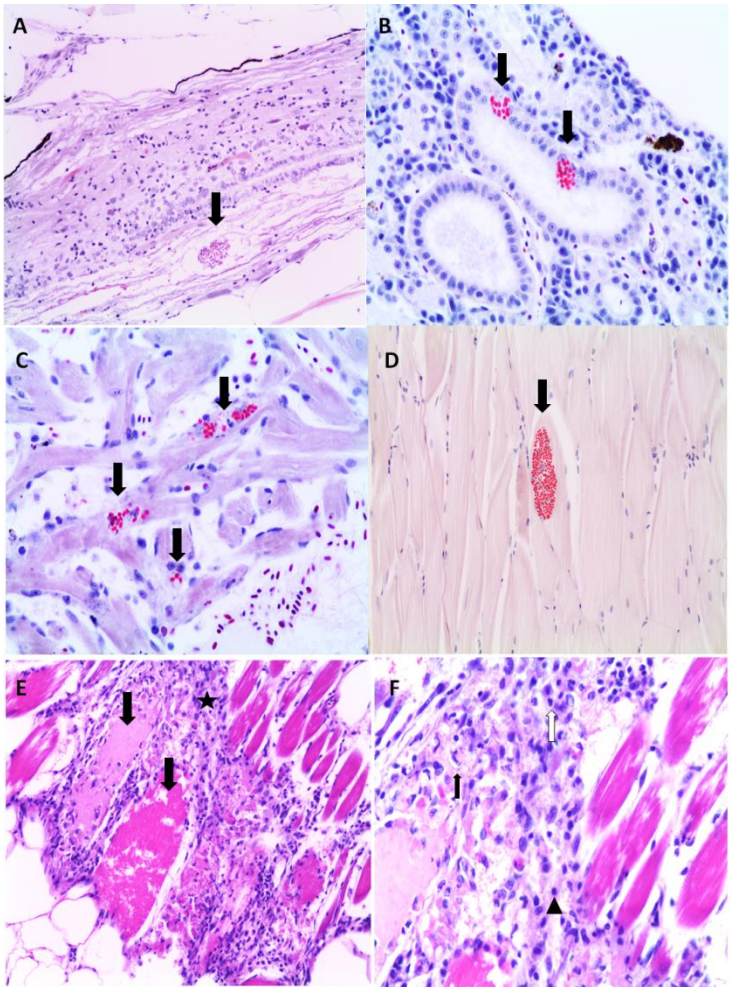

FIG 3. Histologic characteristics of *P. neurophilia* infections in irradiated fish. A. *P. neurophilia* infection within the central nervous system. Commonly, within the spinal cord, parasites form intra-axonal clusters containing all life stages of the parasite, often with minimal to no inflammation (arrow). Luna stain. 200x. B. Extraneuronal infection within the kidney in an irradiated fish. Parasite clusters are observed within renal tubular epithelial cells with no associated inflammation (arrows). Luna stain. 400x. C. Extraneuronal infection within the heart in an irradiated fish. Parasite clusters are observed within cardiomyocytes with no associated inflammation (arrows). Luna stain. 400x. D. Extraneuronal infection within skeletal muscle in an irradiated fish. Parasite clusters are observed within skeletal muscle cells, often with no associated inflammation (arrow). Luna stain. 200x. E. Myositis in an irradiated, infected fish. Skeletal muscle cells exhibit fragmentation and a loss of cross-striations and infiltration by inflammatory cells (arrows). Note the severe infiltration composed of grnulocytes and monocytes adjacent (star) H&E. 200x. F. Myositis in an irradiated infected fish; magnified image of (E). The inflammatory infiltrate associated with myositis is composed primarily of macrophages (black arrow) and granulocytes (white arrow) with fewer scattered lymphocytes (arrowhead). H&E. 400x.

5.4.2 Task 2: Total body irradiation followed by *P. neurophilia* exposure

A significant amount of intertank heterogeneity was detected between replicate tanks of the Pn+G+ treatment group so these tanks were analyzed separately. Similar to Task 1, the Pn+G+ fish showed lower survival (overall 50% at 8 wk PE), compared to the other groups when fish were collected at 8 wk PI (Pn-G- = 100%, Pn+G- = 93.3%, Pn-G+ 94.1%), and hence mortality in the Pn+G+ group was significantly greater than the other groups (Fig 2. Tank 1 $p < 0.0001$, Tank 2 $p< 0.0001$, Tank 3 $p < 0.01$, log-rank test).

Histological examination also showed a similar pattern as in Task 1, with a similar prevalence of infection at 8 wk post-exposure in both parasite exposed groups, but about 3 times the prevalence of non-CNS infections. However, in this experiment, both groups of infected fish showed similar levels of myositis. More than half of the Pn+G+ fish died before the 8 wk sample time, with a mean time to death of 4.5 wk. Although not collected at predetermined times, we included moribund fish in our analysis to provide a larger data set. Exclusion of clinically infected fish (fish with spinal deformities, profound emaciation or abnormal swimming behavior) from the analysis showed about 1.5 times the abundance of parasite clusters, and a slight increase in myositis (64 vs 58 %) and essentially the same prevalence of non-CNS infections (43 vs 42%).

The regression analysis performed using the Cox Proportional Hazards model showed that fish with myositis and extraneural (non-CNS) infections were approximately 3 times more likely to suffer clinical signs and death (1.18-7.66 95% CI, $p < 0.05$; 1.23-7.16 95% CI, $p <0.05$, respectively). The prevalence of *P. neurophilia* was similar within exposed treatment groups and thus this was found to not be a significant predictor of mortality. Overall parasite abundance in the CNS was about 3 times greater in the Pn+G+ treated fish than Pn+G-, but this dithis difference was not determined to be significant ($p=0.17$, U-test). There was no difference in myositis, but Non-CNS infections were about 3.5 times greater in gamma irraditated fish, which was marginally significant ($P=0.08$).

5.5 Discussion

Here we showed that neither infection nor gamma exposure alone was associated with significantly increased mortality. In contrast, when combined, either infection followed by irradiation or irradiation followed by *P. neurophilia* exposure resulted in increased mortality. Concurrent with increased mortality, we observed more parasites and more extensive infections outside of the CNS, and more myositis in infected, irradiated fish. Myositis is particularly associated with immunosuppression with *Pseudoloma*-infected zebrafish and is often more common in fish experiencing clinical signs associated with *P. neurophilia* infections (Ramsay et al. 2009; Spagnoli et al. 2015).

Microsporidiosis has been associated with immunosuppression in a wide variety of hosts including rabbits, fish, and dogs, and was not recognized as a significant pathogen in humans until the AIDS pandemic (Percy and Barthold 2007; Carr et al. 1998; Sanders et al. 2010). Ramsay et al. (2009) showed that fish with higher cortisol levels due to crowding stress had more myositis, which is consistent with our finding of immunosuppression due to gamma irradiation was associated with more myositis (Ramsay et al. 2009). Likewise, gamma irradiation of zebrafish with subclinical *M. marinum* infections exhibited more chronic inflammation and granuloma formation in concordance with more severe infections (Parikka et al. 2012).

The Parikka study is particularly interesting due to the elegant exploration of the mechanisms behind activation of latent *M. marinum* infections with regard to irradiation-induced immune suppression. Similar to the observations regarding immune-deficiency associated microsporidiosis enhancement, human mycobacteriosis appears to be enhanced by immune suppression (Parikka et al. 2012), keeping in mind that this study utilized different radiation and sedation protocols than ours. In fact, distinct latent and active phases for *Mycobacterium tuberculosis* and *Mycobacterium marinum* have both been well-described (Parikka et al. 2012). The Parikka study found that immune suppression in zebrafish via gamma-irradiation was associated with a transition from the latent to active phase of *M. marinum* and an associated increase in mortality and severity of infection (Parikka et al. 2012). Ramsay et al. reported very similar finding, in which high mortalities were induced in zebrafish exposed to crowding stress which had underlying *M. marinum* infections (Ramsay et al. 2009). While it is generally well-known that adaptive immunity is necessary to the inducement and maintenance of *M. marinum*, using flow cytometry of kidney homogenates, Parikka et al. were able to determine exactly

which immune cells were affected by gamma-irradiation (Parikka et al. 2012). They found that leukocyte numbers reached a nadir at 6-7 days post-irradiation (at which point numbers begin to recover) and that lymphocytes were depleted by approximately 80% while monocyte and granulocyte numbers were only depleted by about 47%. Specifically evaluating lymphocytes, B cells were reduced by 99% while T cells were only reduced by 67%. Therefore, it appears that, at least in the case of mycobacteriosis, activation of latent bacteria following gamma irradiation is driven primarily by lymphocyte depletion.

Although the specific interactions between the immune system and *P. neurophilia* are not yet described, the fact that gamma irradiation and infection are associated with increased mortality, extra-CNS infections, and myositis compared to either irradiation or infection alone could indicate some parallels between the mechanism of immune-suppression enhanced microsporidiosis and mycobacteriosis. Both microsporidia and mycobacteria are intracellular organisms. We also see a parallel between the concept of latent and active infections and the switch that occurs during immunosuppression in both infections. Although "latent" and "active" phases have not been identified for *P. neurophilia* as they have for *M. marinum*, the general pattern of subclinical, minimally-inflammatory infections in immunocompetent animals appears to at least partially parallel the mycobacterium narrative. Taking this parallel further, Parikka *et al.*'s finding that lymphoid depletion caused by gamma irradiation was primarily responsible for activation of latent mycobacterial infections could explain our own observations that irradiation results in an increased incidence of myositis, a severe inflammatory response microscopically dominated by granulocytes and macrophages (Parikka et al.2012). Whereas we do not know the exact mechanism of microsporidial myositis in zebrafish, the general reaction of the body to ruptured *P. neurophilia* parasite clusters resembles so-called "foreign body" responses in mammals, which are primarily mediated by innate, rather than adaptive, immunity, meaning that lymphocytes may be minimally involved with myositis lesions, at least in their acute, active presentation[24]. Another possible explanation for the increased inflammatory lesions seen in our study was that most of our fish were evaluated at several weeks after irradiation. If the nadir of the immune system occurs at 7 d post-irradiation (Parikka et al. 2012), it is possible that *P. neurophilia* overgrowth began at the immune system's lowest point and continued to progress as the immune system recovered. As more leukocytes were produced and the immune system began to reactivate, it is possible that the severe inflammation we observed in irradiated, infected

fish was the result of a recovering immune system attacking overgrown, ruptured parasite clusters that had been allowed to flourish during the period of immunosuppression. Because lymphocytes appear to be the primary target of gamma irradiation (Parikka et al. 2012) and because the incidence of myositis was increased in irradiated animals, adaptive immunity likely contributes to the initial entry of the parasite into the nervous system as well as the absence of myositis in most immune competent cases. As seen with chronic inflammation associated with ruptured xenomas of *Loma salmonae* in salmon gills (Kent et al. 2005), the major component of myositis in *P. neurophilia* is likely macrophages, and this cell lineage was likely spared to a large extent following irradiation (Parikka et al. 2012). Another interesting feature of our experiment was that animals in task 2 (irradiation followed by infection) were four times as likely to have extraneural infections than animals in task 1. This observation could indicate that that *P. neurophilia's* distribution throughout the body, particularly its clearance from peripheral tissues and concentration in the nervous system, could be mediated by adaptive, rather than innate immunity.

Gamma irradiation is not the only immunosuppressive experimental modality used in zebrafish. Studies of stress involving introduction of cortisol or exogenous stressors can be directly immunosuppressive and result in increased mortality and infection severity due to microsporidiosis (Ramsay et al. 2009). N-ethyl-N-nitrosurea (ENU) is an alkylating agent and potent mutagen used in many zebrafish studies to produce random mutations for various genetic studies. Among the studies utilizing ENU are experiments that study immune-suppression in induced mutants (Trede et al. 2004). Furthermore, repeated, low level ENU exposure is immunotoxic in male CD1 mice (Jayasekara et al. 1989). Therefore, the potential for ENU to directly induce immune suppression in zebrafish exists and may be further complicated by *P. neurophilia* infection. Contamination of a zebrafish colony with *P. neurophilia* could also potentially confound observations of embryonic-lethal mutations in progeny from ENU-treated individuals that would otherwise provide valuable information concerning genetic immunosuppression in living individuals.

Gamma irradiation and increased mortality due to secondary infections is well-documented in species other than fish, particularly in humans exposed to the horrors of the atom. Both humans and animals present at such tragedies as Chernobyl, Hiroshima, and Nagasaki have

innumerable reported incidences of severe immunodeficiency and secondary infection following ionizing radiation exposure, primarily due to thymic suppression (Yablokov 2005; Elliott and Ledney 1989). These phenomena are so well-documented that the syndromes gain such colorful monikers as "Chernobyl AIDS" (Yablokov 2009). The effects of gamma irradiation on secondary infections is also well-documented in the laboratory mouse, which is susceptible to both latent infection activation and novel infection by otherwise low-virulence pathogens (Van Diepen et al. 2005; Duran-Struuck and Dysko 2009).

There was a general trend of increased severity of infection based on abundance of CNS parasites, non-CNS infections, and myositis. However, even with 2-3 times increase on particular endpoints the change was not always statistically significant. This was likely due to the significant variability within treatment groups. Including moribund or dead fish in the analysis likely underestimates the differences in parasite burden, myositis, and extra-CNS infections as all of these would have likely had higher values if they survived to the appropriate time of collection. This is supported by the study of Ramsay *et al.*, which showed that parasite prevalence and burden increases with time and that there is little if any recovery, at least out to 20 wk PE (Ramsay et al. 2009). Removal of the moribund or dead fish, which had a mean time to death of 4.5 wk, resulted in an increase in parasite abundance but not myositis and CNS infections. This is was likely because examination of only healthy fish at 8 wk represented a group that had more time to develop visible infections. In contrast, myositis and extra-CNS infections were strongly associated with mortality, and thus removing the moribund or dead fish from the analysis probably compensated for any increase in these parameters that would be expected to be related with longer standing infections (Ramsay et al. 2009).

In conclusion, our study showed that the combination of *P. neurophilia* with gamma irradiation was associated with considerably more mortality than either alone. This provides an explanation for the variable mortality observed in a facility that irradiates fish and has an underlying infection by *P. neurophilia*. This is yet another example of the potential impacts on research when subclinical or chronic infections are wide spread in zebrafish used for research.

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5.6 References

Kent ML, Shaw RW, and Sanders J. 2014. Fish Microsporidia. Chapter 20. In Microsporidia: Pathogens of Opportunity In Weiss, LM and Becnel, JJ (eds). Wiley Enterprise pp 493-520.

Percy DH, and Barthold SW, eds. Pathology of laboratory rodents and rabbits, third edition. Blackwell, Iowa. 2007.

Didier ES and Weiss LM. Microsporidiosis: current status**.** Curr Opin Infect Dis 2006;19:485-492.

Carr A, Marriott D, Field A, Vasak E, and Cooper DA. Treatment of HIV-1 associated microsporidiosis and cryptosporidiosis with combination antiretroviral therapy. Lancet. 1998;351:256-261.

Matthews JL, Brown AM, Bishop-Stewart JK, Rogers P, and Kent ML. *Pseudoloma neurophilia* n.g., n. sp., a new microsporidium from the central nervous system of the zebrafish (*Danio rerio*). J Eukaryot Microbiol. 2001;48:227-233.

Sanders JL, Lawrence C, Nichols DK, Brubaker JF, Peterson TS, Murray KN, and Kent ML. 2010. Pleistophora hyphessobryconis (Microsporidia) infecting zebrafish Danio rerio in research facilities. Dis Aquat Org. 2010;91:47-56.

Kent ML, Harper C, and Wolf JC. Documented and potential research impacts of subclinical diseases in zebrafish. ILAR J. 2012;53:126-134.

Spagnoli S, Xue L, Murray KM, Chow F, and Kent ML. *Pseudoloma neurophilia*: A retrospective and descriptive study of nervous system and muscle infections, with new implications for pathogenesis and behavioral phenotypes. Zebrafish, 2015;12:189-201.

Sanders J, Watral V, Clarkson K, and Kent ML. Verification of intraovum transmission of a microsporidium of vertebrates: Pseudoloma neurophilia infecting the zebrafish, *Danio rerio*. PLoS ONE. 2013;8

Ferguson J, Watral V, Schwindt A, and Kent ML. Spores of two fish Microsporidia (*Pseudoloma neurophilia* and *Glugea anomola*) are highly resistant to chlorine. Dis Aquat Org. 2007;76:205-214.

Murray K. Summary of submissions to the ZIRC Diagnostic Service on The Zebrafish International Resource Center webpage. URL: http://zebrafish.org/health/diseaseManual.php#SUMMARY OF SUBMISSIONS TO THE ZIRC DIAGNOSTIC SERVICE.

Spagnoli S, Xue L, Kent ML. The common neural parasite *Pseudoloma neurophilia* is associated with altered startle response habituation in adult zebrafish (*Danio rerio*): Implications for the zebrafish as a model organism. Behav Brain Res. 2015;291:351-360.

Ramsay JM, Watral V, Schreck CB, and Kent ML. Pseudoloma neurophilia infections in zebrafish Danio rerio: effects of stress on survival, growth, and reproduction. Dis Aquat Organ. 2009;88:69-84.

Trede NS, Langenau DM, Traver D, Look AT, Zon LI. The use of zebrafish to understand immunity. Immunity. 2004; 20(4): 367-379.

Traver D, Winzeler A, Stern HM, Mayhall EA, Langenau DM, Kutok JL, Look AT, and Zon LI. Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. Blood. 2004; 104(5): 1298-1305.

Paik EJ and Zon LI. Hematopoietic development in the zebrafish. Int J Dev Biol. 2010; 54: 1127-1137.

Parikka M, Hammare´n MM, Harjula S-KE, Halfpenny NJA, Oksanen KE, et al. Mycobacterium marinum Causes a Latent Infection that Can Be Reactivated by Gamma Irradiation in Adult Zebrafish. PLoS Pathol. 2012;8.

Miller GW, Truong L, Barton CL, Labut EM, Bebold KM, Traber MG, and Tanguay RL. The influences of parental diet and vitamin E intake on the embryonic zebrafish transcriptome. Comp Biochem Physiol D Genom Proteom. 2014;10: 22-29

Peterson TS, Spitsbergen JM, Feist SW, and Kent ML. The Luna stain, an improved selective stain for detection of microsporidian spores in histologic sections. Dis Aquat Org. 2011;95: 175–180.

R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Therneau T (2015). A Package for Survival Analysis in S. version 2.38, <URL: http://CRAN.R-project.org/package=survival>).

Therneau TM and Grambsch PM (2000). Modeling Survival Data: Extending the Cox Model. Springer, New York. ISBN 0-387-98784-3.

Ramsay JM, Watral V, Schreck CB, and Kent ML. Husbandry stress exacerbates mycobacterial infections in adult zebrafish, *Danio rerio* (Hamilton). J. Fish Dis. 2009;32:931-941.

Tang L and Eaton JW. Fibrin(ogen) mediates acute inflammatory responses to biomaterials. J Exp Med. 1993;178:2147-2156.

Kent ML and Speare DJ. Review of the sequential development of *Loma salmonae* (Microsporidia) based on experimental infections of rainbow trout (*Oncorhynchus mykiss*) and Chinook salmon (*O. tshawytscha*). Folia Parasitol. 2005;52:63-68.

Jayasekara S, Sharma RP, and Brown DB. Immunotoxic potential of N-ethyl, N-nitrosourea (ENU) in CD1 mice. Clin Exp Immunol. 1989;77:294-298.

Yablokov AV. Nonmalignant Diseases after the Chernobyl Catastrophe, in Chernobyl: Consequences of the catastrophe for people and the environment. Yablokov AV, Nesterenko VB, and Nesterenko AV, eds. Annals of the New York Academy of Sciences, vol. 1181. 2009, pp 58-161. Blackwell Publishing. Boston, Mass.

Elliott TB and Ledney GD. Therapy for bacterial infections following ionizing radiation injury *in* Medical consequences of radiological and nuclear weapons. Lenhart MK, *ed*. Office of the Surgeon General, Borden Institute. Fort Detrick, MD. 1989.

Van Diepen, A, van de Gevel JS, Koudijs MM, Ossendorp F, Beekhuizen H, Janssen R, and van Dissel JT. Gamma irradiation or CD4+ T cell depletion causes reactivation of latent *Salmonella enterica* serovar Typhimurium infection in C3H/HeN Mice. Infection and Immunity. 2005; 73(5): 2857-2862.

Duran-Struuck R, and Dysko RC. Principles of bone marrow transplantation (BMT): Providing optimal veterinary and husbandry care to irradiated mice in BMT studies. Jour Am Ass Lab An Sci. 2009;48:11-22.

Chapter 6

Discussion

Pseudoloma neurophilia is remarkably common in zebrafish research facilities, and my experiments clearly demonstrate that underlying infections have a significant impact on behavioral research and experiments that utilize. As a demonstration of the relevance and topicality of this dissertation, I present the following narrative of histological interpretations that I recently provided for a research project.

While writing on an unusually sunny morning in late-March, I was awaiting the response to an email I had sent about three hours previously concerning a collaborative study of neurotoxicity in zebrafish. Without revealing too many incriminating details, this was a study funded by a major federal organization involving a number of international collaborators including myself that aimed to examine whether or not a certain toxicant caused a peripheral axonopathy and behavior changes in adults. After four months of coordination, back-and-forth emailing, a delayed international shipment of fixed fish, and about two thousand dollars in histopathology fees, I finished reading the slides. At first I was thrilled when I detected the telltale signs of bilateral muscle atrophy that could provide evidence of a peripheral axonopathy in several fish. I read the slides blinded to treatment status, but the lesions were not subtle. Then again, neither was the labeling: C and T groups…Control and Treatment presumably. "But maybe not," I told myself, "maybe they switched the letters to avoid bias." Nevertheless, both the C and T fish had similar lesions. Then there was the gliosis. Gliosis and bilateral muscle atrophy! This was going to very neatly confirm the researchers' hypothesis.

And there they were: Spores in the ventral white matter, cheerfully stuffing an axon.

I stopped reading the slides for about two days, trying to figure out how I was going to tell these researchers that they had made the very mistake my entire dissertation has been designed to prevent. Once I had worked up the courage, or at least the apathy, to finish reading them all, the final result was not good. Out of 56 fish, 30 contained *Pseudoloma* spores identified via histopathology, and 12 had lesions without identifiable spores. That left only 14 fish without lesions or spores, but still with the potential for nonlesional infection. Both T and C fish were equally represented between these three groups, so even if toxicant-associated lesions were more consistently present in the treatment group, they would have been completely

obscured by the infection. All that work, time, and effort had been tainted- ruined- by parasite contamination. Considering my thesis research, this "non-protocol induced variation" could be considered serendipitous, if it wasn't so tragic.

I'd strongly suspected from the outset that the work I was doing was important—otherwise I don't think I could have brought myself to continue—but being involved in a project that had to be completely scrapped hammered home both the importance of good biosecurity practices and the prevalence of *P. neurophilia* within the international zebrafish community. I would like to say that the odds of receiving infected fish in a neuropathological and behavioral study from halfway across the globe were low, but *P. neurophilia* still remains one of the most commonly diagnosed laboratory contaminants worldwide (Murray 2015).

What has struck me so distinctly since beginning my work with zebrafish is how none of these concepts are new, nor should the results be surprising. Aside from the particulars regarding *P. neurophilia*, which has only been considered a distinct species since 2001 (Matthews et al. 2001), the basic idea that laboratory animals can develop non-protocol related infections just as easily as their wild counterparts, is well recognized. A laboratory animal is, after all, an animal and not a piece of lab equipment. The astronomical rise of SPF rodent lines and biosecurity measures in mammalian laboratories since the 1950s stands testament to the dual necessity of both recognizing model organisms as holistic systems and the need to introduce some level of homogeneity to those systems (Percy and Barthold 2007).

The zebrafish community is not alone in its relative naiveté regarding pathogen contamination. On the contrary, the community as a whole has done a remarkable job "cleaning up" the zebrafish as a model organism given the fraction of the time the rodent community has had to accomplish similar goals. Comparative biomedical research, both currently and historically, has been fraught with complications from the catastrophic to the merely confusing. Some of the noteworthy examples include fatal *Salmonella* outbreaks in laboratory mice, the attribution of transitional cell carcinomas in rats infected with the bladder threadworm *Trichosomoides crassicauda* to saccharine consumption, and pharmaceutical trials being compromised by lungworm infection (*Filaroides* sp.) (Percy and Barthold 2007, Weihrauch and Diehl 2004, Hirth and Hottendorf 1973). Aquatic model have also fallen victim to similar mishaps: in medaka, (*Oryzias latipas*), *Mycobacterium marinum* infection has been associated

with increased incidences of hepatic neoplasia in an hepatotoxicity study, (Broussard et al. 2009), Mycobacterial granulomas being mistaken for tumors in toxicology studies (Abner 1994), and increased gut tumors associated with Pseudocapillaria tomentosa infection (Spitzbergen 2012). In all studies, large groups of experimental animals had to be rejected due to the contamination (Kent et al. 2012). While no research using *in vivo* models can be expected to be perfect, the zebrafish community can at least learn from the mistakes of our predecessors in the mammalian community and take the necessary measures to speed up the timetable towards improving our model.

The question then arises as to how we can accomplish this as a community. After having interacted with a number of colleagues specializing in biomedical research, laboratory animal medicine, veterinary medicine, and anatomic pathology, the answer is simple, at least at face value: Collaboration. If you'll indulge me another anecdote, I'd like to recount an incident that I find incredibly representative of the disconnect between researchers toiling in their individual fields. Without naming names once again, a major journal with an astronomical impact factor published a photograph on its cover depicting a binucleate mouse hepatocyte. Now, among lab animal veterinarians and anatomic pathologists, mouse livers are notorious for having a wide range of hepatocellular phenotypes including binucleate hepatocytes, and so such a change would fall under the realm of nonpathogenic variation... "normal abnormalities," so to speak (Percy and Barthold 2007). However, the image had made the cover of this particular issue because the authors of the article considered binucleate hepatocytes to be a novel phenotype associated with a particular genetic knock-out. Following the ensuing letters to the editor, the article was retracted and revised. The lesson of this particular story is that the authors could have avoided an embarrassing, internationally publicized mistake had they simply thought to collaborate with an experienced laboratory animal veterinarian or anatomic pathologist. A second, more ominous, moral is that the international scientific community seems to lack a particularly high opinion of veterinarians and veterinary pathologists—so much so that they are willing to base important conclusions on erroneous pathological data. Yet another example of this disregard was a federally-funded study that had been published in a reputable environmental journal (Mar. Environ. Res.) wherein it was reported that fish associated with the Deep Horizon oil spill site exhibited various toxicopathic changes such as hepatocellular necrosis . However, upon viewing the images in the article, it was obvious that the "lesions" described were due to

post-mortem autolysis and not to any kind of biological process such as necrosis. Moving to the Pacific coast, the Califorina Sea Grant funded a large study to investigate anthropogenic causes of a high prevalence of ovarian tumors in arrow gobies (*Clevelandia ios*), but these lesions were actually massive infections by a novel microsprodium (Sanders et al. 2012).

Whether such errors are due to ignorance of, or an active disregard for, the input of trained veterinary and pathologic professionals, one thing is certain: Bad science thrives in an atmosphere without collaboration. I hope that, through this dissertation, I have contributed to the discussion involving the need for the use of pathogen-free animals in research, understanding the effects when contamination cannot be avoided, and the need for collaboration and integration of multiple disciplines when in vivo models are involved, whether to improve preventative measures or to aid in data interpretation. In a perfect world, it would be standard practice for trained veterinarians and pathologists to collaborate on any and all projects involving gross or microscopic pathology. I like to think that this dissertation is a step in that direction.

Stepping off of the soap box for a bit, certain facts have come to light following the publication of these studies comprising this manuscript that I feel are important to mention. Although I was excited over the potential for *P. neurophilia* to be the first organism larger than a virus to utilize retrograde axonal transport, and although this is still a fascinating avenue for further research, it bears mentioning that there have been other documented cases of relatively large organisms travelling intra-axonally. The bacterium *Listeria monocytogenes*, which causes rhombencephalitis in both ruminants and man, enters the host through peripheral tissues. There is strong evidence that *L. monocytogenes* utilizes intra-axonal transport in order to migrate from peripheral tissues to the rhombencephalon, including the identification of intra-axonal bacteria and the prevention of ascending infection via severing nervous connections between the infection site and the central nervous system (Antal et al. 2001; Dons et al. 2007; Otter and Blakemore 1989). While the phenomenon is similar to what we have observed for *P. neurophilia*, the method of transport may be different. While there is no evidence that *P. neurophilia* is motile beyond its launching of the polar tubule and therefore may need to "hijack" host molecular motors to travel, *L. monocytogenes* can move through cytosol by using assymetric cometic tails and may not need to use host molecular motors for transport (Antal et al. 2001). Although the observation of retrograde axonal transport in organisms larger than viruses has already been

documented, the research involving *L. monocytogenes* provides a good precedent for further study of retrograde axonal transport by *P. neurophilia*.

I would like to conclude with a discussion of variability. Although a highly specific diagnostic methodology, histopathologic examination of tissues on glass slides is not terribly sensitive considering the fact that the pathologist is viewing a single 5 micrometer-thick section of an animal that is, in the case of the zebrafish, about a centimeter wide. That is, everything in the animal that exists outside of that 5 micrometer section will not be viewed by the pathologist, increasing the risk of false negatives during analysis. Furthermore, the fixation process and variable size of the fish along with the difficulty of making a perfectly parallel cut along the animal's spine means that each section is, to a certain degree, variable in terms of its exposure of specific anatomic structures. In the studies described within this manuscript, we used parasite cluster number as a measure of infection intensity, however, it is important to keep in mind that these counts are made from a single section of the fish and may under-represent true parasite loads and over-represent parasite-negative populations of zebrafish. Furthermore, certain anatomic structures are not visible in every secton. For example, in the retrospective study, I attempted to enumerate parasite clusters within the cranial nerves, but cranial nerves were not visible in every section. It would have been better, therefore, to provide a descriptive mention of their involvement rather than a quantitative one. Perhaps a better methodology available in future studies will be to use a highly sensitive micro-CT device that could scan the animal's entire body in three dimensions, providing us with a more accurate count of parasite clusters than a single five micrometer section. The use of a whole-body scan would help to eliminate the variability inherent in histopathologic sectioning.

Future studies may also involve more precise methods of producing dose-dependent infections in order to reduce variability in terms of dose and exposure compared to our cohabitation/effluent water exposure methods. It is, in fact, possible to gavage zebrafish with a known number of spores at known times in order to more precisely track infection and exposure rates. Also, the 5D zebrafish strain has been bred to be as close to wild-type as possible, increasing interfish variability by virtue of their genetics. Utilizing more genetically consistent strains may also help to reduce variability in future studies. The ultimate goal of these studies was to replicate conditions that produce *P. neurophilia* infections "in the wild," that is, in an average laboratory setting. Interestingly, my work described in this manuscript actively sought

to avoid the tight experimental control that makes the zebrafish such a powerful model organism, yet, despite the variability inherent in the fishes' genetic background, disease exposure, and diagnostic analysis, we were still able to provide strong evidence that *P. neurophilia* infections influence both biomedical and neurobehavioral studies. Our statistically significant evidence in the face of this high inherent variability should act as a strong caution to the zebrafish community regarding biosecurity and diagnostic monitoring.

6.1 References

Murray K. Summary of submissions to the ZIRC Diagnostic Service on The Zebrafish International Resource Center webpage. URL: http://zebrafish.org/health/diseaseManual.php#SUMMARY OF SUBMISSIONS TO THE ZIRC DIAGNOSTIC SERVICE. 2015.

Matthews JL, Brown AM, Larison K, Bishop-Steward JK, Rogers P, and Kent ML. *Pseudoloma neurophilia* n.g., n. sp., a new microsporidium from the central nervous system in the zebrafish (*Danio rerio*). J Eukaryot Microbiol. 2001;48: 227–233.

Percy DH, and Barthold SW, eds. Pathology of laboratory rodents and rabbits, third edition. Blackwell, Iowa. 2007.

Weihrauch MR and Diehl V. Artificial sweeteners—do they bear a carcinogenic risk? Ann Oncol. 2004; 15(10):1460-1465.

Hirth RS and Hottendorf GH. Lesions produced by a new lungworm in beagle dogs. Vet Pathol. 1973;10:385-407.

Broussard GW, Norris MB, Schwindt AR, Fournie JW, Winn RN, Kent ML, and Ennis DG. Chronic *Mycobacterium marinum* infection acts as a tumor promoter in Japanese Medaka. 2009;149:152-160.

Abner SR, Frazier CL, Scheibe JS, Krol RA, Overstreet RM, Walker WW, and Hawkins WE. 1994. Chronic inflammatory lesions in two small fish species, medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*) used in carcinogenesis bioassays. In: Stolen JS, Fletcher TC, eds. Modulators of fish immune responses: models for environmental toxicology. Fair Haven (NJ): SOS Publications. p 219-2.33.

Spitsbergen JM, Buhler DR and Peterson TL. Neoplasia and neoplasm-associated lesions in laboratory colonies of zebrafish emphasizing key influences of diet and aquaculture system design. ILAR J. 2012;53: 114-125.

Sanders JL, Myers MS, Tomanek L, Cali A, Takvorian PA, and Kent ML. *Ichthyosporidium weissii* n. sp. (Microsporidia) infecting the arrow goby (*Clevelandia ios*). J Euk Microbiol. 2012;59:258-267.

Antal EA, Loberg EM, Bracht P, Melby KK, and Maehlen J. Evidence for intraaxonal spread of *Listeria monocytogenes* from the periphery to the central nervous system. Brain Pathol. 2001;11:432-438.

Dons L, Jin Y, Kristensson K, and Rottenberg ME. Axonal transport of *Listeria monocytogenes* and nerve-cell-induced bacterial killing. J Neurosci Res. 2007;85:2529-2537.

Otter A and Blakemore WF. Observation on the presence of *Listeria monocytogenes* in axons. Acta Microbiol Hungarica. 1986;36:125-131.

Chapter 7

CONCLUSION

The work described in this dissertation provides conclusive evidence that subclinical *Pseudoloma neurophilia* infections significantly alter experimental data in neurobehavioral experiments and in research utilizing gamma irradiation protocols. This dissertation includes some of the first work to show that subclinical microsporidiosis in zebrafish alters experimental outcomes in common neurobehavioral and biomedical protocols. Because *P. neurophilia* is such a common contaminant of zebrafish facilities worldwide, this dissertation has wide-reaching implications for the research community.

Our retrospective study is the first of its kind performed in zebrafish and aside from providing us with solid footing on which to base our behavioral and biomedical experiments, it provided fascinating insight into the pathogenesis of *P. neurophilia* infection. We discovered that, not only does P. neurophilia infection cause myositis far less frequently than is widely perceived, but also that the severity of infection and nervous system inflammation is not associated with the recognition of clinical signs. This was to be a common theme throughout our work, as neither central nervous system parasite cluster number nor nervous system inflammation was associated with mortality, clinical signs, or altered behavioral profiles in the startle habituation and gamma irradiation experiments. Furthermore, the tropism for descending white matter tracks in the rhombencephalon and nerve roots along with the appearance of multiple parasite clusters in a single axon suggested that retrograde axonal transport may be responsible for the spread of *P. neurophilia* through the nervous system.

Building on our findings from the retrospective study, the startle response habituation study allowed us to show an association between *P. neurophilia* infection and the formation of distinct behavioral syndromes among both infected and uninfected fish in a contaminated tank. This experiment was the first to apply common neurobehavioral protocols to study the subtle effects of subclinical microsporidiosis, and it was the first published report of *P. neurophilia* being associated with potential non-protocol induced variation in a behavior study. It was unclear from the results whether the altered behavior profiles were caused by mortality based selection of infected fish or by the direct influence of the parasite on the nervous system, which

led us to utilize an experimental protocol that could track fish behavior before and after infection: The shoaling experiment.

The shoaling experiment is the first study to show a causative relationship between P. neurophilia infection and altered behavior in zebrafish. Combined with the results of the startle habituation study, the behavioral syndrome of reduced overall habituation, increased capture evasion, and decreased mean interfish distance suggests that *P. neurophilia* infection may produce a high-arousal or hypervigilant behavioral phenotype. While further experiments are needed to specifically identify the true behavioral phenotype caused by infection, our work clearly shows that *P. neurophilia* infection influences results in neurobehavioral studies.

While obviously not a neurobehavioral study, the gamma irradiation experiment was an important part of this dissertation because zebrafish are a commonly used biomedical model and microsporidia are notorious organisms of opportunity. This study was the first to examine the effects of gamma irradiation on microsporidial infections in zebrafish and the increased mortality associated with infection and irradiation compared to either exposure alone is an important finding for any laboratory utilizing immunosuppressive protocols. Furthermore, the the observation of increased myositis associated with gamma irradiation suggests that lymphocytes and adaptive immunity could be responsible for the early distribution of parasites to the nervous system, rather than to the muscle.

The rise of the zebrafish as a versatile model organism continues unabated. As the variety of experimental protocols increases, so too does the risk of subtle but damaging alterations to experimental data caused by subclinical microsporidiosis. This dissertation should act as a call to arms for the zebrafish community to "clean up its act" so to speak: to increase biosecurity measures, to implement rigorous sentinel programs, and to increase the creation and use of specific pathogen free facilities. It is difficult enough to convince lay people, and even other scientists, of the validity of novel model organisms, particularly non-mammalian species. If we, as a community, wish to improve both the perception and robustness of the zebrafish as a biomedical model, we must strive to reach at least the same standards of cleanliness and consistency that are common in the rodent world.

BIBLIOGRAPHY

Abner SR, Frazier CL, Scheibe JS, Krol RA, Overstreet RM, Walker WW, and Hawkins WE. 1994. Chronic inflammatory lesions in two small fish species, medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*) used in carcinogenesis bioassays. In: Stolen JS, Fletcher TC, eds. Modulators of fish immune responses: models for environmental toxicology. Fair Haven (NJ): SOS Publications. p 219-2.33.

Antal EA, Loberg EM, Bracht P, Melby KK, and Maehlen J. Evidence for intraaxonal spread of *Listeria monocytogenes* from the periphery to the central nervous system. Brain Pathol. 2001;11:432-438.

Ariens Kappers CU. The meninges in Cyclostomes, Selachians, and Teleosts, compared with those in man. Proc R Acad Amst. 1924;28:71–80.

Barber I, Hoare D, and Krause J. Effects of parasites on fish behavior: a review and evolutionary perspective. Rev Fish Biol Fish. 2000;10:131–165.

Berdoy M, Webster JP, Macdonald DW. Fatal attraction in rats infected with *Toxoplasma gondii*. Proc Biol Sci. 2000; 267:1591–1594.

Brierly AS and Cox MJ. Shapes of krill swarms and fish schools emerge as aggregation members avoid predators and access oxygen. Curr Biol. 2010;20:1758-1762.

Broussard GW, Norris MB, Schwindt AR, Fournie JW, Winn RN, Kent ML, and Ennis DG. Chronic *Mycobacterium marinum* infection acts as a tumor promoter in Japanese Medaka. 2009;149:152-160.

Bugel SM, Tanguay RL, and Planchart A. Zebrafish: A marvel of high-throughput biology for 21st century toxicology. Curr Environ Health Rep. 2014;1:341-352.

Buske C and Gerlai R. Early embryonic ethanol exposure impairs shoaling and the dompaminergic and seratoninergic systems in adult zebrafish. Neurotox and Teratol. 2011;33:698-707.

Cali A, Kent M, Sanders J, Pau C, and Takvorian PM. Development, ultrastructural pathology, and taxonomic revision of the Microsporidial genus, *Pseudoloma* and its type species *Pseudoloma neurophilia*, in skeletal muscle and nervous tissue of experimentally infected zebrafish *Danio rerio*. J Eukaryot Microbiol. 2012;59:40–48.

Caramillo EM, Khan KM, Collier AD, and Echevarria DJ. P.T.S.D. Modeling In the zebrafish: are we there yet? Behav Brain Res. 2015;276:151–160.

Carr A, Marriott D, Field A, Vasak E, and Cooper DA. Treatment of HIV-1 associated microsporidiosis and cryptosporidiosis with combination antiretroviral therapy. Lancet. 1998;351:256-261.

Caruncho HJ and Pinto Da Silva P. Alterations in the intermediate layer of goldfish meninges during adaptation to darkness. J Anat. 1994;184:355–362.

Cebrian C, Zucca FA, Mauri P, Steinbeck JA, Studer L, Scherzer CR, et al. MHC-I expression renders catecholaminergic neurons susceptible to T-cell mediated degeneration. Nat Commun. 2013;5.

Cheung E, Chatterjee D, and Gerlai D. Subcutaneous dye injection for marking and identification of individual adult zebrafish (*Danio rerio*) in behavioral studies. Behav Res Methods. 2014;46:619-624.

Chow FW, Xue L, Kent ML. Retrospective study of the prevalence of *Pseudoloma neurophilia* shows male gender bias in zebrafish *Danio rerio* (Hamilton-Buchanan). J Fish Dis. 2015. [Epub ahead of print]; DOI: 10.1111/jfd.12328.

Collymore C, Watral V, White JR, Colvin ME, Rasmussen S, Tolwani RJ, et al. Tolerance and efficacy of Emamectin benzoate and Ivermectin for the treatment of *Pseudocapillaria tomentosa* in laboratory zebrafish (*Danio rerio*). Zebrafish. 2014;11:490–497.

Compton SR and Macy JD. Effect of cage-wash temperature on the removal of infectious agents from caging and the detection of infectious agents on the filters of animal bedding-disposal cabinets by PCR analysis. J Am Assc Lab Anim Sci. 2015;54(6): 745-755.

Crofton HD. A model of host-parasite relationships. Parasitology. 1971;63:343–364.

Didier ES and Weiss LM. Microsporidiosis: current status**.** Curr Opin Infect Dis 2006;19:485-492.

Dirks A, Groenink L, Schipholt MI, van der Gugten J, Hijzen TH, Geyer MA, and Oliver B. Reduced startle reactivity and plasticity in transgenic mice overexpressing corticotrophin-releasing hormone. Soc Biol Psychiatry. 2002;51:583–590.

Dons L, Jin Y, Kristensson K, and Rottenberg ME. Axonal transport of *Listeria monocytogenes* and nerve-cell-induced bacterial killing. J Neurosci Res. 2007;85:2529-2537.

Dubey JP. Advances in the life cycle of *Toxoplasma gondii*. Int J Parasitol. 1998;28:1019–1024.

Dulawa SC, Hen R, Scaerce-Levie K, and Geyer MA. Serotonin 1b receptor modulation of startle reactivity, habituation, and prepulse inhibition in wild-type serotonin1b knockout mice, Psychopharmacol. 1997;132:125–134.

Duran-Struuck R, and Dysko RC. Principles of bone marrow transplantation (BMT): Providing optimal veterinary and husbandry care to irradiated mice in BMT studies. Jour Am Ass Lab An Sci. 2009;48:11-22.

Eaton RC, Bombardieri RA, and Meyer DL. The Mauthner-initiated startle response in teleost fish. J Exp Biol. 1977; 66:65–81.

Elliott TB and Ledney GD. Therapy for bacterial infections following ionizing radiation injury *in* Medical consequences of radiological and nuclear weapons. Lenhart MK, *ed*. Office of the Surgeon General, Borden Institute. Fort Detrick, MD. 1989.

Engeszer RE, Da Barbiano LA, Ryan MJ, Parichy DM. Timing and plasticity of shoaling behavior in the zebrafish, *Danio rerio*. Anim Behav. 2007;74.

Ferguson J, Watral V, Schwindt A, and Kent ML. Spores of two fish Microsporidia (*Pseudoloma neurophilia* and *Glugea anomola*) are highly resistant to chlorine. Dis Aquat Org. 2007;76:205-214.

Filby AL, Paull GC, Bartlet EJ, Van Look KJ, and Tyler CR. Physiological and health consequences of social status in zebrafish (*Danio rerio*). Physiol Behav. 2010;101:576–587.

Gatkowska J, Wieczorek M, Dziadek B, Dzitko K, and Dlugonska H. Behavioral changes in mice caused by *Toxoplasma gondii* invasion of brain. Parasit Res. 2012;111:53–58.

Green J, Collins C, Kyzar EJ, Pham M, Roth A, Gaikwad S, Cachat J, Stewart AM, Landsman S, Grieco F, Tegelenbosch R, Noldus LP, and Kalueff AV. Automated high-throughput neurophenotyping of zebrafish social behavior. J Neurosci Methods. 2012:210;266-271.

Grossman L, Utterback E, Stewart A, Gaikwad S, Chung KM, Suciu C, Wong K, Elegante M, Elkhayat S, Tan J, Gilder T, Wu N, DiLeo J, Cachat J, and Kalueff AV. 2010;214:277-284.

Grunwald D and Eisen J. Headwaters of the zebrafish—emergence of a new model vertebrate. Nat Rev: Genetics 2002;3:717-724.

Guidelines for use of Zebrafish in the N.I.H intramural research program. N.I.H. Online only 2013 <http://oacu.od.nih.gov/ARAC/documents/Zebrafish.pdf>.

Hacker C, Howell M, Bhella D, and Lucocq J. Strategies for maximizing ATP supply in the microsporidian *Encephalitozoon cuniculi*: direct binding of mitochondria to the parasitophorous vacuole and clustering of the mitochondrial porin VDAC. Cell Microbiol. 2014;16: 565–579.

Hatta K and Korn H. Physiological properties of the Mauthner system in the adult zebrafish, J Comp Neurol. 1998;395:493–509.

Hirth RS and Hottendorf GH. Lesions produced by a new lungworm in beagle dogs. Vet Pathol. 1973;10:385-407.

Hosoya N, Miyagawa K. Targeting DNA damage response in cancer therapy. Cancer Sci. 2014;105:370–388.

Jayasekara S, Sharma RP, and Brown DB. Immunotoxic potential of N-ethyl, N-nitrosourea (ENU) in CD1 mice. Clin Exp Immunol. 1989;77:294-298.

Kekalainen J, Lai YT, Vainikka A, Sirkka I, and Kortet R. Do brain parasites alter host personality? Experimental study in minnows. Behav Ecol Sociobiol. 2014;68:197–204.

Kent ML and Bishop-Stewart JK. Transmission and tissue distribution of *Pseudoloma neurophilia* (Microsporidia) of zebrafish *Danio rerio*. J Fish Dis. 2003;26:1–4.

Kent ML and Speare DJ. Review of the sequential development of *Loma salmonae* (Microsporidia) based on experimental infections of rainbow trout (*Oncorhynchus mykiss*) and Chinook salmon (*O. tshawytscha*). Folia Parasitol. 2005;52:63-68.

Kent ML, Buchner C, Watral VG, Sanders JL, LaDu J, Peterson TS, et al. Development and maintenance of a specific pathogen free (SPF) zebrafish research facility for *Pseudoloma neurophilia*. Dis Aquat Org. 2011;95:73–79.

Kent ML, Dawe SC, and Speare DJ. Resistance to reinfection in chinook salmon *Oncorhynchus tshawytscha* to *Loma salmonae* (*Microporidia*). 1999;37:205–208.

Kent ML, Harper C, and Wolf JC. Documented and potential research impacts of subclinical diseases in zebrafish. ILAR J. 2012:53;126-134.

Kent ML, Shaw RW, and Sanders J. 2014. Fish Microsporidia. Chapter 20. In Microsporidia: Pathogens of Opportunity In Weiss, LM and Becnel, JJ (eds). Wiley Enterprise pp 493-520.

Kent ML, Spitsbergen JM, Matthews JM, Fournie JW, and Murray KN, Westerfield M. Diseases of Zebrafish in Research facilities. ZIRC Health Services Zebrafish Disease Manual. 2012. Available at http://zebrafish.org/zirc/health/diseaseManual.php.

Koolhaas JM. Coping style and immunity in animals: making sense of individual variation. Brain Behav Immun. 2008;22:662–667.

Kyzar EJ, Collins C, Gaikwad S, Green J, Roth A, Monnig L, El-Ounsi M, Davis A, Freeman A, Capezio N, Stewart AM, and Kalueff AV. Effects of hallucinogenic agents mescaline and phencyclidine on zebrafish behavior and physiology. Prog Neuropsychopharmacol Biol Psychiatry. 2012;37:194-202.

LaVail JH, Tauscher AN, Aghaian E, Harrabi O, and Sidhu SS. Axonal transport and sorting of herpes simplex virus components in a mature mouse visual system. J Virol. 2003;77:6117–6126.

Lawrence C, Ennis DG, Harper C, Kent ML, Murray K, and Sanders GE. The challenges of implementing pathogen control strategies for fishes used in biomedical research. Comp Biochem Physiol C Toxicol Pharmacol. 2012;155:160–166.

Lester R. A review of methods for estimating mortality due to parasites in wild fish populations. Helgol Mar Res 1984; 37:53–64.

Levsen A, Alvik T, and Grotmol S. Neurological symptoms in tricolor shark minnow *Balantiocheilos melanopterus* associated with *Myxobolus balantiocheili* n. sp. infecting the central nervous system. Dis Aquat Org. 2004;59:135–140.

Liu DW and Westerfield M. Function of identified motoneurones and coordination of primary and secondary motor systems during zebrafish swimming. J Physiol. 1988;403:73–89.

Liu KS and Fetcho JR. Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. Neuron. 1999;23:325–335.

Livingston RS, Besch-Williford CL, Myles MH, Franklin CL, Crim MJ, and Riley LK. *Pneumocystis carinii* infection causes lung lesions historically attributed to rat respiratory virus. Comp Med. 2011;61(1):45-59.

Lom J, and Dykova I. Microsporidian xenomas in fish seen in wider perspective. Folia Parasitol (Praha). 2005;69–81.

Lord CJ, Ashworth A. The DNA damage response and cancer therapy. Nature 2012;481:87–294.

Mathuru AS and Jesuthusasan S. The medial habenula as a regulator of anxiety in adult zebrafish. Front Neural Circuits. 2013;7:1–3.

Matthews JL, Brown AM, Bishop-Stewart JK, Rogers P, and Kent ML. *Pseudoloma neurophilia* n.g., n. sp., a new microsporidium from the central nervous system of the zebrafish (*Danio rerio*). J Eukaryot Microbiol. 2001;48:227-233.

Maxie MG, and Youssef S: Nervous system. In: Jubb, Kennedy, and Palmer's Pathology of Domestic Animals Volume 1. Maxie MG (ed), pp. 281–298, Elsevier Limited, Philadelphia, PA, 2007.

Maximino C, de Brito TM, da Silva Batista AW, Herculano AM, Morato S, and Gouveia A Jr. Measuring anxiety in zebrafish: a critical review. Behav Brain Res. 2010;214:157–171.

Megret F, Prehaud C, Lafage M, Moreau P, Rouas-Freiss N, Carosella ED, et al. Modulation of HLA-G and HLA-E expression in human neuronal cells after rabies virus or herpes virus simplex type 1 infections. Hum Immunol. 2007;68:294–302.

Miller GW, Truong L, Barton CL, Labut EM, Bebold KM, Traber MG, and Tanguay RL. The influences of parental diet and vitamin E intake on the embryonic zebrafish transcriptome. Comp Biochem Physiol D Genom Proteom. 2014;10: 22-29

Miller NY and Gerlai R. From schooling to shoaling: patterns of collective motion in zebrafish (*Danio rerio*). PLoS one. 2012.

Miller NY and Gerlai R. Shoaling in zebrafish: what we don't know. Rev Neurosci. 2011;22:17-25.

Moles A and Heifetz J. Effects of the brain parasite *Myxobolus arcticus* on sockeye salmon. J Fish Biol. 1998;52:146–151.

Morris JA. Zebrafish a model system to examine the neurodevelopmental basis of schizophrenia. Prog Brain Res. 2009;179:97–106.

Murray K. Summary of submissions to the ZIRC Diagnostic Service on The Zebrafish International Resource Center webpage. URL: http://zebrafish.org/health/diseaseManual.php#SUMMARY OF SUBMISSIONS TO THE ZIRC DIAGNOSTIC SERVICE. 2015.

Murray KN, Dreska M, Nasiadka A, Rinne M, Matthews JL, Carmichael C, Bauer J, Varga ZM, and Westerfield M. Transmission, diagnosis, and recommendations for control of *Pseudoloma neurophilia* infections in laboratory zebrafish (*Danio rerio*) facilities. Comp Med. 2011;61:322–329.

O'Malley DM, Kao YH, and Fetcho JR. Imaging the functional organization of zebrafish hindbrain segments during escape behaviors. Neuron. 1996;17:1145–1155.

Oliveira RF. Mind the fish: zebrafish as a model in cognitive social neuroscience. Front Neural Circuits. 2013;8:131.

Otter A and Blakemore WF. Observation on the presence of *Listeria monocytogenes* in axons. Acta Microbiol Hungarica. 1986;36:125-131.

Paik EJ and Zon LI. Hematopoietic development in the zebrafish. Int J Dev Biol. 2010;54:1127-1137.

Parikka M, Hammare´n MM, Harjula S-KE, Halfpenny NJA, Oksanen KE, et al. Mycobacterium marinum Causes a Latent Infection that Can Be Reactivated by Gamma Irradiation in Adult Zebrafish. PLoS Pathol. 2012;8.

Percy DH, and Barthold SW, eds. Pathology of laboratory rodents and rabbits, third edition. Blackwell, Iowa. 2007.

Peterson TS, Spitsbergen JM, Feist SW, and Kent ML. Luna stain, an improved selective stain for detection of microsporidian spores in histologic sections. Dis Aquat Organ. 2001;95:175–180.

Pham M, Raymond J, Hester J, Kyzar E, Gaikwad S, Bruce I, Fryar C, Chanin S, Enriquez J, Bagawandoss S, Zapolsky I, Green J, Stewart AM, Robison BD, and Kalueff AV. 2012. Assessing social behavior phenotypes in adult zebrafish: Shoaling, social preference, and mirror biting tests. In Zebrafish Protocols for Neurobehavioral Research. Kalueff AV and Stewart AM, eds. Springer, New York.

Phillips JB and Westerfield M. Zebrafish models in translational research: tipping the scales toward advancements in human health. Dis Models Mech. 2014;7(7):739-743.

Pittman JT and Lott CS. Startle response memory and hippocampal changes in adult zebrafish pharmacologically-induced to exhibit anxiety/depression-like behaviors. Physiol Behav. 2014;123:174–179.

Poulin R. Explaining variability in parasite aggregation levels among host samples. Parasitology. 2013;140:541–546.

R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/.

Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML and Schreck CB. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. Aquaculture. 2006;258:565-574.

Ramsay JM, Watral V, Schreck CB, and Kent ML. Husbandry stress exacerbates mycobacterial infections in adult zebrafish, *Danio rerio* (Hamilton). J. Fish Dis. 2009;32:931-941.

Ramsay JM, Watral V, Schreck CB, and Kent ML. *Pseudoloma neurophilia* infections in zebrafish *Danio rerio*: effects of stress on survival, growth, and reproduction. Dis Aquat Organ. 2009;88:69–84.

Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2015.

Richier B and Salecker I. Versatile genetic paintbrushes: Brainbow technologies. Wiley Interdiscip Rev Dev Biol. 2015;4(2):161-180.

Riehl R, Kyzar E, Allain A, Green J, Hook M, Monnig L, Rhymes K, Roth A, Pham M, Razavi R, DiLeo J, Gaikwad S, Hart P, and Kalueff AV. Neurotoxicol and Teratol. 2011;33:658-667.

Salinas S, Schiavo G, and Kremer EJ. A hitchiker's guide to the nervous system: the complex journey of viruses and toxins. Nat Rev Microbiol. 2010;9:645–655.

Sanders GE. Zebrafish housing, husbandry, health and care: IACUC considerations. ILAR J 2012;53:205–207.

Sanders J, Peterson T, and Kent M, Early development and tissue distribution of *Pseudoloma neurophilia* in the zebrafish, *Danio rerio*. J Eukaryot Microbiol. 2014;61:238–246.

Sanders J, Watral V, Clarkson K, and Kent ML. Verification of intraovum transmission of a microsporidium of vertebrates: *Pseudoloma neurophilia* infecting the zebrafish, *Danio Rerio*. PLoS One 2013;8(9).

Sanders JL, Lawrence C, Nichols DK, Brubaker JF, Peterson TS, Murray KN, and Kent ML. 2010. Pleistophora hyphessobryconis (Microsporidia) infecting zebrafish Danio rerio in research facilities. Dis Aquat Org. 2010;91:47-56.

Sanders JL, Myers MS, Tomanek L, Cali A, Takvorian PA, and Kent ML. *Ichthyosporidium weissii* n. sp. (Microsporidia) infecting the arrow goby (*Clevelandia ios*). J Euk Microbiol. 2012;59:258-267.

Sanders JS, Watral V, and Kent ML. Microsporidiosis in zebrafish research facilities colonies. ILAR J. 2012;53:106–113.

Spagnoli S, Xue L, and Kent ML. The common neural parasite *Pseudoloma neurophilia* is associated with altered startle response habituation in adult zebrafish (*Danio rerio*): Implications for the zebrafish as a model organism. Behav Brain Res. 2015;291:351-360.

Spagnoli S, Xue L, Murray KM, Chow F, and Kent ML. *Pseudoloma neurophilia*: a retrospective and descriptive study of nervous system and muscle infections with new implications for pathogenesis and behavioral phenotypes. Zebrafish. 2015;12:189-201.

Spence R, Gerlach G, Lawrence C, and Smith C. The behavior and ecology of the zebrafish, *Danio rerio*. Biol Rev Camb Philos Soc. 2008;83:13–34.

Spence R, Smith C. Mating preference of female zebrafish, *Danio rerio*, in relation to male dominance. Behav Ecol. 2006;17:779–783.

Spitsbergen JM, Buhler DR and Peterson TL. Neoplasia and neoplasm-associated lesions in laboratory colonies of zebrafish emphasizing key influences of diet and aquaculture system design. ILAR J. 2012;53: 114-125.

Stewart A, Gaikwad S, Kyzar E, Green J, Roth A, and Kalueff AV. Modeling anxiety using adult zebrafish: a conceptual review. Neuropharmacol. 2012;62:135–143.

Stewart A, Wong K, Cachat J, Gaikwad S, Kyzar E, Wu N, Hart P, Piet V, Utterback E, Elegante M, Tien D, and Kalueff AV. Zebrafish models to study drug abuse-related phenotypes, Rev. Neurosci. 2011; 22:95–105.

Stewart AM, Nguyen M, Wong K, Poudel KW, and Kalueff AV, Developing zebrafish models of autism spectrum disorder (asd). Prog Neuropsychopharmacol Biol Psychiatry. 2014;50:27–36.

Tang L and Eaton JW. Fibrin(ogen) mediates acute inflammatory responses to biomaterials. J Exp Med. 1993;178:2147-2156.

Therneau T (2015). A Package for Survival Analysis in S. version 2.38, <URL: http://CRAN.R-project.org/package=survival>).

Therneau TM and Grambsch PM (2000). Modeling Survival Data: Extending the Cox Model. Springer, New York. ISBN 0-387-98784-3.

Thomas F, Adamo S, and Moore J. Parasitic manipulation: where are we and where should we go. Behav Process. 2005;68:185–199.

Toms CN and Echevarria DJ. Back to basics: searching for a comprehensive framework for exploring individual differences in zebrafish (*Danio rerio*) behavior. Zebrafish. 2014;11:325–340.

Tran S and Gerlai R. Individual differences in activity levels in zebrafish (*Danio rerio*), Behav Brain Res. 2013;257:224–229.

Traver D, Winzeler A, Stern HM, Mayhall EA, Langenau DM, Kutok JL, Look AT, and Zon LI. Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. Blood. 2004;104(5): 1298-1305.

Trede NS, Langenau DM, Traver D, Look AT, Zon LI. The use of zebrafish to understand immunity. Immunity. 2004;20(4):367-379.

Valsamis B and Schmid S. Habituation and prepulse inhibition of acoustic startle in rodents. J Vis Exp. 2011.

Van Diepen, A, van de Gevel JS, Koudijs MM, Ossendorp F, Beekhuizen H, Janssen R, and van Dissel JT. Gamma irradiation or CD4+ T cell depletion causes reactivation of latent *Salmonella enterica* serovar Typhimurium infection in C3H/HeN Mice. Infection and Immunity. 2005; 73(5): 2857-2862.

Vyas A, Kim SK, Giacomini N, Boothroyd JC and Sapolsky RM. Behavioral changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. PNAS. 2007;104:6442-6447.

Wang JH, Short J, Ledent C, Lawrence AJ, and van den Buuse M. Reduced startle habituation and prepulse inhibition in mice lacking the adenosine A2A receptor. Behav Brain Res. 2003;143:201–207.

Weihrauch MR and Diehl V. Artificial sweeteners—do they bear a carcinogenic risk? Ann Oncol. 2004; 15(10):1460-1465.

Whipps CM, Lieggie C, and Wagner R. Mycobacteriosis in zebrafish colonies. ILAR J. 2012;53:95–105.

Willemsen R, Hasselaar W, van der Linde H and Bonifati V. Zebrafish as a new model organism for Parkinson's disease. Proc Meas Behav. 2008;5:0–1.

Wulliman MF, Barbara R, and Reichert H. Neuroanatomy of the Zebrafish Brain: A Topological Atlas, Birkhaüser, Boston, 1996.

Yablokov AV. Nonmalignant Diseases after the Chernobyl Catastrophe, in Chernobyl: Consequences of the catastrophe for people and the environment. Yablokov AV, Nesterenko VB, and Nesterenko AV, eds. Annals of the New York Academy of Sciences, vol. 1181. 2009, pp 58-161. Blackwell Publishing. Boston, Mass.

Zebrafish Protocols for Neurobehavioral Research. Kalueff AV and Stewart AM (Eds). Springer, New York, 2012.

Zottoli SJ, Newma BC, Rieff HI and Winters DC. Decrease in occurrence of fast startle responses after selective Mauthner cell ablation in goldfish (*Carassius auratus*). J Comp Physiol A. 1999;184:207–218.

APPENDIX

Stress in fish as model organisms

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Chapter 13 in: Biology of Stress in Fish.

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Introduction.

The use of aquatic models as part of an integrative approach to biomedical research has expanded rapidly in the last few decades (Lieschke & Currie 2007; Allen & Neely 2010; Harland and Grainger 2011; Phillips & Westerfield 2014). This is perhaps most strikingly exemplified by the zebrafish (*Danio rerio*), as the Zebrafish Model Organism Database (ZFIN) web site (http://zfin.org) now lists approximately 1000 laboratories that employ zebrafish, and a 2014 search of the U.S. National Institutes of Health RePORTER website using the term "zebrafish" revealed a list of 735 grants using this model. In fact, zebrafish recently surpassed *Drosphilia* in PubMed listings (2,279 versus 2,265 PubMed listings in 2011).

It is important to keep both the researchers' and aquaculturists' primary goals in mind when shifting between food production, conservation, and laboratory settings. For much of this book, stress has been discussed mostly as it relates to fish "performance," which is a single word with numerous meanings depending on context. In a production setting, "performance" can be measured in terms of fecundity, growth, and survival. In a conservation setting, "performance" can be measured in terms of population stability, recovery, and homing and straying rates. In the laboratory, the definition of "performance" becomes entirely fluid, changing based on the

objectives of the research at hand. Fortunately, there are some fundamental goals that apply to all experimental organisms: similarity of subjects apart from experimental manipulations, animal welfare, and robustness of the model under study—both in general and as it pertains to specific research endpoints.

For many years, researchers have used adult trout (Bailey *et al.* 1992; Bailey *et al.* 1996; Williams *et al.* 2003), medaka (Hawkins *et al.* 1990; Takeda & Shimida 2010), and swordtail X platyfish hybrids (Schartl *et al.* 1997; Schartl1995; Patton et al. 2010) (Ostrander 2000) as models for basic biomedical science. Zebrafish experimentation was initially led by developmental geneticists whose primary interests concerned embryos or larval fish. As researchers have explored the utility of the zebrafish as a model for aging, chronic disease, and complex neurobehavioral syndromes, increasing focus has been placed on juvenile and adult-stage zebrafish (Dooley and Zon 2000). Challenges in experiments utilizing embyros or newly hatched larvae have many similarities with research using post-larval through adult fish because both require appropriate rearing and population maintenance. However, the latter type of research requires additional diligence in order to avoid non-protocol induced variation in in vivo experiments. The emergent challenges involved in rearing and maintaining populations of juvenile and adult zebrafish compared to embryos and larvae has led to a heightened need for greater involvement by veterinary specialists trained in comparative pathology and laboratory animal medicine.

Specific areas of study utilizing juvenile and adult zebrafish are varied and include infectious disease susceptibility and immune system function (Sullivan and Kim 2008; Novoa & Figueras 2012; van der Vaart 2012), aging (Gerhard 2007; Kishi et al. 2009), toxicology (Mathew et al. 2009; Truong et al. 2011, 2014), oncology (Liu & Leach 2011; Ceol et al. 2011), and behavior (Sisson et al. 2007; Wong et al. 2010; Stewart et al. 2012). Regarding infectious diseases, some 30 different bacterial pathogens have been studied using the zebrafish model (Meijer & Spaink 2011). A number of programs rely on experimental infection of zebrafish with *Mycobacterium* spp. (e.g., Prouty et al. 2003; Meijer et al. 2005; Swain et al. 2006), which could be confounded by underlying, preexisting infections. Diverse work on fish in the field of cancer research includes the establishment of xenotransplantation models, where human tumors are transplanted into immune compromised zebrafish (Patton et al. 2011; White et al. 2008; Patton & Zon 2005; Taylor & Zon 2009).

Other aquatic species commonly used as model organisms include three-spined sticklebacks (*Gasterosteus aculeatus*), Japanese medaka (*Oryzias latipes*), fathead minnow (*Pimephales promelas*), and several *Xipophorus* species. Sticklebacks are considered a robust model for studies concerning evolution and ecology, specifically phenotypic and genomic variation, speciation, and natural selection (Hendry et al 2013). Medaka have been indispensable to the fields of genomics and toxicology, and were the first fish in which transgenesis was achieved (Shima and Mitani 2004). Fathead minnows have been used in numerous toxicology studies (Ankley and Villeneuve 2006) and are used as bioindicator species.(EPA 2002) *Xipophorus* species are excellent models for melanomas and sexual development disorders (Schartl 2014). Other common research species include common carp (*Cyprinus carpio*) in environmental toxicology studies (Henshel et al. 1999), channel catfish (*Ictalurus punctatus*) in studies of evolutionary genomics (Liu 2003) and Mozambique tilapia (*Oreochormis mossambicus*) in osmoregulatory research (Gardell et al. 2013).

Despite the wide utility of these organisms, investigators are only just now becoming aware of the potential impact that underlying stress and chronic disease may have on research utilizing post-larval fish in medium- to long term experiments. With the dramatic rise of fish as a model organisms for neurobehavioral experiments (Sission et al. 2007; Rihel & Schier 2012; Spence 2011; Bailey et al. 2013), it is likely that chronic stress may exert unwanted effects on studies with behavioral or psychological endpoints. Kent et al. (2012) reviewed the impacts of chronic diseases as causes of non-protocol induced variation in zebrafish research. This chapter will expand on this theme to highlight potential and demonstrated impacts of stress in model laboratory fish and to provide a general overview of stress in these animals.

Indicators of Stress in Laboratory Fish.

The relationship between researchers and aquaculturists is a paradox: Researchers house animals adapted to natural environments in totally artificial habitats to eliminate potentially confounding variables, including environmental olfactory cues, local environmental microbiota, and wild-type nutritional profiles, which causes stress (Wedemeyer, 1997). Aquaculturists consider that what may be best for the fish in terms of health, welfare, and environmental adaptations may introduce confounding variables in the forms of unspecified bacterial flora, altered behavioral syndromes, and environmental variability associated with more “natural”

settings. When working with fish in the laboratory, one must integrate the knowledge of the animals' natural histories and potential adaptation to the laboratory environment over generations of artificial rearing with the demands of the research at hand in order to produce robust scientific models without sacrificing animal welfare. Indeed, close attention to animal welfare can reduce or eliminate stress as a confounding variable, and the first step in reducing stress for fish in the laboratory is the ability to measure and monitor stress responses (Sneddon et al. chapter 12, this volume).

The most commonly used indicator of stress in fish is cortisol, a readily measured component of the primary neuroendocrine stress response (Ellis et al. 2012, Spokinka et al., chapter 11, this volume). Elevation of plasma cortisol is a well-documented indicator of stress in fishes, although there are many other hormonal and physiological changes associated with stress. (Martinez-Porchas et al. 2009; Ellis et al. 2012, Schreck and Tort, chapter 1, this volume). Whole body cortisol concentrations have been used in small fish when blood volumes are inadequate to provide measurements for plasma cortisol (de Jesus et al. 1991; Barry et al. 1995; Feist and Schreck 2001). This approach has been extended to zebrafish (Canavello et al. 2011, Pottinger and Calder; Ramsey et al. 2006; 2009; Egan et al. 2012). Pavlidis et al. (2011, 2013) used a similar approach with zebrafish, but confined the analysis to the "trunk" portion of the body. More recently, Gesto et al. (2015) proposed the use of gill biopsies as an in vivo alternative to whole or partial body homogenates, the advantages being that gill biopsy can be obtained without euthanasia of the fish and that sampling induces far less stress than blood draws. Laboratory fishes, such as zebrafish, are usually held in relatively small tanks, and assays have been developed to test cortisol levels in the water (Ellis et al. 2004, Gronquist and Berges 2013), which correlate well with plasma cortisol values (Félix et al. 2013). This non-lethal approach also has the advantage of providing an overall assessment of whole-tank stress using only a single test. These studies evaluating cortisol/stress associations have shown that crowding, brief handling (netting), or underlying chronic infections by two zebrafish pathogens commonly found in research facilities (*Pseudoloma neurophilia* or *Mycobacterium marinum*) are all associated with elevated cortisol levels.

Recently, Aerts and co-authors demonstrated that the cortisol content in both ontogenetic and regenerated scales of common carp (*Cyprinus carpio*) could be used as a reliable and quantitative biomarker of chronic stress in this species (Aerts et al. 2015). As conventional

methods for quantification of cortisol in fishes from plasma, feces, and water are only able to provide information relative to the short-term response of a given animal or set of animals, this new approach will allow for assessment of stress levels in fish over much longer periods of time.

Cortisol, while easily the most quantifiable indicator of stress reactions, does not necessarily tell the whole story. Stress, from perception to processing to response, involves the coordination of dozens of neural circuits and endocrine loops (Maximino et al. 2010). While each strand in the web is still being explored, behavior, one of the end products of the stress response, can be used as both a quantitative and qualitative indicator. Behavior is a summation and synthesis of all facets of the stress response, and because zebrafish have relatively robust and repeatable responses to certain stimuli, behavior can be employed as a "soft indicator" of stress. Whereas not as specific as cortisol, behavioral responses may be a better way determining fish preferences in vivo, leading to improved welfare and experimental consistency.

Most stress-associated behaviors tend to be conserved among vertebrates, with species specific natural histories guiding individual reactions. In the case of zebrafish, having evolved as a prey species in its range in the major river drainages in India, Bangladesh and Nepal, it is no great stretch to surmise that the animal prefers darkness and depth—the better to hide from predators with—under stressful conditions (Engeszer et al. 2007). Therefore, Egan et al. (2012) found that behaviors such as reduced tank exploration, scototaxis (light avoidance), thigmotaxis (wall hugging), erratic swimming, diving, and freezing (sudden, brief immobility) are correlated with increased cortisol levels and are indicative of stress. The authors also showed that different strains of zebrafish exhibited different inherent anxiety levels, which is particularly important in genetically modified animals: Knowing the background strain of a fish may help to account for differential levels of stress. To make matters even more complicated, there can be a great deal of variability in behavioral syndromes (sometimes referred to as 'personalities') between individual fish, leading to a potentially high level of variability in stress responses (Dugatkin et al 2004; Conrad et al 2011).

Although impractical as diagnostic tests for stress in the laboratory, the use of pharmacological substances that are specifically associated with stress and anxiety allow researchers to identify stress-induced behavioral preferences and to correlate these behaviors with stress-associated cortisol levels. For example, in a preference experiment where a zebrafish must choose between a white walled and a black walled compartment, fish treated with

anxiolytic (anxiety-reducing) compounds such as selective serotonin reuptake inhibitors (SSRIs), spend more time in the white compartment while fish treated with anxiogenic (anxiety-enhancing) compounds such as caffeine and substituted amphetamines spend more time in the black compartment than controls (Araujo et al. 2012). Extending the correlation between behavior and cortisol levels to larval zebrafish, Tudorache et al. (2015) showed 8 day old larvae that hatched early exhibited higher cortisol peaks, but faster recovery times, than those hatching later. The close evaluation of stress-related behaviors in laboratory animals is particularly important in the use of zebrafish as model organisms for drug discovery, particularly in the arena of pharmaceutical screening. If certain behaviors associated with stress are confirmed, then behavioral responses following pharmaceutical exposure can be used to characterize the neurobehavioral effects of the drug (Stewart et al. 2015). Behavioral indicators of stress also have the advantage of being non-invasive to both the individual and to the tank as a whole when measurements are taken.

Factors Impacting Stress in Laboratory Fish

Handling.

Handling of fish by humans, which usually entails removing them from water, is probably the greatest stressor most laboratory fish will face during their lifetimes (Ghisleni et al. 2012). Hence, handling events should be kept to a minimum. Because experimentation requires handling at various times during a laboratory fish's life, understanding responses to handling can help reduce stress and to increase post-handling survival. Fortunately, because the perception and anticipation of stress make up a large component of the stress response, manipulation of perception and anticipation can greatly reduce overall stress in some species. For example, anesthetization of yearling chinook salmon prior to netting can reduce cortisol levels and increase post-netting survival (Schreck and Strange, 1978) Regarding non-salmonids commonly used in research, serum cortisol levels in goldfish are not affected by rapid capture with a net, ice immobilization, tricaine methanesulfonate (MS 222) anesthesia or electric immobilization (Singley and Chavin 1975).

In contrast, zebrafish exhibit rapid cortisol elevation and subsequent recovery from acute netting stress (Ramsay et al. 2009; Tran et al. 2015). Brydges and Braihewait (2009) found that

scooping fish in water, rather than netting, resulted in an attenuated cortisol response. There are several reports of handling associated with reduced fecundity, which are discussed later.

Fish handling in a laboratory setting frequently involves more elaborate and stressful events than netting: laboratory fishes are often exposed to pathogens or chemical agents by immersion, gavage or various routes of injection. Therefore, understanding the stress involved in these procedures is important, but data are extremely limited and can only be inferred from overall recovery and/or post-procedure survival rates. Kahl et al. (2001), in one of the few such studies available, found that anesthesia with MS-222 and intraperitoneal (IP) injections with corn oil and 10% ethanol was not correlated with behavioral or secondary sex changes, reduced survival, or fecundity.

Housing.

Fish used in research are most typically housed in aquaria that are on either flow-through or recirculating aquaculture systems (RAS; Lawrence & Mason 2012). While literature documenting differential effects of different types of aquatic systems on stress is sparse, it has been suggested that animals held in RAS might experience elevated stress as a result of metabolite, cortisol, and alarm substance accumulation in intensively stocked systems (Martins et al. 2011). This hypothesis was tested in Nile tilapia, but the results of the study were inconclusive (Martins et al 2009). Because of the limited data available, the subject deserves further examination, especially because laboratory species like zebrafish are almost exclusively maintained in RAS.

Parker et al. (2012) examined the effect of varying pre-housing conditions on stress reactivity measured by the novel tank exploration test. They found that group size, water changes, and especially the ability to make visual contact with conspecifics influenced novel tank exploration in zebrafish. They also showed that basal cortisol levels were lower in individually housed than in group-housed fish. These data support the idea that housing conditions mediate the stress response in this species, and therefore need to be standardized in order to improve study design.

Other housing characteristics have been evaluated relative to their impact on stress and anxiety in zebrafish. Blaser et al. (2012) found that tank depth and wall color elicited different responses in depth preference and scototaxis assays. An aspect of housing vital to fish husbandry

that generally goes unnoticed due to its constant presence is the sound of air and water pumps. Some work has been done on the subject of noise in salmonids: specific frequencies produce avoidance responses in juvenile Atlantic salmon (*Salmo salar* L.) (Knudsen et al 1992 and 1993). While specific research regarding the effects of husbandry-associated noise on zebrafish behavior is in its infancy, Smith et al. (2003) demonstrated that goldfish (*Carassius auratus*), a cyprinid like *D. rerio*, may be susceptible to stress and hearing loss in the face of chronic noise exposure. More recently, Neo et al. (2015) found that moderate sound levels alter group cohesion, swimming velocity, and depth preference in laboratory zebrafish. Interestingly, the same study found that there was no avoidance response to chronic, loud sound levels. These data provide further evidence that housing configuration can be an important contributor to zebrafish stress and behavioral responses.

The effects of external acoustics on fish behavior have been explored in a number of model and non-model organisms aside from zebrafish, with overall results indicating a subtle and potentially insidious influence of sound on survival and ecology. Radford and Purser (2011) found that while captive three-spined sticklebacks exposed to brief and prolonged noise had no reduction in total feed consuption, the fish had increased incidences of food-handling errors and decreased discrimination between food- and non- food items, indicating a "distraction" effect. Similarly, European eels (*Anguilla anguilla*) showed reduced anti-predator behaviors following exposure to anthropogenic noise (Simpson et al. 2014). Furthermore, there is ample evidence to suggest that anthropogenic noise interferes with acoustic communication between fish, another important survival strategy associated with social behavior (Radford et al. 2014).

Density.

Most studies utilizing fish in the laboratory are not concerned with production in terms of fecundity or feed efficiency, except in nutrition or husbandry studies. However, the number of animals present per unit volume along with the water exchange rate is closely related to fish stress levels, and may contribute to unexpected non-protocol induced variation in studies measuring other criteria, including immune, metabolic, and behavioral responses.

Relationships between fish number per unit volume, water flow, and stress may be neither direct nor intuitive. Generally, reduction of animal numbers among zebrafish housed at

initially high densities correlates with reduced cortisol levels (Ramsay et al. 2006; Pavlidis et al. 2013), but only to a certain point. There exists a lower limit of stocking density past which cortisol levels begin to increase as the number of fish decreases (Figure 1).. This may be due to the establishment of dominance hierarchies among small shoals lacking the capacity to spread chasing and aggression behavior throughout a large population of individuals (Filby et al. 2010; Pavlidis et al. 2013). Strikingly, despite the fact that zebrafish are a shoaling species, Parker et al. (2012) and Giacomini et al. (2015) showed that individuals held in isolation actually had reduced cortisol, presumably due to reduced interfish aggression, The latter authors also suggested that this reduced stress response may be related to the absence of behavioral and chemical cues from other fish held in the same housing unit. Correspondingly, zebrafish exposed to chemical cues released from dead fish showed elevated cortisol (Oliveira et al. 2014). This should be of particular note in zebrafish husbandry, since the vast majority of research laboratories use recirculating systems and pheromones such as alarm substance could persist in and spread through these systems, causing elevated levels of inter-tank stress.

A survey of the literature shows that the relationship between stocking density and stress is extremely variable and complex. For example, in contrast to all of the observations mentioned above, Pagnussat et al. (2013) found that zebrafish held in isolation had higher cortisol levels than those held in small groups of three. Likewise, another study found no correlation between stocking density and tank water cortisol levels (Gronquist and Beges 2013). Although densities were the same (4 fish/L), Ramsay et al. (2006) found that fish crowded within 76 L aquaria resulted in higher cortisol levels than those held in 4 L tanks. These studies show that differences in environmental conditions, husbandry methods, and study design may result in very different stress responses, especially when cortisol is used as the primary indicator

As discussed earlier, cortisol levels may not tell the whole story of stress and social interactions. A behavioral shoaling preference is well-described in zebrafish, which, when presented with a choice between an empty tank and a tank full of conspecifics, tend to spend more time near the shoal (Stewart et al. 2012). Furthermore, minimal numbers of conspecifics are considered environmentally enriching by many animal care and use protocols (ACUPs). Even though individual fish may have lower cortisol levels in isolation, there is a behavioral preference for the shoal, which may indicate that some degree social stress may be a positive, rather than a negative source of stress from the fish's point of view.

Interestingly, there is evidence that stocking density may have an effect on zebrafish behavior as early as the egg stage. Steenbergen et al. (2011) found that embryos raised in isolation had different responses to a dark pulse assay than embryos raised in batches of 25. This effect was rather elegantly proven to be due to contact between embryonated eggs as the behavioral effects of isolation could be reversed by artificial tactile stimulation during the first two days of embryogenesis. Therefore, consistency in embryo stocking densities may be important to the development of larval, and possibly even adult, behavioral phenotypes.

Enrichment.

A considerable proportion of this chapter has been devoted discussing practices concerning the optimization of "production" in laboratory fish. It is necessary to remember that a "happy" fish is often productive fish and that animal welfare is the cornerstone of improved production, especially in a laboratory setting. Optimizing husbandry goes beyond simply meeting an animal's physiological needs such as stocking density, nutrition, and water quality. In this regard, animal care committees and regulatory oversight bodies in research institutions require that laboratory mammals be provided with various forms of environmental enrichment (Baumans and Van Loo 2013, Hutchinson et al. 2005, Institute of Laboratory Animal Resources Committee 2011). The advantages of providing structural enrichment – for all animals - is very much species and context dependent and a number of studies have shown that enriched environments affect certain behavioral syndromes in fish (Brydes and Braiwaite 2009). For example, Kistler et al. (2011) found that zebrafish were attracted to structures (artificial plants), but that the attraction response was stronger in checkered barbs (*Puntius oligolepis*). In zebrafish, spatial complexity tends to reduce aggression, and decreased aggression is associated with increased fecundity (Carfagnini et al. 2009). However, a direct correlation between environmental enrichment and stress reduction has not been demonstrated. For example, Wilkes et al. (2012) found that glass rods simulating plant stems did not reduce cortisol levels in zebrafish. In another study, Keck and co-authors (2015) found that while the provision vegetation in the form of plastic plants did not reduce cortisol levels in pair-housed zebrafish, it did reduce mortality rates associated with aggression in these settings. In contrast, Pavlidis et al. (2013) showed that darker tanks (dark backgrounds) resulted in reduced cortisol compared to

zebrafish held in tanks with light backgrounds. The findings of these, and similar studies, serve to highlight the complexity of fish behavior as it relates to husbandry. More research is needed to explore the specific environmental enrichment needs of laboratory fish in order to optimize their well-being and their utility as model organisms.

Light/Dark Cycle

Fish, like all animals, are profoundly influenced by photoperiod (Pavlidis et al. 1999;Valenzuela et al. 2012; El-Sayed & Kawanna 2004; Leonardi & Klempau 2003) (Boeuf & Le Bail 1999), so it is critically important to understand and regulate light cycles in the housing environment of captive fishes. Given that photoperiod exerts such profound influence on the fish physiology, it is not surprising that variations in 'day' length beyond the animal's evolved preferences can be a source of distress. For example, African catfish (*Clarias gariepinus*) exhibit elevated cortisol levels when held under extended daylight conditions (Almazán-Rueda et al. 2005). Furthermore, Mustapha et al. (2014) found that Nile tilapia and African catfish exhibited increased mortality when maintained under constant light without a dark period (Mustapha et al., 2014). Female *Astyanax bimaculatus* exposed to longer light cycles showed elevated cortisol, higher aggression rates and decreased survival than fish held under constant darkness (Navarro et al., 2014).

Whereas it is well established that zebrafish are photoperiodic breeders (Lawrence 2007; Westerfield 2007), there is little data available concerning the effects of day length on stress. Existing photoperiod studies do not address stress specifically, but their results tend to be relatively consistent: larval fish show increased rates of swimming activity in darkness as opposed to light (de Esch et al. 2012) and both larval and adult fish show elevated activity levels when lights are abruptly turned off during light/dark challenge tests (Vignet et al. 2013). It can be inferred from these studies that light and darkness have neurobehavioral consequences for zebrafish, but more research is needed to specifically address potential correlations between photoperiod and stress.

Feeding and Stress.

Many animal care committees consider daily feeding of fish to be a requirement for humane treatment. With small tanks, especially static aquaria, this may cause issues with water quality and increase the adverse effects of high stocking density. Salmonids may exhibit increased cortisol when feeding is abruptly ceased, probably due to increased aggression (Brännäss et al. 2003). In contrast, Gronquist and Berges (2013) showed a clear reduction in cortisol levels in zebrafish that were subjected to food deprivation for up 9 d, possibly due to increased water quality outstripping metabolic needs in relatively "sedentary" fish. Another possibility is that captive fish without natural activity patterns are chronically overfed, which could also interfere with experimentation. Conversely, stress inhibits food intake in many species (Steenbergen 2011). Interestingly, the timing of feeding for groups of cichlid fish accustomed to being fed at predictable intervals induced a rise in cortisol levels (Galhardo et al. 2011), suggesting that changes in feeding routines might be a source of stress in fish held under laboratory conditions.

Sex and Hierarchies.

Zebrafish in aquaria, as well as a number of other species including salmon, establish dominance hierarchies, which are more pronounced in males (Spence et al. 2008; Paull et al. 2010; Ejicke et al. 1980). Filby et al. (2010) showed that subordinate males exhibited a greater rise in cortisol than subordinate females, potentially due to the more prevalent dominance hierarchies observed in males.

In laboratory zebrafish, physiological factors associated with dominance include animal size and aggressive behavior (Paull et al. 2010). Subordinate animals overall appear to experience more stress than dominants across species. For example, non-territorial male African cichlids (*Haplochromis burtoni*) tend to have higher cortisol levels than territorial males (Fox et al. 1997). This is also true of juvenile rainbow trout, in which the submissive behavioral phenotype is considered to be a chronic stressor. Interestingly, elevated cortisol and submissive behavior likely have an interactive relationship of positive reinforcement wherein submission causes stress and the resulting elevated cortisol results in increased submissive interactions due to poor health, small size, etc (Gilmour et al. 2005). The question of what makes a fish more likely to be dominant or submissive may be related to genetic predispositions to elevated cortisol

responses to external stimuli, although this hypothesis is still being explored (Ramsay et al. 2009).

In order to improve behavioral consistency and reduce hierarchy-related stress, adequate feeding has been suggested as a potential mitigating factor in zebrafish as it promotes consistent size distributions within a tank while reducing the need for aggression to compete for food. Crowding, of course, goes hand in hand with resource management and hierarchy formation and so appropriate stocking density may help to reduce hierarchy-induced stress (Ramsay et al. 2009).

Sex determination and reversal.

Fish are often used in studies wherein gonadal phenotype is an endpoint, as in the evaluation of endocrine-disrupting compounds. The Japanese medaka (*Oryzias latipes*) is an important species in toxicology research. Hayashi et al. (2010) showed that cortisol caused female-to-male sex reversal and that metyrapone (an inhibitor of cortisol synthesis) inhibited HT-induced masculinization of female medaka. As cortisol has also been implicated in influencing gonadal fate in other species, including pejerrey (*Odontesthes bonariensis*) (Hattori et al. 2009) and Japanese flounder (*Paralichthys olivaceus*), the possbility that the hormone may have similar effects in laboratory species such as zebrafish should be considered.

Stress, Cortisol and Reproduction.

The tremendous increase in the use of fish in biomedical research has been primarily focused on early developmental stages, particularly the embryo. This is especially true of the zebrafish, which is well-suited to high throughput experimental protocols due to its high fecundity, external fertilization, rapid development, and larval whole-body transparency compared to traditional mammalian models (Murphey & Zon 2006). Many laboratories, therefore, use adult fish solely for embryo production and the effects of stress on fecundity and embryogenesis are of particular concern in this setting.

Because fecundity is one of the major production endpoints in aquaculture, most studies regarding the relationship between stress and fecundity have been done with rainbow trout and other salmonids. These studies were extensively reviewed by Schreck et al. (2001). Similar

studies on laboratory fish species are far less common, however, Cloud (1981) found that medaka hatching rates were accelerated when eggs were incubated with deoxycorticosterone.

A discussion of stress and fecundity must focus on the concept of maternal stress effects on offspring, most of which are epigenetic. Schreck and Tort (Chapter 1, this volume) have already discussed the data concerning the maternal effects of stress on salmonid offspring, but data on its effects on laboratory fish are sparse. Nesan and Vijayan (2012) found that pre-hatching exposure to cortisol in zebrafish embryos induced cardiac dysfunction and other developmental defects. In this study, injection of cortisol into single celled eggs was meant to mimic maternal cortisol delivery to eggs because independent cortisol production does not occur in zebrafish until after hatching. In threespine sticklebacks, maternal exposure to predation risk was associated with increased embryonic transcription of genes associated with metabolism, epigenetic inheritance, and neuronal development. The embryos of predator risk-exposed mothers also tended to be larger than control embryos (Mommer and Bell 2014).

The consequences of larval and embryonic stress can extend into adulthood: Rainbow trout exposed to stress at early embryonic stages, both at stages involving maternal and endogenous cortisol, actually resulted in lowered stress responses as adults (Steenbergen et al. 2011). These studies highlight that consistency in maternal sourcing and husbandry, as well as consistent care for embryos and larvae are necessary for reducing the impact of stress as a source of non-protocol induced variation.

Anesthetics.

Laboratory fishes are often anesthetized to allow for various procedures, including injections, tagging, and implants. Palic et al. (2006) evaluated the effects of MS-222, metomidate and eugenol on cortisol levels in fathead minnows. Fish anesthetized with metomidate or eugenol (but not MS-222) showed reduced cortisol compared to controls following handling and crowding. Recently, two separate studies found that zebrafish perceive the most commonly used anesthetic agent in fish research, MS-222, as aversive. "Aversive" in this case means that fish attempt to avoid the substance under study—given the choice, they prefer water with lower concentrations of the substance or places where it is absent entirely. Using behavioral analysis software that quantified spatial preference based on swimming behaviors, Readman et al. (2013) showed that adult zebrafish displayed aversive behavior when

exposed to 7 of 9 commonly used anesthetics, including MS-222 and benzocaine (Readman et al. 2013). They suggested the use of etomidate as a possible alternative for this species. In a separate study, Wong et al. (2014) used a conditioned place avoidance paradigm test to demonstrate zebrafish aversion to MS222 (Wong et al. 2014). Whereas these data apply only to zebrafish, they clearly demonstrate that the choice of anesthetic should be carefully considered depending on the species and experimental application. Also worth consideration is the balance of a brief aversive stimulus in the form of anesthesia compared to the pain and stress of handling or surgery without anesthesia. We do not suggest that anesthesia should not be used prior to experimental manipulation—in fact it is necessary to survival in many cases—merely that broader options for less aversive or stressful anesthesia methods should be explored.

Underlying Diseases.

Kent et al. (2012) reviewed the documented and potential impacts that underlying lesions and infections have or may have on research using zebrafish. In line with the topic of this book, stress and underlying diseases have a symbiotic relationship, with disease potentially elevating incipient stress levels and stress exacerbating pathogenicity in turn. For example, *Pseudoloma neurophilia* is extremely common in zebrafish facilities (Sanders et al. 2012), and we recently showed that this microsporidium, which targets the central nervous system, can infect many laboratory fishes, such as medaka and fathead minnows (Sanders et al. 2015). Most infections are subclinical (Spagnoli et al. 2015), but fish subjected to crowding stress develop more severe infections (Ramsay et al 2009). *P. neurophilia* infections have been associated with altered responses to assessments of stress and anxiety, indicating that infections may result in high-responding behavioral phenotypes (Spagnoli et al. 2015). Zebrafish with underlying *Mycobacterium marinum* infections subjected to crowding stress exhibited higher infection-associated mortality than unstressed controls. This was not due to increasing horizontal transmission, as this pathogen requires ingestion of infected tissue or invertebrates for transmission amongst laboratory fishes (Peterson et al. 2013). Rather, the increased mortality was attributed to the exacerbation of pre-existing infections by stress in individual fish. There are numerous examples of stress, crowding, and elevated cortisol enhancing the proliferation and virulence of pathogens within the fish host, since cortisol is notoriously immunosuppressive

(Cortés et al. 2013). Conversely, the presence of underlying parasite infections can be stressors themselves, resulting in increased cortisol levels (Hoole and Williams 2004). The importance of cortisol as an immune suppressor is particularly important in studies concerning inflammatory and immune responses with regards to underlying fish stress (see Yada and Tort, Chapter 10, this volume).

Consistency

Whereas all of the specific aspects of husbandry discussed above are important individually, there is one factor that should unite them all and direct the decision-making process of anyone working with these animals: consistency in care and husbandry. Changes are obviously necessary as the fish grows from a larva to an adult, but steps should be taken to minimize drastic changes in factors like feeding, temperature, ambient noise, and water quality once the specific husbandry practices of that life stage are established. A fascinating study by Piato et al (2011) found that zebrafish have the potential to be an excellent model for unpredictable chronic stress in humans. When exposed to unpredictable multiple stressors given at irregular intervals and unpredictable time points, zebrafish showed increased anxiety and decreased cognitive function as well as increased cortisol after 7 days of exposure. Whereas this is excellent news for the zebrafish as a model for human neurobehavioral disease, it could mean that successive, random changes in husbandry practices could both produce undue stress and alter responses to neurobehavioral research. Therefore, husbandry changes or frequent random stress events such as equipment repair or replacement, as well as alarms or lighting changes, should be avoided whenever possible.

Conclusion and Key Unknowns

Future research should explore the exact effects of specific husbandry manipulations on stress and its capacity to influence the fish as a model organism. It is likely that, as more data are gathered on the subject, the scientific community will begin to unearth some heretofore unknown complicating factors. For example, domestication selection in salmonids is a well-established phenomenon (Araki et al. 2008) with marked genetic and behavioral changes occurring as quickly as a single generation. It is likely, therefore, that in a totally artificial laboratory

environment, domestication selection could strongly influence behavioral syndromes of model fishes. To support this concept, behavioral differences have been observed between AB and TU/TL zebrafish lines (Varga 2011). This artificial selection may actually be beneficial in that it could reduce genetic variability among fish within a laboratory, however, inadvertent selection for distinct behavioral phenotypes could produce variable stress responses between different laboratories and breeders. For example, the behavioral shifts associated with *P. neurophilia* infection observed by Spagnoli et al. (2015) may have been due to the selective pressure of the parasite killing off fish with a particular behavioral phenotype. If this is the case, then it is possible that laboratories contaminated by this very common parasite could be inadvertently selecting for zebrafish with high-responding, non-mortality-associated behavioral phenotypes.

This chapter has provided an extensive discussion of the sources, measurability, and effects of stress in laboratory fishes. It is important here to reiterate the three basic goals of animal husbandry for research: consistency of subjects, robustness to the model under study, and animal welfare. Stress is a fundamental neurophysiological state that links the brain and body in all vertebrates. Therefore, the myriad of experimental endpoints utilizing laboratory fish, whether they are neurobehavioral, pathologic, molecular or metabolic, can all be influenced by stress. A thorough understanding of the causes and consequences of stress in laboratory fish will ultimately lead to the development of husbandry protocols that marry animal welfare with experimental consistency.

Literature Cited

Allen JP and Neely MN. Trolling for the ideal model host: zebrafish take the bait. Future Microbiol. 2010;5:563-569.

Almazán-Rueda P, Van Helmond AM, Verreth JJ, and Schrama JW. Photoperiod affects growth, behaviour and stress variables in *Clarias gariepinus*. J Fish Biol. 2005;67:1029–1039.

Ankley GT and Villeneuve DL. The fathead minnow in aquatic toxicology: Past, present and future. Aquat Toxicol. 2006;78: 91-102.

Araki H, Berejikian BA, Ford MJ, and Blouin MS. Fitness of hatchery-reared salmonids in the wild. Evol Appl. 2008;1:342-355.

Araujo J, Maximino C, de Brito TM, da Salva AWB, Oliveira KRM, Batista EJO, Morato S, Herculano AM, and Gouveia Jr A. 2012. Behavioral and pharmacological aspects of anxiety in the light/dark preference test *in:* Zebrafish Protocols for Neurobehavioral Research. (Kalueff AV and Stewart AM, Eds.) pp. 191-203. Springer, New York.

Bailey GS, Williams DE, and Hendricks JD. Fish models for environmental carcinogenesis: The rainbow trout. Environ Health Perspect. 1996;104:5-21.

Bailey J, Oliveri A, and Levin ED. Zebrafish model systems for developmentaneurobehavioral toxicology. Birth Defects Research Part C - Embryo Today:Reviews. 2013;99:14–23.

Baumans V and Van Loo PP. How to improve housing conditions of laboratory animals: The possibilities of environmental refinement. Vet Journ. 2013;195:24–32.

Blaser RE and Rosemberg DB. Measures of anxiety in zebrafish (*Danio rerio*): Dissociation of black/white preference and novel tank test. PLoS ONE. 2012;7.

Boeuf G and Le Bail PY. Does light have an influence on fish growth? Aquaculture. 1999;177:129–152.

Brännäss ES and Lundqvist, H. Influence of food abundance in individual behaviour strategy and growth rate in juvenile Brown Trout (*Salmo trutta*). Can. J. Zool. 2003;81:684–692.

Brydges NM and Braithwait A. Does environmental enrichment affect the behaviour of fish commonly used in laboratory work? Appl Anim. Behaviour Sci. 2009;118:137-143.

Canavello PR, Cachat JM, Beeson EC, Laffoon AL, Grimes C, Haymore WM, Elegante MF, Bartels BK, Hart PC, Elkhayat SI, Tien DH, Mohnot S, Amri H, and Kalueff AV. 2011. Measuring endocrine (cortisol) responses of zebrafish to stress *in*: Zebrafish Neurobehavioral Protocols. (Kalueff AV and Stewart AM, Eds.) pp. 135-142. Springer, New York.

Canavello PR, Cachat JM, Beeson E, Laffoon AL, Grimes C, Haymore W, Elegante MF, Bartels BK, Hart PC, Elkhayat SI, Tien DH, Mohnot SM, and Kalueff AV. Measuring endocrine (cortisol) responses of zebrafish to stress. Neuromethods. 2011;51:135–142.

Carfagnini AG, Rodd FH, Jeffers KB, and Bruce E. The effects of habitat complexity on aggression and fecundity in zebrafish (*Danio rerio*). Environ Biol Fish 2009;86:403–409

Ceol CJ, Houvras Y, Jane-Valbuena J, Bilodeau S, Orlando DA, Battisti V, Fritsch L, Lin WM,

Hollmann TJ, Ferré F, Bourque C, Burke CJ, Turner L, Uong A, Johnson LA, Beroukhim R, Mermel CH, Loda M, Ait-Si-Ali S, Garraway LA, Young RA, and Zon LI. The histone methyltransferase SETDB1 is recurrently amplified in melanoma and accelerates its onset. Nature. 2011;471:513-517.

Cloud JG. Deoxycorticosterone-induced precocious hatching of teleost embryos. J. Exp. Zool. 1981;216:197-199.

Conrad JL, Weinersmith KL, Brodin T, Saltz JB, and Sih A. Behavioural syndromes in fishes: a review with implications for ecology and fisheries management. J Fish Biol. 2011;78:395-435.

Cortés R, Teles M, Tridico R, Acerete L, and Tort L. Effects of cortisol administered through slow release implants on innate immune responses in rainbow trout (*Onchorhynchus mykiss*). Int J Genomics. Online edition. 2013.

De Esch C, van der Linde H, Slieker R, Willemsen R, Wolterbeek A, Woutersen R, and DeGroot D. Locomotor activity assay in zebrafish larvae: Influence of age, strain and ethanol. Neurotoxicol Teratol. 2012;34:425–433.

Dugatkin L, McCall MA, Gregg RG, Cavanaugh A, Christensen C, Unseld M. Zebrafish (*Danio rerio*) exhibit individual differences in risk-taking behavior during predator inspection. Ethology Ecology and Evolution 2004;17(1).

Dooley K and Zon LI. Zebrafish: a model for the study of human disease. Curr opin genet dev. 2001;10:252-256.

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat, SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, and Kalueff AV. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res. 2009;205:38-44.

Ejike C and Schreck CB. Stress and social hierarchy rank in coho salmon. Transactions American Fisheries Society. 1980;109:423-426.

Ellis T, James JD, Stewart C, Scott AP. A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. J Fish Bio. 2004;65(5):1233-1252.

Ellis T, Yildiz HY, Lopez-Olmeda J, Spedicato MT, Tort L, Overli O, and Martins CI. Cortisol and finfish welfare. Fish Physiol Biochem. 2012;38:163–188.

El-Sayed AM and Kawanna M. Effects of photoperiod on the performance of farmed Nile tilapia *Oreochromis niloticus: I.* Growth, feed utilization efficiency and survival of fry and fingerlings. Aquaculture. 2004;231:393–402.

Engeszer RE, Patterson LB, Rao AA and Parichy DM. Zebrafish in the wild: a review of natural history and new notes from the field. Zebrafish. 2007;4:21-40.

Félix AI, Faustino AI, Cabral EM and Oliveira RF. Noninvasive measurement of steroid hormones in zebrafish holding-water. Zebrafish. 2013;10:110-115.

Filby AL, Paull GC, Bartlett EJ, Van Look KW and Tyler CR. Physiological and health consequences of social status in zebrafish (*Danio rerio*). Physiol. Behav. 2010;101:576-587.

Fox HE, White SA, Kao MHF, Fernald RD. Stress and dominance in a social fish. Jour Neurosci. 1997;17:663-669.

Galhardo L, Vital J and Oliveira RF. The role of predictability in the stress response of a cichlid fish. Physiol and Behav. 2011;102:367–372.

Gardell AM, Yang J, Sacchi R, Fangue NA, Hammock BD, and Kultz D. Tilapia (*Oreochromis mossambicus*) brain cells respond to hyperosmotic challenge by inducing myo-inositol biosynthesis. J Exp Bio. 2013;216:4615-4625.

Gerhard GS. Comparative aspects of zebrafish (*Danio rerio*) as a model for aging research. ExpGerontol. 2003;38:1333-41.

Gesto M, Hernandez J, Lopez-Patino MA, Soengas JL, Miguez JM. 2015. Is gill cortisol concentration a good acute stress indicator in fish? A study in rainbow trout and zebrafish. 2015. Comp Biochem Physiol A Mol Integr Physiol. 188:65-69.

Ghisleni G, Capiotti KM, Da Silva RS, Oses JP, Piato AL, Soares V, Bogo MR, and Bonan CD. The role of CRH in behavioral responses to acute restraint stress in zebrafish. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2012;36:176-182.

Giacomini AC, de Abreu MS, Koakoski G, Idalencio R, Kalichak F, Oliveira TA, da Rosa JG, Gusso D, Piato AL, and Barcellos LJ. My stress, our stress: Blunted cortisol response to stress in isolated zebrafish. Physiol and Behavior. 2015;139:182-187.

Gilmour KM, DiBattista JD and Thomas JB. Physiological causes and consequences of social status in salmonid fish. Integ and Comp Biol. 2005;45:263-273.

Gimeno S and Bowmer CT. 1999. Ecological risk assessment of substances with suspected estrogenic activity using standard laboratory fish tests in Environmental Toxiology and Risk Assessment: Standardization of biomarkers for endocrine disruption and environmental assessment. 8th volume. Henshel DS, Black MC, and Harrass MC, eds. American society for testing and materials. Toronto.

Gronquist D and Berges JA. Effects of aquarium-related stressors on the zebrafish: A comparison of behavioral, physiological, and biochemical indicators. J Aquat Animal Health. 2013;25:53-65.

Hattori RS, Fernandino JI, Kishii A, Kimura H, Kinno T, Oura M, Somoza GM, Yokota M, Strussmann CA, and Watanabe S. Cortisol-induced masculinization : Does thermal stress affect

gonadal fate in pejerrey, a teleost fish with temperature-dependent sex determination? PLOSone. 2009;4.

Harland RM and Grainger RN. *Xenopus* research: metamorphosed by genetics and genomics. Trends Gen. 2011;27:507-515.

Hayashi Y, Kobira J, Yamaguchi T, Shiraishi E, Yazawa T, Hirai T, Kamei Y, Kitano T. High temperature causes masculinization of genetically female medaka by elevation of cortisol. Mol Rep Develop. 2010;77:679-686.

Hendry AP, Peichel CL, Matthews B, Boughman JW, and Nosil P. Stickleback research: the now and the next. Evol Ecol Res. 2013;15.

Hirose K. Endocrine control of ovulation in medaka *Oryzias latipes*. and *ayu Plecoglossus altielis*. J Fish Res Board Can. 1976;33:989–994.

Hoole D and Williams GT. 2004. The role of apoptosis in non-mammalian host-parasite relationships *in*: Host-Parasite Interactions (G. Fli and G. Wiegertjes eds.). SEB symp. 55. pp 13-45. BIOS Sci Publ.

Hutchinson E, Avery A, and Van de Woude S. Environmental enrichment for laboratory rodents. ILAR Journal. 2005;46:148–161.

Institute of Laboratory Animal Resources Committee, 2011. Guide for the Care and Use of Laboratory Animals.

Kahl MD, Jensen KM, Korte JJ, and Ankley GT. Effects of handling on endocrinology and reproductive performance of the fathead minnow. J Fish Biol. 2001;59:515–523.

Kent ML, Harper C, and Wolf JC. Documented and potential research impacts of subclinical diseases in zebrafish. ILAR J. 2012;53:126-134.

Kishi S, Slack BE, Uchiyama J, and Zhdanova IV. Zebrafish as a genetic model in biological and behavioral gerontology: Where development meets aging in verterates – A mini-review. Gerontology. 2009;55:430–441.

Kistler C, Hegglin D, Würbel H, and König B. Preference for structured environment in zebrafish (*Danio rerio*) and checker barbs (*Puntius oligolepis*). Appl Animal Behav Sci. 2011;135:318-327.

Lawrence C and Mason T. Zebrafish housing systems: A review of basic operating principles and considerations for design and functionality. ILAR J. 2012;53:179–191.

Lawrence C. The husbandry of zebrafish (*Danio rerio*): A review. Aquaculture. 2007;269:1-4.

Leonardi MO and Klempau AE. Artificial photoperiod influence on the immune system of

juvenile rainbow trout (*Oncorhynchus mykiss*) in the Southern Hemisphere. Aquaculture. 2003;221:581–591.

Lieschke GJ, and Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet. 2007;8:353-367.

Liu S and Leach SD. Zebrafish models for cancer. Ann Rev Pathol Mech Dis. 2011;6:71–93.

Martinez-Porchas M, Martinez-Cordova LT, and Ramos-Enriquez R. Cortisol and Glucose: Reliable indicators of fish stress ? J Aquat Sci. 2009;4:158–178.

Liu Z. A review of catfish genomics: progress and perspectives. Comp Funct Genom. 2003;4:259-265.

Martins CM, Eding EH and Verreth JJ. Stressing fish in Recirculating Aquaculture Systems (RAS): Does stress induced in one group of fish affect the feeding motivation of other fish sharing the same RAS? Aquaculture Research. 2011;42:1378–1384.

Martins CM, Ochola D, Ende SW, Eding EH, and Verreth JJ. Is growth retardation present in Nile tilapia *Oreochromis niloticus* cultured in low water exchange recirculating aquaculture systems? Aquaculture. 2009;298:1-2.

Maximino C, deBrito TM, da Silva Batista AW, Herculano AM, Morato S, and Gouveia A Jr. Measuring anxiety in zebrafish: a critical review. BehavBrain Res. 2010;214:157-171.

Meijer AH and Spaink HP. Host-pathogen interactions made transparent with the zebrafish model. Curr Drug Targets. 2011;12:1000-1017.

Meijer AH, Verbeek FJ, Salas-Vidal E, Corredor-Adamez M, Bussman J, van der Sar, AM, Otto GW, Geisler R, Spaink HP. Transcriptome profiling of adult zebrafish at the late stage of chronic tuberculosis due to *Mycobacterium marinum* infection. Mol Immunol. 2005;42:1185–1203.

Mommer BC and Bell AM. Maternal experience with predation risk influences genome-wide embryonic gene expression in threespined sticklebacks (*Gasterosteus aculeatus*). PLoS One. 2014;9.

Murphey RD and Zon LI. Small molecule screening in the zebrafish. Methods. 2006;39:255–261.

Murphey RD and Zon LI. Small molecule screening in the zebrafish. Methods. 2006;39:255–261.

Mustapha MK, Oladokun OT, Salman MM, Adeniyi IA, and Ojo D. Does light duration (photoperiod) have an effect on the mortality and welfare of cultured Oreochromis niloticus and Clarias gariepinus? Turk J Zool. 2014;38:466-470.

Navaro F, Navarro FP, Navarro RD, Murgas R., and Felizardo VO. Efeito do fotoperíodo na avaliação do estresse e na atividade locomotora em fêmeas de lambari (*Astyanax bimaculatus*).

Ciênc agrotec 2009;38:173-180.

Neo YY, Parie L, Bakker F, Snelderwaard P, Tudorache C, Schaaf M, and Slabbekoorn H. Behavioral changes in response to sound exposure and not special avoidance of noisy conditions in captive zebrafish. Front Behav Neurosci. 2015.

Nesan D, Vijayan MM. Embryo exposure to elevated cortisol level leads to cardiac performance dysfunction in zebrafish. Mol Cell Endocrinol. 2012;363:85-91.

Novoa B and Figueras A. Zebrafish: model for the study of inflammation and the innate immune response to infectious diseases. Adv Exp Med Biol. 2012;946:253-275.

Oliveira TA, Koakoski G, da Motta AC, Piato AL, Barreto RE, Volpato GL, Barcellos, LJ. Death-associated odors induce stress in zebrafish. Horm Behav. 2014;65:340-344.

Palić D, Herolt DM, Andreasen CB, Menzel BW, Roth JA. Anesthetic efficacy of tricaine methanesulfonate, metomidate and eugenol: Effects on plasma cortisol concentration and neutrophil function in fathead minnows (*Pimephales promelas* Rafinesque, 1820). Aquaculture. 2006;254:675-685.

Pangnussat N, Piato AL, Schaefer IC, Blank M, Tamborski AR, Guerim LD, Bonan CD, Vianna MR, and Lara DR. One for all and all for one: the importance of shoaling on behavioral and stress responses in zebrafish. Zebrafish. 2013;10:338-342.

Parker MI, Millington ME, Combe FJ, and Brennan CH. Housing conditions differentially affect physiological and behavioural stress responses of zebrafish, as well as the response to anxiolytics. PLoS One. 2012;7.

Patton EE, Mitchell DL, and Nairn RS. Genetic and environmental melanoma models in fish. Pigment cell melanoma res. 2010;23:314-337.

Patton EE and Zon LI. Taking human cancer genes to the fish: a transgenic model of melanoma in zebrafish. Zebrafish, 2005;1:363–368.

Patton EE, Mathers ME and Schartl M. Generating and Analyzing Fish Models of Melanoma. Methods in Cell Biology. 2011;105:339–366.

Paull GC, Filby AL, Giddins HG, Coe TS, Hamilton PB and Tyler CR. Dominance hierarchies in zebrafish (*Danio rerio*) and their relationship with reproductive success. Zebrafish. 2010;7:109-117.

Pavlidis M et al. The effect of photoperiod on diel rhythms in serum melatonin, cortisol, glucose, and electrolytes in the common dentex, *Dentex dentex*. General and comparative endocrinology. 1999;113:240–250.

Pavlidis M, Digka N, Theodoridi A, Campo A, Brasakis K, Skouradakis G, Samaras A, and

Tsalafouta A. Husbandry of zebrafish, *Danio rerio*, and the cortisol stress response. Zebrafish. 10:524-531.

Peterson TS, Ferguson JA, Watral VG, Mutoji KN, Ennis DG, and Kent ML. *Paramecium caudatum* enhances transmission and infectivity of *Mycobacterium marinum* and *Mycobacterium chelonae* in zebrafish (*Danio rerio*). Dis Aquat Org. 2013;106:229-239.

Phillips JB and Westerfield M. Zebrafish models in translational research: tipping the scales towards advancements in human health. Dis Model Mech. 2014;7:739-743.

Piato AL, Capiotti KM, Tamborski AR, Oses JP, Barcellos LG, Bogo MR, Lara DR, Vianna MR, and Bonan CD. Unpredictable chronic stress model in zebrafish (*Danio rerio*): behavioral and physiological responses. Prog in neuro-psychopharm and biol psyc. 2011;35:561-567.

Prouty MG, Correa NE, Barker LP, Jagadeeswaran P, and Klose KE. Zebrafish- *Mycobacterium marinum* model for mycobacterial pathogenesis. FEMS Microbiol Lett. 2003;225:177–182.

Purser J and Radford AN. Acoustic noise induces attention shifts and reduces foraging performance in three-spined sticklebacks (*Gasterosteus aculeatus*). PLOS one. 2011.

Radford AN, Kerridge E, Simpson SD. Acoustic communication in a noisy world: can fish compete with anthropogenic noise? Behav Ecol. 2014;25(5):1022-1030.

Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML, and Schreck CB. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. Aquaculture. 2006;258:565-574.

Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML, and Schreck CB. Rapid cortisol response of zebrafish to acute net handling stress. Aquaculture. 2009;297:157-162.

Ramsay JM, Watral V, Schreck CB, and Kent ML. Husbandry stress exacerbates mycobacterial infections in adult zebrafish, *Danio rerio* (Hamilton). J Fish Dis. 2009;32:931-941.

Ramsay JM, Watral V, Schreck CB, and Kent ML. *Pseudoloma neurophilia* (Microsporidia) infections in zebrafish (*Danio rerio*): Effects of stress on survival, growth and reproduction. Dis Aquat Org. 2009;88:69-84.

Readman GD, Owen SF, Murrell JC, and Knowles TG. Do fish perceive anaesthetics as aversive? PLoS ONE. 2013;8.

Rihel J and Schier AF. Behavioral screening for neuroactive drugs in zebrafish. Develop Neurobio. 2012;72:373–385.

Sanders JL, Watral V and Kent ML, Microsporidiosis in zebrafish research facilities. ILAR J. 2012;53:106–113.

Schartl, M. 2014. Beyond the zebrafish: diverse fish species for modeling human disease. Dis Model Mech. 7:181-192.

Schreck CB, Contreas-Sanchez W, and Fitzbatrick MS. Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture. 2001;197:3-24

Shima A and Mitani H. Medaka as a research organism: past, present and future. Mech of devel. 2004;121:599-604.

Singley JS and Chavin W. Serum cortisol in normal goldfish (*Carassius auratus L.*). Comp Biochem Physiol Part A Physiol. 1975;50:77-82.

Sisson M, Cawker J, Buske C, and Gerlai R. Fishing for genes of vertebrate behavior: zebra fish as an upcoming model system. Lab Anim. 2006;35:33–39.

Simpson SD, Purser J, and Radford AN. Anthropogenic noise compromises antipredator behavior in European eels. Glob Change Bio. 2014;21(2);586-593.

Smith ME, Kane AS, and Popper AN. Noise-induced stress response and hearing loss in goldfish (*Carassius auratus*). J Exp Biol. 2003;207:427-435.

Spagnoli S, Xue L, and Kent ML. The common neural parasite *Pseudoloma neurophilia* is associated with altered startle response habituation in adult zebrafish (*Danio rerio*): Implications for the zebrafish as a model organism. Behav Brain Res. 2015;291.

Spagnoli S, Xue L, Murray KM, Chow F, and Kent ML. *Pseudoloma neurophilia*: A retrospective and descriptive study with new implications for pathogenesis and behavioral phenotypes. Zebrafish. 2015;12:189-201.

Spence R. Zebrafish Models in Neurobehavioral Research. NeuroMethods. 2011;52:211–222.

Spence R, Gerlach G, Lawrence C and Smith C. The behaviour and ecology of the zebrafish, *Danio rerio*. Biol Rev Cambridge Philosoph Soc. 2008;83;13-34.

Spitsbergen JM and Kent ML. The state of the art of the zebrafish model for toxicology and toxicologic pathology research - advantages and current limitions. Toxicol Pathol. 2003;31(suppl.):62-87.

Steenbergen PJ, Richardson MK, and Champagne DL. The use of the zebrafish model in stress research. Prog neuro-psychopharm and biol psych. 2011;35:1432-1451.

Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., and Kalueff, A.V. 2012. Modeling anxiety using adult zebrafish: A conceptual review. Neuropharmacol. 62:135-143.

Stewart AM, Gerlai R, and Kalueff, AV. Developing higher throughput zebrafish screens for in-vivo CNS drug discovery. Front Behav Neurosci. 2015;9:1-8.

Swaim LE, Connoly LE, Volkman HE, Humbert O, Born DE, and Ramakrishnan L. *Mycobacterium marinum* infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. Infect Immun. 2006;74:6108-6117.

Takeda H and Shimada A. The art of medaka genetics and genomics: What makes them so unique? Ann Rev Gen. 2010;44:217-241.

Taylor AM and Zon LI. Zebrafish tumor assays: The state of transplantation. Zebrafish. 2009;6:339-346.

Tran S, Chatterjee D, and Gerali R. Acute net stressor increases whole-body cortisol levels without altering whole-brain monoamines in zebrafish. Behavioral Neuroscience. 2014;128:621-624.

Truong L, Harper SL, and Tanguay RL. Evaluation of embryo toxicity using the zebrafish model. Methods Mol Biol. 2011;691:271-279.

Tudorache C, Ter Braake A, Tromp M, Slabbekoom H, and Schaaf MJ. Behavioral and physiological indicators of stress coping styles in larval zebrafish. Stress. 2015;18:121-128.

Varga ZM. Aquaculture and husbandry at the zebrafish international resource center. Methods Cell Biol. 2011;104:453-478.

Valenzuela A, Campos V, Yanez F, Alveal K, Gutierrez P, Rivas M, Contreras N, Klempau A, Fernandez I, and Oyarzun C. Application of artificial photoperiod in fish: A factor that increases susceptibility to infectious diseases? Fish Physiology and Biochemistry. 2012;38:943–950.

Vignet C, Begout ML, Pean S, Lyphout L, Leguay D, and Cousin X. Systematic screening of behavioral responses in two zebrafish strains. Zebrafish. 2013;10:365–75.

Wedemeyer GA. Rearing conditions: effects on fish in intensive culture. 1997. In: Fish Stress and Health in Aquaculture. (Iwama, G.K., Pickering, A.D., Sumpter, J.P., and Schreck, C.B., Eds.) pp. 35-73. Cambridge University Press, New York.

Westerfield M. 2007. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), 5th Edition. University of Oregon Press, Eugene

White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, Borque C, Dovey M, Goessling W, Burns CE, and Zon LI. Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell. 2008;2:183–189.

Wilke L, Owen SF, Readman GD, Sloman KA, and Wislon RW. Does structural enrichment for toxicology studies improve zebrafish welfare? Appl Animal Behav Sci 2012;129:143-150.

Williams DE, Bailey GS, Reddy A, Hendricks JD, Oganesian A, Orner GA, Pereira CB, and

Swenberg JA. 2003. The rainbow trout (*Oncorhynchus mykiss*) tumor model: recent applications in low-dose exposures to tumor initiators and promoters. Toxicol Pathol. 31:58-61.

Wong D, von Keyserlingk MA, Richards JG, and Weary DM. Conditioned place avoidance of zebrafish (*Danio rerio*) to three chemicals used for euthanasia an\anaesthesia. PLOSone. 2014.

Wong K, Eegante M, Bartels B, Elkhayat S, Tien D, Roy S, Goodspeed J, Suciu J, Tan J, Grimes C, and Chung A. Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). Brain Res. 2010;208:450–457.

Figure 1. Cortisol levels in zebrafish related to density in adult zebrafish (adapted from Harper and Lawrence, 2010).