AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved:

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Zebrafish, *Danio* rerio, are widely used as vertebrate research organisms yet little is know about their responses to husbandry stressors. This dissertation examined the whole-body cortisol responses of zebrafish to husbandry stress. Additionally, the effects of stress on the susceptibility to mycobacteriosis and microsporidiosis (*Pseudoloma neurophilia*) were determined including effects on growth and reproduction. In Chapter 2, crowding increased cortisol but this was modulated by fasting and feeding; fasted but not fed fish elevated cortisol when crowded. Fish weight was inversely related to cortisol level in fasted, crowded fish. In Chapter 3, zebrafish rapidly responded to net handling stressors with a 6-fold increase in whole-body cortisol which peaked at 15 min post-net-stress. Cortisol recovered to control levels by 60 min. The variability in resting cortisol increased with fish age. In Chapter 4, fish infected with *Mycobacterium marinum* and subjected to husbandry stressors had 14% cumulative mortality over 8 weeks while no mortality occurred among infected fish without stress. Stressed fish, injected with *M. chelonae* were 15-fold more likely to be infected than non-stressed fish. Sub-acute, diffuse infections were more common among infected fish that were stressed than those not stressed. In Chapter 5, we examined the effects of stress on pre-existing and experimental *Pseudoloma* infections and the subsequent effects on survival, growth, and reproduction. Stress increased mortality and myositis in fish with pre-existing infections. There was no mortality among fish experimentally infected with *Pseudoloma* regardless of stress treatment. The onset of infection occurred sooner in stressed fish and stress

significantly increased the mean intensity of infection. Weight decreased in stressed and experimentally infected groups from week 13 to 20 post-exposure but not in the control group. Fecundity was negatively related to mean parasite area among *Pseudoloma*-infected and stressed fish. Whole-body cortisol is an indicator of crowding and handling stress in zebrafish. Husbandry stressors exacerbated mycobacteriosis and microsporidiosis in zebrafish with associated reductions in growth and fecundity. Zebrafish are hardy and well adapted to a laboratory environment. Nevertheless, reducing stress should aid in preventing disease outbreaks and maintaining growth and fecundity of this popular laboratory fish species.

Husbandry Stress of Zebrafish, *Danio rerio*

by
Jennifer Mara Ramsay

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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.

Jennifer Mara Ramsay, Author

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CONTRIBTUION OF AUTHORS

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DEDICATION

I dedicate this dissertation to my late grandmother, E. Marjorie Ramsay, who asked me questions from a very young age and encouraged me to do the same. I have been inspired by her strength and creativity, and her love of learning and the natural world.

Small fish in a dying lake

Gasping for breath

What have we done?

- E. Marjorie Ramsay, 1991-

CHAPTER 1: GENERAL INTRODUCTION

There has been increasing concern about husbandry stress, infectious diseases, and reproductive fitness of laboratory fishes, including zebrafish, *Danio rerio* (Lawrence 2007; Markovich et al. 2007; Kent et al. in press). There is a paucity of information regarding the response of zebrafish to husbandry stressors and the subsequent effects on susceptibility to diseases and fitness. Many of the assumptions regarding stress, disease, and reproduction in zebrafish are borrowed from other fish species and often mammalian laboratory animals (Baker 2003; Kent et al. in press). The hardy nature of zebrafish, which has greatly contributed to its success as a research model, has perhaps delayed the examination of husbandry stress in this species. Nevertheless, husbandry stress may affect disease prevalence and reproduction and serve as a potential source of variation during experimentation. It was the goal of this dissertation to examine the response of laboratory zebrafish to husbandry stressors and the subsequent effects on disease susceptibility and reproductive fitness.

Zebrafish

Zebrafish are cyprinids native to the Indian subcontinent and were first described by Hamilton (1822) in his survey of fishes of the Ganges River. The domestication of zebrafish began over a century ago. Zebrafish were first imported to Europe for the aquarium trade in 1905 and gained popularity in the 1930s as a valuable experimental and teaching organism (Creaser, 1934). Subsequently, zebrafish were used in physiological studies, particularly in breeding experiments (Eaton and Farley, 1974; Laale, 1977). Dr. George Streisinger developed zebrafish as a model to study the genetics of vertebrates (Streisinger 1981).

Over the past 30 years, zebrafish have been widely used as vertebrate research organisms, primarily for developmental genetics, and increasingly for toxicological and environmental monitoring, cancer, aging, behavioral, and disease studies (Kishi 2004; Trede et al., 2004; Parng 2005; Wright et al. 2006; Beckman 2007, Scholz and Mayer 2008). The small size, high fecundity, transparent embryos, rapid development, and ease of culture in the laboratory have made zebrafish highly favorable research models

(Westerfield 2007; Matthews et al. 2002). Availability of the whole genome sequence and numerous mutant lines facilitates studies of development and physiology (Mullins 2002; Phelps and Neely 2005). Similarities between zebrafish and mammalian genetics suggest that the zebrafish is an excellent vertebrate model of human developmental and disease processes (Fishman 2001; Epstein and Epstein 2005). In addition, zebrafish may prove to be a useful genetic model for aquaculture species (Dahm and Geisler 2006).

Despite the wide use of zebrafish in research, little is known about the optimal rearing environment for this species. Poor environmental conditions have been implicated in disease outbreaks and reproductive impairment at zebrafish facilities (Matthews 2004; Lawrence 2007; Westerfield 2007). However, the roles of specific husbandry parameters have not been adequately assessed, suggesting a further need to measure and elucidate how husbandry stressors affect zebrafish health and fitness (Lawrence 2007).

Biological Stress

The concept of biological stress was first introduced by Hans Selye who described it as *the non-specific responses of the body to any demand* (Selye 1936; 1950). The idea for stress arose from the concept of homeostasis: a relatively constant steady state maintained within certain tolerable limits (Cannon 1929) and the concept of a *milieu intérieur* or internal environment of the body in which cells are nourished and maintained in a state of equilibrium (Bernard 1865). Since its original introduction, the concept of stress has evolved and our ideas about stress continue to change. More recently stress has been described as *the physiological cascade of events that occurs when an organism is attempting to resist death or re-establish homeostatic norms in the face of a perceived threat* (Schreck et al. 2001).

Stress of Fishes

The stress response of fishes is similar to that of other vertebrates (Wendelaar Bonga 1997). Three phases of the stress response have been described by the General Adaptation Syndrome paradigm (Selye 1950), including alarm, resistance, compensation and recovery or exhaustion and death. The initial neural response to stress involves the production of catecholamines, including epinephrine and norepinephrine, as a primary

alarm response to a perceived threat. Catecholamines produce secondary effects such as increased respiratory and cardiovascular function and the mobilization of energy resources (Reid et al. 1998).

Cortisol, the primary corticosteroid of fishes, is produced in primary response to a stressor and is widely used as an indicator of stress in fishes (Schreck 1981; Wendelaar Bonga 1997; Barton 2002). Control of the secretion of cortisol is a complex process which is regulated by the hypothalamic-pituitary interrenal (HPI) axis (Yao and Denver 2007). Sensory information is translated to neural pathways which converge in the pre-optic area of the hypothalamus eliciting the production of the neurohormone corticotropin-releasing hormone (CRH). CRH elicits production of adrenocorticotropin (ACTH) by the anterior pituitary which, in turn, elicits production of cortisol from the interrenal tissue of the kidney (Flik et al. 2006). Cortisol circulates in the blood, and binds to receptors in various tissues to mobilize energy, and modulate hydromineral and immune balance (Takei and Loretz 2006; Bury and Sturm 2007).

Regulation of the HPI axis is achieved by negative feedback. Cortisol binds to receptors in the pituitary and hypothalamus to inhibit production of ACTH and CRH while ACTH feeds back on the hypothalamus to inhibit CRH production. Additionally, cortisol may also feedback on the interrenal to inhibit further cortisol production (Bradford et al. 1992). Further control of cortisol secretion and may be achieved by adrenergic pathways (catecholamines; Reid et al. 1998). Negative feedback is also controlled by neural pathways and gene expression but much of what controls cortisol secretion is still largely unknown for fishes (Alsop and Vijayan 2008).

Circulating cortisol is typically measured to determine stress levels in individual fish (Barton 2002). Whole-body cortisol has been measured in developmental stages of fishes due to inadequate blood volume for accurate analyses of circulating cortisol (de Jesus et al. 1991; Pottinger and Mosuwe 1994; Barry et al. 1995; Feist and Schreck 2002). Whole-body corticosteroids have also been measured in smaller adult fishes including, three-spined sticklebacks, *Gasterosteus aculeatus* (Pottinger et al. 2002), as well as zebrafish (Pottinger and Calder 1995).

Cortisol is associated with the alarm and resistance phases of the stress response and has a broad spectrum of secondary effects in fish, particularly in energy mobilization

as well as in the regulation of hydromineral and immune balance (Schreck 1996; Barton 2002; Dhabhar 2002). Under conditions of short-term or acute stress, compensation or recovery may occur as the fish adapts to the stressor (Schreck 1981). However, the tertiary effects of chronic elevation of cortisol include reduced growth rate, impaired reproduction and suppressed immune function (Schreck 1996; Schreck et al. 2001; Barton 2002). Additionally, altered behavior, such as decreased learning and predator avoidance and impaired migration, may occur as a result of exposure to stressors. Although a stress response is necessary to enable an organism to adapt and overcome the threat, under conditions of chronic stress, the adaptive value of the stress response is typically lost, as indicated by reduced health and fitness (Schreck 2000).

Diseases of Zebrafish

Transmission of pathogens and exacerbation of diseases in fish populations involves a dynamic interplay of host, pathogen, and environmental factors (Reno 1998). Diseases typically emerge in aquaculture with increases in production and trade of the cultured species between farms or laboratories (Murray and Peeler 2005). Increased stocking density increases contact between members of a cohort and facilitates the transmission of pathogens. Furthermore, crowding, handling, transport during breeding, grading, vaccination, and experimental manipulation are associated with increased stress in cultured fishes (Schreck 1981, 2000).

Stress and elevated cortisol levels have been demonstrated to exacerbate diseases of fishes. Cultured fishes are susceptible to infectious diseases, the severity of which is often associated with environmental or husbandry stressors (Meyer 1991). There are currently no effective treatments for the most common diseases of laboratory zebrafish. In addition, stress is believed to exacerbate disease in zebrafish colonies (Matthews 2004) but this has not been empirically examined. Better understanding husbandry stressors and diseases of zebrafish may prove to be an effective means of control.

There are several diseases which affect laboratory zebrafish. Opportunistic environmental bacteria, ciliates, and dinoflagellates can cause dermal and systemic infections (Matthews 2004). Additionally, intestinal capillarid nematodes (*Pseudocapillaria tomentosa*) have been associated with chronic wasting and neoplasms

of the intestine (Kent et al. 2002). Water moulds and fungi may also present disease problems in poorly managed facilities (Matthews 2004).

The two most common and problematic diseases of zebrafish are mycobacteriosis and microsporidiosis both of which have been associated with chronic progressive disease, although fish often appear clinically healthy (Astrofsky et al. 2000; Matthews et al. 2001; Kent and Bishop-Stewart 2003; Kent et al. 2004). Chronic diseases in other laboratory animals that do not cause clinical disease have been demonstrated to alter host physiology and affect research results (Baker 2003). Similar chronic infections in food fishes may be acceptable as long as mortality is low and growth not affected; however, such diseases in laboratory fishes, such as zebrafish, may alter the physiology of the host and introduce variation into experiments (Kent et al. in press). It is essential to better understand how zebrafish physiology is affected by chronic diseases and what role stress plays in this dynamic interaction.

Mycobacteriosis

Mycobacteriosis commonly affects both wild and cultured fishes (Decostere et al. 2004). *Mycobacterium marinum, M. chelonae, M. abscessus*, and *M. fortuitum* are the most common species of mycobacteria infecting fishes (Chinabut 1999). Chronic proliferative mycobacteriosis is characterized by the formation of granulomas, while sub-acute and acute forms of the disease are associated with necrosis and acid-fast bacilli scattered diffusely among the kidney, liver, spleen, and often all visceral organs (Ferguson 2006). Often no external signs are present until advanced stages of the disease occur (Whipps et al. 2008), at which time non-specific signs present including emaciation, hemorrhagic and dermal lesions, lethargy, and death (Gauthier and Rhodes in press).

Infection of zebrafish with *M. marinum* isolates has resulted in 100% infection with mortality between 30 and 100% whereas infection with *M. chelonae, M. peregrinum*, or *M. abscesuss* isolates resulted in low to moderate infection prevalence with negligible mortality (Watral and Kent 2007). *Mycobacterium haemophilum* has been identified as the causative agent of outbreaks in zebrafish facilities resulting in high mortality (Whipps et al. 2007). Conversely, *M. chelonae* usually causes minimal

mortality in well-maintained laboratories, even when fish exhibit histological changes and a relatively high prevalence of infection (Whipps et al. 2008). Although differences in the virulence and pathogenicity of *Mycobacterium* spp. play a significant role in subsequent outbreaks of disease (Watral and Kent 2007) the rearing environment may also be important.

Stress has been demonstrated to suppress the immune response in animal models for mycobacteria. The acute inflammatory response of autoimmune-prone mice infected with *M. avium* was reduced during swimming stress (Martins and Águas 1995). Restraint stress impaired the activation of T-cells in mice with mycobacterial infections (Zwilling et al. 1992). Host resistance to *M. bovis* was reduced in hamsters with increased serum cortisol levels (Righi et al. 1999; Palermo-Neto et al. 2001). Zebrafish *rag1* mutants, lacking fully functional T and B cells, were hyper-susceptible to infection with *M. marinum*, failing to control bacterial growth (Swaim et al. 2006).

Many *Mycobacterium* species are ubiquitous in the aquatic environment, making control by avoidance of these pathogens very difficult. Furthermore, there is no effective treatment for mycobacteriosis in zebrafish. A better understanding of the pathogenesis of *Mycobacterium* spp., including factors affecting host susceptibility, may enable zebrafish researchers to manage this pathogen and prevent potential disease outbreaks through effective husbandry practices.

Microsporidiosis

Microsporidia are spore-producing obligate intracellular fungal parasites infecting a wide variety of animals including protozoa, fishes, birds, and mammals (Canning and Lom 1986; Hirt et al. 1999; Shaw and Kent 1999; Didier and Weiss 2006). Approximately 150 species of microsporidia are infective to fishes with a wide distribution in freshwater and marine habitats (Lom and Nilsen 2003). The spores of microsporidia are quite resistant and capable of surviving outside the host for weeks and often months (Amigó et al. 1996; Shaw et al. 2000a).

Transmission of fish microsporidia can occur via ingestion of infected fish tissue containing spores, cohabitation with infected individuals, or vertically from parent to offspring (Shaw et al. 1998; Speare et al. 1998; Phelps and Goodwin 2008). After

ingestion, spores swell during germination in the gut and a polar tubule, everted from each spore, pierces the host gut epithelium and the infective sporoplasm passes directly into the host cell cytoplasm (Cali and Takvorian 1999). The parasite undergoes developmental stages known as merogony, during which the parasite increases numbers, and sporogony, during which resistant spores are produced (Canning and Lom 1986).

Spores may be contained within host-parasite complexes known as xenomas (Chatton 1920). Xenoma formation is characterized by hypertrophy of the host cell and the formation of granulomatous tissue (Dyková 1995; Shaw and Kent 1999). Upon completion of the life-cycle of the parasite, the xenoma ruptures releasing spores into the tissue which may be ingested and destroyed by phagocytes but are often released into the environment to infect susceptible hosts (Canning and Lom 1986). Hyperplasia of tissue surrounding the xenoma may also occur. Tissue repair may occur but the original function of the tissue is often not restored.

Xenoma-forming microsporidia which infect fish include the genera *Glugea*, *Loma*, *Tetramicra*, and *Pseudoloma* (Shaw and Kent 1999; Matthews et al. 2001). Microsporidian infections may restrict fish growth and limit productivity in fisheries and aquaculture (Dyková 1995). Significant mortality from microsporidiosis may occur in environments which favor transmission, such as aquaculture (Shaw and Kent 1999). Microsporidiosis is becoming an increasing problem in laboratory fishes, including zebrafish, as their culture increases.

Microsporidiosis of zebrafish

Microsporidiosis was first reported in zebrafish almost 30 years ago (de Kinkelin 1980), and this microsporidium was recently assigned to a new genus and species, *Pseudoloma neurophilia* (Matthews et al. 2001), which we henceforth refer to in this chapter as *Pseudoloma*. Microsporidiosis is the most common disease of laboratory zebrafish, affecting the central nervous system and somatic muscle, and is associated with emaciation, spinal deformity, and morbidity (Matthews et al. 2001; Kent and Bishop-Stewart 2003). The spores of *Pseudoloma* are contained within xenomas and infections are characterized by multiple xenomas in the hind brain, spinal cord, nerve roots, and occasionally within the somatic muscle (Matthews et al. 2001). Free spores are found

within phagocytes and are associated with severe, chronic myositis, meningitis, and encephalitis (Matthews et al. 2001; Kent and Bishop-Stewart 2003). Ovarian tissue is often infected and occasionally eggs may harbor *Pseudoloma* spores indicating potential for vertical transmission (Kent and Bishop-Stewart 2003).

Stress has been implicated in the exacerbation of microsporidiosis in zebrafish but has yet to be examined. In other animal models exposure to corticosteroids generally increases the intensity of microsporidiosis. Nude rats, *Rattus norvegicus*, and gerbils, *Meriones unguiculatus*, infected with *Enterocytozoon bieneusi*, and given the synthetic corticosteroid, dexamethasone (DEX), produced more spores than infected animals given a placebo (Feng et al. 2006). Clinical signs of encephalitozoonosis were associated with reduced antibody titers and only apparent in infected mice treated with DEX (Lallo et al. 2002; Herich et al. 2006). In turbot, *Scophthalmus maximus*, injected with *Microgemma caulleryi*, DEX treatment reduced the ability of macrophages and neutrophils to ingest spores (Leiro et al. 2000). *Loma salmonae*–infected rainbow trout, *Oncorhynchus mykiss*, had higher infection intensities when treated with DEX (Lovy et al. 2008).

There is currently no effective treatment for *Pseudoloma*. Identifying factors affecting the exacerbation of *Pseudoloma* infections may aid in controlling outbreaks of disease in this intensively cultured biomedical research model (Reno 1998; Lawrence 2007).

Stress, Disease, and Fish Reproduction

In addition to increasing susceptibility to disease, stress also affects the reproduction of captive fishes (Brooks et al. 1997). Developmental biology is the cornerstone of zebrafish research and a consistent supply of good quality eggs is fundamental to a productive zebrafish laboratory (Lawrence 2007; Westerfield 2007). Stress has been implicated when breeding is poor or during high mortality of embryos. However, the effects of stress on zebrafish reproductive fitness have not been well documented.

Stress has a variable effect on reproduction, which is dependent upon numerous factors, including the species, gender, reproductive strategy, and the nature of the stressor (Schreck et al. 2001; Wingfield and Sapolsky 2003). Rainbow trout and brown trout,

Salmo trutta, subjected to chronic crowding stress in the months prior to spawning had increased plasma cortisol and produced smaller eggs and offspring with reduced survival (Campbell et al.1994). Increased maternal cortisol in damselfish, *Pomacentrus amboinensis*, was associated with reduced larval size (McCormick 1998, 2006). The timing of stressors is important in determining the effects on reproduction. Rainbow trout subjected to random stressors during late maturation or during the entire maturation period ovulated 2 weeks earlier than control fish with no significant effects on fecundity or fertilization (Contreras-Sánchez et al. 1998). Stress during early vitellogenesis resulted in smaller eggs and fry but there was no significant difference in juvenile weight.

Fish pathogens have also been demonstrated to reduce the reproductive fitness of fishes. A negative relationship between ovum mass and the intensity of cestode infection has been demonstrated for sticklebacks, *Gasterosteus aculeatus* (Heins and Baker 2003). Among microsporidia, infections are associated with failure to spawn, a reduction in early-life fecundity, and an inability to produce viable offspring (Summerfelt and Warner 1970; Wiklund et al. 1996; Futerman et al. 2005). Sometimes pathogenic infections have no effect on reproductive fitness (Candolin and Voigt 2001) or may result in early maturation and spawning (Haine et al. 2004). This suggests that host reproduction may be altered to facilitate vertical or horizontal transmission to other susceptible hosts.

The impact of disease on zebrafish reproduction has not been examined. Both mycobacteria and microsporidia affect the ovaries of fish suggesting that the reproduction of zebrafish may be affected by these diseases (Matthews 2004). Furthermore, the energetic cost of combating infection may leave insufficient resources for reproduction (Schreck 1996; Schreck et al. 2001). In combination with husbandry stress, disease may significantly impact the reproductive potential of zebrafish.

Aims of Research

Prior to initiating this dissertation research a single study had examined the zebrafish cortisol response to handling and transport stress (Pottinger and Calder 1995). There was a lack of information on the role of husbandry stress on infectious diseases of laboratory zebrafish. Furthermore, it was not know how stress and infectious diseases affected zebrafish reproduction.

This dissertation was designed to better understand the nature of husbandry stress with respect to the whole-body cortisol response, the susceptibility to infectious diseases, and the reproductive fitness of laboratory zebrafish. The overall goal of this dissertation was to draw together the following 3 specific aims of the research in order to recommend management suggestions which will optimize zebrafish husbandry and minimize the impacts of disease while ensuring optimal growth and reproductive fitness.

The specific aims of this research were:

1) To determine the whole-body cortisol response of adult zebrafish to acute stressors (i.e., handling) and chronic stressors (i.e., tank density), and their relationships with feeding regimen, fish weight, and gender

2) To determine the effects of husbandry stressors on mycobacteriosis caused by virulent (*M. marinum*) and non-virulent (*M. chelonae*) species in adult zebrafish

3) To determine the sequential development of *Pseudoloma* infections in stressed and non-stressed zebrafish, including the effects of the infection, stress, and stress combined with infection, on mortality, infection intensity, growth, and reproduction

CHAPTER 2: WHOLE-BODY CORTISOL IS AN INDICATOR OF CROWDING STRESS IN ADULT ZEBRAFISH, *DANIO RERIO*

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Abstract

Plasma cortisol levels have been used to evaluate the stress response in a variety of cultured fish species. However, little is known about the stress response of zebrafish, *Danio rerio*, despite its extensive use as a laboratory research organism. Due to its small size, evaluation of whole-body cortisol has provided a means to assess zebrafish stress levels. Understanding the role of crowding on zebrafish whole-body cortisol would allow researchers to optimize various rearing procedures. The aim of this study was to determine the zebrafish whole-body cortisol response to crowding stress. Baseline cortisol values have not been established for zebrafish; therefore, we compared specific treatment groups acclimated to similar conditions. We crowded zebrafish at a high density (40 fish/L) for 3 h (acute stress), or 5 days (chronic stress). Crowding resulted in a four-fold increase in mean whole-body cortisol level in both groups compared to zebrafish maintained at a much lower density (0.25 fish/L). Additional experiments demonstrated that the cortisol response to crowding was modulated by fasting, feeding, and density. In large glass aquaria (76 L), fasted, crowded fish (40 fish/L) had significantly higher cortisol compared to fasted, control fish (0.2 fish/L). Furthermore, weight was inversely related to cortisol level in fasted, crowded fish held in large glass aquaria. For fed fish, crowding did not significantly increase cortisol level, suggesting an interaction between feeding and crowding. In small tanks (4 L), crowding (40 fish/L) did not significantly increase cortisol compared to control fish held at 4 fish/L. Our results suggest that whole-body cortisol is a useful indicator of crowding stress in fasted, adult zebrafish. Understanding how crowding and other environmental conditions affect zebrafish fitness could aid in optimizing zebrafish growth, health, and reproduction as well as improving the consistency and reproducibility of *in vivo* studies that use this popular vertebrate model.

Keywords: Cortisol; Crowding; Density; Feeding; Husbandry; Stress; Zebrafish

Introduction

Successful fish culture depends upon providing a rearing environment suitable to the species of interest (Colt, 1991; Klontz, 1995). Suboptimal conditions may result in chronic stress in fish culture facilities (Schreck, 1981, 2000; Barton and Iwama, 1991). Understanding how the rearing environment affects the stress response of cultured fishes is critical for optimizing production and maintaining health and welfare (Pickering, 1992; Schreck, 2000; Ellis et al., 2002).

The stress response has been described for numerous fish species (Schreck, 1981, 2000; Barton and Iwama, 1991; Wendelaar Bonga, 1997; Barton, 2002). Stress has been defined as a physiological cascade of events that occurs when an individual attempts to re-establish homeostatic norms in the face of a perceived threat (Schreck et al., 2001). The immediate response to stress is believed to be adaptive, allowing the organism to respond to the perceived threat, whereas chronic stress is considered to be maladaptive, with impacts on growth, reproduction, and the immune response (Maule et al., 1989; Pickering, 1992; Schreck et al., 2001). Stress also has implications for fish welfare, a growing concern for national, regional, and institutional animal care and use organizations (Ellis et al., 2002; Erickson, 2003).

The steroid hormone cortisol is widely accepted as an indicator of stress in fish generally increasing after exposure to physical stressors (Schreck, 1981; Barton and Iwama, 1991; Wendelaar Bonga, 1997; Barton, 2002). Handling and severe crowding greatly increase the cortisol levels of numerous fish species (Strange et al., 1978; Redding and Schreck, 1983; Vijayan et al., 1997; Arends et al., 1999). Fish crowded at moderate densities typically respond with smaller but significant elevations in cortisol (Schreck et al., 1985; Patiño et al., 1986).

Circulating cortisol levels are typically measured to determine the stress condition of an individual fish (Redding et al., 1984; Montero et al., 1999). Alternatively, whole-body cortisol levels have been used to assess the stress response of developing salmonids and flatfish because blood volumes are insufficient to provide measurements of circulating cortisol (de Jesus et al., 1991; de Jesus and Hirano, 1992; Pottinger and Mosuwe, 1994; Barry et al., 1995; Feist and Schreck, 2002). Similarly, whole-body corticosteroids have been measured in smaller adult fishes including, three-spined

sticklebacks, *Gasterosteus aculeatus* (Pottinger et al., 2002), as well as zebrafish, *Danio rerio* (Pottinger and Calder, 1995).

Environmental parameters, including temperature, time of day and feeding regimen, have been shown to affect the cortisol response to crowding and other stressors (Barton et al., 1988; Lankford et al., 2003; Davis, 2004). In the absence of other stressors, fasting of fish may result in increased (Peterson and Small, 2004), decreased (Barton et al., 1988) or unchanged (Czesny et al., 2003; Pottinger et al., 2003) cortisol levels depending upon species. Different feeding regimens may alter the cortisol response to stressors. Feeding gilthead sea bream, *Sparus aurata*, high levels of arachidonic acid reduced the cortisol response to crowding (Van Anholt et al., 2004). Similarly, vitamin E supplementation reduced density-dependent cortisol increases in the teleost, *Piaractus mesopotamicus* (Belo et al., 2005). Feeding regimen should be considered in stress studies, particularly when little is known about the stress response of the species being examined (Schreck, 1981, 1993, 2000).

Zebrafish are used extensively for studies of vertebrate genetics, development, and physiology (Grunwald and Eisen, 2002; Rubinstein, 2003). Zebrafish have been favored as research models due to their small size, high fecundity, transparent embryos, rapid development, and ease of culture in the laboratory (Westerfield, 2000; Matthews et al., 2002). Availability of the whole genome sequence and numerous mutant lines facilitates studies of development and physiology (Mullins, 2002; Phelps and Neely, 2005). Similarities between zebrafish and mammalian genetics suggest that the zebrafish is an excellent vertebrate model of human developmental and disease processes (Fishman, 2001; Epstein and Epstein, 2005).

Despite the wide use of zebrafish in research, little is known about the optimal rearing environment for this species. Poor environmental conditions, including poor water quality, overcrowding, high stress, and poor diet, have been implicated in disease outbreaks at zebrafish facilities (Matthews, 2004). Interestingly, the role of these environmental parameters in the etiology of disease has not been well examined. Poor water quality, including hypoxia and exposure to estrogenic compounds, has been demonstrated to impair both reproduction and development of zebrafish (Nash et al., 2004; Shang and Wu, 2004); however, the roles of specific husbandry parameters have

not been adequately assessed. The stress response of zebrafish has not been characterized, although there have been suggestions that reducing stress will improve production (Westerfield, 2000; Matthews et al., 2002; Matthews, 2004).

To date, a single study has examined physiological stress and whole-body corticosteroids in zebrafish during toxicological procedures (Pottinger and Calder, 1995). Whole-body corticosteroids were found to be a suitable indicator of transport stress; zebrafish corticosteroids rapidly declined within 1 h of transport but increased after periodic netting during a 96-h semi-static test procedure (Pottinger and Calder, 1995). The effects of crowding on zebrafish whole-body cortisol levels have not been determined. Better understanding how crowding and other husbandry parameters affect the whole-body cortisol levels of zebrafish will provide further insight into rearing stress in this widely used fish model.

The primary aim of this study was to determine the whole-body cortisol response of adult zebrafish to crowding stress. A secondary aim was to determine if laboratory setting, tank density, feeding regimen, fish weight and gender affected whole-body cortisol level. We tested the specific hypotheses that: (1) crowding increases whole-body cortisol and (2) laboratory setting, density, feeding, weight, and gender modulate the cortisol response to crowding.

Methods

Zebrafish (adult, wild-type, AB strain), aged 9 to 12 months, were provided by the Zebrafish International Resource Center (ZIRC) at the University of Oregon in Eugene, Oregon, USA and were maintained at 28– 29 °C.

Experiment 1: conducted at the Fish Performance and Genetics Laboratory

Ninety fish (two-thirds male, one-third female) were transported from the ZIRC to the Fish Performance and Genetics Laboratory (FPGL) at Oregon State University (OSU) in Corvallis, Oregon, USA. Fifteen fish were randomly allocated to each of six tanks (60 L) in a 6000-L recirculated freshwater system, equipped with large capacity rotating drum filters (10% freshwater exchange/day), and maintained at 28–29 °C; ammonia (0 mg/L), nitrite (0 mg/L) and pH (~7) were measured daily. Fish were acclimated for a

period of 7days and fed to satiation once daily withTetramin Tropical Flakes aquarium fish food (Tetra Holding U.S. Inc., Blacksburg, VA, USA). Photoperiod was 14-h light/10-h dark.

The crowding stressor consisted of brief netting and crowding of fish in modified, roughly cube-shaped, acrylic boxes (~ 500 mL volume) which were subsequently placed into the acclimation tanks. The aim of this study was to test the effects of crowding, not poor water quality. Therefore, to ensure the water quality of the crowded group was similar to that of the noncrowded (control) groups, numerous 3 mm holes were drilled in the walls of the boxes to allow water to flow through; the rough edges of the holes were sanded to prevent injury of the fish. The effective volume of the boxes was 375 mL resulting in a density of 40 fish/L. The control density was 15 fish in the 60-L acclimation tanks (0.25 fish/L). Control fish were not handled (netted) after acclimation to minimize stress and cortisol response (Schreck, 1981).

We used replicate tanks for each of two crowding durations: an acute crowding period of 3 h or a chronic crowding period of 5 days (Table 2.1). These crowding durations were chosen based on previous crowding studies done with other teleosts (Schreck et al., 1985; Barton et al., 1988; Vazzana et al., 2002). We sampled one control tank at each of the 3-h and 5-day sampling times. We stopped feeding all groups, including the controls, the day before crowding began; the fish were not fed during the experiment. We sampled control fish by dip netting them from the 60-L tank. Sampling time for all control fish was less than 30 s to minimize a possible stress response (increased cortisol) from netting (Schreck, 1981). We sampled the crowded fish by lifting the crowding boxes out of the 60-L tank. Crowded and control fish were immediately euthanized with an overdose of buffered tricaine methane sulfonate (500 mg/L MS-222). Individual fish were blotted on paper towels to remove excess water, immediately frozen in liquid nitrogen for 10–30 s, and placed in individual 2.0 mL plastic, screwcap centrifuge tubes. The samples were stored at −80 °C until we extracted the cortisol. All procedures were carried out in accordance with the Institutional Animal Care and Use Committee at Oregon State University (LAR# 2446).

Experiment 2: conducted at the Zebrafish International Resource Center

Experiment 1 was repeated at the ZIRC to validate the use of whole-body cortisol as an indicator of crowding stress in a zebrafish research facility. The ZIRC employs a large recirculated freshwater system (~37 000 L; 10% freshwater exchange/day), filtered through large sand filters and biofilters, with consistently monitored water quality parameters; ammonia (0 mg/L), nitrite (0 mg/L), nitrate (5 mg/L), pH (7.2– 7.4); dissolved oxygen (7 mg/L) (D. Lains, personal communication). Repeating the experiment under different husbandry conditions allowed us to compare the responses of the same genetic strain reared in two different environments and to assess the general applicability of whole-body cortisol as a stress indicator for zebrafish.

To examine how crowding in different tank types and volumes affects whole-body cortisol response, we made a few modifications to the methods used in Experiment 1. We used clear, rectangular, 4 L plastic tanks with an effective volume of 3.7 L (Thoren Aquatics model 91007) and 76 L glass aquaria, both of which are commonly used in zebrafish research laboratories (Westerfield, 2000). Crowding was achieved using the same small acrylic boxes used in the Experiment 1. Replicate crowded and control fasted 4 L tanks were used (tank n=4). Both fasted and fed replicate control and crowded 76 L tanks were used (tank n=8) (Table 2.1).

Fifteen fish were randomly allocated to each tank and acclimated (9 days) prior to beginning the experiment. Fish were fed twice daily (Tetramin Tropical Flakes and brine shrimp nauplii, *Artemia* sp.) during acclimation according the ZIRC feeding protocols (Westerfield, 2000). Roughly equal numbers of male and female, 9–12-month-old AB wild-types were randomly allocated to each tank for a total of 15 fish per tank. The gender of each fish was recorded upon sampling to determine whether there were any gender-specific effects on cortisol levels.

We included fed and fasted control and crowded treatment tanks to measure the effect of feeding on whole-body cortisol (76 L aquaria only). Fed groups were fed brine shrimp twice daily. Crowded fish were fed brine shrimp via a tube placed into the crowding container. Feeding was ceased the day before sampling. We used replicate tanks of each treatment combination for a total of 12 tanks (Table 2.1). A total of 180 fish were used for Experiment 2.

Sampling of fish, including euthanasia and freezing, was performed as described in Experiment 1. Samples were transported on dry ice to OSU and stored at −80 °C until analyzed. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Oregon (Protocol # 03-01R).

Whole-body cortisol extraction

The term whole-body cortisol was used to describe the portion of corticosteroid extracted and measured by a cortisol specific radioimmunoassay (RIA). It is possible that the cortisol antibody may be recognizing other cortisol metabolites in the whole-body fraction that have not yet been identified (Pottinger et al., 1992). Nevertheless, we feel confident based on previous studies that the majority of the corticosteroid recognized by the assay was cortisol. Thus, we have chosen the term “whole-body cortisol” as a descriptor (Redding et al., 1984; Feist and Schreck, 2002).

Whole-body cortisol was extracted using a modification of a method used with Chinook salmon, *Oncorhynchus tshawytscha*, eggs and embryos (Feist et al., 1990 as modified by Feist and Schreck, 2002). Individual whole, frozen zebrafish were thawed, individually weighed, and placed in a glass test tube (15×85 mm). Each fish was homogenized in deionized water (0.5 mL) for 45–75 s using a Tissue Tearor (BioSpec Products, Inc. model: 985370-04). Homogenate on the Tissue Tearor probe was rinsed into the sample tube with an additional 0.5 mL aliquot of deionized water. The homogenized samples were vortexed briefly and placed on ice. The probe was cleaned between samples with distilled water, 100% ethanol, and then rinsed again with distilled water. The homogenized contents of the glass test tube were transferred to a larger test tube (16×125 mm) to facilitate extraction. The original tube was rinsed with diethyl ether (Mallinckrodt Baker, Inc. 0848-10) which was transferred to the larger tube for subsequent extraction.

Each sample was extracted twice with 8 vol. (8 mL) of diethyl ether. For each extraction, tubes were vigorously vortexed with ether for 30 s and briefly centrifuged (2700 rpm for 2–3 min) to separate the aqueous and ether layers. The bottom layer (aqueous homogenate) was frozen in liquid nitrogen for 20 s and the top ether layer was poured into a new test tube (16×100 mm) and dried in a Speed-Vac centrifuge

(Savant Instruments, Inc. SVC100H). Extraction efficiencies were determined for each extraction by adding tritiated cortisol [hydrocortisone ([1,2,6,7-3H]-Cortisol; Dupont NEN #NET-396)] to homogenized samples (n=4) and extracting the samples using the above methods. All sample cortisol values were corrected for extraction efficiency. Following the second extraction, the samples were dried in a fume hood overnight, reconstituted with 1 mL phosphate-buffered saline with 1% gelatin (PBS-G), vortexed for 30 s, and stored at 4 °C for at least 24 h (not more than 96 h) before measurement of cortisol.

The average extraction efficiencies of cortisol from whole zebrafish ranged from 75% to 78%. This was similar to the extraction efficiencies obtained by other studies examining whole body cortisol levels in Chinook salmon (Feist and Schreck, 2002), and chum salmon, *Oncorhynchus keta* (de Jesus and Hirano, 1992).

Cortisol radioimmunoassay

Cortisol in the whole-body extracts was measured using an RIA from the method of Redding et al. (1984), as modified by Feist and Schreck (2002). Known cortisol concentrations (Sigma 4001) ranging from 3.125 pg/tube to 1250 pg/tube were used to create a standard curve to which sample cortisol values could be compared. Replicates of each sample were measured to give an average sample cortisol concentration. The cortisol concentrations obtained from the RIAs were validated by verifying that serial dilutions of sample extracts matched the standard curve. Whole-body extracts spiked with known high and low concentrations of cortisol were measured to verify consistency between RIAs. Intra-assay and inter-assay variation was accepted at no more than 10%. The cross-reactivity of the cortisol antibody with cortisone was 3.3% (determined from previous studies).

The detection limit of the cortisol RIA was 6.25 pg/tube. The majority of the samples were well above the detection limit but several of the control fish had cortisol values below the detection limit. Fish below detection limits were assigned the detection limit value (6.25 pg); this equated to between 0.7 and 2.0 ng cortisol per gram of fish weight. The optimal volume of whole-body extract for the RIA was determined to be 25 μL from a 1-mL suspension. The raw cortisol values (pg/tube) were corrected for

extraction efficiency, dilution volume, and weight of the fish. The average weights of the thawed fish were 301 mg (±6.5 mg) for Experiment 1 and 309 mg (±6.9 mg) for Experiment 2.

Statistical analyses

Statistical analyses were performed using a statistical software program (S-Plus 2000, MathSoft Inc., Seattle WA, USA). Replicates of each treatment were compared using Student's t-tests. Treatment replicates were pooled if there was no significant difference. For Experiment 1, treatments were compared using an analysis of variance (ANOVA) with post-hoc Fisher's LSD. For Experiment 2, treatments in the 76-L aquaria were also compared using an ANOVA (Fisher's LSD); control and crowded fish in the 4-L tanks were compared using a Student's t-test. Non-parametric tests (Wilcoxon rank test or Kruskal-Wallis rank test) were used for comparison if the sample variances were not equal. Gender was included in a linear model to determine its significance on cortisol level. The relationship between cortisol and fish weight was evaluated using a linear model ($y=mx+b$; where y=cortisol, x=weight) with linear, least squares fit.

Significance differences between treatment groups were reported at $\alpha=0.05$.

Results

Sampling, extraction and cortisol RIA

There was no morbidity or mortality of the fish during Experiment 1 or 2. A few fish from each of the control tanks were not sampled because we were unable to net these individuals in less than 30 s. Although the sample variances were not equal in all cases, similar results were obtained using parametric and non-parametric methods; therefore, we used parametric methods for our comparisons. Therefore, the number of fish sampled was slightly less for the control tanks for both experiments. Comparisons of the cortisol values of replicate tanks using the same treatment indicated no significant differences (Experiment 1: $p>0.415$; Experiment 2: $p>0.1641$). Therefore, we pooled data from replicate treatment tanks.

Experiment 1

Both the 3-h and 5-day crowded groups had mean whole-body cortisol levels approximately four-fold greater than that of the control group ($p<0.0025$; Fig. 2.1). There was no significant difference between the 3-h and 5-day crowded groups ($p=0.302$). Fish weight was not a significant factor predicting cortisol level in any treatment group.

Experiment 2

Fasted, crowded fish had a mean cortisol level four-fold higher than that of fasted, control fish ($p=0.0002$), similar to the results of Experiment 1; however, among the fed groups of fish, crowding did not significantly increase mean cortisol level (Fig. 2.2). There was no significant difference in the cortisol levels of fasted versus fed control fish. There was also no significant difference between the mean cortisol levels of crowded versus control fish held in 4 L tanks, even though the crowded density was ten-fold greater than the control density ($p=0.264$; Fig. 2.2). Gender was not a significant factor determining whole-body cortisol.

Fish weight was significantly related to whole-body cortisol level among fasted, crowded fish (pooled) held in 76 L aquaria ($p=0.0043$; Fig. 2.3). Regression analysis revealed an inverse relationship between cortisol and fish weight: Cortisol = 22.0674 − 0.0434_weight; R^2 =0.3035. Fish weight was not predictive of cortisol level in any of the other treatment groups.

Discussion

To optimize reproduction, reduce disease outbreaks in zebrafish facilities, and increase the reproducibility of zebrafish studies, it is important to monitor stress and establish standards for rearing conditions. We have demonstrated significant increases in whole-body cortisol with crowding. Our study as well as a previous study of zebrafish whole-body corticosteroids during toxicological procedures (Pottinger and Calder, 1995), suggest that whole-body cortisol is useful as a general indicator of stress in zebrafish. Whole-body cortisol techniques have been previously used for evaluating stress of small fishes (Pottinger et al., 2002) and may be useful for optimizing production and evaluating stress in other small fish species.

Zebrafish whole-body cortisol versus previous studies

Previous measurements of zebrafish whole-body corticosteroids were found to be about 1 ng/g fish in control (non-stressed) fish (Pottinger and Calder, 1995), very close to those we have reported in the current study. This suggests a relatively consistent resting level among laboratory zebrafish populations. Among adult three-spined stickleback held under control conditions mean whole-body corticosteroid levels ranged from 2 to 8 ng/g fish (Pottinger et al., 2002), within the range of cortisol values we reported in zebrafish.

The mean cortisol levels of both control and crowded zebrafish were much higher than those measured in developing salmonids subjected to stressors. Whole-body cortisol for developing Chinook salmon ranged from 1.5 to 4 ng/g fish (Feist and Schreck, 2002), 2 to 10 times less than adult zebrafish. A number of differences between these studies could contribute to different cortisol levels, including differences in species, life-stage, and rearing temperature. For example, the ability to respond to stress, as indicated by increased cortisol level, generally increases as fish grow older (Pottinger and Mosuwe, 1994; Barry et al., 1995; Feist and Schreck, 2002; Szisch et al., 2005). Differences in the type, severity, and timing of stressors may also contribute to differences in cortisol responses between these studies (Schreck, 1981).

Whole-body cortisol levels in developing Japanese flounder, *Paralichthys olivaceus* (2.5–11 ng/g fish; de Jesus et al., 1991) and chum salmon (2.5–20 ng/g; de Jesus and Hirano, 1992) are more similar to those we found in zebrafish. A recent study examining the ontogeny of cortisol in gilthead sea bream showed a similar range of values that increased during development (Szisch et al., 2005). Interestingly, these studies reported whole-body cortisol levels in the absence of stressors, further suggesting that life stage, species, and rearing environment are important in determining fish cortisol levels (Barton, 2002).

Whole-body cortisol response to crowding

Crowding in 76 L aquaria resulted in a significant increase in whole-body cortisol levels, whereas crowding in 4 L tanks did not. Both groups of fish were crowded at the same density (40 fish/L). However, fish in smaller 4 L tanks were acclimated at a higher density (4 fish/L) compared to those in larger 76 L aquaria (0.2 fish/L). Fish crowded in

large aquaria experienced a 200-fold increase in density from acclimation, whereas fish crowded in small tanks experienced only a 10-fold increase in density. The greater relative change in density in the larger aquaria may have been perceived as a greater threat, thus eliciting a greater cortisol response (Schreck, 1981). Because acclimation differed between experiment and tank type (Experiment 2) comparisons are limited. Nevertheless, our results suggest that tank type or size may influence the whole-body cortisol response to crowding.

Whole-body corticosteroids of mildly stressed zebrafish (transported and briefly handled) were found to be in the range of 9 ng/g fish (Pottinger and Calder, 1995), very similar to what we found in the current study. This suggests that the whole-body cortisol response to mild stress may be similar across different populations of zebrafish held under different laboratory conditions. Conversely, the whole-body cortisol response to chronic crowding (15 fish in 1500 mL for 5 days) of fasted three-spined sticklebacks was considerably higher than our study, at 35 ng/g fish (Pottinger et al., 2002). Rearing environment as well as species differences in the cortisol response to crowding may explain differences between the studies (Barton, 2002).

Feeding, social aspects and effects of fish size

The diets fed during acclimation differed between Experiments 1 (formulated feed) and 2 (live and formulated feed). Conceivably, there could be an effect of acclimation diet on the stress response (Schreck, 1993, Van Anholt et al., 2004). However, it was beyond the scope of this study to determine such effects of diet on the subsequent response to stress. Interestingly, we did see similar significant increases in fish acclimated to different diets but subjected to similar chronic crowding (fasted) conditions in Experiments 1 and 2.

Feeding did not significantly affect the cortisol levels of non-crowded fish in the current study. Cortisol levels of fed versus fasted fish vary widely among fish species. Fasting did not significantly affect the plasma cortisol levels of rainbow trout, *Oncorhynchus mykiss* (Pottinger et al., 2003) or juvenile walleye, *Stizostedion vitreum* (Czesny et al., 2003). However, fasted juvenile Chinook salmon had significantly lower plasma cortisol levels compared to fed fish (Barton et al., 1988). Conversely, fasting of

channel catfish, *Ictalurus punctatus*, resulted in a significant increase in plasma cortisol (Peterson and Small, 2004).

Interestingly, among the fed groups, we did not see a significant increase in mean cortisol levels with crowding. One explanation may be that a significant source of energy ameliorates the stress of crowding and, perhaps, better enables the fish to cope with this stressor compared to fish that are unfed. Although there was no effect of feeding among fish maintained at the control densities, the interactive effects of feeding and crowding on cortisol levels suggest an important role of the feeding regimen.

Biliary clearance may be greater in fed fish than fasted fish resulting in a greater accumulation of corticosteroid metabolites in the bile of fasted fish, indirectly increasing whole-body cortisol levels (Pottinger et al., 1992, 2002). This may have been more evident among crowded fish as opposed to controls, particularly among the fasted groups. Despite this possibility, the specificity of the RIA antibody for cortisol (not its metabolites), the fact that our extraction procedure should eliminate conjugated steroids, and the validation of the extracts for parallelism, reduced the possibility of detecting these metabolites even if they were present in the extracts.

There was an inverse relationship between body size and cortisol level among fasted, crowded fish, reared in 76 L aquaria. Larger fish are typically socially dominant (Øverli et al., 2004). Socially dominant fish generally have much lower cortisol levels than subordinate fish reared under the same conditions (Ejike and Schreck, 1980; Øverli et al., 1999; Sloman et al., 2001). Zebrafish display aggression and establish dominance hierarchy with tank cohorts (Gerlai, 2003). While dominance hierarchies may breakdown under extreme crowding (Li and Brocksen, 1977), hierarchies may persist under mild crowding conditions (Sloman and Armstrong, 2002). Food deprivation has been demonstrated to increase aggressive behavior, suggesting enhancement of dominance hierarchies of Atlantic salmon, *Salmo salar* (Symons, 1968). It is conceivable that mild crowding, in the absence of food, enhanced the dominance hierarchies resulting in the inverse relationship between fish weight and cortisol level among crowded and fasted fish.

Individual variation and baseline cortisol values

There was a wide variation in individual cortisol levels in all treatment groups, with the exception of the fasted control groups. This is not uncommon in crowding and other stress studies with fish (Wendelaar Bonga, 1997; Barton, 2002; Feist and Schreck, 2002). Genetic factors and social hierarchies in fish have been demonstrated to affect individual responses to stress. There is a genetic basis of the stress response that has allowed for selection of individuals with high and low cortisol responses (Pottinger and Carrick, 2001; Fevolden et al., 2002). It is reasonable to assume that a genetically unselected zebrafish population will show a broad range of cortisol responses to crowding, density, and feeding regimen based on both genetic and social factors.

Baseline cortisol values for zebrafish have not been established. Fish reared under control conditions (not crowded) in Experiments 1 and 2, demonstrated whole-body cortisol levels ranging from 2.1 to 4.7 ng/g fish (Fig. 2.4), which could be considered a baseline cortisol range for AB adult zebrafish. Additionally, Pottinger and Calder (1995) found control (baseline) whole-body corticosteroids for zebrafish to be in the range of 0.4–1.0 ng/g fish. Differences in rearing facility (FPGL vs. ZIRC), feeding regimen (fasted vs. fed), density (0.2, 0.25, 4 fish/L), and tank size (4 L, 60 L, 76 L) may have accounted for the range of cortisol values in the different groups of control fish. We are cautious in suggesting that rearing zebrafish at a particular density is stressful. Cortisol, albeit at low, non-stress levels, is needed for normal development and growth (Schreck, 1993). Further studies are needed to correlate cortisol levels with zebrafish fitness parameters such as reproduction and susceptibility to disease. This will aid in establishing baseline cortisol levels for optimal zebrafish health and welfare.

Conclusions

The hardy nature of zebrafish was demonstrated during this study as reflected by the lack of morbidity and mortality as well as the presence of normal feeding behavior during the 5-day crowding period. Despite the absence of any apparent ill effects due to crowding, inconsistent laboratory environments may affect the quality and consistency of research with laboratory fishes including zebrafish. Furthermore, the rearing environment should be considered a critical component to fish welfare, an increasing concern for fish

husbandry. Whole-body cortisol could be used as a tool to assess stress, optimize rearing conditions, and improve the health and welfare of this popular vertebrate model. This technique could also be useful for assessing stress in other small laboratory fishes.

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Table 2.1: Treatment specifications for Experiment 1 conducted at the Fish Performance and Genetics Laboratory (FPGL) and Experiment 2, conducted at the Zebrafish International Resource Center (ZIRC).

Treatment	**Experiment (location)**	**Density (fish/L)**	**Tank Type**	**Fed**
Control	1 (FPGL)	0.25	60 L fiberglass	No
3-hour Crowding	1 (FPGL)	40	60 L fiberglass	No
5-day Crowding	1 (FPGL)	40	60 L fiberglass	No
Fasted Control	2 (ZIRC)	0.2	76 L glass	No
Fasted Crowded	2 (ZIRC)	40	76 L glass	No
Fed Control	2 (ZIRC)	0.2	76 L glass	Yes
Fed Crowded	2 (ZIRC)	40	76 L glass	Yes
Fasted Control	2 (ZIRC)	4	4 L plastic	No
Fasted Crowded	2 (ZIRC)	40	4 L plastic	No

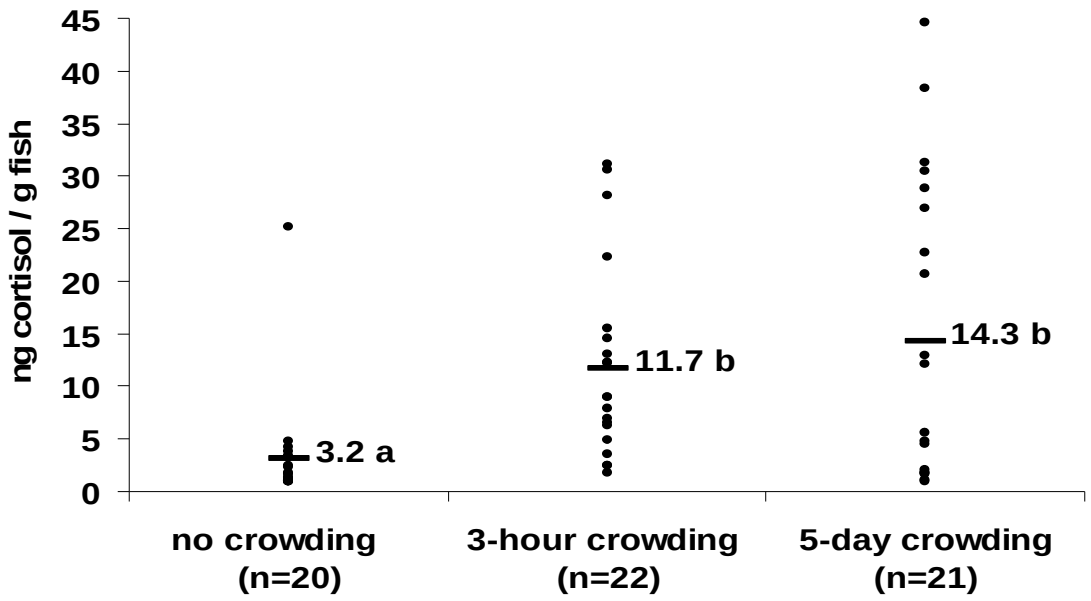

Figure 2.1: *Experiment 1*: Crowding significantly increases cortisol levels. Individual whole-body cortisol (ng/g fish) of adult zebrafish subjected to no crowding, 3-h or 5-day crowding. The dash (-) indicates the mean value of each treatment group. Different letters indicate a significant difference between the mean cortisol values of the treatment groups ($p<0.0025$).

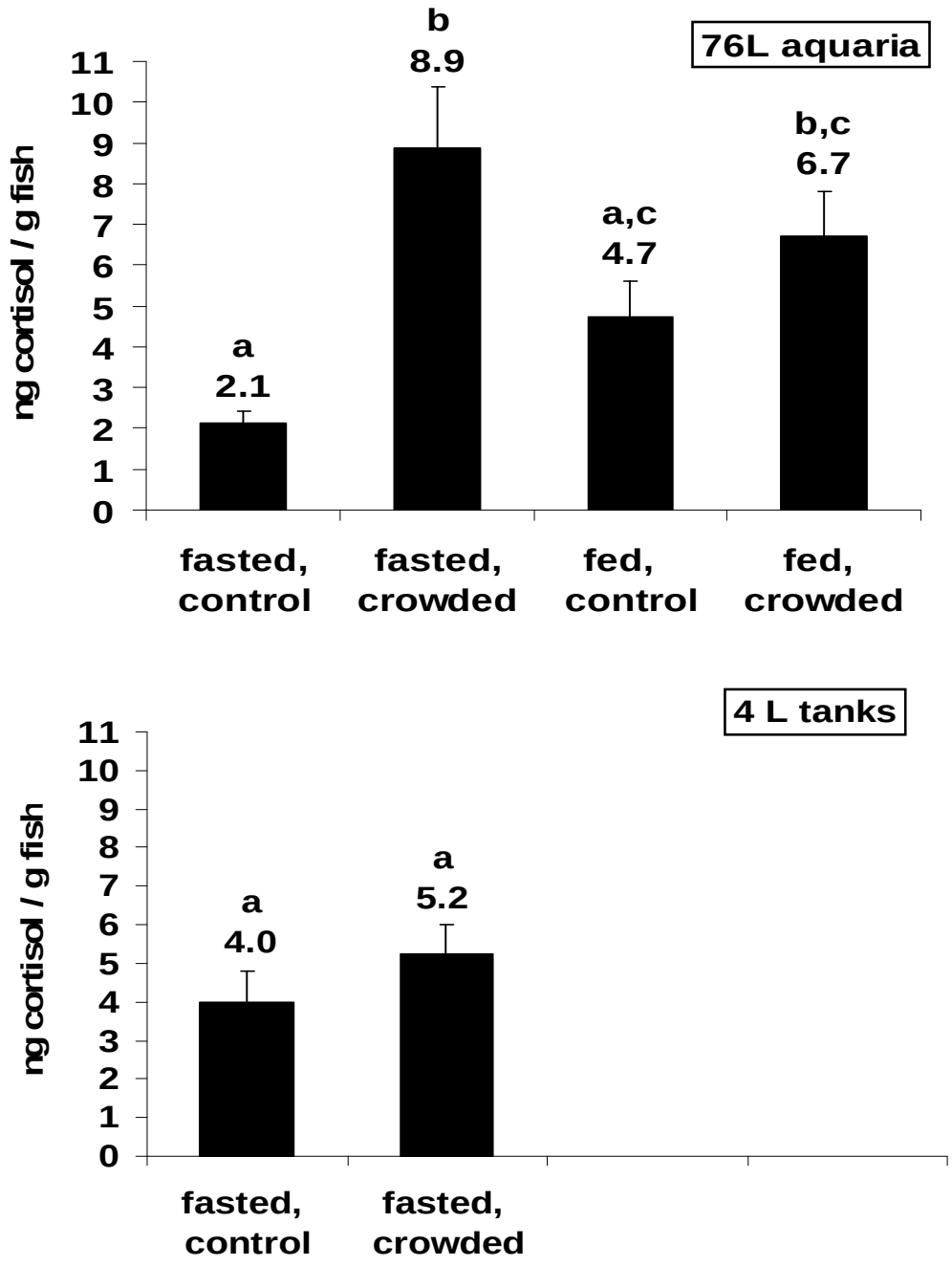

Figure 2.2: *Experiment 2*: Crowding increases cortisol levels in large, but not small tanks. Mean whole-body cortisol (± SEM) (ng/g fish) for 76 L glass aquaria and 4 L plastic tank treatments. Different letters above the standard error bars indicate a significant difference between treatment groups ($p=0.0002$; $n=24$-25).

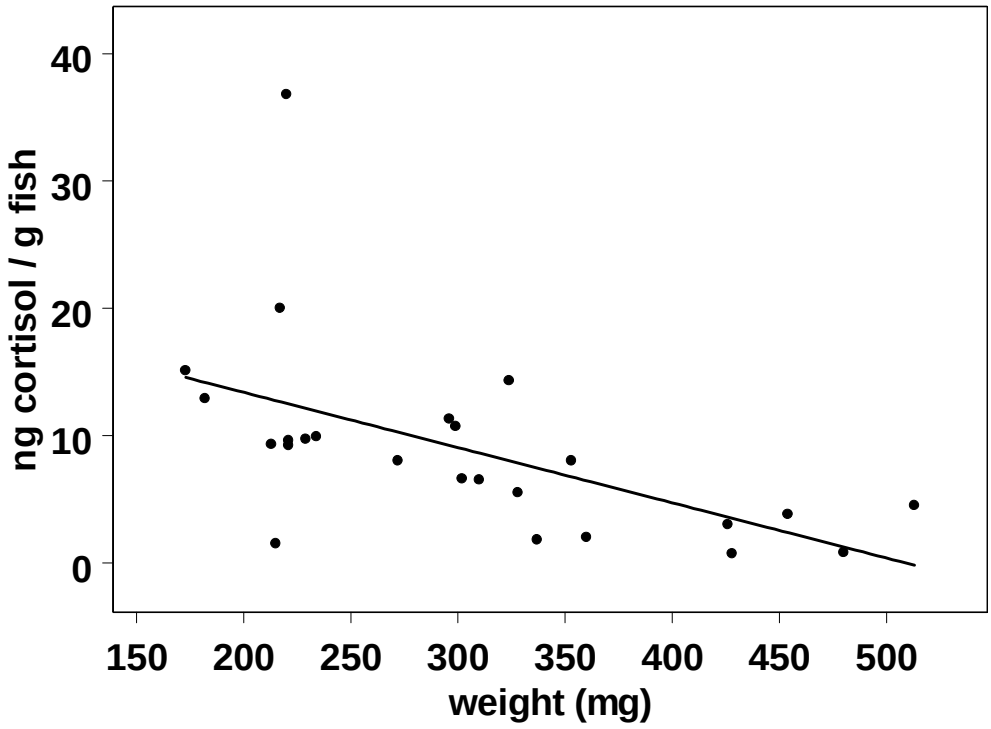

Figure 2.3: *Experiment 2*: Weight is a significant predictor of cortisol. Cortisol (ng/g) versus weight (mg) of fasted, crowded zebrafish (76 L aquaria); Least squared fit (Cortisol = 22.0674 – 0.0434_weight; R^2 = 0.3035; p=0043).

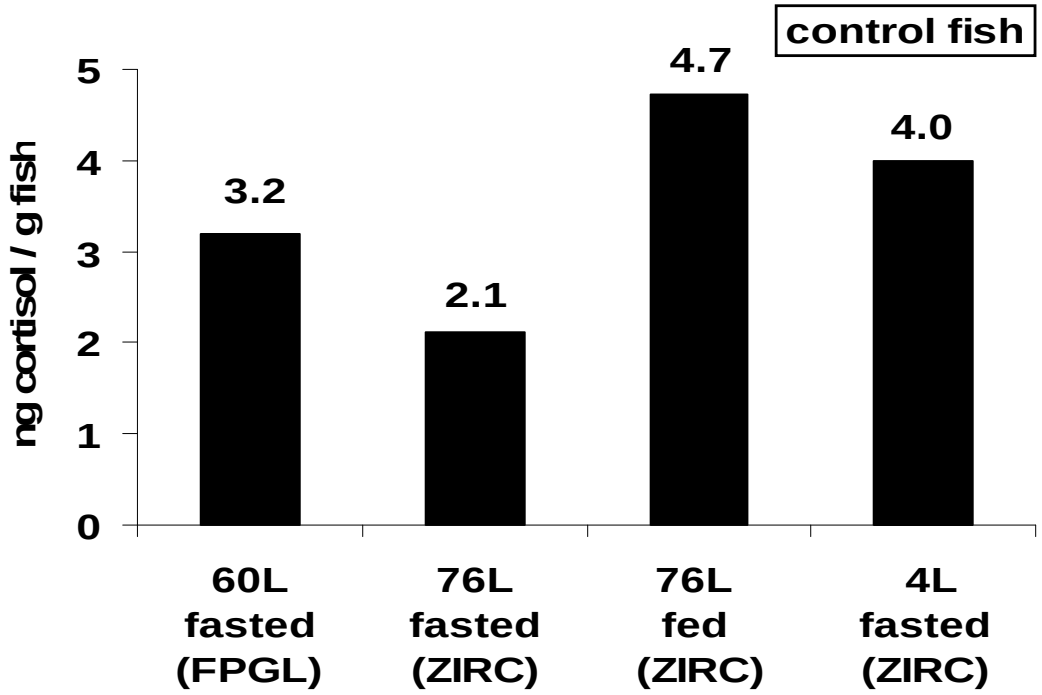

Figure 2.4: Whole-body cortisol levels of control fish from *Experiments 1* and *2* reared under a variety of non-crowded husbandry conditions including different laboratories (FPGL and ZIRC), densities (0.2, 0.25, 4 fish/L), tank sizes (4 L, 60 L, 76 L), and feeding regimes (fasted and fed). Baseline cortisol ranged from 2.1 to 4.7 ng/g fish.

CHAPTER 3: RAPID CORTISOL RESPONSE OF ZEBRAFISH TO NET HANDLING STRESS

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Abstract

Zebrafish, *Danio rerio*, are frequently handled during husbandry and experimental procedures in the laboratory, yet little is known about the physiological responses to such stressors. We measured the whole-body cortisol levels of adult zebrafish subjected to net stress and air exposure at various intervals over a 24 h period; cortisol recovered to control levels by about 1 h post-net-stress (PNS). We then measured cortisol at frequent intervals over a 1 h period. Cortisol levels were more than 2-fold higher in net stressed fish at 3 min PNS and continued to increase peaking at 15 min PNS, when cortisol levels were 6-fold greater than the control cortisol range. Mean cortisol declined from 15 to 60 min PNS, and at 60 min, net-stressed cortisol was within the control range. Because the age of fish differed between studies, we examined resting cortisol levels of fish of different ages (3, 7, 13, and 19 months). There was wide variation in resting cortisol among tanks with the same age fish. The variability in resting cortisol increased with age and older fish tended to have higher resting cortisol suggesting that the age of fish may contribute to variation during experimentation. The rapid response and recovery of cortisol after net handling likely reflect the intensive domestication of zebrafish which would select for rapid responses to net handling.

Keywords: Age; Biomedical model; *Danio rerio*; Fish husbandry; Stressors

Introduction

Cultured fishes are confined, crowded, captured, handled, and transported during routine husbandry (Piper et al., 1992; Klontz et al., 1995). These procedures are stressful and elicit physiological changes in response to perceived threats (Schreck, 1981; Davis, 2006). Changes may be rapid or delayed, long-term or short-term depending upon numerous factors including the species and life stage of the fish, the nature of the stressor, and other environmental factors (Schreck, 2000; Barton, 2000, 2002).

Stress has been described as a cascade of physiological events that occurs after perception of a threat, during which an organism attempts to restore homeostatic norms (Schreck et al., 2001). The hormone cortisol is a primary stress indicator in fishes, which typically increases during exposure to acute or chronic stressors (Schreck, 1981; Wendelaar Bonga, 1997; Barton, 2002; Ramsay et al., 2006). Plasma cortisol levels are typically used as an indicator in larger fish species (Barton and Iwama, 1991, Wendelaar Bonga 1997), whereas whole-body cortisol is often used for smaller fishes, due to inadequate blood volume to accurately measure plasma cortisol (Feist and Schreck, 2002; Pottinger et al., 2002; Ramsay et al., 2006). Short-term increases in cortisol are believed to be adaptive, whereas repeated or chronic increases in cortisol are maladaptive resulting in decreased growth, impaired reproduction, and increased susceptibility to infectious diseases (Maule et al., 1989; Schreck et al., 2001; Saeij et al., 2003; Jentoft et al., 2005).

Handling of fishes typically elicits a cortisol response, but the nature of this response is quite variable. The cortisol response to common husbandry stressors such as crowding and handling has been described for numerous species, particularly salmonids (Schreck, 1981, 1982; Barton, 2000). After a 30 s acute handling stress, salmonids can elevate cortisol levels 20 to 100 fold within 1 h (Schreck, 1981; Barton, 2000); however, the magnitude of this response and recovery time to control levels varies greatly with species (Barton, 2002). Juvenile white suckers, *Catostomus commersoni*, elevate plasma cortisol levels after 5 min of chasing with a net and recover to control levels 6 h later (Bandeen and Leatherland, 1997). In contrast, juvenile pallid sturgeon, *Scaphirhynchus albus*, exhibit a low cortisol response to acute handling stress (Barton et al., 2000) even

though this is the predominant steroid produced by this species in response to stress (Webb et al., 2007).

Although the cortisol response to husbandry stressors has been described for many aquatic species, less is known about the response of fishes commonly used for biomedical research. Zebrafish, *Danio rerio*, are widely used as vertebrate research organisms, primarily for developmental genetics, and increasingly for toxicological, cancer, behavioral, and disease studies (Trede et al., 2004; Dahm and Giesler, 2006; Wright et al., 2006; Beckman, 2007). Few studies have examined the effects of husbandry stress on zebrafish (Ramsay et al., 2006; Lawrence, 2007). Stress is often implicated when breeding is poor and during outbreaks of disease (Westerfield, 2007) suggesting a further need to measure and elucidate how husbandry stressors affect zebrafish.

Pottinger and Calder (1995) described recovery times of zebrafish whole-body corticosteroids after transport; whole-body corticosteroids were highest upon arrival in the laboratory but recovered significantly 1 h following transfer to aquaria. Increases in the whole-body cortisol of zebrafish, in response to direct and visual contact with a predator, have also been reported (Barcellos et al., 2007). We have recently described increases in the whole-body cortisol of zebrafish in response to crowding (Ramsay et al., 2006). However, the cortisol response to net handling and air exposure has not been examined.

The aim of this study was to describe the whole-body cortisol response of adult zebrafish to an acute handling stressor. Determining how zebrafish respond to common husbandry stressors will allow researchers to improve handling of fish, and ultimately, the health and fitness of their fish, while minimizing husbandry-associated sources of variation in experiments.

Methods

Zebrafish (adult, AB wild types) were provided by the Zebrafish International Resource Center (ZIRC) at the University of Oregon in Eugene, Oregon, USA. Acrylic tanks (4 L, Thoren Aquatics®, 91007) were each stocked with 10 fish, maintained in a recirculation system at the ZIRC (Ramsay et al., 2006; Westerfield, 2007). A total of 260

fish (age: 7 months) were used for the 24 h Study; 200 fish (age: 13 months) were used for the 1 h Study; 120 fish (ages: 3, 7, 13, and 19 months) were used for the Age Study. An acclimation period of 7 to 10 d was used prior to initiating experiments. Fish were fed twice daily (ZIRC Mastermix and brine shrimp nauplii, *Artemia* sp.) during the acclimation and experimental periods according the ZIRC feeding protocols (Westerfield 2007).

Net stress protocol and sampling

All sampling commenced at approximately the same time of day (10:00 – 11:00 h) in order to minimize the possibility of fluctuations in cortisol due to natural circadian rhythms (Schreck, 1981).

24 hour study

Net stress was administered to each stress treatment tank. All fish were netted, suspended in the air for 3 min, returned to their original tanks for 3 min, and suspended in the air for an additional 3 min. Duplicate tanks of fish were sampled at 9 min, 39 min, 69 min, 3 h, 8 h, 12 h, and 24 h post-net-stress (PNS; Fig. 3.1A). Duplicate control tanks, to which no net stress had been administered, were sampled at similar intervals over 24 h.

One hour study

The dynamics of the cortisol response following net stress was examined over more frequent intervals during a 1 h period. The same net stress protocol was administered as for the 24 h study. Duplicate treatment tanks of 10 fish were sampled at 3, 6, 9, 15, 22, 30, and 60 min PNS (Fig. 3.1B). Duplicate control tanks were sampled at 0, 15, and 60 min PNS.

Age study

We used 7 month old fish for the 24 h Study and 13 month old fish for 1 h Study. Differences in control cortisol levels between the Studies suggested that age may affect resting cortisol levels. To investigate this further, triplicate tanks of fish aged 3, 7, 13, or 19 months were held under similar control conditions (10 fish in a 4 L acrylic tank) for a period of 2 weeks after which they were sampled for whole-body cortisol.

Sampling procedures

Sampling of fish, including euthanasia and freezing, was performed as described by Ramsay et al. (2006). Upon sampling, fish were euthanized with buffered tricaine methane sulfonate (500 mg/L MS-222), blotted on paper towels to remove excess water, immediately frozen in liquid nitrogen, and stored at -80^{o}C until analyzed. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Oregon (Protocol # 03-01R).

Whole-body cortisol extraction and measurement

Whole-body cortisol was extracted using the method of Ramsay et al. (2006). Whole zebrafish were thawed, weighed, homogenized, and extracted twice with 8 mL of diethyl ether. Extraction efficiency was determined for each extraction by adding tritiated cortisol to homogenized samples (n = 4) and extracting the samples as described above. Average extraction efficiency was 73%, similar to previous studies that examined whole body cortisol levels in zebrafish (Ramsay et al., 2006), Chinook salmon, *Oncorhynchus tshawytscha* (Feist and Schreck, 2002), and chum salmon, *O. keta* (de Jesus and Hirano, 1992).

Cortisol in the whole-body extracts of individual fish was measured using a radioimmunoassay (RIA) from the method of Redding et al. (1984), modified and validated by Ramsay et al. (2006). Consistency between assays was verified by measuring cortisol in whole-body extracts spiked with known concentrations of cortisol. Intra-assay and inter-assay variation was accepted at no more than 10%. The detection limit of the cortisol RIA was 3.125 pg/tube. All whole-body cortisol values were above the detection limit. Cortisol values (pg/tube) were corrected for dilution factors, weight of fish, and extraction efficiency. The average weight of the thawed fish was 224 mg ($\pm$ 2.7 mg).

Statistical analyses

Statistical analyses were performed using the statistical software program S-PLUS 7 (Insightful Corp., 2005, Seattle, Washington). For each study, the weights of fish in each treatment were examined and compared using an analysis of variance (ANOVA).

For the 24 and 1 h Studies, replicates of each treatment time PNS were compared using a Welch's modified t-test. The cortisol data from treatment replicates were pooled if there was no significant difference. Two 95% confidence intervals of control cortisol values were reported as a control cortisol range for each the 24 and 1 h Studies. Net-stressed cortisol values at each time PNS were compared to those of each control cortisol range using an ANOVA. Non-parametric tests (Wilcoxon rank test or Kruskal-Wallis rank test) were used for comparison if the sample variances were not equal. For the 1 h Study, relationships between cortisol and time PNS were evaluated using a linear regression model ($y= mx + b$; where y=cortisol, x=time PNS) to describe the increase in cortisol and another linear regression model to describe the decrease in cortisol.

For the Age Study, an ANOVA was used to compare mean cortisol values between triplicate tanks within each age group. Treatment tanks of the same age were pooled if there was no significant difference and the means of different age fish were compared using ANOVA.

Significance differences were reported at $\alpha=0.05$.

Results

There was no observed morbidity or mortality of the fish during the experiments. Comparisons of the cortisol values of replicate tanks subjected to the same treatment indicated no significant differences in either the 24 h or 1 h Study. Therefore, data from control tanks was presented as a control cortisol range to which net-stressed groups were compared. For the Age Study, significant differences between triplicate tanks of the same age group did not allow us to pool data from replicate tanks. Although the sample variances were not equal in all cases, similar results were obtained using parametric and non-parametric methods; therefore, we reported only results from parametric comparisons.

Age study

Mean resting cortisol values varied widely (Fig. 3.2A). The range of cortisol values tended to increase with age and was most variable among the oldest fish (19 months old; Fig. 3.2B). Three month old fish weighed significantly less (212 ± 14 mg)

than fish at the other ages. There were no significant differences between the weights of the 7, 13, and 19 month old fish (367 $\pm$ 13 mg).

One hour study

We found that 3 to 15 min after netting stress, cortisol levels increased linearly from 2.5 to 6 fold higher than control levels. Then, cortisol decreased linearly to control levels. Sixty minutes after netting stress, we did not detect a significant difference between control and net stressed fish (Fig. 3.3).

The positive (1) and negative (2) linear relationships between cortisol and time PNS were explained by the models:

(1) cortisol = 7.26 + 1.76 x time (R^2 = 0.591; p=0; Fig. 3.3).

(2) cortisol = 39.5 – 0.56 x time (R^2 = 0.556; p=0; Fig. 3.3).

There were no significant differences in the weights of fish in any of the treatment groups.

24 h study

At 9 min PNS, mean cortisol increased almost 3-fold in net-stressed fish compared to the control fish (Fig. 3.4). By 39 min, cortisol was about 50% greater in net-stressed fish compared to controls. Cortisol levels of the net stressed individuals were significantly lower compared to controls 69 min PNS. The mean cortisol values 12 h after net stress was significantly higher than control. The cortisol values at all other time points did not differ between the net-stressed groups and control range. There were no significant differences in the weights of any of the fish in the treatment groups.

Discussion

Our study describes the cortisol response of adult zebrafish to acute handling stress; this complements our previous study that examined the cortisol response of zebrafish to chronic crowding stress (Ramsay et al., 2006). Despite the wide use of zebrafish as a research organism, there are few data regarding the stress response as it relates to husbandry (Lawrence, 2007). Better understanding how zebrafish respond to handling will allow researchers to create a rearing environment for optimal health and reproduction while minimizing husbandry-associated sources of variation. Additionally,

zebrafish may be developed as a model of stress for aquaculture species (Dahm and Geisler, 2006; Alsop and Vijayan, 2008).

There was no mortality during or after netting. Similar stressors often result in mortality in other fishes, notably salmonids (Schreck, 1982; Schreck et al., 1995) and marine fishes (Davis et al., 2001; Suski et al., 2007). In addition, zebrafish responded and recovered rapidly from the acute stress. An extensive history of domestication and physiological adaptations favorable to laboratory culture may explain the ability of zebrafish to endure husbandry stressors without significant mortality (Creaser, 1934; Eaton and Farley, 1974; Laale, 1977; Dahm and Geisler, 2006). Domesticated zebrafish have a reduced startle response compared to zebrafish obtained from wild populations (Robison and Rowland, 2005). Behavioral differences between wild and domesticated zebrafish reflect differences in genetic profiles (Wright et al., 2006). In addition, the cortisol response is highly heritable in fishes (Øverli et al., 1999; Pottinger and Carrick, 2001; Fevolden, et al., 2002). Continuous spawning and short generation time of zebrafish likely facilitated rapid selection of traits favorable to survival in a laboratory environment, including the ability to respond to and recover from husbandry stressors such as net handling.

Rapid linear increase in whole-body cortisol after net handling

Zebrafish rapidly increase whole-body cortisol levels upon exposure to net stress with peaks occurring 9 min after netting. When examined at more frequent intervals over a 1 h period, cortisol peaked at an even higher level at 15 min, confirming that zebrafish rapidly produce cortisol in response to net stress and air exposure. In a similar study examining transport stress in zebrafish, whole-body corticosteroids peaked 30 min after transfer from holding tanks to transport containers (Pottinger and Calder, 1995). This was the first measurement following transfer. Therefore, it is not possible to know whether or not this value would have been higher 15 minutes after transfer. Peak whole-body cortisol values in our study ranged from 28 to 30 ng/g. This is much greater than the peak values found by Pottinger and Calder (1995) which ranged from 4 to 9 ng/g. The nature of the stressor (brief handling and transfer rather than severe net handling and air exposure) may have resulted in a much greater cortisol response in our study. Additionally,

differences in temperature between studies (28.5 versus 23^{o}C) may explain differences in the magnitude and timing of peak cortisol response (Schreck, 2000).

Zebrafish demonstrated a linear increase in whole-body cortisol over time. Davis and Small (2006) showed a similar linear increase in plasma cortisol values among sunshine bass, *Morone chrysops* x *M. saxatilis*, exposed to a 15 min low water stressor with plasma cortisol levels peaking at 20 to 30 min post-stressor. Differences in the nature and duration of the stressor as well as species and temperature differences may have resulted in differences in the timing and magnitude of the cortisol response. Nevertheless, the similar linear increase in cortisol suggests that a better understanding of the dynamics of the cortisol response following exposure to handling stressors could be examined to create husbandry protocols that minimize stress in aquaculture.

Recovery from net handling: clearance of cortisol

Whole-body cortisol levels recovered to that of the control range by 1 h after net stress for both the 24 h and 1 h Studies. Similarly, whole-body corticosteroids of adult zebrafish had recovered to baseline values 1 h following transport (Pottinger and Calder, 1995). Pottinger and Calder (1995) also noted that zebrafish are less sensitive to changes in environment and recover relatively rapidly from transport stress compared to salmonids. Zebrafish are frequently handled during routine laboratory procedures (breeding, tank cleaning, etc.). Stress conditioning has been shown to attenuate the cortisol response of Chinook salmon, *Oncorhynchus tshawytscha,* to subsequent stressors (Schreck et al., 1995). Previous experience with similar handling stressors may have facilitated the rapid recovery of elevated cortisol to control levels.

Interestingly, at 69 min after net stress, cortisol levels among the net stressed fish were significantly lower than the control range suggesting an increased rate of cortisol clearance in the first 69 min after net stress. The clearance rate of cortisol was increased in coho salmon, *Oncorhynchus kisutch*, after netting and crowding stress or chronic administration of exogenous cortisol (Redding et al., 1984). Bradford et al. (1992) demonstrated evidence for ultra-short-loop negative feedback of cortisol secretion by the interrenal of coho salmon in the presence of elevated levels of exogenous cortisol. Elevated whole-body cortisol as a result of severe net stress may have increased the

clearance rate of cortisol as well as suppressed cortisol secretion, resulting in significantly lower cortisol levels at 69 min after net stress.

The cortisol of the net stressed group at 12 h PNS was significantly higher than the control cortisol range. The 12 h group was sampled at approximately 22:00 h, during which time the facility is typically void of activity and people. Barcellos et al. (2007) have demonstrated increased cortisol in zebrafish upon visual contact with a predator. It is possible that unanticipated visual contact with the sampler just prior to sampling elicited a slight but significant elevation in the cortisol of previously stressed zebrafish, perhaps in anticipation of another net stressor.

The relationship between cortisol and time from 15 to 60 min was linear. Davis and Small (2006) also demonstrated a decrease in plasma cortisol among channel catfish, *Ictalurus punctatus*, and sunshine bass of a similar linear nature. After a 30 s handling stressor, coho salmon showed a linear decrease in plasma cortisol from 1 to 3 h post-stress; however, subsequent measurements at 6, 12, and 24 h post-stress did not reflect a linear decrease (Patiño et al., 1987). The nature of stressor, species of fish and environmental conditions of the experiment all influence the recovery of cortisol levels to baseline.

Resting cortisol: effects of age

Cortisol values varied widely between tanks of fish of the same age, indicating a possible tank effect. We were not able to discern any effect of tank position as demonstrated in other studies (e.g. Speare et al., 1995) but could not pool the data from replicate tanks for comparison. Nevertheless, the trend was toward increased variability in resting cortisol in older fish. The 3 and 7 month old fish tended to have lower cortisol than the 13 and 19 month old fish. As animals age, basal cortisol secretion typically increases and is associated with decreased health and increased disease problems (Sapolsky et al. 1986; Butcher and Lord 2004). Zebrafish may experience similar age-related increases in cortisol which may subsequently affect the health of the fish.

Differences in the cortisol levels of the control groups between the 24 h and 1 h Studies may also be the result of slight differences in sampling procedures during each experiment. During the 24 h Study, sampling activities were performed in closer

proximity to the tanks than during the 1 h Study. The transparent acrylic tanks allowed the fish to see activity outside of the tank, which may have been perceived as a threat. Zebrafish rely on visual cues to avoid threats such as predators (Fleisch and Neuhauss, 2006; Barcellos et al., 2007; Spence et al., 2008). The close proximity of treatment tanks to control tanks allowed control fish to see distressed treatment fish which may have elicited a slight but significant increase in whole-body cortisol among the control fish. Although there were slight differences in control cortisol levels between the 24 h and 1 h Studies, the cortisol levels of the net stressed fish were much greater than control groups.

Conclusions

Zebrafish responded and recovered rapidly to the net stress protocol used in our study. Extensive domestication suggests selection of traits favorable for survival in a laboratory environment, including the ability to endure net handling and air exposure. Despite the ability of zebrafish to recover rapidly from handling stressors, increasing the frequency and duration of handling may alter the dynamics of the cortisol response and necessitate longer recovery periods. Differences in handling procedures among laboratories may increase the husbandry-associated sources of variation in experiments. Additionally, reproduction and disease resistance may be affected long after cortisol levels have recovered. Older zebrafish tended to have higher variation in resting cortisol levels than younger zebrafish suggesting that age is an important factor in the dynamics of the cortisol response of this species. Better understanding how handling stressors and age affect whole-body cortisol, reproduction, and diseases of zebrafish may allow improvements in the husbandry, health, and consistency of studies that use this important model.

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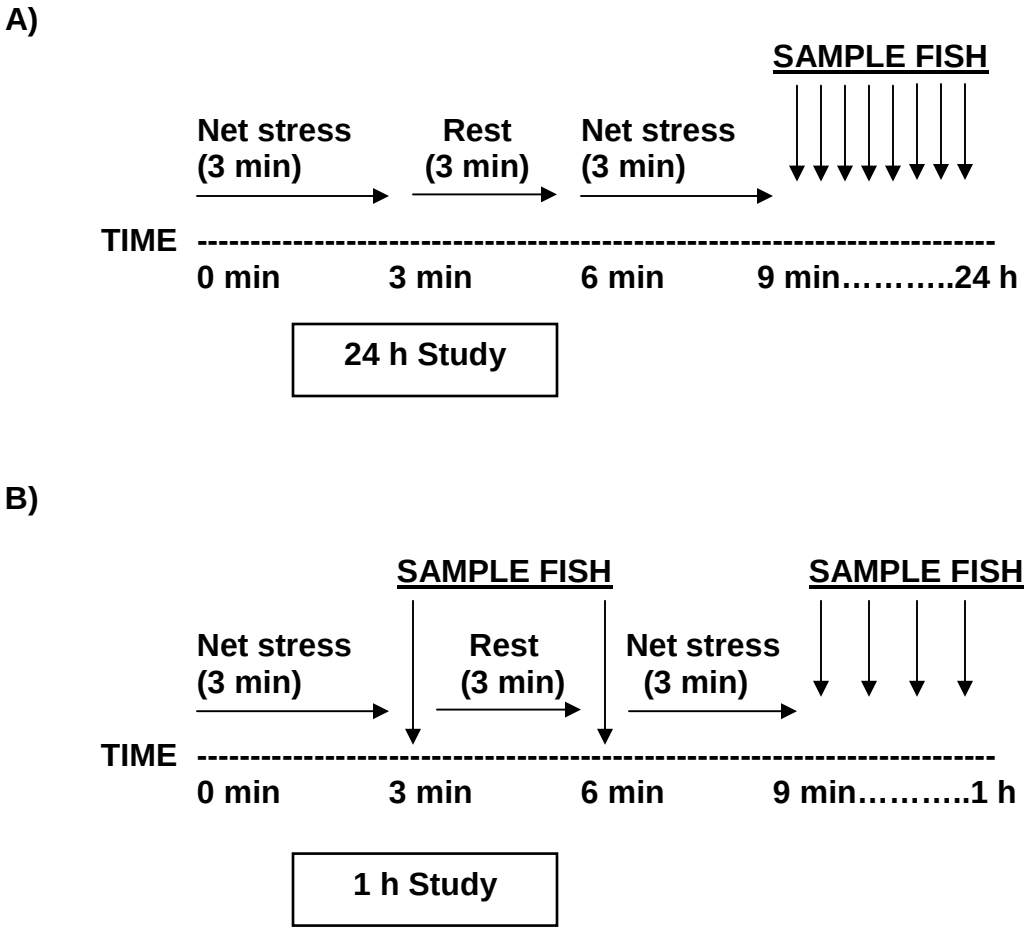

Figure 3.1: Timeline of net stress protocol. Fish were netted from their tanks, suspended in the air for 3 min, returned to their original tanks to rest for 3 min and subjected to a second 3 min net stressor. For the 24 h Study, duplicate tanks (n=10) were sampled at 9 min, 39 min, 69 min, 3 h, 8 h, 12 h, and 24 h after the netting. For the 1 h Study, duplicate tanks (n=10) were sampled 3, 6, 9, 15, 22, 30, and 60 min after netting.

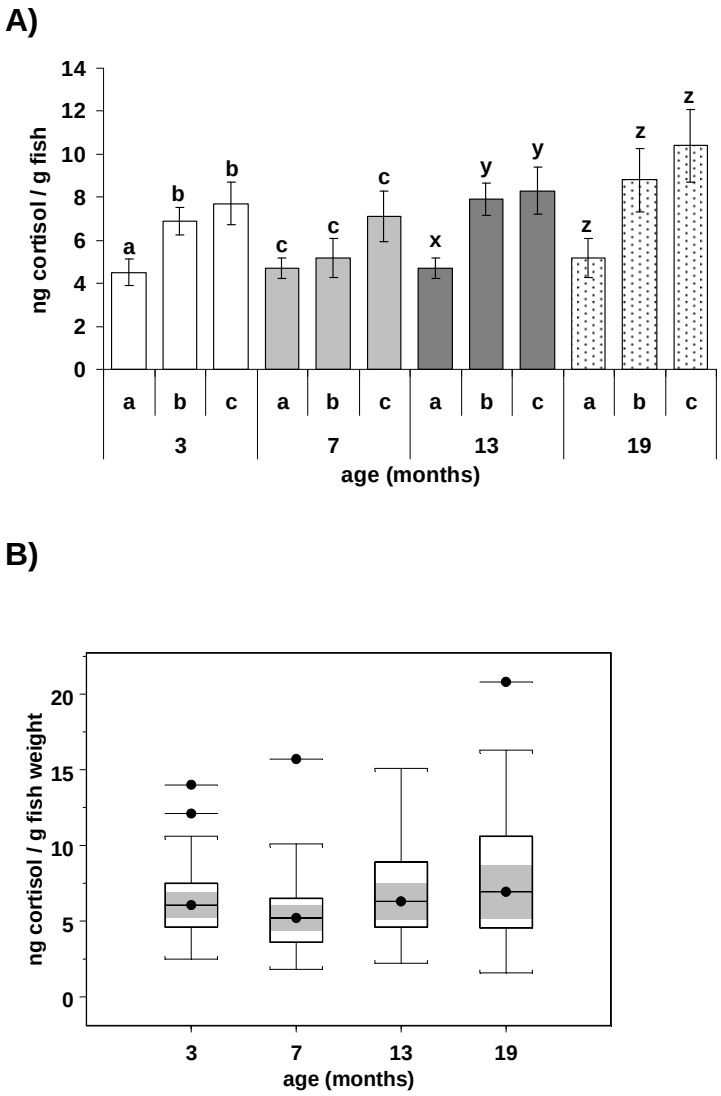

Figure 3.2: A) Mean whole-body cortisol (ng/g fish weight; ± SEM) of triplicate tanks (a,b,c) of different aged fish (3, 7, 13, 19 months) held at the same density (2.5 fish/L). Cortisol values were not pooled because of significant differences between triplicate tanks of the same age group. Different letter above the standard error bars indicate a significant difference between cortisol values within each age group. B) Box-plot of whole-body cortisol (ng/g) of zebrafish of different ages. The bottom and top of the boxes represent the interquartile range (IQR) of the data (25^{th} - 75^{th} percentile). The line through each box represents the median for each age group. The shaded area of the box represents the 95% confidence interval for each age group. The whiskers extend to 1.5*IQR; data points beyond this range are represented by dots with lines and are outliers.

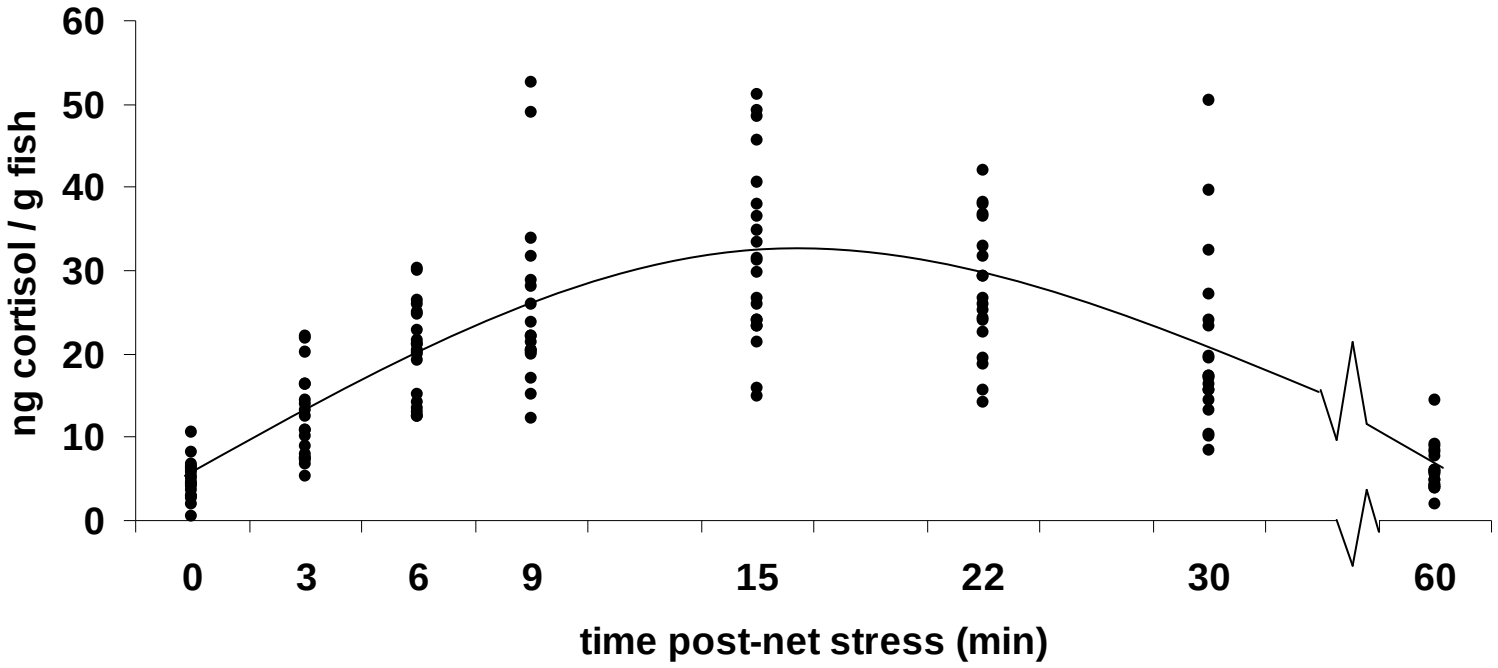

Figure 3.3: Individual whole-body cortisol values (ng/g fish weight) over time (min) after net stress (1 h Study). Net-stressed fish (●) were held in a net for 3 min, allowed to recover for 3 min and suspended again in a net for 3 min. Fish at time 0 min received no net stress prior to sampling. Mean cortisol values at each time post-net stress (PNS) are represented by a line passing through the points. There was a positive linear relationship between cortisol and time from 0 to 15 min PNS (cortisol = 7.3 +1.76 x time; R^2 = 0.595, p=0) and a negative linear relationship between cortisol and time from 15 to 60 min PNS (cortisol = 39.5-0.56 x time; R^2 = 0.56, p=0).

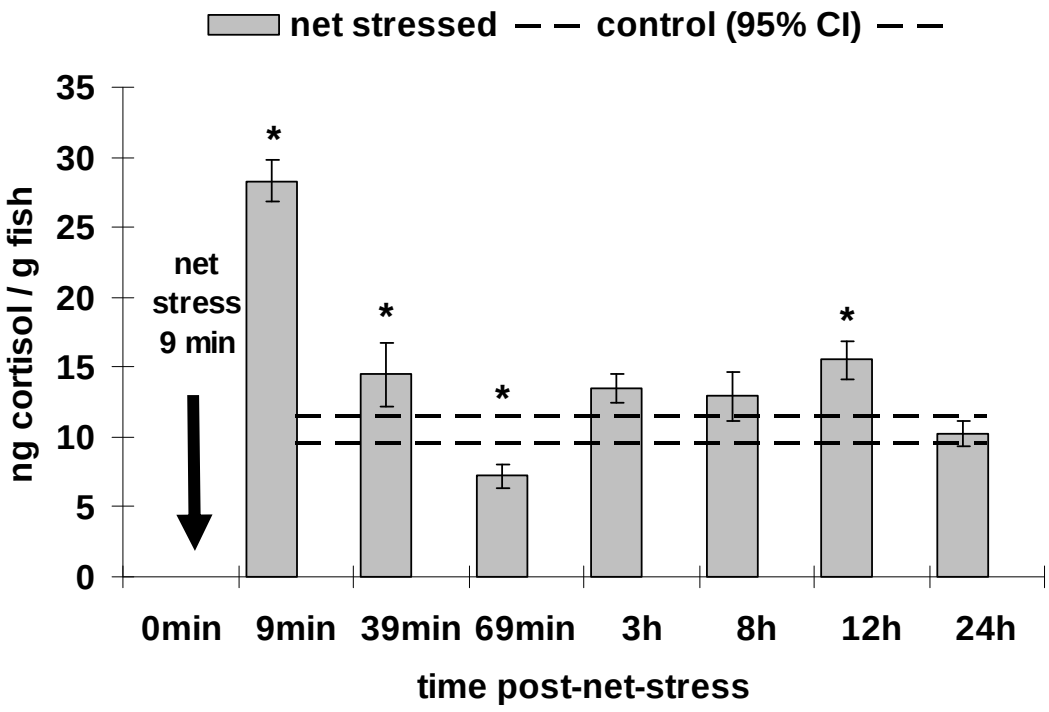

Figure 3.4: Mean whole-body cortisol (ng/g fish weight; ±SEM; n=20) over time (24 h Study). Net-stress groups (gray bars) were held suspended in a net for 3 min, allowed to rest in their original tanks for 3 min and then suspended in a net for 3 min. The control cortisol range (- - -) is summarized as a 95% confidence interval (controls received no net stress prior to sampling. Significant differences ($p<0.05$) between the control and net stressed groups are indicated with an asterisk (*).

CHAPTER 4: HUSBANDRY STRESS EXACERBATES MYCOBACTERIAL INFECTIONS IN ADULT ZEBRAFISH, *DANIO RERIO* (HAMILTON)

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Abstract

Mycobacteria are significant pathogens of laboratory zebrafish, *Danio rerio* (Hamilton). Stress is often implicated in clinical disease and morbidity associated with mycobacterial infections but has yet to be examined with zebrafish. The aim of this study was to examine the effects of husbandry stressors on zebrafish infected with mycobacteria. Adult zebrafish were exposed to *Mycobacterium marinum* or *M. chelonae*, two species that have been associated with disease in zebrafish. Infected fish and controls were then subjected to chronic crowding and handling stressors and examined over an 8-week period. Whole-body cortisol was significantly elevated in stressed fish compared to non-stressed fish. Fish infected with *M. marinum* ATCC 927 and subjected to husbandry stressors had 14% cumulative mortality while no mortality occurred among infected fish not subjected to husbandry stressors. Stressed fish, infected with *M. chelonae* H1E2, from zebrafish, were 15-fold more likely to be infected than non-stressed fish at week 8 post-injection. Sub-acute, diffuse infections were more common among stressed fish infected with *M. marinum* or *M. chelonae* than non-stressed fish. This is the first study to demonstrate an effect of stress and elevated cortisol on the morbidity, prevalence, clinical disease, and histological presentation associated with mycobacterial infections in zebrafish. Minimizing husbandry stress may be effective at reducing the severity of outbreaks of clinical mycobacteriosis in zebrafish facilities.

Keywords: Mycobacteria, Stress, Cortisol, Zebrafish, Husbandry

Introduction

Mycobacteriosis is a common disease of wild and cultured fishes (Decostere, Hermans & Haesebrouck 2004). Among the more common species infecting fishes are *Mycobacterium marinum*, *M. chelonae*, *M. abscessus* and *M. fortuitum* (Chinabut 1999). Piscine mycobacteriosis is a chronic progressive disease. There are often no external signs until advanced stages of the disease during which non-specific signs are present including emaciation, hemorrhagic and dermal lesions, lethargy, and death (Gauthier & Rhodes in press). The chronic proliferative form of the disease is characterized by granulomas, while sub-acute forms of the disease are associated with necrosis and acid-fast bacilli scattered diffusely among affected tissues including the kidney, liver, spleen, and often all visceral organs (Ferguson 2006).

Aquarium fishes are particularly susceptible to mycobacterial infections (Pate, Jenčič, Žolnir-Dovč & Ocepek 2005; Zanoni, Florio, Fioravanti, Rossi & Prearo 2008) and mycobacteria have been identified as significant pathogens of zebrafish, *Danio rerio* (Astrofsky, Schrenzel, Bullis, Smolowitz & Fox 2000; Kent, Whipps, Matthews, Florio, Watral, Bishop-Stewart, Poort & Bermudez 2004; Seok, Koo, Kasuga, Kim, Lee, Lee, Park, Baek, Lee, Kim, Lee, Lee, Cho & Park 2006; Whipps, Dougan & Kent 2007). Zebrafish are excellent hosts for these bacteria and have been increasingly used to study the pathogenesis of mycobacteriosis (Prouty, Correa, Barker, Jagadeeswaran & Klose 2003; Tobin & Ramakrishnan 2008). The increasing popularity of zebrafish as a research model (Dahm & Geisler 2006; Lieschke & Currie 2007) has resulted in a significant increase in the number of laboratories rearing, breeding, and transporting zebrafish. This increases the potential for dissemination and exacerbation of infectious diseases, such as mycobacteriosis (Kent, Feist, Harper, Hoogstraten-Miller, Law, Sánchez-Morgado, Tanguay, Sanders, Spitsbergen & Whipps in press).

The pathogenesis of *Mycobacterium* spp. from zebrafish research facilities has recently been examined (Watral & Kent 2007). Infection with *M. marinum* isolates resulted in 100% infection with mortality between 30 and 100%. Infection with *M. chelonae*, *M. peregrinum* or *M. abscesuss* isolates resulted in low to moderate infection prevalence with negligible mortality. Recently, *M. haemophilum* has been identified as

the causative agent of outbreaks resulting in high mortality in zebrafish facilities (Whipps et al. 2007). Conversely, in well-maintained laboratories *M. chelonae* usually causes minimal mortality, even when fish exhibit a relatively high prevalence of infection and histological changes (Whipps, Matthews & Kent 2008). Clearly differences in the virulence and pathogenicity of *Mycobacterium* spp. play a significant role in subsequent outbreaks of disease (Watral & Kent 2007), but the rearing environment may also play an important role.

Husbandry stress is often considered a factor in exacerbating diseases of fishes. Stress is a physiological cascade of events that occurs when organisms attempt to maintain homeostatic balance after perception of a threat (Schreck, Contreras-Sánchez & Fitzpatrick 2001). The response to stress typically includes elevation of the stress hormone cortisol (Barton 2002). Stress is adaptive under acute conditions but maladaptive under conditions of chronic or repeated stress (Schreck 2000). Chronically stressed fish tend to be more susceptible to pathogens and diseases that non-stressed fish (Schreck 1996).

We have recently described increases in whole-body cortisol levels of zebrafish exposed to chronic crowding or acute handling stressors (Ramsay, Feist, Varga, Westerfield, Kent & Schreck 2006; Ramsay, Feist, Varga, Westerfield, Kent & Schreck in review). Chronic stress and elevation of cortisol is generally immunosuppressive and often a factor contributing to increased disease prevalence and morbidity in fish populations (Kent & Hedrick 1987; Maule, Tripp, Kaattari & Schreck 1989; Saeij, Verburg-van Kemenade, van Muiswinkel & Wiegertjes 2003; Dror, Sinyakov, Okun, Dym, Sredni & Avtalion 2006), and stress has been implicated in exacerbating mycobacteriosis in zebrafish (Harriff, Bermudez & Kent 2007; Westerfield 2007).

In animal models for mycobacteria, stress has been demonstrated to suppress the immune response. Swimming stress decreased the acute inflammatory response of autoimmune-prone mice infected with *M. avium* (Martins & Águas 1995). Restraint stress impaired the activation of T-cells in mice with mycobacterial infections (Zwilling, Brown, Christner, Faris, Hilburger, McPeek, Van Epps & Hartlaub 1992). Host resistance to *M. bovis* was reduced in hamsters with increased serum cortisol levels (Righi, Pinheiro, Guerra & Palmero-Neto 1999; Palermo-Neto, Santos, Guerra, Santos &

Pinheiro 2001). Zebrafish *rag1* mutants, lacking fully functional T and B cells, were hyper-susceptible to infection with *M. marinum* (strain M) because of a failure to control bacterial growth (Swaim, Connolly, Volkman, Humbert, Born & Ramakrishnan 2006). Similar impairments to adaptive immunity may result with chronic stress (Schreck 1996) suggesting that stress may increase susceptibility to mycobacterial infections in zebrafish.

Many *Mycobacterium* species are ubiquitous in the aquatic environment, making control by avoidance of these pathogens very difficult. Furthermore, there is no effective treatment for mycobacteriosis in zebrafish. A better understanding of the pathogenesis of *Mycobacterium* spp., including factors affecting host susceptibility, may enable zebrafish researchers to manage this pathogen and prevent potential disease outbreaks through effective husbandry practices. The aim of this study was to examine the role of husbandry stressors on the susceptibility of adult zebrafish to mycobacterial infections. We chose the type strain of *M. marinum* ATCC 927 because it resulted in a high infection prevalence and low mortality in previous studies in our laboratory (Ostland, Watral, Whipps, Austin, St-Hilaire, Westerman & Kent 2008; Watral & Kent 2007) and *M. chelonae* H1E2 because it resulted in a moderate infection prevalence, negligible mortality, and is commonly found in apparently healthy fish in zebrafish facilities (Whipps et al. 2008).

Materials and methods

Bacteria and growth conditions

Mycobacterium marinum ATCC 927 was originally obtained from the American Type Culture Collection (Manassas, VA USA), first isolated over 80 years ago from aquarium fish (Aronson 1926). Prior to infection of the fish in our study, *M. marinum* was passed through hybrid striped bass (*Morone saxatilis* x *M. chrysops*; Ostland et al. 2008), re-isolated on Lowenstein-Jensen medium (Remel, Lenexa KS USA) and used for an *in vivo* pathogenesis study (Watral & Kent, 2007; Ostland et al. 2008). The *M. chelonae* H1E2 was isolated from zebrafish (TU strain) during routine screening at a zebrafish facility (Whipps et al. 2008).

The growth conditions of each *M. marinum* and *M. chelonae* were identical. Fresh colonies of bacteria were suspended in Middlebrook 7H9 broth containing 1% albumin-

dextrose-catalase (ADC, Becton Dickson, Sparks, MD) with 0.1% Tween (Sigma-Aldrich, St. Louis MO) to prevent clumping of the bacteria. The cultures were placed on a rocker at 28 ^{o}C for 5 days. The presence of mycobacteria in the cultures was verified by acid-fast staining via the Kinyoun method (Hardy Diagnostics, Santa Maria CA, USA). Concentrations of the inocula were adjusted using McFarlane's standards and verified by plating on Middlebrook 7H10 media with 10% oleic acid-albumin dextrose-catalase (OADC, Becton Dickson, Sparks MD).

Fish husbandry and experimental design

Zebrafish (AB strain; 15-19 months old; 2/3 males, 1/3 females) from the Zebrafish International Resource Center in Eugene, OR USA were randomly allocated to acrylic tanks (10 L) in a Bio-safety Level 2 Laboratory at Oregon State University in Corvallis, OR USA. Tanks were filled with static de-chlorinated city water and fish were allowed to acclimate for 2 weeks at a density of 1.5 – 2 fish/liter. Water temperature was maintained at 27-28 ^{o}C. Ammonia and nitrite levels were monitored daily using test kits and water changes were performed periodically. Additionally, box-type aquarium filters with porous lava rock were placed into each tank for biological filtration. Fish were fed twice daily (Zeigler adult zebrafish diet, Zeigler Bros. Inc., Gardners, PA USA) during the acclimation and experimental periods.

M. marinum-stress study

Treatments were randomly assigned to 12 tanks each containing 15 fish (180 fish total; average wet weight: 541 $\pm$ 30 mg). Four tanks were assigned to be *M. marinum*-infected tanks; two of these tanks were assigned to be stressed (*M. marinum*-stress) and two were assigned to receive no stress (*M. marinum*-control). The remaining eight tanks were assigned to be sham-infected; four of these tanks were assigned to be stressed (sham-stress) and four were assigned to receive no stress (sham-control). Fish in all four of the *M. marinum*-infection tanks were processed for histology and cultured for bacteria. Fish in two sham-stress and two sham-control tanks were processed for histology and cultured for bacteria. Fish in the remaining two sham-stress and two sham-control tanks were examined for whole-body cortisol only.

M. chelonae-stress study

We repeated the protocol used for the *M. marinum* study using *M. chelonae*, but we did not measure whole-body cortisol during this study. We, therefore used four tanks injected with *M. chelonae* (2 *M. chelonae*-stress, 2 *M. chelonae*-control) and four sham-injected (2 sham-stress, 2 sham-control). Each tank contained 17-20 fish; a total of 109 fish were used for this study. The average wet weight of fish was 484 mg (± 20 mg).

Infection of fish and stress protocol

Exposure to the bacteria was performed as described by Watral & Kent (2007). Briefly, fish were anesthetized with 150 mg/L buffered tricaine methane sulfonate (MS-222; Argent, Redmond WA, USA). Each fish from the infection tanks was intraperitoneally (IP) injected with 50 µL inoculum to achieve a target dose of 5×10^4 colony-forming units (cfu) per fish. The actual dose received by fish in the *M. marinum* study was 1×10^2 cfu/fish while fish from the *M. chelonae* study received the target dose of 5×10^4 cfu. Fish from the infection-control tanks were sham-injected IP with sterile distilled water (50 µL).

One week following injection, stressors were administered to the stress tanks. A continuous crowding stressor was administered for the duration of the studies. Fish were crowded in rectangular breeding cage inserts, with a false bottom, placed into the 10 L tanks so the edges of the breeding cage rested on the top edges of the tank; the crowding density was 30 fish/L. In addition to continuous crowding, various handling stressors were administered randomly to ensure the fish did not acclimate to the stressors. Fish were exposed to air by lifting the breeding cage out of the water for various durations from 30 sec to 3 min. This was often repeated, either immediately or later in the day (within 2-4 h). Rapid repeated emersion and immersion of fish out of and into the water was also performed for periods up to 3 min. Low water handling was performed by holding the fish in the breeding cages so their backs were out of the water for periods up to 3 min. Handling of fish was performed at least once daily for the duration of the experiment. *M. marinum*-control and sham-control groups were maintained at the acclimation density (1.5 – 2 fish/L). Whole-body cortisol levels of sham-control and

sham-stress groups were measured at one week (7 fish/tank) and two weeks (8 fish/tank) after stressors were initiated.

Cumulative mortality was measured over an 8 week experimental period which started after the fish were injected. Moribund fish were removed from the tank just prior to death. Mycobacterial infections were evaluated by histology and culture for *M. marinum* study and by histology alone for the *M. chelonae* study. The number of infected fish in each group was compared at week 4 (5 fish/tank) post-injection (PI) and the remaining fish were sampled and evaluated at week 8 PI. Additionally, the numbers of fish with diffuse (sub-acute) infection were compared between groups at week 4 and 8 PI.

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Oregon State University (ACUP #3144).

Histology

Upon sampling, fish were euthanized with buffered MS-222 (500 mg/L), placed in Dietrich's fixative (Gray 1954) and processed for histology. Prior to processing, preserved fish were de-calcified using 5% trichloroacetic acid in Dietrich's fixative. Mid-sagittal sections were cut and stained using a modified Kinyoun's cold acid-fast stain to identify mycobacteria. Briefly, slides were de-paraffinized, rehydrated and placed into carbol-fuchsin (8 g basic fuchsin HCl, 40 mL 95% Ethanol, 200 mL deionized water, 16 mL liquefied phenol) for 15 minutes. De-staining was performed in a 1% hydrochloric acid solution in 70% ethanol for 2 minutes. Methylene blue was used to counter-stain.

Bacterial culture

For the *M. marinum* study, prior to processing for histology, bacteria were cultured from each fish. The spleen and a portion of the liver were removed from each fish and placed in 100 μL phosphate-buffered saline (PBS). A 1% cetylpyridinium chloride solution (100 μL) was added to this preparation. The tissues were manually homogenized and incubated overnight at room temperature to remove contaminants. The samples were rinsed twice with PBS, re-suspended with 100 μL PBS and plated on Middlebrook 7H10 plates. The plates were incubated for 7-10 days at 28 ^{o}C and bacterial growth (cfu) was recorded. Bacteria were not cultured for the *M. chelonae* study as a

previous study with this same isolate in our laboratory showed an excellent correlation between culture and histology using acid fast staining (Whipps et al. 2008).

Whole-body cortisol

Whole-body cortisol was measured using the method of Ramsay et al. (2006). Whole zebrafish were homogenized and extracted for cortisol using diethyl ether. Samples were corrected for extraction efficiency and weight. Cortisol was measured in the extracted samples using a radioimmunoassay. Consistency between assays was verified by measuring cortisol in whole-body extracts spiked with known concentrations of cortisol. Intra-assay and inter-assay variation was accepted at no more than 10%.

Statistical analyses

Statistical analyses were performed using S-PLUS 7 (Insightful Corp., 2005, Seattle, Washington). Whole-body cortisol levels of duplicate treatment tanks were compared using a Welch's modified t-test and pooled if there was no significant difference between the duplicates. Pooled stressed and non-stressed groups at week 1 and 2 post-stress were then compared using an analysis of variance (ANOVA) with Fisher's LSD.

Fisher's Exact Tests were used to determine the strength of associations between treatment groups and prevalence of infection or prevalence of diffuse infection. An odds ratio (OR) was used to determine whether the odds of being infected were different between stressed and non-stressed groups. Odds ratios were also used to determine if the odds of having a diffuse infection were greater between stressed and non-stressed groups. The odds were considered significant at OR>2 (Ludbrook 2008). Comparisons were made within each study but not between studies.

Significance differences between treatment groups were reported at $\alpha=0.05$.

Results

Background infection with Pseudoloma neurophilia

During the *M. marinum* study, the microsporidium, *Pseudoloma neurophilia*, a parasite of the central nervous system of zebrafish, was found in many of the histological

sections of the fish. The prevalence of *P. neurophilia* was 57% in the sham-injected tanks and 81% in the *M. marinum*-injected tanks (Table 4.1). There was a strong association between infection group (*M. marinum*-injected vs. sham-injected) and the prevalence of *P. neurophilia* infection (p=0.0076). *Pseudoloma neurophilia* was not detected in the fish from the *M. chelonae* experiment.

Whole-body cortisol (M. marinum study)

There were no significant differences between replicates of the same treatment; therefore the data were pooled. Whole-body cortisol was significantly higher in the stressed groups compared to the control groups (Fig. 4.1). There was no difference between the cortisol of the control groups from week 1 to week 2 post-stress. However, among the stressed groups cortisol was significantly lower at week 2 compared to week 1 post-stress.

Histopathology and culture

Infected fish from both the *M. chelonae* and *M. marinum* studies exhibited characteristic mycobacteriosis including granulomas containing acid-fast bacilli in the visceral organs and kidney, (Fig. 4.2 A,B,E,F; Fig. 4.5, Appendix Figure A4.1). Additionally, diffuse infections associated with inflammation caused by acid-fast bacilli scattered in the kidney, liver and gastrointestinal tract were identified in numerous individuals, particularly among stressed fish (Fig. 4.2 C,D,G,H, Fig. 4.5, Appendix Figure A4.2).

Acid-fast bacterial colonies were recovered from 20 infected fish from both the *M. marinum*-stress and *M. marinum*-control groups. Colony counts ranged from 1×10^2 cfu to 7×10^4 cfu (Fig. 4.3). Individuals with the highest bacterial counts were from the stressed groups. Histopathology was more sensitive at identifying *M. marinum* infections than culture (Table 4.2).

Cumulative mortality over time

During the *M. marinum*-study, there was no morbidity among the *M. marinum*-control tanks. Cumulative mortality was 14% among the *M. marinum*-stress tanks (Fig. 4.4; Table 4.1). Interestingly, there was 3% mortality in both the sham-control and sham-

stress tanks but there was no evidence of mycobacterial infection in any individuals from these groups. However, many of the individuals in these sham-infected tanks were infected with *P. neurophilia* (Table 4.1).

For the *M. chelonae*-stress study, all fish in one of the control-stress tanks unexpectedly died at week 5 PI. There were no mycobacteria or other pathogens identified from this group. There was no additional mortality among any of the other groups.

Prevalence of mycobacterial infections

None of the control fish in the *M. marinum*-stress study became infected with mycobacteria. Conversely, almost all of the fish that were injected with *M. marinum* were infected at both week 4 and 8 PI (Table 4.1). Therefore, odds ratios were not compared for the *M. marinum*-stress study because the odds of being infected, among the *M. marinum* groups, were the same.

For the *M. chelonae*-stress study there was a single fish in one of the sham-stress tanks that had acid-fast mycobacterial rods in the swim bladder. There was no further histological evidence of mycobacteria in any visceral tissues and no internal or external signs of mycobacteriosis.

Among the *M. chelonae*-infected groups, at week 4 PI there was no association between stress treatment and infection ($p=0.473$). However, at week 8 PI, there was a positive association between stress treatment and infection ($p=0.03$); the stressed group was over 15-fold more likely to be infected than the non-stressed group ($OR=15.11$).

Prevalence of diffuse infections

There was a strong association between stress treatment and diffuse infections for fish infected with *M. marinum* ($p=0.0012$; Table 4.1). Stressed fish infected with *M. marinum* were almost 7-fold more likely to have diffuse infections compared to fish that were not stressed ($OR = 6.97$). At week 4 PI, there was no association between stress treatment and diffuse infections for *M. chelonae*-infected fish ($p=0.285$). However, by week 8 PI, there was a strong association between stress treatment and *M. chelonae*-

infection (p=0.0045); stressed fish were over 11-fold more likely to have diffuse infection than non-stressed fish (OR = 11.43).

Discussion

Mycobacteriosis is a significant disease of laboratory zebrafish (Astrofsky et al. 2000; Kent et al. 2004; Whipps et al. 2007), with no effective treatments currently available. Indeed, zebrafish are a highly susceptible species based on laboratory transmission studies comparing zebrafish to medaka, *Oryzias latipes* (Broussard & Ennis 2007) and hybrid striped bass, *Morone chrysops* x *Morone saxatilis* (Ostland et al. 2008). Better understanding the factors affecting dissemination and exacerbation of this disease may aid in controlling outbreaks in zebrafish colonies. We have demonstrated husbandry stress exacerbates mycobacterial infections in adult zebrafish. To our knowledge this is the first study linking stress and disease in laboratory zebrafish.

We detected background infection with the microsporidium, *P. neurophilia* during the *M. marinum* study. *Mycobacterium* spp. and *P. neurophilia* are both common pathogens of laboratory zebrafish and co-infection is not uncommon (M.L. Kent, V. Watral and J.M. Ramsay, personal observations). *P. neurophilia* infects the central nervous system and somatic muscle of zebrafish and is associated with emaciation, spinal deformity, and low-level chronic mortality (Matthews, Brown, Larison, Bishop-Stewart, Rogers & Kent 2001; Kent & Bishop-Stewart 2003). The low-level mortality among the sham-control and sham-stress groups, which were negative for mycobacteria, may be attributable to *P. neurophilia*. Interestingly we found a higher prevalence of microsporidiosis in zebrafish infected with *M. marinum*. Microsporidiosis tends to be more prevalent in immune-suppressed individuals (Didier & Weiss 2006) and mycobacteriosis has been demonstrated to suppress immunity (Fenton & Vermeulen 1996; Moura & Mariano 1997). *Loma salmonae* (Microsporidia)–infected rainbow trout, *Oncorhynchus mykiss* (Walbaum), treated with the immune-suppressing synthetic steroid dexamethasone, had increased parasitic infections compared to untreated fish (Lovy, Speare, Stryhn & Wright 2008). Further investigation on the effects of co-infection with mycobacteria and microsporidium may be useful in controlling these common pathogens of zebrafish

In the *M. chelonae* study, we found one fish with a mycobacterial infection of the swim bladder. Similar background levels of mycobacteria have been found in previous studies in our laboratory (Harriff et al. 2007). Environmental mycobacteria, such as *M. chelonae* and *M. fortuitum*, are common in freshwater aquaria (Zanoni et al. 2008), and *M. fortuitum* is commonly isolated from fish without causing disease (Beran, Matlova, Dvorska, Svastova & Pavlik 2006). Our laboratory has examined hundreds of histological sections of zebrafish stained with acid-fast through our diagnostic service of the NIH Zebrafish International Resource Center (http://zebrafish.org/zirc/health/index.php), and we frequently see acid-fast bacteria only in the lumina of the intestine and swim bladder. The swim bladder can easily be colonized via the gut as zebrafish are physostomus. Moreover, infection of this organ is a common finding in zebrafish mycobacteriosis (Whipps et al. 2008).

Mycobacterial infections were more readily identified by histopathology than culture for the *M. marinum* study. *Mycobacterium marinum* is fastidious in culture making this method of identification less sensitive than histology for this particular species of mycobacteria (Kent et al. 2004). Conversely, there is a strong correlation between culture and histopathology with *M. chelonae* (Whipps et al. 2008). *M. chelonae* exhibits more rapid growth than *M. marinum* which could account for the better correlation between culture and histopathology (Whipps et al. 2008).

Among the *M. marinum*-injected group we were able to achieve almost 100% infection after injection with 1 x 10^2 cfu with no mortality among groups not subjected to chronic stressors. Similarly, 100% of zebrafish were infected at a dose of 10^3 cfu, with no mortality (Prouty et al. 2003). Our dosage used in the present study was much lower than that used in previous virulence trials with zebrafish (Watral & Kent 2007; Ostland et al. 2008), where injection of the same strain of *M. marium* caused 100% infection and about 30% mortality in non-stressed fish. Goldfish, *Carassius auratus*, injected with 10^7 cfu *M. marinum* all survived 8 weeks PI (Talaat, Reimschussel, Wasserman & Trucksis 1998). Leopard frogs, *Rana pipiens*, injected with 23 cfu *M. marinum* (M) developed chronic disease with no mortality (Ramakrishnan, Valdivia, McKerrow & Falkow 1997). Identifying the minimum and maximum dose required to achieve a chronic infection with

no mortality may be useful for studying the effects of stress during chronic mycobacteriosis.

Whole-body cortisol levels were higher for stressed than non-stressed groups, and consistent with our other studies (Ramsay et al. 2006; Ramsay et al. in review). Stressed groups, infected with *M. marinum*, experienced 14% cumulative mortality while non-stressed groups experienced no mortality. This agrees with studies with other species. Daily handling significantly increased plasma cortisol levels and reduced the survival of carp, *Cyprinus carpio* L., injected with *Trypanoplasma borreli* (Saeij et al. 2003). Goldfish infected with *Aeromonas salmonicida* and subjected to handling stress had significantly elevated plasma cortisol levels associated with decreased survival among stressed groups (Dror et al. 2006). Chronic cortisol injection of leopard frogs injected with *M. marinum* (M) resulted in 70% mortality over a 19 week period while frogs not treated with cortisol experienced no mortality (Ramakrishnan et al. 1997). Mice injected with *M. tuberculosis* experienced similar increases in mortality when injected with corticosteroids (Batten & McCune 1957). In a previous study, zebrafish injected with 5 x 10^4 cfu of the same strain of *M. marinum* (ATCC 927) had 30% mortality over an 8 week period (Watral & Kent 2007) suggesting that chronic stress and elevated cortisol may lower the dose of *M. marinum* which will result in mortality.

Fish injected with *M. chelonae* did not experience any mortality over an 8-week period. *M. chelonae* is typically found at background levels in zebrafish facilities and has been associated with low levels of mortality (<20%) which are often attributed to poor husbandry and stress (Astrofsky et al. 2000; Kent et al. 2004; Whipps et al. 2008). A greater percentage of stressed catfish, *Ictalurus punctatus*, were positive for *Edwardsiella ictaluri* compared to those not stressed (Small & Bilodeau 2005). Although stressed groups infected with *M. chelonae* did not experience any mortality in our study, stressed individuals were 15-fold more likely to be infected and show histological lesions compared to non-stressed individuals. Although the infected-stressed fish did not die over the 8 week study, the increased prevalence of infection by this common, ubiquitous bacterium probably increases the likelihood of mortality associated with infection. Moreover, with fish used in research, underlying chronic conditions that cause low-grade or subclinical infections should be avoided because they may add non-protocol induced

variables, including inadvertent alterations in the physiology of the host, which were not accounted for in the original experimental design; this may confound research results (Baker 2003; Kent et al. in press).

Diffuse infections were greater in stressed groups than in non-stressed groups for both *M. marinum* and *M. chelonae* infected fish. This was likely due to impaired ability to sequester the bacteria in granulomas. For example, zebrafish (*rag1*) with impaired immunity failed to control mycobacterial growth resulting in increased diffuse infections (Swaim et al. 2006). In leopard frogs, injection with cortisol resulted in impaired formation of granulomas and increased replication of mycobacteria in tissues (Ramakrishnan et al. 1997). Stress generally reduces the host response (acute inflammatory, T-helper cell activation) to mycobacterial infections subsequently increasing host susceptibility to infection (Zwilling et al. 1992; Martins & Águas 1995; Palmero-Neto et al. 2001).

In conclusion, husbandry stress increased clinical disease associated with mycobacterial infections. With *M. marinum*, stressors increased mortality and diffuse infections. With *M. chelonae* (a less virulent bacterium), stressors increased the prevalence of infection and diffuse infections, but did not cause mortality. Mycobacteria are ubiquitous in the environment making eradication of this pathogen very difficult. Effective management of mycobacteria by minimizing husbandry stress in the zebrafish laboratory may be a reasonable means of controlling outbreaks of disease and ensuring continued success in zebrafish research.

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Table 4.1: Zebrafish were injected with *Mycobacterium marinum* or *M. chelonae* or distilled water (DW; sham-injection) and some were exposed to stressors over an 8-week period. Replicate tanks of the same treatment are indicated by the letters A and B. Morbidity (proportion of fish) was measured over an 8 week period. The prevalence of infection was evaluated by culture or histology at week 4 and 8 post-injection (PI) The proportion of fish with diffuse infections was measured at week 4 and 8 PI. The proportion of fish which were positive for *Pseudoloma neurophilia* via histology was measured. * Unexplained mortality of a sham-injected tank in *M. chelonae* Study at week 5 PI (no mycobacteria present).

					Positive (culture or histology)			Diffuse infections			
Injection	Tank	Inoculum (x 10^2)	Stressor	Moribund	Week 4 (PI)	Week 8 (PI)	Moribund	Week 4 (PI)	Week 8 (PI)	Moribund	*Pseudo -loma*
M. marinum	A	1	Y	1/15	5/5	8/9	1/1	3/5	5/9	1/1	13/15
M. marinum	B	1	Y	3/14	5/5	6/6	3/3	2/5	5/6	3/3	14/14
M. marinum	A	1	N	0/14	5/5	9/9	NA	1/5	2/9	NA	10/14
M. marinum	B	1	N	0/15	5/5	10/10	NA	1/5	2/10	NA	10/15
DW	A	0	Y	1/15	0/5	0/5	0/1	NA	NA	0/1	5/15
DW	B	0	Y	0/15	0/5	0/5	NA	NA	NA	NA	9/15
DW	A	0	N	1/15	0/5	0/5	0/1	NA	NA	0/1	10/15
DW	B	0	N	0/15	0/5	0/5	NA	NA	NA	NA	9/15
M. chelonae	A	500	Y	0/18	4/9	9/9	NA	3/9	9/9	NA	0/17
M. chelonae	B	500	Y	0/16	4/7	8/9	NA	4/7	7/9	NA	0/18
M. chelonae	A	500	N	0/14	5/7	4/7	NA	5/7	2/7	NA	0/14
M. chelonae	B	500	N	0/18	5/8	5/10	NA	5/8	5/10	NA	0/18
DW	A	0	Y	0/21	1/10	0/11	NA	0/10	NA	NA	0/21
DW	B	0	Y	11/21*	0/10	0/11	NA	NA	NA	NA	0/10
DW	A	0	N	0/21	0/10	0/11	NA	NA	NA	NA	0/21
DW	B	0	N	0/21	0/10	0/11	NA	NA	NA	NA	0/21

Table 4.2: Proportion of fish positive for mycobacteria by culture (acid-fast colonies) and histology (acid-fast bacterial rods) at weeks 4 and 8 post-injection (PI).

	Week 4 PI		Week 8 PI	
	Culture	Histopathology	Culture	Histopathology
M. marinum-stress tank A	2/5	2/5	3/9	5/9
M. marinum-stress tank B	1/5	4/5	3/5	3/5
M. marinum-control tank A	0/5	4/5	2/9	6/9
M. marinum-control tank B	0/5	4/5	8/10	7/10

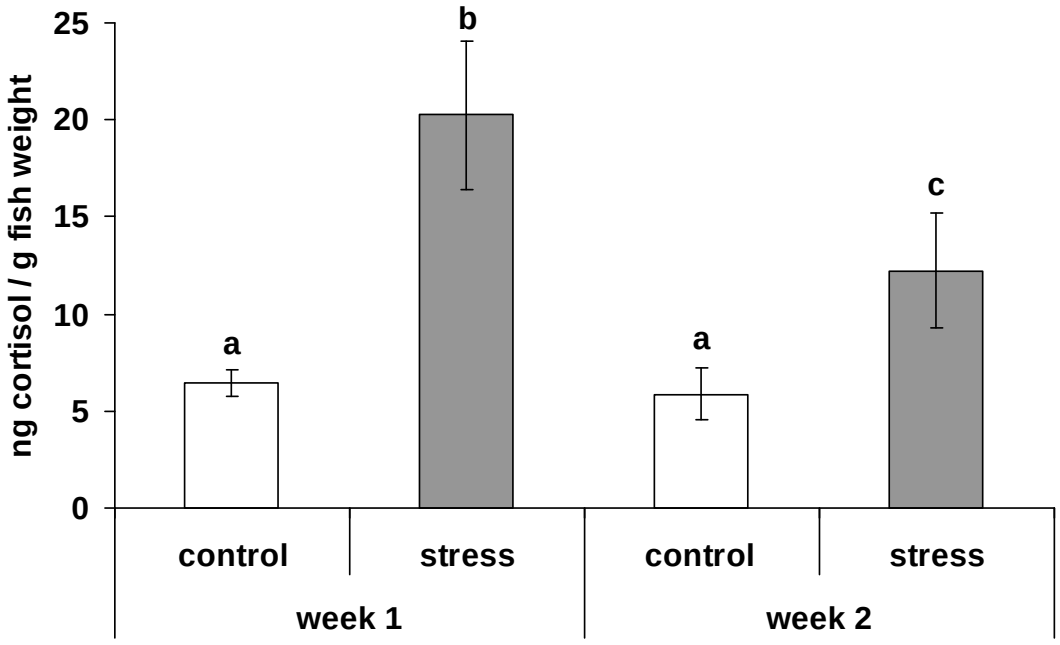

Figure 4.1: *M. marinum*-stress experiment. Mean whole-body cortisol (ng/g fish; +SEM; n=15) of control groups (no stressors) and stress groups (chronic crowding and random daily handling) one and two weeks after the stressors were initiated. Replicate tanks of the same treatment were pooled because they were not significantly different. Different letter above the standard error bars indicate a significant difference between treatment groups ($p<0.05$).

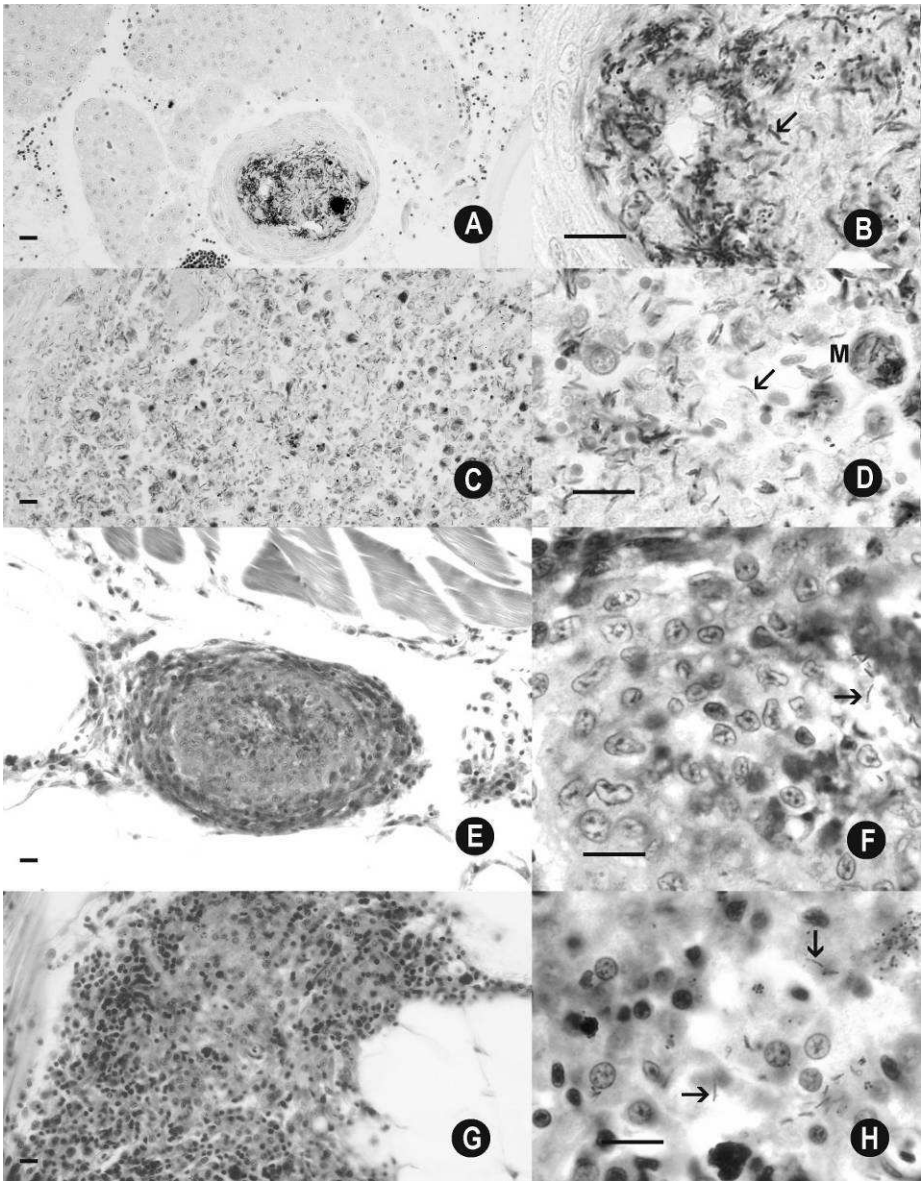

Figure 4.2: Histological sections of zebrafish infected with *Mycobacterium* spp. (acid-fast staining). Arrows = individual bacteria in lesions Bars = 10µm. A) *M. marinum.* Granuloma in liver. B) High magnification of A. C) *M. marinum*, diffuse infection in anterior kidney, with numerous bacteria within phagocytes. D) High magnification of C, M = macrophage replete with bacteria. E) *M. chelonae* – granuloma in mesenteries ventral to kidney. F) High magnification of E showing bacteria. G. *M. chelonae*, diffuse infection in mesenteries. H) High magnification of G showing bacteria.

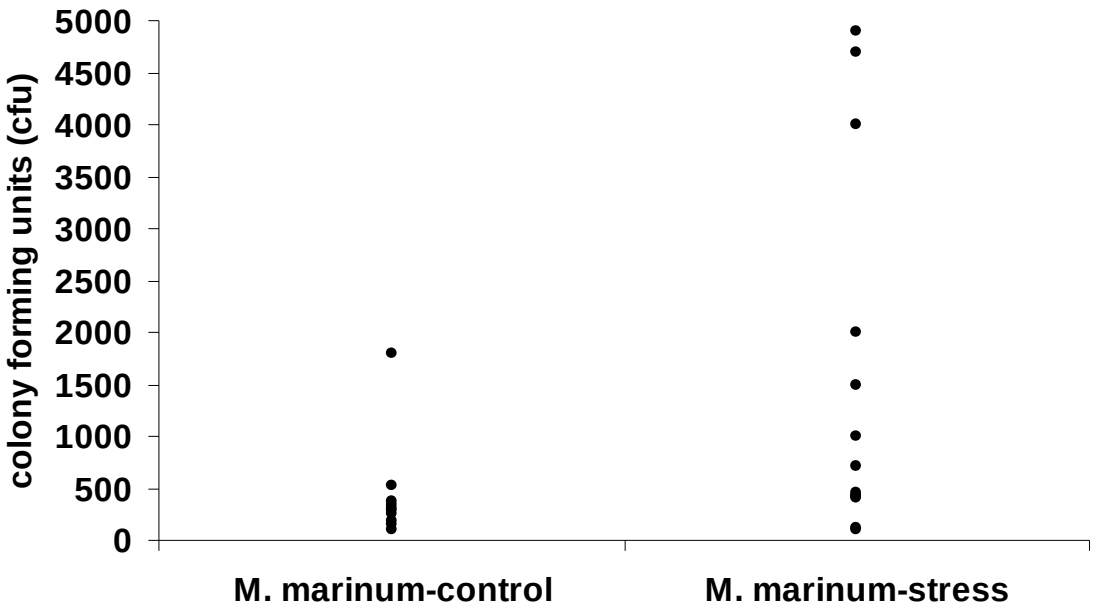

Figure 4.3: Bacterial colony counts individual fish from the *M. marinum* Study. Counts ranged from 100 to 70000 colony-forming units (cfu). One individual in the *M. marinum*-stress group had a colony count 70000 cfu (not shown on Figure).

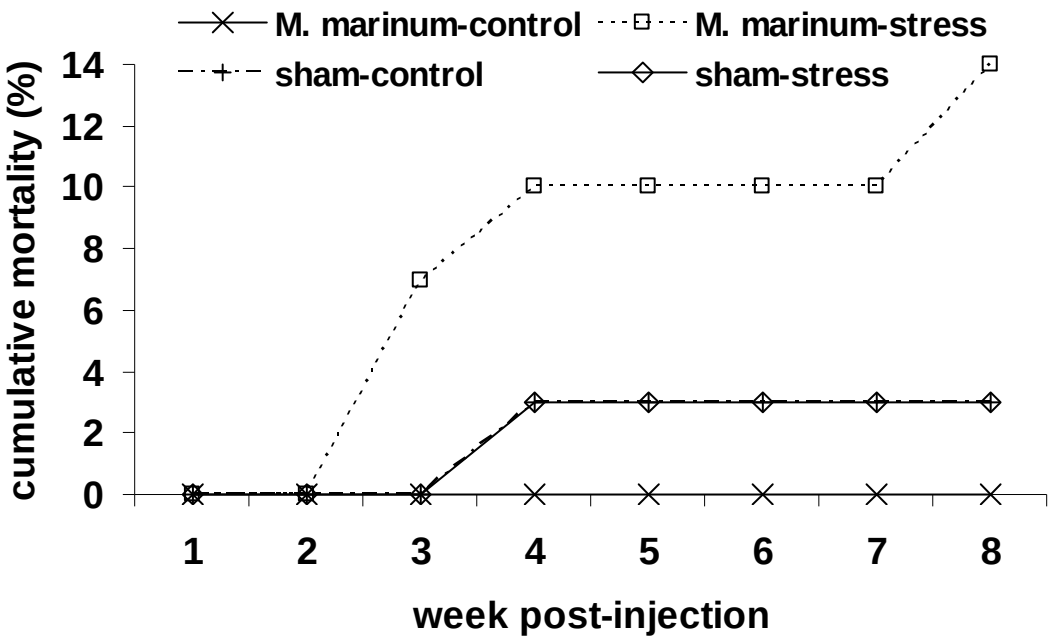

Figure 4.4: Cumulative mortality (%) of *M. marinum*-injected fish subjected to stress and not stressed (control) and sham-injected fish subjected to stress and not stressed (control) over time (week post-injection).

CHAPTER 5: SEQUENTIAL DEVELOPMENT OF *PSEUDOLOMA NEUROPHILIA* (MICROSPORIDIA) IN JUVENILE ZEBRAFISH (*DANIO RERIO*): EFFECTS OF STRESS ON SURVIVAL, GROWTH AND REPRODUCTION

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Abstract

Pseudoloma neurophilia (Microsporidia) is a common disease of zebrafish, *Danio rerio*, including those used as research models. We conducted a study comprised of three separate experiments to determine the effects of stress on pre-existing and experimental *Pseudoloma* infections and the subsequent effects on survival, growth and reproduction. In fish (AB strain) with pre-existing infections, stress or feeding cortisol increased mortality over 7 weeks compared to no stress or cortisol treatment. In contrast, no mortality was observed with experimentally-exposed fish (TL strain) over 10 weeks. A third experiment involved experimental exposure of AB fish to *Pseudoloma* and exposure to crowding and handling stress. No mortality was associated with *Pseudoloma* regardless of stress treatment over a 20 week period. However, the onset of infection occurred sooner in stress-treated fish. Stress significantly increased the mean intensity of infection (described as xenoma area / spinal cord area in histological sections) at week 20 PE (post-exposure). In fish with pre-existing infections, myositis, was significantly greater in stressed and cortisol-treated fish than those not stressed. With experimental exposure of AB fish, weight decreased in stressed and infected groups from week 13 to 20 PE but not in the control group. Regarding fecundity, the number of larvae hatched at 5 days post fertilization was negatively associated with mean infection intensity among *Pseudoloma*-infected and stressed AB fish. These three experiments are the first to show empirically that *Pseudoloma* can be associated with reduced weight and fecundity, and that stress can exacerbate the severity of the infection.

KEYWORDS: *Pseudoloma neurophilia*, Stress, Growth, Reproduction, Mortality, Microspora

Introduction

Microsporidia are spore-producing obligate intracellular parasites with over 150 species infective to fishes (Shaw and Kent 1999; Lom and Nilsen 2003). Microsporidiosis was first reported in zebrafish, *Danio rerio*, almost 30 years ago (de Kinkelin 1980), and this microsporidium was recently assigned to a new genus and species, *Pseudoloma neurophilia* (Matthews et al. 2001), which we henceforth refer to in this chapter as *Pseudoloma*. Microsporidiosis, the most common disease of laboratory zebrafish, affects the central nervous system and somatic muscle, and is associated with emaciation, spinal deformity and morbidity (Matthews et al. 2001; Kent and Bishop-Stewart 2003). The spores of *Pseudoloma* are contained within host-parasite complexes known as xenomas (Chatton 1920; Lom and Dyková 2005). *Pseudoloma* infections are characterized by multiple xenomas in the hind brain, spinal cord, nerve roots, and occasionally within the somatic muscle. Free spores (probably from ruptured xenoma) are found within phagocytes and are associated with severe, chronic myositis, meningitis and encephalitis (Matthews et al. 2001; Kent and Bishop-Stewart 2003). Ovarian tissue is often infected and occasionally eggs may harbor *Pseudoloma* spores indicating potential for vertical transmission (Kent and Bishop-Stewart 2003; Fig. 5.12, Appendix 2).

Zebrafish are popular biomedical and environmental research models (Dahm and Geisler 2006; Scholz and Mayer 2008). The number of laboratories rearing, breeding, and transporting zebrafish has increased, subsequently increasing the potential for dissemination and exacerbation of diseases such as microsporidiosis (Kent et al. in press). Control of infectious diseases in zebrafish laboratories typically involves quarantine and chlorine disinfection of eggs (Westerfield 2007). Microsporidian spores are durable and remain infective for long periods (Shaw et al. 2000a) and chlorine treatments used for disinfecting zebrafish eggs have proven to be ineffective for killing *Pseudoloma* spores (Ferguson et al. 2007). Optimizing rearing conditions may aid in controlling diseases of zebrafish such as microsporidiosis (Lawrence 2007). Infected fish often appear clinically healthy (Matthews 2004; Whipps and Kent 2006) suggesting that another factor, such as rearing environment, may play a key role in the severity of *Pseudoloma* infections.

Husbandry stress is often implicated in infectious diseases of cultured fishes (Schreck 1996). Stress is an adaptive and dynamic physiological state which occurs after an organism perceives a threat and attempts to restore physiological balance (Schreck et al. 2001). Chronic stress and elevation of cortisol is generally maladaptive resulting in immune suppression and increased susceptibility to infectious diseases (Schreck 1996), and cortisol is typically used as an indicator of stress in fishes (Barton 2002). In addition, stress has been demonstrated to reduce growth and reproductive fitness of fishes (Campbell et al. 1994; Schreck 2000; Schreck et al. 2001).

Under conditions of chronic stress and elevation of cortisol, immune suppression is typical and often contributes to increased disease prevalence and morbidity in fish populations (Kent & Hedrick 1987; Maule et al. 1989; Saeij et al. 2003). Stress has been suggested to exacerbate microsporidiosis in zebrafish but this has yet to be examined (Westerfield 2007). We described increases in zebrafish whole-body cortisol following chronic crowding or acute handling stress (Ramsay et al. 2006; Ramsay et al. in review).

In other animal models exposure to corticosteroids generally increases the intensity of microsporidiosis. Nude rats, *Rattus norvegicus*, and gerbils, *Meriones unguiculatus*, infected with *Enterocytozoon bieneusi* and given the synthetic corticosteroid dexamethasone (DEX), produced more spores than infected animals given a placebo (Feng et al. 2006). Clinical signs of encephalitozoonosis were associated with reduced antibody titers and only apparent in infected mice treated with DEX (Lallo et al. 2002; Herich et al. 2006). In turbot, *Scophthalmus maximus*, injected with *Microgemma caulleryi*, DEX treatment reduced the ability of macrophages and neutrophils to ingest spores (Leiro et al. 2000). *Loma salmonae*–infected rainbow trout, *Oncorhynchus mykiss*, had higher infection intensities when treated with DEX (Lovy et al. 2008).

In addition to increasing susceptibility to disease, stress also affects reproduction. Developmental biology is the cornerstone of zebrafish research and a consistent supply of good quality eggs is fundamental to a productive zebrafish laboratory (Lawrence 2007; Westerfield 2007). Stress has been implicated when breeding is poor or during high mortality of embryos However, the effects of stress on zebrafish reproductive fitness have not been well studied.

Stress has a variable effect on reproduction, which is dependent upon numerous factors, including the species, gender, reproductive strategy, and the nature of the stressor (Schreck et al. 2001; Wingfield and Sapolsky 2003). Rainbow trout and brown trout, *Salmo trutta*, subjected to chronic crowding stress in the months prior to spawning had increased plasma cortisol and produced smaller eggs and offspring with reduced survival (Campbell et al.1994). Increased maternal cortisol in damselfish, *Pomacentrus amboinensis*, was associated with reduced larval size (McCormick 1998, 2006). The timing of stressors is important in determining the effects on reproduction. Rainbow trout subjected to random stressors during late maturation or during the entire maturation period ovulated 2 weeks earlier than control fish with no significant effects on fecundity or fertilization (Contreras-Sánchez et al. 1998). Stress during early vitellogenesis resulted in smaller eggs and fry but there was no significant difference in juvenile weight.

In addition to the effects of stress on reproduction, pathogens may impair reproductive fitness. The microsporidia are one of the few groups of parasites known to disrupt the endocrine system of fishes resulting in reproductive impairment. Heavy infections of *Pleistophora mirandellae* in the gonads of roach, *Rutilus rutilus,* were associated with intersex, and infected fish were unable to produce viable offspring (Wiklund et al. 1996). *Drosophila* spp. infected with *Tubulinosema kingi* had up to 55% reduction in early-life fecundity with increased larval mortality and delayed larval development (Futerman et al. 2005). Golden shiners infected with *Ovipleistophora ovariae,* had reduced fecundity and failed to spawn due to destruction of the ovaries (Summerfelt and Warner 1970). Conversely, in some cases pathogenic infections do not negatively affect reproductive fitness as with laboratory male sticklebacks infected with cestodes (Candolin and Voigt 2001). Moreover, amphipods, *Gammarus roeseli*, infected with microsporidia, matured and spawned earlier than those not infected (Haine et al. 2004), suggesting that pathogens may manipulate host reproduction in order to facilitate vertical or horizontal transmission to other susceptible hosts.

There is currently no effective treatment for *Pseudoloma*. Identifying factors affecting the exacerbation of *Pseudoloma* infections may aid in controlling outbreaks of disease in this intensively cultured biomedical research model (Reno 1998; Lawrence 2007). Furthermore, elucidating how husbandry-associated factors affect reproductive

fitness will aid researchers in optimizing the reproductive output of zebrafish. The aims of this study, comprised of three separate experiments, were to examine the effects of *Pseudoloma* on growth and reproduction. Also, we attempted to elucidate the role of husbandry stressors or feeding cortisol on *Pseudoloma* infections, including the effects of stress on the sequential development of the parasite and the severity of associated disease.

Materials and Methods

Fish husbandry and sampling

Experiments were conducted at the Zebrafish Disease Laboratory (ZDL) at Oregon State University, Corvallis OR, USA, which used a flow-through system of de-chlorinated, de-gassed city water maintained at 28 ^{o}C (ammonia, nitrite, chlorine: 0 mg/L; pH: 6.5-7.2). Box aquarium filters with porous lava rock were placed into each tank for biological filtration. Photoperiod was 14 h light: 10 h dark. A 7-14 day acclimation period was used prior to initiating experiments.

Non-lethal sampling of fish consisted of anesthesia in 150 mg/L buffered tricaine methane sulfonate (MS-222; Argent, Redmond WA, USA). Lethal sampling of fish consisted of an overdose of MS-222 (500 mg/L). Wet weights (mg) and fork lengths (mm) were measured at various intervals during each experiment. Condition factor (CF) was calculated using the formula 100 * weight (mg) / length (mm)^{3}.

Stress treatment

Various stressors, including crowding, net handling with air exposure, and simulated transport, were administered as stress treatments during the experiments. These stressors have all been demonstrated to elevate zebrafish whole-body cortisol (Pottinger and Calder 1995; Ramsay et al. 2006; in review). Low water crowding involved removing the majority of the water from a tank, leaving the fish just enough water to stay upright; fish were typically crowded in this manner for 1-4 h. Fish were also temporarily crowded in a net, by netting all of the fish from a tank and placing the net, containing fish, back into the tank; fish were typically crowded in a net for 1-2 h. Acute net handling was also performed in which all of the fish were netted out of the tank and held suspended in the air for various periods ranging from 1-3 min; this procedure was often

repeated after short periods of recovery (e.g., in net for 3 min, recover in tank for 3 min, in net for 3 min, etc.). Transport stress was administered by netting all fish out of the tank into a smaller tank. This tank was then transported to another laboratory to simulate transport experienced during sampling. Fish were occasionally, anesthetized with MS-222 (150 mg/L) and allowed to recover prior to returning them to their tanks. These stressors were administered a random intervals and durations at least 5 days per week over the entire experimental periods (specified below) to ensure that the fish did not acclimate to them.

Whole-body cortisol

Whole-body cortisol was measured by the methods of Ramsay et al. (2006). First, whole zebrafish were homogenized and the lipid portion extracted using diethyl ether. The efficiency of extraction efficiency was determined by adding tritiated cortisol to homogenized samples, extracting the samples and measuring the amount of tritiated cortisol recovered. All samples were corrected for extraction efficiency and weight. Cortisol was measured in the extracted samples using a radioimmunoassay. Consistency between assays was verified by measuring cortisol in whole-body extracts spiked with known concentrations of cortisol. Intra-assay and inter-assay variation was accepted at no more than 10%.

Histology

Fish were euthanized and placed in Dietrich's fixative (Gray 1954) and processed for histology. Preserved fish were de-calcified prior to processing using 5% trichloroacetic acid in Dietrich's fixative. Mid-sagittal sections were cut and stained using a modified Kinyoun's cold acid-fast method to identify *Pseudoloma* spores and associated histological changes because acid-fast stains have been demonstrated to be effective at detecting microsporidian spores (Joseph et al. 2006). The method used was similar to that used by the NIH Zebrafish International Resource Center (ZIRC) diagnostic service (http://zebrafish.org/zirc/health/diseaseManual.php), except de-staining was reduced to less than one minute to enhance red staining of spores.

Infection intensity was normalized by measuring the area of parasite (xenomas) occupying visible spinal tissue using SPOT^{TM} Advanced imaging software (Diagnostic Instruments Inc., Sterling Heights, MI, USA). The area of visible spinal tissue was measured followed by the area of xenomas and spores in the spinal tissue. The percent area occupied by *Pseudoloma* was then calculated to give xenoma area. Myositis in individual fish was evaluated using a scoring system (0 = no myositis, 1 = one area of myositis, 2 = two areas of myositis, 3 = three or more areas of myositis).

Experiments

Cortisol-dose Experiment (AB Strain) - A preliminary study was performed to determine what dose of cortisol, administered by feeding, would elevate zebrafish whole-body cortisol levels. Adult zebrafish (AB strain; 13 months old; 120 fish) were obtained from a zebrafish facility. We randomly allocated fish to 4 acrylic tanks (30 fish per 10 L tank) and acclimated for one week. Fish in each tank were exposed to different doses of cortisol by feeding (0, 5, 33 and 100 µg cortisol/g feed). Cortisol was dissolved in ethanol and sprayed onto Zebrafish diet (Zeigler Bros. Inc., Gardners, PA USA) at the appropriate dose. Cortisol-treated feed was allowed to dry overnight in a fume hood and stored at –20 ^{o}C. The dose of cortisol was verified by the methods of Ramsay et al. (2006). Fish were fed to satiation twice daily and feeding behavior was monitored. Whole-body cortisol was measured from 5 fish per tank before beginning the experiment (week 0) and at week 1, 2, 4, and 6 post-exposure (PE).

Pre-existing Infection (AB strain) - A population of zebrafish (AB strain; 13 months old; 2/3 male, 1/3 female) with a pre-existing *Pseudoloma* infection was obtained from a zebrafish facility. Six acrylic tanks (16 L) were each stocked with 50 fish. The following treatments were administered to the tanks over a 7 wk period. STRESS: two tanks received the stress treatment, described above. CORTISOL-FED: Two tanks were fed cortisol-treated zebrafish diet. Cortisol feed was prepared at a dose of 10 µg cortisol /g feed as described in the preliminary study. The dose of cortisol was determined from the Cortisol-dose Experiment examining changes in whole-body cortisol in zebrafish fed different doses of cortisol over a 7 wk period (Fig. 5.1A). CONTROL: the remaining two

tanks were held at the acclimation conditions (3 fish/L). The stressed and control tanks were fed zebrafish diet sprayed with absolute ethanol and air-dried overnight.

We evaluated morbidity and mortality over a 7 wk period as well as changes in clinical disease associated with *Pseudoloma* infection. On Day 1, five fish were lethally sampled from each tank and processed for histology to determine the baseline indices of disease for the population. At wk 1 and 6, whole-body cortisol was measured in 5 fish /tank; in the stress tanks, cortisol was measured 15 min following net handling and air exposure in order to measure peak cortisol response (Ramsay et al. in review). Fish were weighed and measured on Day 1 and at wk 7 of the experiment to determine differences in growth between treatments.

Experimental Exposure and Growth (TL strain) - A preliminary experiment was conducted to evaluate the effects of *Pseudoloma* infection on body weight. A total of 66 fish (TL strain; 5 months old) were initially divided into two 16 L tanks. One tank of fish was exposed to *Pseudoloma* by placing the carcasses of 20 *Pseudoloma*-infected fish (minus viscera) into the tank for a period of 4 days. The second tank was exposed in a similar manner to carcasses from *Pseudoloma*–free fish. Each tank was divided (2 controls and 2 exposed), and held for 10 wk. Fish were then weighed and the infection determined by screening spinal cords in tissue smears using Fungi-Fluor chitin stain (Kent and Bishop-Stewart 2003). Length data are not provided as the tails of TL fish are long and variable in length making accurate comparisons difficult.

Experimental Exposure and Stress (AB strain) - Approximately 600 juvenile zebrafish (AB strain; 4 wk old) from *Pseudoloma*-negative adults were obtained from a zebrafish facility and transported to the ZDL. These fish were randomly allocated to 8 tanks and acclimated for 2 wk. During the acclimation period fish were fed brine shrimp nauplii, *Artemia* sp., and fed zebrafish larval diet (Westerfield 2007). Diets were changed during the experiment as fish grew. Adult zebrafish diet, in addition to brine shrimp nauplii, was fed to fish 3 months and older. Prior to initiating the experiment, 20 fish were lethally sampled from the population, to determine the baseline, weight and length, and identify

any existing *Pseudoloma* by histology. The following 4 treatments were randomly assigned to the tanks: *Pseudoloma*, stress, *Pseudoloma*-stress, control. Stress treatment, described above, was administered starting at week 2 PE to *Pseudoloma* (or sham-exposure) and continued for the duration of the experiment, until week 20 PE. Control fish were maintained at the acclimation density (4 fish/L) and not crowded, handled, or transported, except during spawning.

We exposed fish by harvesting spores from zebrafish infected with *Pseudoloma* and feeding the spores to our infection groups. Forty *Pseudoloma*-infected fish were euthanized. The brains and spinal cords were removed, minced in sterile deionized water, and passed through sterile needles of decreasing size (18, 23, 26 gauge) to break up the tissue. The tissue was then passed through a 40 μm cell strainer and centrifuged (2000 rpm) for 20 minutes. The pellet, obtained from centrifuging, was re-suspended and the centrifugation repeated. The spores in the re-suspended pellet were counted using a hemocytometer. Fish in the tanks assigned to be infected with *Pseudoloma* were fed spores at a dose of 10 000 spores/fish. During exposure, water flow into the tanks was turned off and fish were fed brine shrimp to promote ingestion of spores. At this time, control and stress tanks were sham-exposed by feeding brine shrimp with the water to the tanks turned off.

Histology, growth measurement, and spawning – At week 4 post-exposure (PE), 10 fish were removed from each tank, euthanized, weighed, measured, and processed for histology. Fish were paired spawned at week 8, 13, and 20 PE prior to being sampled. We evaluated the effects of each treatment on fecundity (number of eggs spawned) and the number of larvae hatched at 5 days post fertilization (dpf). We chose 5 dpf because first feeding occurs at about this time (Westerfield 2007). Feeding is highly variable among larvae and we did not want differences in feeding among larvae to be a factor affecting survival.

Spawning was performed according to protocols used at the ZIRC (Westerfield 2007). We spawned five pairs of fish from each treatment tank (10 pairs/treatment) at each 8, 13, and 20 week PE. From each tank, 5 males and 5 females were randomly selected and paired. In the afternoon (15 00 – 16 00 h) of the day before the spawning

was to occur, each pair was placed into a crossing tank with screen insert and divider (Thoren Aquatics, Inc., Hazelton, PA); the male was placed on one side of the divider and the female was placed on the other side of the divider. When the lights came on the next morning (08 00 h), the divider was removed from the crossing cage allowing the pairs to spawn. Pairs were left to spawn for up to 4 hours. If fish did not spawn within 2 hours, water changes were performed to facilitate spawning (as suggested by Westerfield 2007).

After the fish had spawned, the fish were removed from the crossing tank, euthanized, weighed and measured, and placed into Dietrich's fixative for histology. Eggs were carefully rinsed with clean water from our fish system and placed into Petri dishes containing embryo media (Westerfield 2007) at a density of 50 eggs per dish. Dead eggs were removed daily and the number of hatched larvae was recorded up to 5 dpf.

Group spawning (non-lethal) of each tank was performed at weeks 6, 10, and 15 PE to ensure fish did not become egg-bound (as suggested by Westerfield 2007). Spawning was set up according the methods of the ZIRC (Westerfield 2007). In the afternoon on the day before the spawning was to occur all fish were netted out of their tanks. Tank inserts with a false bottom were placed into each treatment tanks in order to allow eggs to fall through the bottom. Each group of fish was returned to their respective tanks containing the tank inserts. Spawning occurred the following day after the lights came on. The tank inserts were then removed from the tanks and the fish were placed back into the tanks.

Statistics

Statistical analyses were performed using S-PLUS 7 (Insightful Corp., 2005, Seattle, Washington). For the preliminary cortisol-feed dose-response study, we compared median cortisol values using a Kruskal-Wallis non-parameter test due to the small number of fish sampled at each time point. For whole-body cortisol, weight, length, xenoma area, and myositis score, duplicate treatment tanks within each study were compared using a Welch's modified t-test and pooled if there was no significant difference between the duplicates. Pooled cortisol, weight, length, xenoma areas, and

myositis scores of different treatments within each study were compared using an analysis of variance (ANOVA) with Fisher's LSD. We used Fisher's exact tests to evaluate the association between treatment group and prevalence of infection, mortality, and, spawning success. We compared the number of eggs produced and number of hatched larvae 5 dpf produced by each treatment group using an ANOVA. The relationship between the number of hatched larvae and infection intensity (xenoma area) was determined using regression analysis. The data were explained by fitting a regression model to the data (y=mx+b), where y=hatched larvae and x=xenoma index, m=slope of the line and b=y-intercept. Significant differences were reported at α=0.05.

Results

Cortisol-dose Experiment (AB strain)

Median wole-body cortisol was highest among fish fed 5 or 33 μg/g cortisol for 7 weeks (Fig. 5.1A). We did not calculate the standard errors for the median values due to non-normal distribution of the data which reduces the accuracy of the standard error values. Feeding activity was reduced in the 100 μg/g cortisol group, and this group had whole-body cortisol levels similar to those of the control dose (0 μg/g cortisol) at week 6 PE. Therefore, we selected a dose of 10 μg/g cortisol (in between 5 and 33 μg/g cortisol) for the Pre-existing Infection – AB experiment in order to ensure consumption of cortisol-feed and elevation of whole-body cortisol.

Pre-existing Infection – AB experiment

Whole-body cortisol - Replicate tanks of the same treatment were not significantly different; therefore we pooled the data. Whole-body cortisol was significantly higher in the stressed groups compared to the control and cortisol-fed groups at both week 1 and 6 PE (Fig. 5.1B). There was no significant difference between the control and cortisol-fed groups at either week 1 or 6 PE. Among stressed fish cortisol was significantly lower at week 6 compared to week 1 PE.

Weight, length, and condition factor - The overall mean initial weight of fish was 509 $\pm$ 11 mg and the overall mean final weight of fish was 544 $\pm$ 16 mg; with no significant

difference between treatment tanks at either time. Additionally, the initial and final weights did not differ significantly from one another, although there was a trend toward increasing weight over time (p=0.058). The mean length of fish did not differ between treatments. Final length (34 ± 0.3 mm) was significantly greater than initial length (33 ± 0.2 mm). Condition factor did not differ significantly between treatment groups or over time (mean = 1.35 ± 0.01).

Cumulative mortality, prevalence, and indices of infection - There was some mortality in all tanks (Fig. 5.2). Mortality occurred earliest among the stressed and cortisol-fed groups during the first week after the treatments were administered. A second wave of mortality occurred in the stress and cortisol-fed groups during the third and fourth week of treatment. The final phase of mortality occurred from week 5 to 7 in all groups including the controls. At week 7 PE, there was a significant association between treatment and mortality with greater mortality occurring among the stressed-treated fish compared to control fish (p=0.018).

Histological examination of infected fish from the Pre-existing Infection - AB experiment exhibited characteristic microsporidiosis including multiple xenomas in the central nervous system, particularly the spinal tissue, as well as xenomas in the muscle tissue, along with myositis associated with chronic inflammation of the somatic muscle (Fig 5.3 A,B,C; Fig, 5.10, 5.11, Appendix Figure A5.1). Additional tissue reactions included meningitis associated with rupturing xenomas. An example of the SPOT analysis used on histological sections to determine the xenoma area occupying visible spinal tissue is provided (Fig 5.3A; Fig. 5.10, Appendix A5.2). All fish were positive for *Pseudoloma*, which precluded any examination of differences in the prevalence of infection between treatment groups. The xenoma area did not differ between any of the treatment groups (overall mean=0.85 ± 0.1%). However, the stress group had the highest mean myositis score followed by the cortisol-fed group. The control group has a mean myositis score that was not significantly different from the baseline group (Fig 5.4).

Experimenta Exposure - TL experiment

Ten weeks after exposure, *Pseudoloma*-exposed fish were visibly smaller than controls, and females in the control tanks were particularly more rotund than those in the exposed tanks. *Pseudoloma* spores were observed in the spinal cords or hind brains of all exposed fish using Fungi-Fluor stained smears, whereas none were detected in the control fish. No significant difference in weight or length was found between the duplicate treatment tanks so the weights and lengths were pooled for each treatment. The control group weighed 27% more than the *Pseudoloma*-exposed group (control= 484 $\pm$ 23 mg; *Pseudoloma*= 354 $\pm$ 14 mg).

Experimental Exposure and Stress – AB experiment

Weight, length, and condition factor - The mean initial weight of zebrafish at the time of infection was 30 $\pm$ 0.5 mg. Weight increased over time from week 4 to 13 PE in all groups (Fig 5.5A). From week 13 to 20 PE, weight increased in the control group but was decreased in all other groups; and the control group weighed the most (Fig 5.5A). The initial length of zebrafish was 15.1 $\pm$ 1.6 mm and the final length was 31.5 $\pm$ 0.27, with no significant difference between treatment groups at each time. Condition factor increased over time from 1.04 to 1.1 and did not differ over between treatments within each time. Interestingly, at week 20 PE, none of the control fish had a CF less than 1 whereas several individuals from the stress, *Pseudoloma* and *Pseudoloma*-stress groups had a CF less than 1 (Fig. 5.5B).

The individual weights, lengths, and CFs of the fish remaining at weeks 40 and 52 PE are reported in Fig. 5.6. Generally, fish from the *Pseudoloma*-infected tank were smaller than that of the uninfected tanks.

Cumulative mortality, prevalence, and indices of infection - Accidental mortality occurred on occasion during stress protocol or tank cleaning but no *Pseudoloma*-associated mortality occurred in any group until almost one year after exposure. No *Pseudoloma* was identified in any of the fish prior to experimental exposure. Histological presentations of the infection were similar to those seen in the Pre-existing Infection – AB experiment. One fish in the *Pseudoloma* group was infected at week 4 PE. At week 8

PE, there was a significant association between stress treatment and infection prevalence; 55% of the *Pseudoloma*-stress group and 16% of the *Pseudoloma* group were positive for the microsporidium. By week 13, almost 100% of these experimentally infected fish were positive for *Pseudoloma* regardless of stress treatment group. At week 20 PE, 95% of the *Pseudoloma* group and 100% of the *Pseudoloma*-stress group were positive (Fig. 5.7). None of the control or stress tanks had any infection from week 4 to 20 PE.

At week 4 and 8 PE, only a few fish were infected from the *Pseudoloma* tanks and mean xenoma area was not significantly different. Among the *Pseudoloma* tanks, mean xenoma area did not differ between treatment groups at week 13 PE, but at week 20 PE the *Pseudoloma*-stress group had a higher xenoma area than did the *Pseudoloma* group (Fig. 5.8A). There was a trend toward increased mean myositis score with stress ($p=0.09$; Fig. 5.8B). However, due to the large amount of variation in myositis score, there was no significant difference between stressed and non-stressed groups.

Among the fish remaining one year PE in the single *Pseudoloma*-stress tank, 16 of 18 fish were positive for *Pseudoloma* with a median xenoma area of 0.5%. Additionally, 3 fish in the control tanks were positive for *Pseudoloma*.

Fecundity and its relationship to infection indices - Fecundity and larvae hatched at 5 dpf are reported in Table 5.1. There was no effect of treatment (control, stress, *Pseudoloma*, *Pseudoloma*-stress) on the number of pairs that successfully spawned at any of time PE. We did not find any effects of treatment on the mean or median number of eggs laid. We did not see any effects on the number of larvae hatched at 5 dpf. Within each treatment group, there was no effect of time (week 8, 13, 20 PE) on egg production or larvae hatched. The raw data are presented in Table 5.2. Spawning success, the number of eggs laid, the number of larvae hatched at 5 dpf, and the percent larvae hatched at 5 dpf for each individual pair at week 8, 13, and 20 PE are presented in Appendix Table A5.1.

At week 20 PE, there was a negative natural logarithmic relationship between the number of eggs laid and xenoma area in the *Pseudoloma*-stress group, with males and females combined, described by the following equation: eggs laid = 80 – 61 ln (xenoma area); $R^2 = 0.25$; $p=0.025$ (Fig. 5.9A). There was a similar relationship between the number of larvae hatched at 5 dpf described by the following equation: 61 – 63 ln

(xenoma area); R^2 = 0.3; p=0.01 (Appendix Figure A5.3). At week 20 PE fish from the *Pseudoloma*-stress group with a xenoma area greater than 0.8% produced fewer than 75 larvae hatched at 5 dpf. Although there was no significant relationship between the percent larvae hatched at 5 dpf, there was a trend toward a negative natural logarithmic relationship (p=0.06; Appendix Figure A5.3). Among male fish, only the were similar trends toward a negative relationship between eggs laid, larve hatched, and percent hatched and xenoma area described by: eggs laid = 90 – 73 ln (xenoma area), R^2=0.3, p=0.1; larvae hatched = 72-70 ln (xenoma area), R^2=0.3, p=0.09; percent hatched = 53 - 26 ln (xenoma area), R^2=0.3, p=0.1. Among female fish these relationships were described by: eggs laid =71 – 56 ln (xenoma area), R^2=0.2, p=0.1; larvae hatched = 50-63 ln (xenoma area), R^2=0.3, p=0.1; percent hatched = 48 - 15 ln (xenoma area), R^2=0.1, p=0.3. Among the *Pseudoloma* group (no stress) at week 20 PE, there were no significant relationship between fecundity and xenoma area (Fig 5.9B; Appendix FigureA5.4).

Fish remaining after week 20 PE - Due to a lack of mortality, a number of fish remained in each tank after the week 20 PE sampling. We continued to monitor these groups for infection. However, at week 23 PE, all fish in 5 of the 8 tanks died from an unexpected failure of the tank water system. We continued to monitor fish from the surviving tanks (one control tank, one stress tank, one *Pseudoloma*-stress tank) but no longer administered stress treatment. Fish from these tanks were all weighed and measured at week 40 PE. Fish from the *Pseudoloma*-infected tank started to die between week 40 and 52 PE; all fish were euthanized, weighed and measured and examined by histology at week 52 PE. Weight, length, and CF data are reported, including median values, but the treatments were not compared using statistical tests due to a lack of tank replication

Background infection with Mycobacterium spp.

A small number of fish were positive for *Mycobacterium* spp. by histology. The majority of mycobacteria were found in the swim bladder and ovaries. In the Pre-existing Infection – AB experiment, 22 out of 202 fish (11%) were positive for mycobacteria. With Experimental Exposure and Stress – AB experiment, a single fish was positive for mycobacteria out of over 400 sampled.

Discussion

Pseudoloma is the most prevalent disease of laboratory zebrafish. The infection is often associated morbidity (De Kinkelin 1980; Matthews et al. 2001), but infected fish are often asymptomatic. This is based on examination of hundreds of zebrafish from many laboratories submitted to a diagnostic service provided by the National Institutes of Health (NIH) Zebrafish International Resource Center, Eugene, OR (http://zebrafish.org/zirc/health/index.php) over the past ten years, which included apparently healthy fish for routine health checks (Matthews 2004).

Size and infection

Disease tends to limit the growth potential and condition of fishes. *Pseudoloma*-infected zebrafish are often referred to as having "skinny disease" (Matthews et al. 2001) suggesting that microsporidiosis is associated with weight loss or poor growth. Here, we present the first empirical data supporting this observation. Our two laboratory transmission experiments with TL and AB zebrafish demonstrated that *Pseudoloma* is associated with reduced size. There are many examples of reduced growth associated with chronic parasite infections (Crompton 1986; Beamish et al. 1996, Barber and Svensson 2003). Specifically, Speare et al. (1998) showed that infections by *L. salmonae* in rainbow trout caused reduced growth, but growth was compensated following recovery. Furthermore, *L. salmonae*-associated reductions in growth were associated with xenoma onset and accompanied by a reduction in feed intake (Ramsay et al. 2004). It is not known whether zebrafish recover from *Pseudoloma* or if auto-infection occurs as is believed to occur for some fish-infecting microsporidia (Rodriguez-Tovar et al. 2003; Matos et al. 2003; Kent and Speare 2005). With *Pseudoloma* infections, zebrafish did not recover over the duration of the study and they continued to show smaller size as the infection progressed. Perhaps the most analogous infection to *Pseudoloma* is *Encephalitozoon cuniculi*, which infects rabbits and other mammals (Wasson and Peper 2000). Both species of microsporidia are wide spread in their respective hosts, including within research laboratories. Both cause encephalitis and meningitis, but many animals are asymptomatic. Infections by *E. cuniculi* are associated with stunted growth, particularly in young dogs, and other clinical changes (Wasson and Peper 2000).

Although data were derived from two separate experiments, the TL strain showed greater impacts on size associated with the parasite than the AB strain. This is consistent with observations in our laboratory with other exposure studies and from our diagnostic service. Although not investigated in zebrafish, there are numerous examples of differences in susceptibility to parasites amongst strains of the same species of fish (Jones et al. 2001). With fish microsporidia, different strains of Chinook salmon, *O.tshawytscha*, have shown differences in susceptibility to *Loma salmonae* (Shaw et al. 2000b).

Stress and growth

We also investigated the effects of stress on growth and *Pseudoloma* infection, but first we had to evaluate our stressors. In our preliminary cortisol dose experiment, we determined a dose between 5 and 33 µg/g cortisol was best for increasing whole-body cortisol for the 7 week duration of the experiment. Fish fed the highest dose of cortisol (100 µg/g) had reduced feed intake and failed to elevate whole-body cortisol. Stress and exogenous cortisol has been demonstrated to reduce feed intake (Bernier et al. 2004; Peterson and Small 2005) which may explain why feed intake was reduced in our study.

Whole-body cortisol was significantly elevated among stressed fish compared to the control and cortisol-fed groups at both week 1 and 6 PE , confirming our earlier studies (Ramsay et al. in review) indicating that *Pseudoloma* infections were impacted by persistent crowding coupled with handling inducing chronic stress and elevated cortisol levels. Interestingly, cortisol was significantly lower at week 6 than week 1 PE among the stressed group, suggesting that fish were acclimating to the random stressors we were administering. Whole-body cortisol was not significantly elevated among the cortisol-fed groups compared to the control groups despite the fact that the dose we used (10 µg cortisol/g feed) was within the dose range (5 and 33 µg/g) in our preliminary cortisol-dose experiment. A possible explanation for this lack of effect include an increased clearance rate of cortisol as has been demonstrated for salmonids exposed to chronic stress or exogenous cortisol (Redding et al. 1984). Negative feedback loops suppress cortisol secretion in the presence of exogenous cortisol (Bradford et al. 1992), which may explain why whole-body cortisol among cortisol-fed fish was not elevated.

Size and stress

Both disease and stress alter metabolism and oxygen consumption, often resulting in less energy available for both growth and reproduction, and may explain the smaller size of the *Pseudoloma*-infected group (Barton and Schreck 1987; Mommsen et al. 1999; Heins and Baker 2003; Leef et al. 2007). We did not see any effect of stress or feeding cortisol on changes in weight, length, or condition factor among fish in the Pre-existing Infection – AB experiment, perhaps because the fish were near fully grown when the treatments were initiated. Accordingly, it has been suggested that the finite size of zebrafish may limit their use as a model to study growth (Mommsen 2001). Interestingly, we did see a reduction in weight associated with both stress and infection in the Experimental Exposure and Stress - AB experiment, but here infection was initiated in juvenile fish (6 weeks old). Young zebrafish are particularly susceptible to *Pseudoloma* (Ferguson et al. 2007), and perhaps the impact of the infection on growth is most evident if the infection is initiated when fish are young. Weights at week 20 PE were significantly lower than at week 13 PE, among the stress, *Pseudoloma*, and *Pseudoloma*-stress groups, while the control group increased weight during this period. Stressed and *Pseudoloma*-infected groups had a number of individuals with a condition factor below 1, while all of the control fish had condition factors above 1. Stress and elevated cortisol are associated with metabolic costs that have been demonstrated to reduce growth and condition factor in fishes (Barton and Schreck 1987; Gregory and Wood 1999; Bernier et al. 2004) which may explain our results.

Mortality and stress

Mortality in the Pre-existing Infection – AB experiment was greatest amongst the stressed group followed by the cortisol-fed and then the control groups. Shipping stress has been demonstrated to increase mortality in parasitized fish (Goulding et al. 2004; Kåll et al. 2004), and handling stress tends to increase mortality of diseased fish (Saeij et al. 2003; Dror et al. 2006). Additionally, rainbow trout, infected with the myxosporean PKX and implanted with cortisol, experienced increased mortality compared to parasitized fish not exposed to cortisol (Kent and Hedrick 1987).

Mortality was not related to *Pseudoloma* infection in both experiments with laboratory exposure. Juvenile fish were used for the Experimental Exposure and Stress – AB experiment, whereas adult fish were used for the Pre-existing Infection – AB experiment. Life history stage is important in determining stress and immune responsiveness (Schreck 1996) and may have influenced the stress-related mortality in *Pseudoloma*-infected fish. While young fish may be more susceptible, this chronic infection may require many months before clinical disease is evident. We began to see mortality between week 40 and 52 PE. This indicates that *Pseudoloma*-associated mortality tends to occur later after infection, which is consistent with observations from the Pre-existing Infection – AB experiment in that it is probable that fish from this population had *Pseudoloma* for many months before we initiated our experiment.

Histology and prevalence of infection

We observed some differences in the infection associated with stress at a histological level. Stress was associated with earlier detection of infection in experimentally exposed fish subjected to stress. At week 8 PE, 16% of fish from the *Pseudoloma* group and 55% of fish from the *Pseudoloma*-stress group were positive. Among catfish, *Ictalurus punctatus*, infected with *Edwardsiella ictaluri*, stress increased the overall percentage of infected fish (Small and Bilodeau 2005). By week 13 and 20 PE almost all fish from both *Pseudoloma* groups were positive in this experiment. This concurs with Kent and Bishop-Stewart (2003) where 30% of fish were positive for *Pseudoloma* at week 8 PE and 100% were positive at week 20 PE. Husbandry stress or feeding cortisol exacerbated pre-existing *Pseudoloma* infections in adult zebrafish. Although stress did not affect the size of fish in the Pre-existing Infection – AB experiment, it was associated with increased myositis. Indeed, most histological changes occur as the infection progresses from the central nervous system to the muscle, where the most prominent inflammatory changes are observed. This is consistent with our observations from diagnostic cases; when microsporidiosis is the diagnosis for the cause of morbidity, it is most often associated with chronic myositis. The sequence of infection and progression of lesions with *Pseudoloma* in zebrafish is similar to gill infections by *Loma salmonae* in salmonids (Kent and Speare 2005). In this case, intact xenomas in the

gills are associated with little inflammation and clinical disease. This is followed by rupture of the xenomas and severe chronic branchitis, with accumulation of released spores within phagocytes.

Infection intensity (xenoma area and myositis)

Husbandry stress increased the intensity of infection during the Experimental Exposure and Stress – AB experiment. Mean xenoma area was greater among stressed fish experimentally infected with *Pseudoloma* compared to non-stressed fish at week 20 PE. Stress and cortisol-treatment has been demonstrated to increase the intensity of infection in diseased fish. In Northern pike, *Esox lucius*, the number of gill arteries infected with nematodes increased after transport stress (Kåll et al. 2004), and cortisol-treated rainbow trout had an increase in the density of PKX spores in the interstitium of the kidney (Kent and Hedrick 1987). Interestingly, the mean myositis score was not significantly affected by stress treatment, although there was a trend toward increasing myositis among stressed fish at week 20 PE. The large amount of variation in the data may explain why we did not see any difference. Additionally, differences in myositis between stressed and non-stressed groups may have been more apparent past week 20 week PE as the infection progressed, but with lack of replicates beyond 20 weeks PE we could not evaluate this.

There was no effect of stress or feeding cortisol on the mean xenoma area of fish with pre-existing *Pseudoloma* infections. The fish used in the Pre-existing Infection – AB experiment had a baseline mean parasite area of 0.91%, whereas during the Experimental Exposure and Stress – AB experiment, the mean parasite area was 0.61% for the *Pseudoloma* group and 1.2% for the *Pseudoloma*-stress group. In the former experiment, fish were stressed long after the infection was established. There may be a maximum area of tissue that can be occupied by *Pseudoloma*, after which the host either resists the parasite or succumbs to disease. Alternatively, the short duration of exposure to stressors or cortisol (7 weeks) may not have been sufficient to change the mean parasite area in fish with existing *Pseudoloma* infections.

At week 52 PE, a few fish from the control and previously stressed groups were positive for *Pseudoloma*. Inadvertent infection of these *Pseudoloma*-control groups may

have been due to aerosol transmission of the parasite from adjacent tanks containing *Pseudoloma*-infected fish. Control tanks were intermixed with the infected tanks on the same racks to randomize differences in extraneous stressors such as light and the position of the tank in the room. Aerosol transmission has been reported for fish pathogens including *Aeromonas salmonicida* and *Ichthyophthirius multifiliis* (Wooster and Bowser 1996). Microsporidian spores are capable of surviving relatively long periods outside the host (Shaw et al. 2000a) and this may have facilitated transmission of *Pseudoloma* to the control and stress tanks. It is not possible to know when these tanks were exposed to *Pseudoloma*. Although these infections appeared to be incidental, the inadvertent transmission of *Pseudoloma* demonstrates the persistence of this parasite and the potential difficulty in controlling it in zebrafish facilities.

Fecundity

In fish infected with *Pseudoloma*, increasing infection intensity was associated with decreasing reproductive fitness. Although stressed and infected fish may have produced fewer eggs, this was not verified statistically as the number of eggs spawned and larvae hatched at 5 dpf was extremely variable within groups. This is typical for zebrafish; females produced eggs and spawn many times throughout the year, but their egg production can be highly variable (Westerfield 2007). There are relatively few studies examining the effects of environmental parameters on zebrafish reproductive output (Eaton and Farely 1974; Laale 1977), and no studies that have examined the effects of stress or *Pseudoloma* on reproduction in zebrafish. The lack of any direct effects on fecundity may reflect the need to better understand the dynamics of reproduction with respect to husbandry (Lawrence 2007).

Whereas stress and infectious diseases have been documented to reduce fecundity (Barber et al. 2000; Schreck et al. 2001), there are examples where the reverse occurs (Gowaty et al. 2007). Candolin and Voigt (2001) reported an inhibitory effect of cestode tapeworms on reproduction in stickleback males in the natural environment but not in the laboratory, suggesting that the favorable laboratory environment, free of predation risk and fluctuating environment, mitigates the negative effects parasitism has on reproduction. We observed a negative natural logarithmic relationship between the both

the number of eggs laid and parasite area at week 20 PE among the *Pseudoloma*-stress group; a similar relationship was seen for the number of larvae surviving to 5 dpf. Only stressed fish had heavy infections (> 1% xenoma area), and these fish were consistently poor spawners. Infection level, not stress treatment, determined spawning success which was highly variable in lightly infected or uninfected fish and poor in heavily infected fish. This was even the case for the non-stressed fish; only one fish in this group had a heavy infection (>1% xenoma area) precluding statistical analyses. Interestingly fecundity was similarly reduced whether males or females were infected. This suggests a possible effect of the parasite on spawning behavior or the neurological control of sperm release as *Pseudoloma* affects the CNS and nerve roots (Matthews et al. 2001).

Fish with severe *Pseudoloma* infections are typically emaciated and the females lack large numbers of eggs typical of zebrafish (Matthews et al. 2001). *Pseudoloma* is a chronic persistent disease, and perhaps examining reproduction in fish with higher infection intensities later in the infection may have resulted in more significant effects of the parasite on fecundity and offspring survival.

Mycobacteria

A small number of fish were infected with *Mycobacterium* spp. Mycobacteria are common pathogens of zebrafish (Astrofsky et al. 2000; Kent et al. 2004) and have been found at background levels in previous studies (Harriff et al. 2007). Environmental mycobacteria are common to freshwater aquaria and frequently isolated from fish without clinical signs of disease (Beran et al. 2006; Zanoni et al. 2008; Whipps et al. 2008). The majority of mycobacteria we saw were in the swim bladder or ovaries. Acid-fast bacteria are frequently only seen in the lumina of the intestine and swim bladder, which can be easily colonized from the gut, and are not associated with clinical mycobacteriosis (Kent et al. 2004; Whipps et al. 2008). Fortunately, only a few experimental fish had concurrent mycobacteriosis, and thus it is unlikely that this compromised our experiment. Nevertheless, as with any animal used in research, it is important to conduct experiments with fishes that do not have underlying chronic infections in order to avoid non-protocol induced variation in *in vivo* experiments (Kent et al. in press).

Conclusion

Pseudoloma is the most common pathogen of laboratory zebrafish, yet little is known about husbandry factors that affect the development and progression of clinical disease. Our study is an initial step in examining the dynamic interactions of stress, reproduction, and *Pseudoloma* in laboratory zebrafish. We have described the sequential development of *Pseudoloma* in zebrafish, showing that it persists for well over 6 months after exposure. Furthermore, we have demonstrated that stress exacerbates clinical disease associated with *Pseudoloma* infections with direct effects on growth.

Fecundity, rather than growth, is usually the most important criterion in zebrafish used for research, particularly when used in developmental genetics research. Reproductive output was negatively related to the intensity of *Pseudoloma* infection in stressed fish with heavy infections. Therefore, we recommend that if avoidance or eradication of the parasite is not an option in a given research laboratory, then particular care should be taken to minimize stress if fecundity is an important laboratory endpoint. Moreover, it should be recognized that spores of *Pseudoloma* are resistant to chlorine at the levels used in research laboratories (Ferguson et al. 2007), and there is a risk of contamination of the next generation or even true vertical transmission within eggs (Kent et al. in press). Further investigation on the interactions of *Pseudoloma* infection and stress and the subsequent effects on survival, growth and reproductive fitness will aid in controlling outbreaks and optimizing zebrafish health and reproduction in order to ensure the continued success of this important model for biomedical research.

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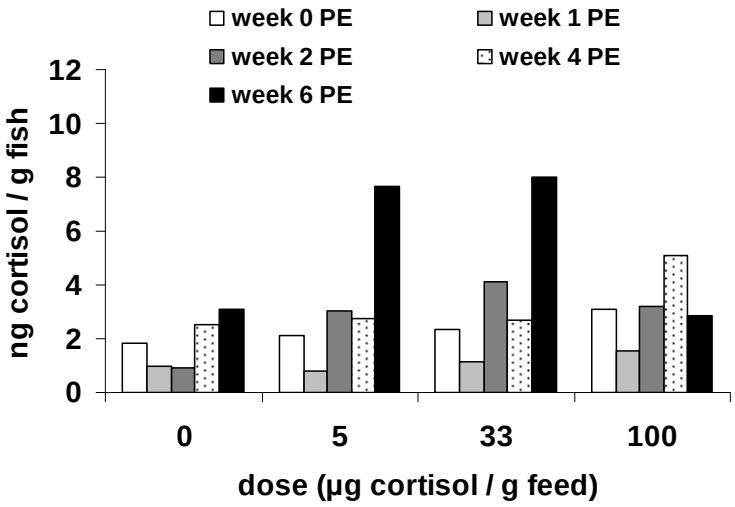
Table 5.1: *Danio rerio*. The number of pairs of zebrafish spawned, eggs laid and hatched and the % viable larvae surviving to 5 days post-fertilization (dpf) at week 8, 13, and 20 post-exposure (PE) to Stress, *Pseudoloma*, *Pseudoloma* and stress and control conditions (no stress and no *Pseudoloma*). Tank A and B indicate replicate tanks within the same treatment.

		Week 8 PE			Week 13 PE			Week 20 PE		
Treatment	Tank	Number of pairs spawned	Number of eggs laid (hatched)	% viable larvae (5 dpf)	Number of pairs spawned	Number of eggs laid (hatched)	% viable larvae (5 dpf)	Number of pairs spawned	Number of eggs laid (hatched)	% viable larvae (5 dpf)
Control	A	5/5	89 (47)	52	5/5	199 (162)	74	2/5	263 (223)	85
Control	B	4/5	102 (80)	84	3/5	136 (67)	41	3/5	63 (39)	70
Stress	A	4/5	72 (31)	45	5/5	193 (91)	48	5/5	135 (124)	91
Stress	B	4/5	83 (64)	71	5/5	151 (65)	32	3/5	53 (32)	56
Pseudoloma	A	4/5	98 (23)	33	3/5	105 (81)	78	5/5	181 (143)	65
Pseudoloma	B	5/5	118 (101)	78	4/5	167 (125)	72	3/5	250 (181)	71
Pseudoloma stress	A	4/5	151 (90)	56	3/5	220 (187)	70	5/5	54 (32)	56
Pseudoloma stress	B	4/5	129 (123)	94	5/5	193 (105)	49	3/5	216 (192)	86

A)



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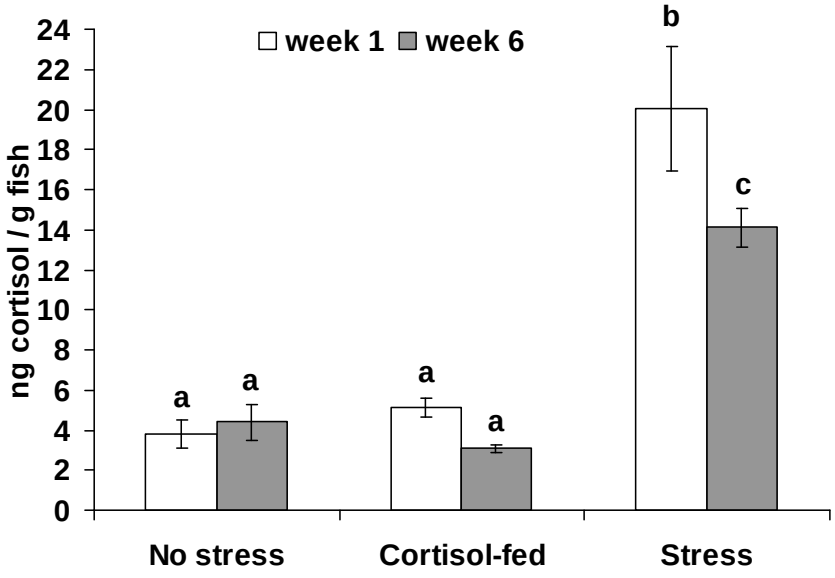


Figure 5.1: *Danio rerio*. A) Median whole-body cortisol (ng/g) after feeding fish 0, 5, 33 and 100 µg cortisol / g feed for 0, 1, 2, 4 and 6 weeks. B) Mean cortisol (ng/g; ± SEM) in groups exposed to no stress, cortisol-fed or exposed to stress for one or six weeks. Different letters over the SEM bars indicate a significant difference between groups ($p<0.05$).

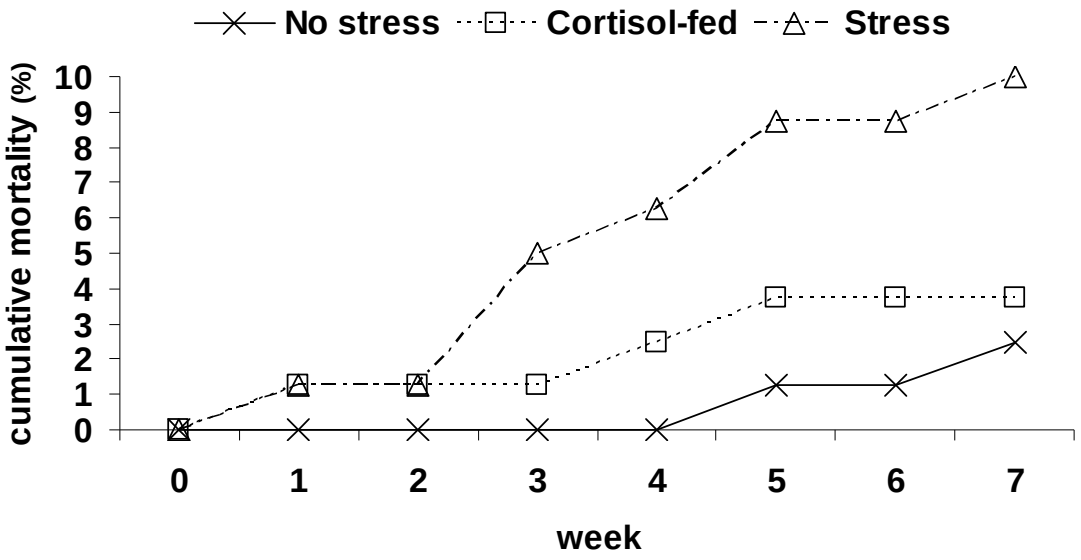


Figure 5.2: *Danio rerio*. Cumulative mortality of zebrafish exposed to no stress, cortisol-fed (10 µg cortisol / g feed) or exposed to random crowding and handling stressors over a 7 week period. At week 7, there was a significant association (p=0.018) between mortality and treatment with the stress-treated group having significantly higher mortality than the control group.

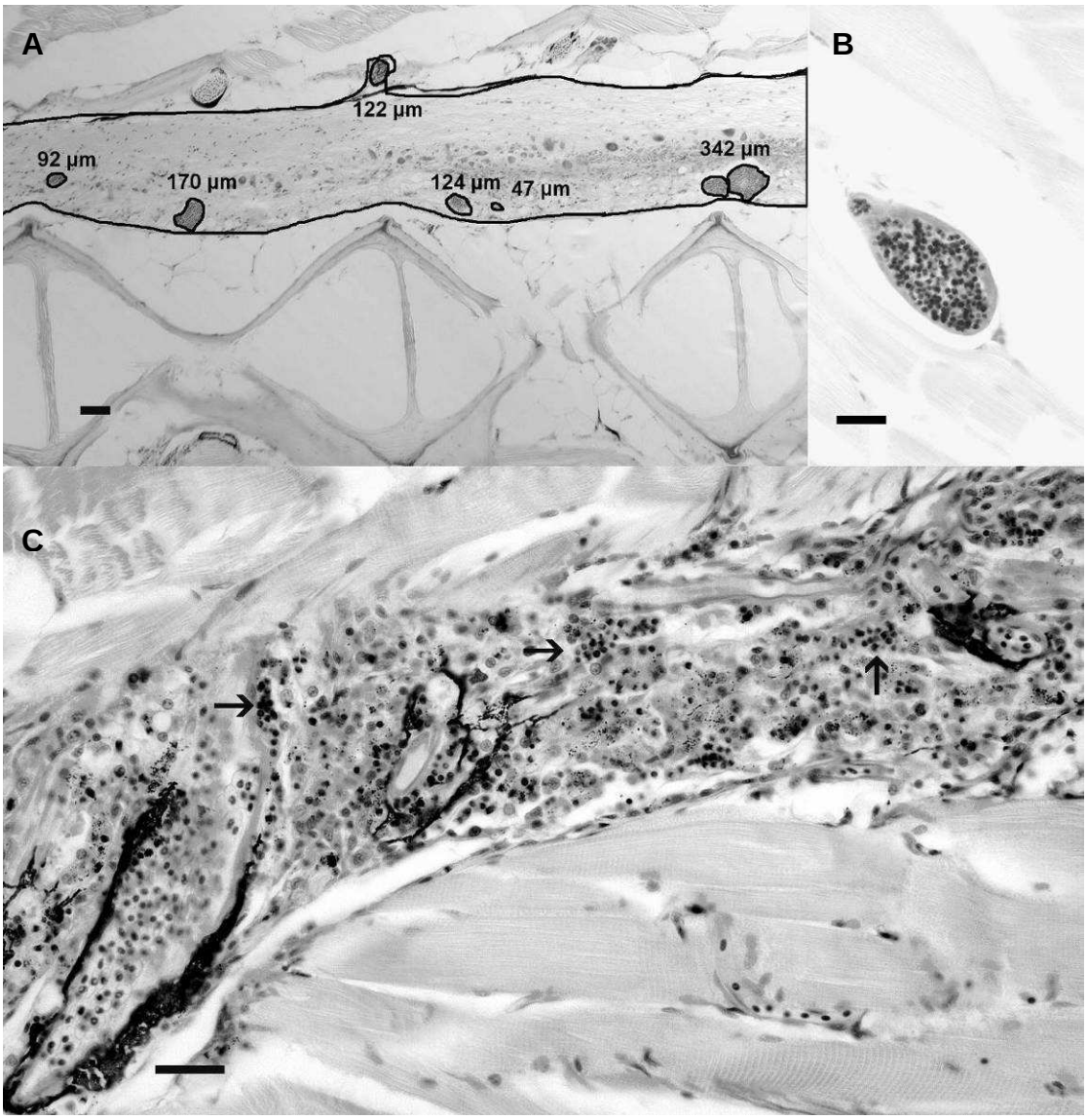


Figure 5.3: *Danio rerio*. Histological sections of zebrafish infected with *Pseudoloma neurophilia*. Acid-fast. A. Sagittal section of spinal cord, with measurements of xenomas (square µm). Spinal cord area is 3 422 sq. µm. Bar = 50 µm. B. Xenoma in somatic muscle. B = Bar 20 um, Focal, chronic myositis with numerous spores (arrows). Bar = 20 µm.

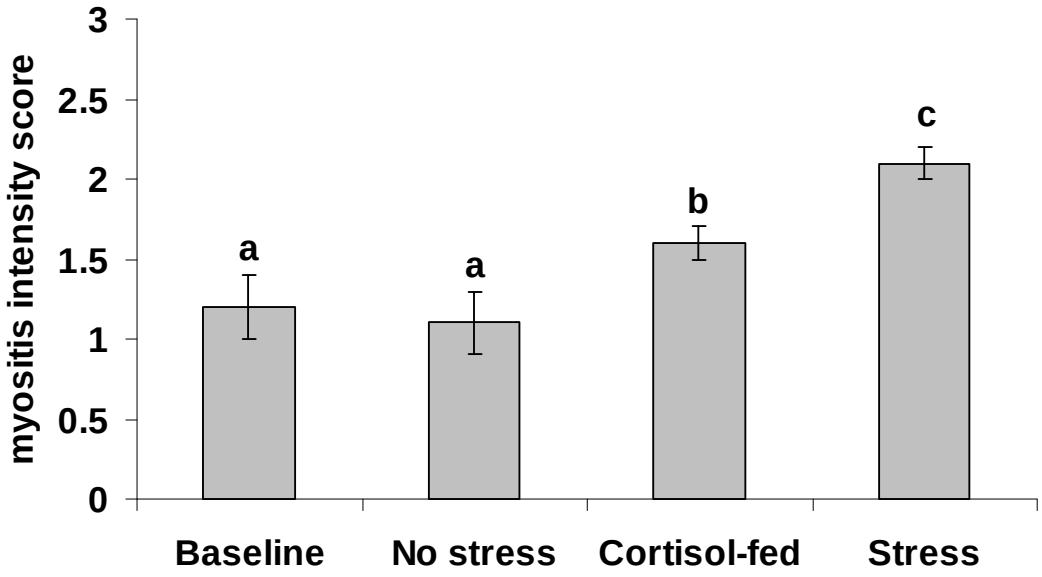


Figure 5.4: *Danio rerio*. Mean myositis intensity score (± SEM) in fish with existing *Pseudoloma* infections. Myositis was assessed by sagittal histological sections of whole-zebrafish. Myositis intensity scoring: 1 = one area of myositis, 2 = two areas of myositis, 3 = three or more areas of myositis. The baseline group was sampled prior to initiating treatments. Treatments included no stress, feeding cortisol or stress for 7 weeks. Different letters over the SEM bars indicate a significant difference between groups ($p<0.05$).

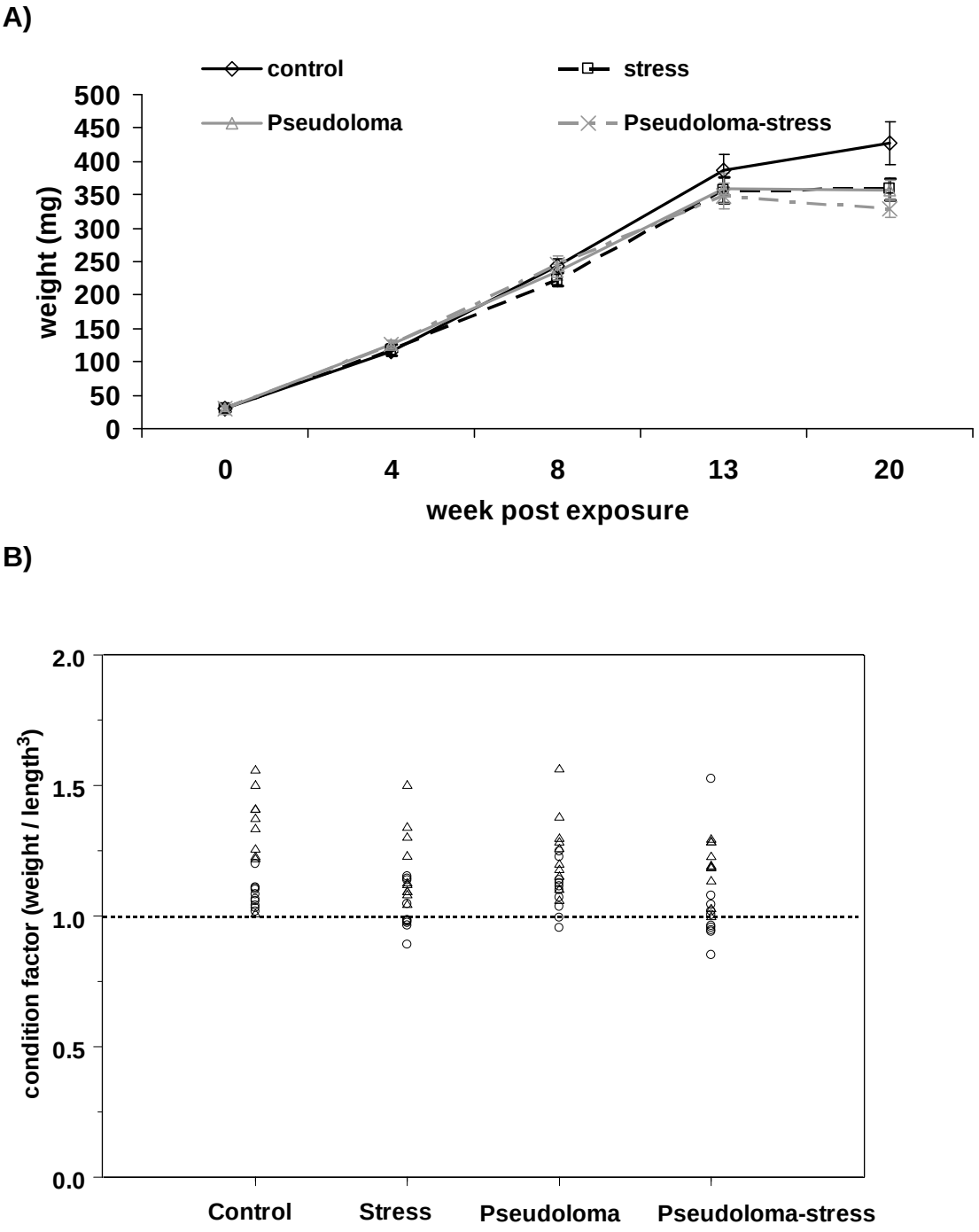


Figure 5.5: *Danio rerio*. A) Mean weight (mg; ± SEM) over time (week) in groups exposed to stress, *Pseudoloma*, *Pseudoloma* and stress, and maintained under control conditions (no stress and no *Pseudoloma*). B) Condition factors of individual fish in control, stress, *Pseudoloma* and *Pseudoloma*-stress groups at week 20 post-exposure to the *Pseudoloma*. The dotted line indicates a condition factor of 1.

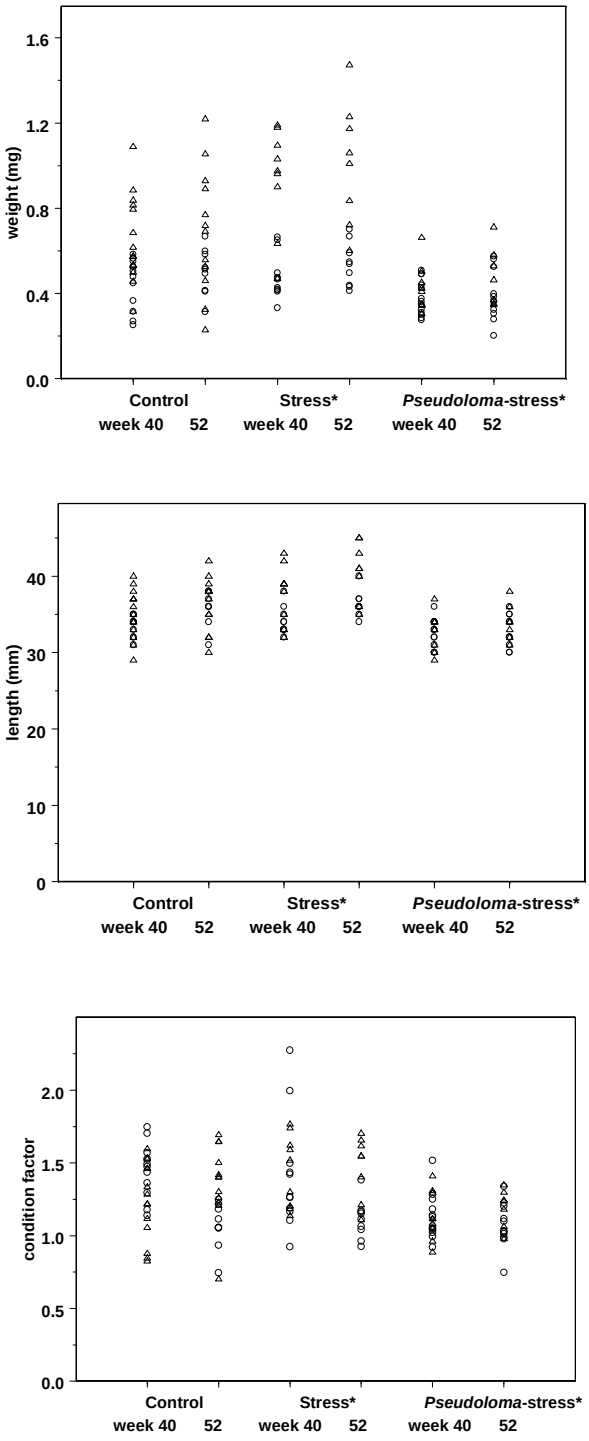


Figure 5.6: *Danio rerio*. Individual weights (mg), Lengths (mm) and condition factors (100* weight/length^3) of fish at week 40 and 52 post-exposure (PE) to *Pseudoloma*. Circles (○) indicate a male fish and triangles (Δ) indicate a female fish. *Stress and *Pseudoloma*-stress groups received stress treatment until week 23 PE

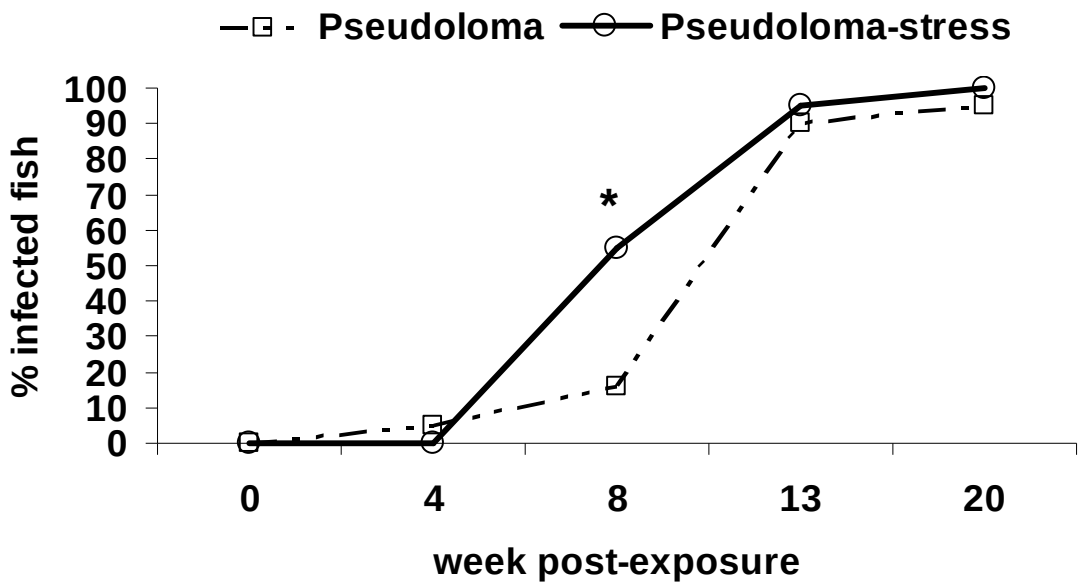
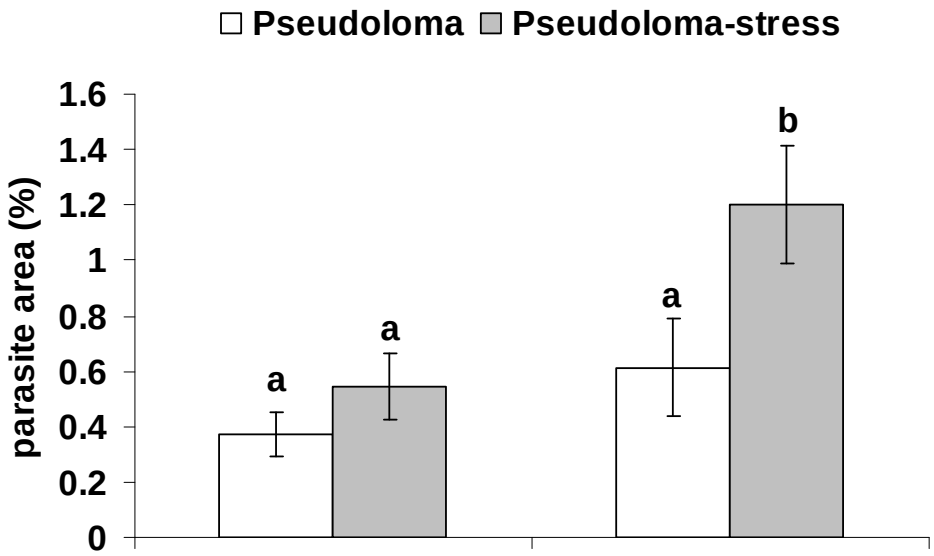


Figure 5.7: *Danio rerio*. Prevalence of *Pseudoloma* infection (% infected fish) over time (week post-exposure) in fish exposed to *Pseudoloma* and *Pseudoloma* and stress. * There was a significant association between prevalence of infection and treatment at week 8 PE ($p<0.05$).

A)



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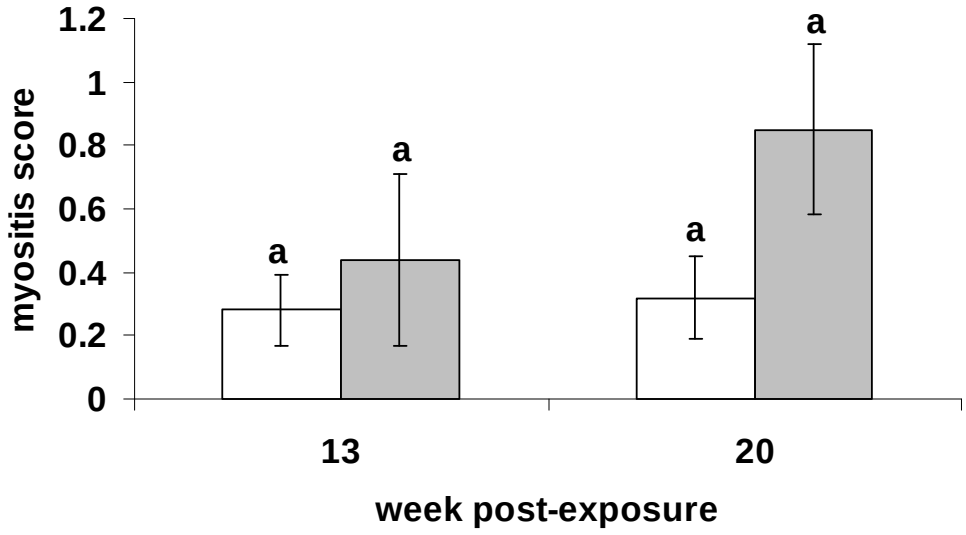

Figure 5.8: *Danio rerio.* A) Mean spinal parasite area (%; ± SEM) and B) mean myositis score (± SEM) with and without stress at week 13 and 20 PE (n=12 *Pseudoloma* parasite area at week 13 PE; n=13 *Pseudoloma*-stress area at week 13 PE) (week 20 PE: n=28 *Pseudoloma* parasite area and n=32: *Pseudoloma*-stress area). Different letters over the SEM bars indicate a significant difference between treatment groups.

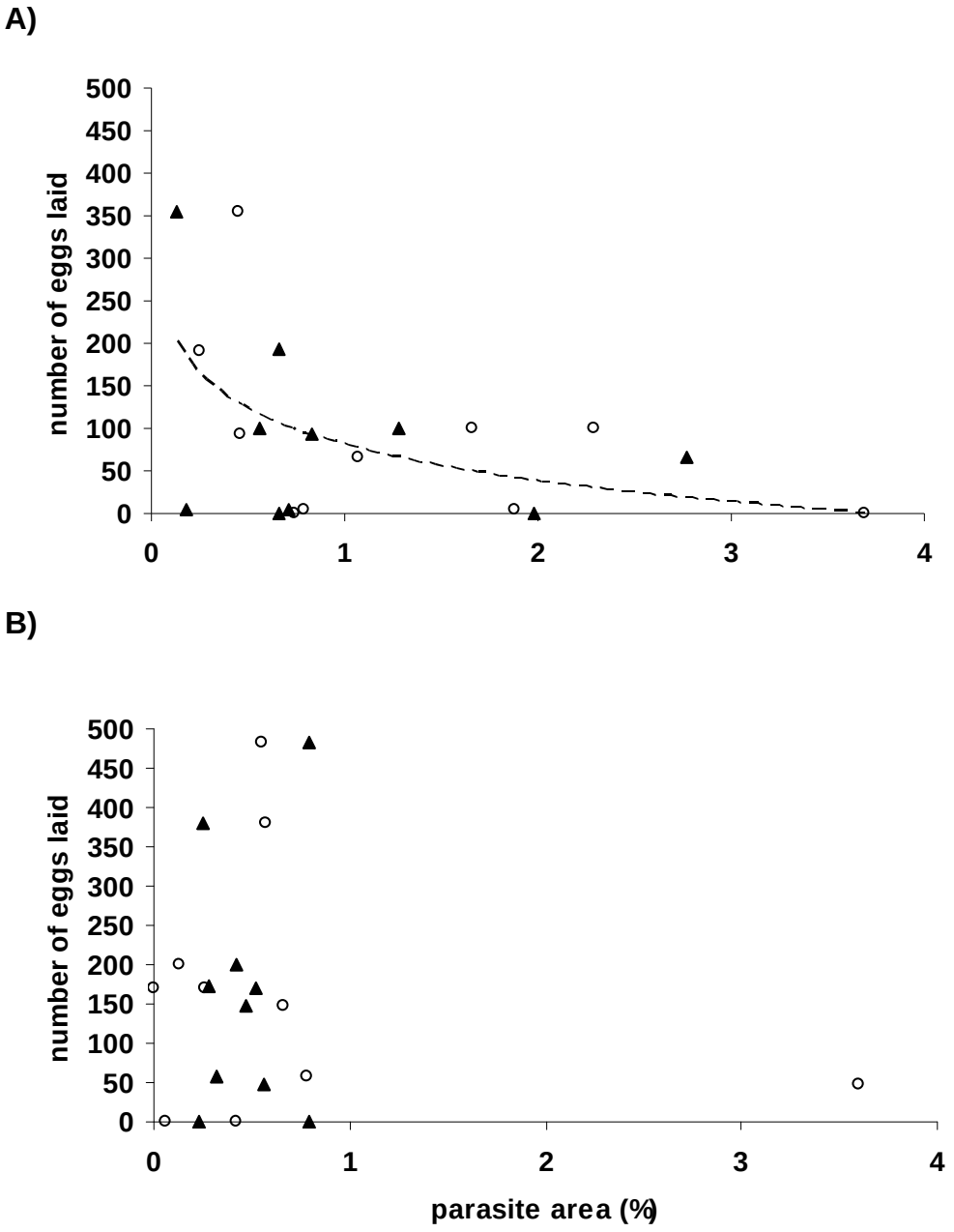

Figure 5.9: *Danio rerio*. Number of eggs laid vs. parasite (xenoma) area (%) at week 20 post-exposure to *Pseudoloma*. A) Among the *Pseudoloma*-infected-stressed fish the relationship was described by the following equation: eggs laid = 80 – 61 ln (xenoma area); $R^2 = 0.25$; p=0.025. B) There was no significant relationship between the the number of eggs laid and parasite area among the *Pseudoloma*-infected group in the absence of stress (p=0.62). Male fish are indicated by an open circle (○). Female fish are indicated by a closed triangle (▲).

CHAPTER 6: GENERAL CONCLUSION

The main objective of this dissertation was to describe the whole-body cortisol response of zebrafish to husbandry stressors and the subsequent effects on disease susceptibility and reproduction. We identified whole-body cortisol as a useful indicator of crowding and handling stress. Additionally, husbandry stress increased the intensity of mycobacterial and microsporidian (*Pseudoloma*) infections. Furthermore, growth and reproductive fitness were impaired with stress and *Pseudoloma* infection.

The use of zebrafish as laboratory research organisms has greatly increased over the past 30 years and is expected to continue to increase into the foreseeable future. It is important to minimize stress and diseases in animals used in research (Baker 2003; Kent et al. in press). The research conducted during this dissertation contributed to the understanding of the responses of zebrafish to husbandry stress. The effects of husbandry stress on diseases and reproduction in laboratory zebrafish has been described for the first time. Additionally, descriptions of the relationship between underlying chronic infections in zebrafish and fitness parameters, including growth and reproduction, were provided. This examination of the dynamic interactions between husbandry stress and fitness of zebrafish provides important data for optimizing husbandry conditions and should serve as a guide for future husbandry stress, disease, and reproduction studies with zebrafish. Additionally, monitoring and minimizing husbandry stress may be useful for adhering to institutional and federal animal care guidelines. My dissertation also provides a foundation for the examination of the role of husbandry stress in diseases and reproduction in other small laboratory fishes, including medaka (*Oryzias latipes*), sticklebacks (*Gasterosteus* spp.), fathead minnows (*Pimephales promelas*), and guppies (*Poecilia reticulata*).

Whole-body Cortisol Responses to Husbandry Stressors

My dissertation research provided a useful tool for assessing stress in zebrafish, whole-body cortisol. Crowded fish had elevated cortisol compared to those not crowded. Net stressed fish responded with rapid increases in cortisol and a similarly rapid recovery

to control cortisol levels. Additionally, the magnitude of the cortisol response was greater during net stress than during crowding. Similar increases in whole-body cortisol were attained during the disease-stress studies, further indicating the suitability of whole-body cortisol as an indicator of stress in adult zebrafish in various laboratory environments.

Feeding regimen was demonstrated to modulate the cortisol response to crowding and smaller fish tended to have greater responses to crowding than larger fish under fasting conditions. Zebrafish should be fed regularly to minimize the cortisol response to mild crowding stressors. Additionally, adequate feeding may reduce size variation within a tank of zebrafish subsequently reducing dominance hierarchies and elevated cortisol levels common among subordinate fish (Ejike and Schreck 1980; Øverli et al. 2004). Feeding may also reduce aggression and dominance hierarchies which can result in elevated cortisol under mild crowding stress (Symons 1968).

There was no mortality associated with stress, demonstrating the hardy nature of zebrafish and why it has thrived as a laboratory research organism. Zebrafish have an extensive history of domestication which likely explains this ability to endure moderate husbandry stressors without mortality. Furthermore the natural history of zebrafish suggest that they evolved in environment characterized by daily and seasonal variation accompanied by dramatic changes in the water quality parameters including temperature, turbidity, flow, and density (Engeszner et al. 2007). As a result, zebrafish have been able to adapt and thrive in dynamic agricultural environments, including rice paddies (Spence et al. 2006). This ability to adapt to changing environments has clearly been advantageous for the survivability of zebrafish in the laboratory. Furthermore, continuous spawning and short generation time have likely facilitated selection of hardy fish with a rapid cortisol response (Fevolden et al. 2002).

There was a large amount of variation in the resting or control cortisol levels of zebrafish. Biological variation in resting cortisol has been demonstrated for numerous species (Hansen et al. 2001; Romero 2002). Resting cortisol may have also been affected by individual differences in genetic profiles, dominance hierarchies, and slight difference in environments (Schreck 1981, 2000; Malisch et al. 2007). Individual variation is part of

normal biological variation (Fraser 2004) but must be considered when designing experiments and interpreting data.

Although age was hypothesized to have an effect on resting cortisol, due to a large amount of variation in the data, statistically significant effects were not demonstrated. However, as age increased the variation in whole-body cortisol also increased. This suggests that the ability to regulate resting cortisol decreases with age. Cortisol generally tends to be elevated in older animals and is associated with an increased susceptibility to diseases (Sapolsky et al. 1986). Therefore, it may be prudent to avoid the use of older zebrafish in research in order to avoid possible elevations in cortisol. This may create a dilemma for zebrafish aging studies which require older fish (Gerhard and Cheng 2002). When it is necessary to use older zebrafish, age should be consistent to avoid introducing non-protocol sources of variation into experiments; furthermore, stress and disease levels should be monitored to ensure proper interpretation of results (Kent et al. in press).

Diseases and Stress of Zebrafish

Husbandry stressors and elevated cortisol were associated with increased clinical mycobacteriosis and microsporidiosis in laboratory zebrafish. In addition, growth and reproduction were impaired by microsporidiosis and stress. Although stress alone did not cause mortality in the studies conducted for Chapters 2 and 3, stress and disease resulted in mortality in the disease-stress studies conducted for Chapters 4 and 5. Furthermore, the impairment of growth and fecundity during infection and stress demonstrated that stress and disease result in decreased productivity of zebrafish.

The persistence and spread of these diseases in zebrafish colonies may have been exacerbated by the great increase in the number of laboratories rearing, breeding, and shipping zebrafish. Infectious diseases tend to increase in aquaculture environments as production, densities, and trade increase (Meyer 1991; Murray and Peeler 2005). Mycobacteria and microsporidia are ubiquitous and capable of surviving long periods outside of their hosts. Once introduced into an aquaculture system these pathogens may be very difficult to eradicate (Francis-Floyd and Yanong 1999: Kent et al. in press).

Mycobacteria, detected in zebrafish systems (Whipps et al. 2008), may serve as a reservoir for future infections. Similarly, fish microsporidia have been detected in fish aquaculture systems (Ramsay et al. 2001) and it is expected that *Pseudoloma* would also be detected in zebrafish systems (Whipps and Kent 2006). Effective management by minimizing stress may be a practical way of controlling disease outbreaks in zebrafish facilities.

In the absence of mortality or apparent signs of clinical disease, we demonstrated a reduction of growth and impairment of reproduction in fish infected with *Pseudoloma* and subjected to stress. The majority of zebrafish studies involve examining various aspects of early development, which arguably makes reproduction the most important production parameter for zebrafish culture. It is unknown how stress and infectious diseases will further affect zebrafish physiology and affect research results. However, close attention should be given to diseases in laboratory fishes such as zebrafish as these may introduce non-protocol sources of variation into experiments (Kent et al. in press). Furthermore, interactions with stress and disease and the subsequent effects of reproductive fitness must be considered.

Recommendations

Recommendations on the control of pathogens and infectious diseases in fish research facilities have recently been published (Kent et al. in press). Additionally, the goal of stress management in fish culture is to ensure optimal performance and production (Schreck 1981). Given the inevitability of stress in zebrafish husbandry together with the persistence of mycobacteria and *Pseudoloma* in zebrafish facilities, we would like to recommend the following management strategies for minimizing the impacts of husbandry stress on underlying chronic infections in order to reduce disease outbreaks and optimize the growth and fecundity of laboratory zebrafish.

1. Determine if fish are stressed

Routine monitoring of whole-body cortisol levels in zebrafish facilities will provide information to researchers regarding the stress level of fish in their facility. We

found a wide range of control cortisol levels across our different studies. Additional information from zebrafish facilities will provide information which could be used to construct range of a baseline cortisol values for laboratory zebrafish. Furthermore, these data could be used to develop husbandry standards that minimize stress. This dissertation has provided data on whole-body cortisol levels in AB strain zebrafish. There are thousands of genetic strains of zebrafish used by researchers. Monitoring stress levels of the more popular genetic strains will discern any effects of genetic strain on the cortisol response and if different husbandry standards need to be established based on strain. Finally, developing non-lethal methods of examining stress levels in zebrafish facilities would be of great advantage, particularly for researchers with valuable stocks of fish. Water cortisol methods have been developed for other fish species (Scott and Ellis 2007). I plan to examine cortisol from the water of stressed zebrafish following the completion of this dissertation.

2. Control pathogens and infectious diseases in zebrafish laboratories

Recommendations for the control of fish pathogens and infectious diseases in fish research facilities have been suggested by Kent et al. (in press). These include: identifying pathogens of interest, employing strategies for avoiding introduction of pathogens, knowing source history of fish, using quarantine methods for fish of unknown history, importing eggs only into a facility, developing specific-pathogen free fish stocks, controlling the spread of pathogens within zebrafish facilities, routinely monitoring fish in the facility for pathogens, and employing sentinel programs. To this list we would like to include minimizing stressful husbandry procedures such as frequent handling and crowding which we have demonstrated to increase the severity of mycobacteriosis and microsporidiosis in laboratory zebrafish. Developing husbandry standards to minimize stress (as suggested in the previous recommendation) would be an ideal way to reduce the potential for disease outbreaks in zebrafish facilities.

3. Understand chronic infections, stress, and reproduction

There is a paucity of information regarding the effects of husbandry stress or chronic infections with mycobacteria or *Pseudoloma* on the reproductive fitness of laboratory zebrafish. We demonstrated a reduction in the number of viable larvae with and increase in the intensity of *Pseudoloma* infections. It would be of further interest to examine if there are any effects of chronic mycobacteria infections on zebrafish fecundity. We were not able to discern any effects of stress alone on the fecundity of zebrafish; however, there was a large amount of variability in the data. We recommend increasing the number of replicates in order to account for this variation. Additionally, reproduction should be examined over the life of the fish to account for age-related differences in reproduction. Furthermore, better understanding the basic biology of zebrafish reproduction may aid in controlling other factors which may have been affecting reproduction in our studies. This will allow the design of studies which will better discern the nature of the relationship between stress, chronic infections, and reproduction. It would also be of interest to examine the effects of stress or chronic infections on zebrafish fecundity and reproduction over multiple generations.

4. Understand and reduce non-protocol sources of variation

Minimizing non-protocol sources of variation associated with underlying chronic infections in laboratory animals is a hallmark of preventive veterinary medicine (NRC 1996). We have demonstrated that zebrafish with underlying chronic microsporidiosis have reduced growth and impaired reproduction. Additionally, stressed zebrafish had reduced growth and increased severity of mycobacteriosis and microsporidiosis. The effects were all demonstrated in apparently healthy animals but would likely have introduced non-protocol sources of variation into experiments. The physiology of zebrafish is likely affected by stress and chronic infections in other ways, not measured during this dissertation. Reducing stress and underlying infections will reduce non-protocol sources of variation in zebrafish experiments.

Concluding Thoughts

The use of zebrafish as a laboratory research model continues to increase as novel methods for examining aspects of physiology and development continue to be discovered. The hardy nature of zebrafish has been a great advantage in its development as a pre-eminent biomedical research model. Husbandry, stress, and chronic diseases have been given little consideration in the culture of zebrafish. Increasing awareness about the potential effects of stress and disease on the growth and reproduction of zebrafish will hopefully prompt researchers to give more credence to the laboratory environment as a potential source for variation in their experiments (Kent et al. in press).

A century ago pathogens occasionally caused death in laboratory rodents but often resulted in persistent underlying chronic infections (Baker 2003). Today, most biomedical researchers demand that laboratory rodents be free of pathogens, even those that only cause chronic disease and minimal mortality. Specific pathogen-free stocks of rodents are available and frequently used by researchers. Similarly, husbandry standards which minimize stress are well established for laboratory rodents (NRC 1996). Continued research into the effects of husbandry stress on zebrafish physiology, including growth, reproduction, and disease susceptibility, will ensure the development of husbandry standards. Better understanding the pathogenesis of diseases of laboratory zebrafish will enable the development of pathogen-free stocks. Both will ensure the continued success of zebrafish research.

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APPENDICES

Appendix Table

Table A5.1: Spawning data for individual pairs of fish in each treatment at each week post exposure (week 8, 13, 20). Includes spawning (yes/no), number of eggs laid, number of larvae hatched at 5 days post-fertilization (dpf), and the percent larvae hatched (5 dpf).

Treatment	tank	week	pair	spawn (Y/N)	number of eggs laid	number of larvae (5dpf)	% hatched (5 dpf)
Control	2	8	1	Y	82	62	73
Control	2	8	2	Y	75	40	56
Control	2	8	3	Y	105	0	0
Control	2	8	4	Y	50	23	46
Control	2	8	5	Y	132	109	83
Control	7	8	1	Y	61	53	87
Control	7	8	2	Y	117	114	97
Control	7	8	3	Y	185	113	61
Control	7	8	4	N	0	n/a	n/a
Control	7	8	5	Y	43	4	91
Stress	3	8	1	N	0	n/a	n/a
Stress	3	8	2	Y	163	61	37
Stress	3	8	3	Y	52	41	79
Stress	3	8	4	Y	28	11	39
Stress	3	8	5	Y	46	11	24
Stress	5	8	1	Y	91	91	100
Stress	5	8	2	Y	63	26	41
Stress	5	8	3	Y	64	34	53
Stress	5	8	4	Y	115	104	90
Stress	5	8	5	Y	0	n/a	n/a
Pseudoloma	1	8	1	Y	127	12	9
Pseudoloma	1	8	2	N	0	n/a	n/a
Pseudoloma	1	8	3	Y	49	39	80
Pseudoloma	1	8	4	Y	86	28	33
Pseudoloma	1	8	5	Y	128	15	12
Pseudoloma	6	8	1	Y	169	158	93
Pseudoloma	6	8	2	Y	33	26	79
Pseudoloma	6	8	3	Y	175	170	97
Pseudoloma	6	8	4	Y	52	19	37
Pseudoloma	6	8	5	Y	160	133	83

Treatment	tank	week	pair	spawn	number of eggs laid	number of larvae (5dpf)	% hatched
Pseudoloma stress	4	8	1	Y	257	161	63
Pseudoloma stress	4	8	2	N	0	n/a	n/a
Pseudoloma stress	4	8	3	Y	83	18	22
Pseudoloma stress	4	8	4	Y	52	37	71
Pseudoloma stress	4	8	5	Y	212	143	67
Pseudoloma stress	8	8	1	Y	89	77	87
Pseudoloma stress	8	8	2	Y	103	101	97
Pseudoloma stress	8	8	3	Y	175	170	97
Pseudoloma stress	8	8	4	N	0	n/a	n/a
Pseudoloma stress	8	8	5	Y	149	142	95
Control	2	13	1	Y	87	61	70
Control	2	13	2	Y	372	344	92
Control	2	13	3	Y	138	81	59
Control	2	13	4	N	0	n/a	n/a
Control	2	13	5	N	0	n/a	n/a
Control	7	13	1	Y	147	4	3
Control	7	13	2	Y	251	207	82
Control	7	13	3	Y	142	116	82
Control	7	13	4	Y	130	3	2
Control	7	13	5	Y	8	3	38
Stress	3	13	1	Y	261	198	76
Stress	3	13	2	Y	269	125	46
Stress	3	13	3	Y	80	7	9
Stress	3	13	4	Y	310	86	28
Stress	3	13	5	Y	47	37	79
Stress	5	13	1	Y	234	86	37

Treatment	tank	week	pair	spawn	number of eggs laid	number of larvae (5dpf)	% hatched
Stress	5	13	2	Y	96	4	4
Stress	5	13	3	Y	228	206	90
Stress	5	13	4	Y	73	5	7
Stress	5	13	5	Y	126	25	20
Pseudoloma	1	13	1	N	0	n/a	n/a
Pseudoloma	1	13	2	Y	58	47	81
Pseudoloma	1	13	3	Y	81	70	86
Pseudoloma	1	13	4	Y	147	141	96
Pseudoloma	1	13	5	Y	133	64	48
Pseudoloma	6	13	1	Y	235	192	82
Pseudoloma	6	13	2	Y	0	n/a	n/a
Pseudoloma	6	13	3	Y	120	54	45
Pseudoloma	6	13	4	Y	0	n/a	n/a
Pseudoloma	6	13	5	Y	145	129	89
Pseudoloma stress	4	13	1	Y	153	95	64
Pseudoloma stress	4	13	2	Y	40	11	28
Pseudoloma stress	4	13	3	Y	56	42	75
Pseudoloma stress	4	13	4	Y	445	408	92
Pseudoloma stress	4	13	5	Y	405	376	93
Pseudoloma stress	8	13	1	Y	301	159	53
Pseudoloma stress	8	13	2	Y	28	10	36
Pseudoloma stress	8	13	3	N	0	n/a	n/a
Pseudoloma stress	8	13	4	Y	249	147	59
Pseudoloma stress	8	13	5	N	0	n/a	n/a
Control	2	20	1	N	0	n/a	n/a
Control	2	20	2	N	0	n/a	n/a

Treatment	tank	week	pair	spawn	number of eggs laid	number of larvae (5dpf)	% hatched
Control	2	20	3	Y	233	210	90
Control	2	20	4	Y	292	236	81
Control	2	20	5	N	0	n/a	n/a
Control	7	20	1	N	0	n/a	n/a
Control	7	20	2	N	0	n/a	n/a
Control	7	20	3	Y	12	12	100
Control	7	20	4	Y	80	9	11
Control	7	20	5	Y	98	96	98
Stress	3	20	1	Y	65	54	83
Stress	3	20	2	Y	4	3	75
Stress	3	20	3	Y	88	48	55
Stress	3	20	4	Y	96	92	96
Stress	3	20	5	Y	10	1	10
Stress	5	20	1	Y	0	n/a	n/a
Stress	5	20	2	N	29	29	100
Stress	5	20	3	Y	80	63	79
Stress	5	20	4	N	0	n/a	n/a
Stress	5	20	5	Y	296	279	94
Pseudoloma	1	20	1	Y	54	2	3
Pseudoloma	1	20	2	Y	147	73	50
Pseudoloma	1	20	3	Y	47	47	100
Pseudoloma	1	20	4	Y	483	457	95
Pseudoloma	1	20	5	Y	170	135	79
Pseudoloma	6	20	1	N	0	n/a	n/a
Pseudoloma	6	20	2	Y	379	280	74
Pseudoloma	6	20	3	N	0	n/a	n/a
Pseudoloma	6	20	4	Y	171	103	60
Pseudoloma	6	20	5	Y	201	159	79
Pseudoloma stress	4	20	1	Y	101	71	70

Treatment	tank	week	pair	spawn	number of eggs laid	number of larvae (5dpf)	% hatched
Pseudoloma stress	4	20	2	Y	66	7	11
Pseudoloma stress	4	20	3	Y	4	4	100
Pseudoloma stresss	4	20	4	Y	5	1	20
Pseudoloma stress	4	20	5	Y	104	75	80
Pseudoloma stress	8	20	1	N	0	n/a	n/a
Pseudoloma stress	8	20	2	Y	354	33	94
Pseudoloma stress	8	20	3	Y	101	75	74
Pseudoloma stress	8	20	4	Y	192	172	90
Pseudoloma stress	8	20	5	N	0	n/a	n/a

Appendix Figures

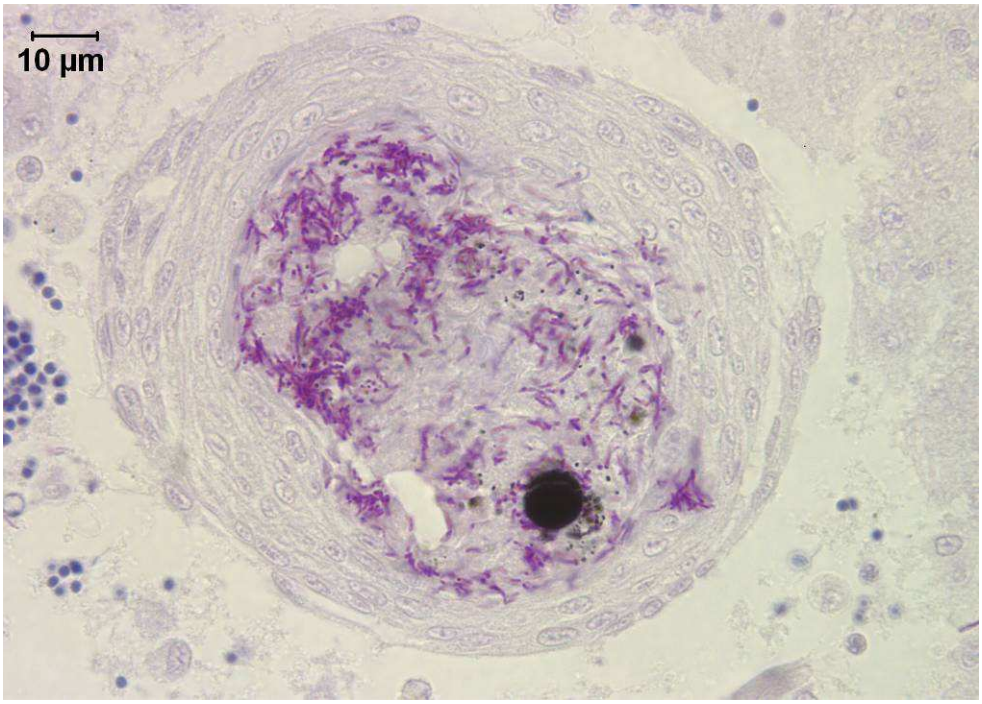


Figure A4.1: Histological section of zebrafish infected with *Mycobacterium marinum* (acid-fast staining). Granuloma of the liver with individual mycobacterial rods contained in the center.

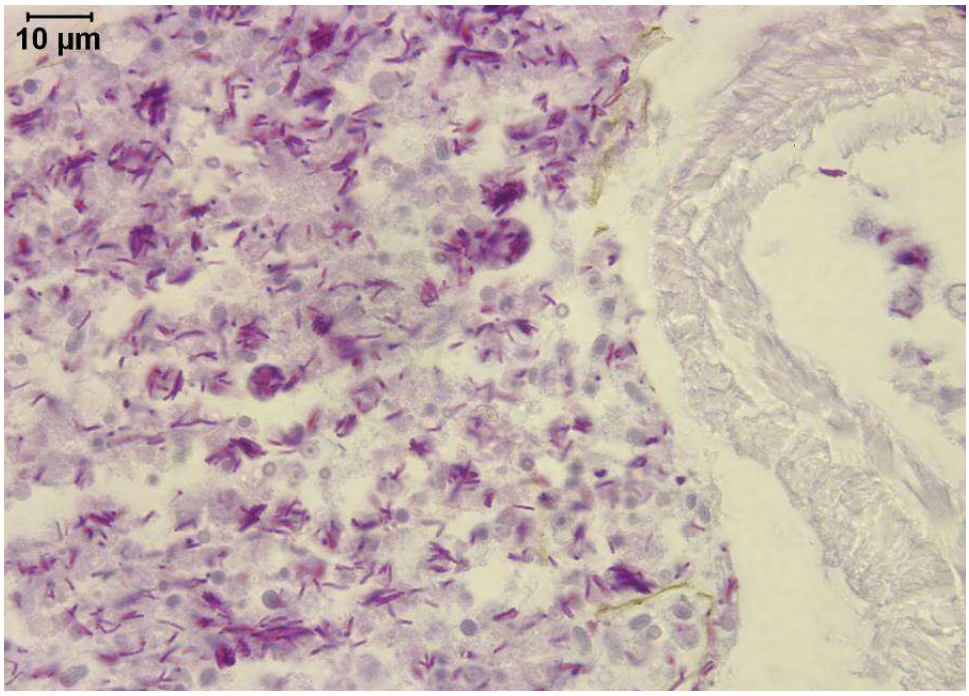
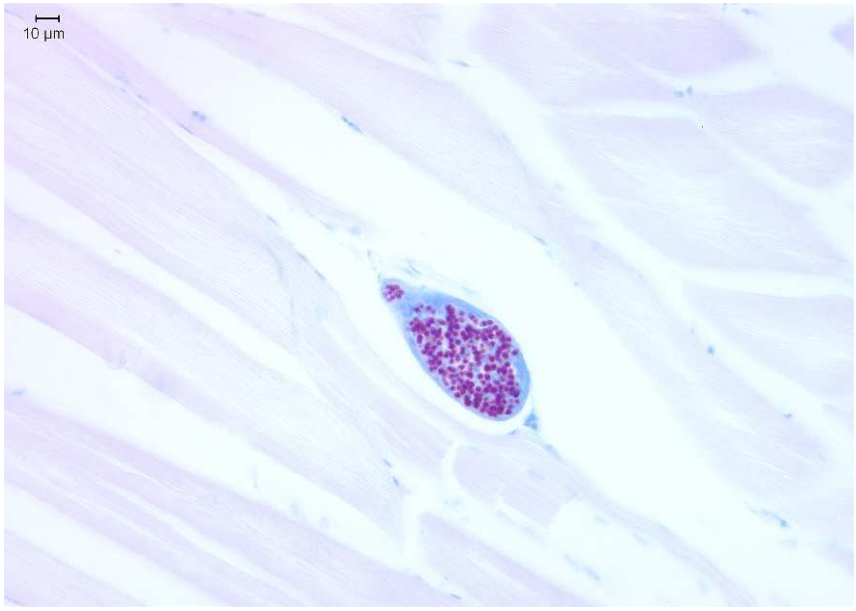


Figure A4.2: Histological section of zebrafish infected with *Mycobacterium marinum* (acid-fast staining). Sub-acute diffuse infection of the intestine with individual mycobacterial rods scattered throughout the tissue.

A)



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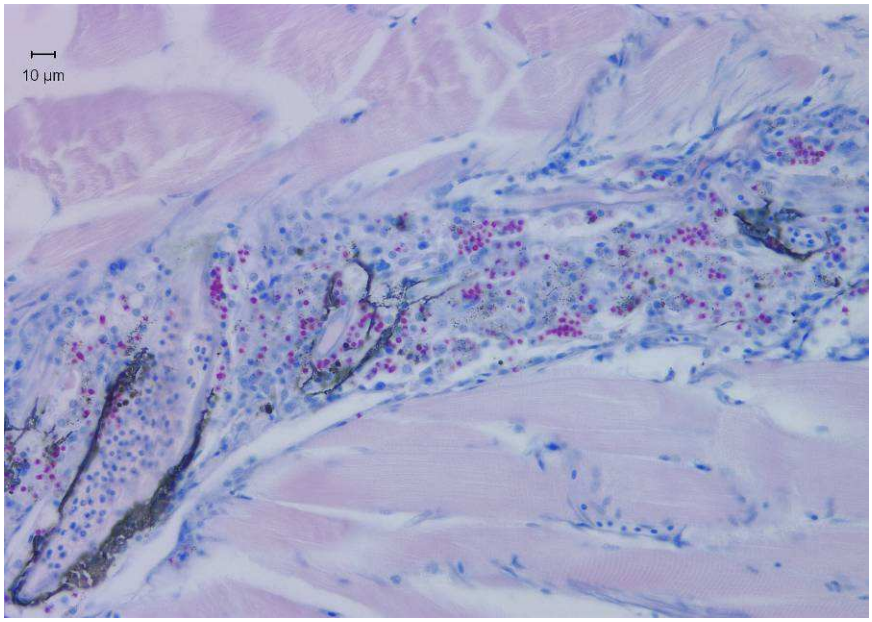
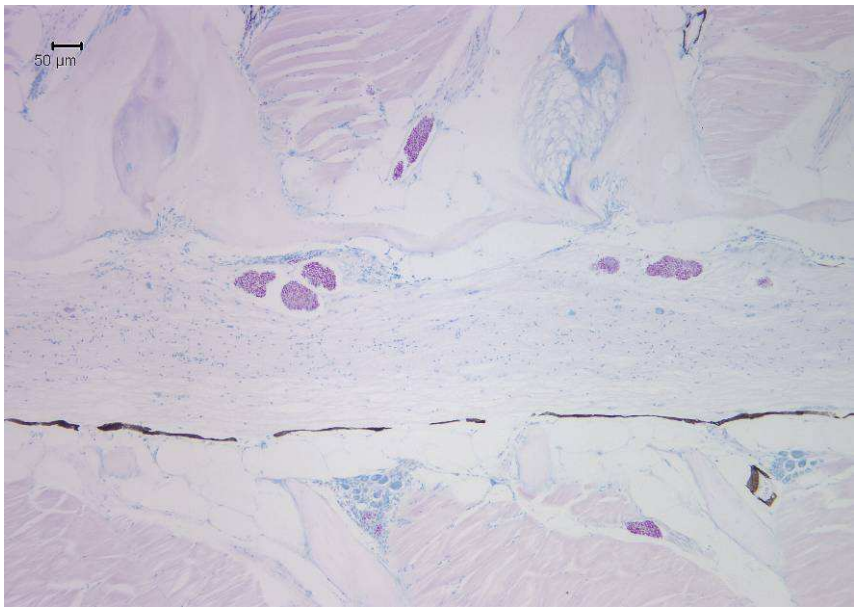


Figure A5.1: Histological sections of the zebrafish muscle tissue infected with *Pseudoloma neurophilia* (acid-fast staining). A) Xenoma containing individual spores in the somatic muscle B) Focal chronic myositis caused by a rupturing xenoma with individual spores scattered throughout the somatic muscle tissue.

A)



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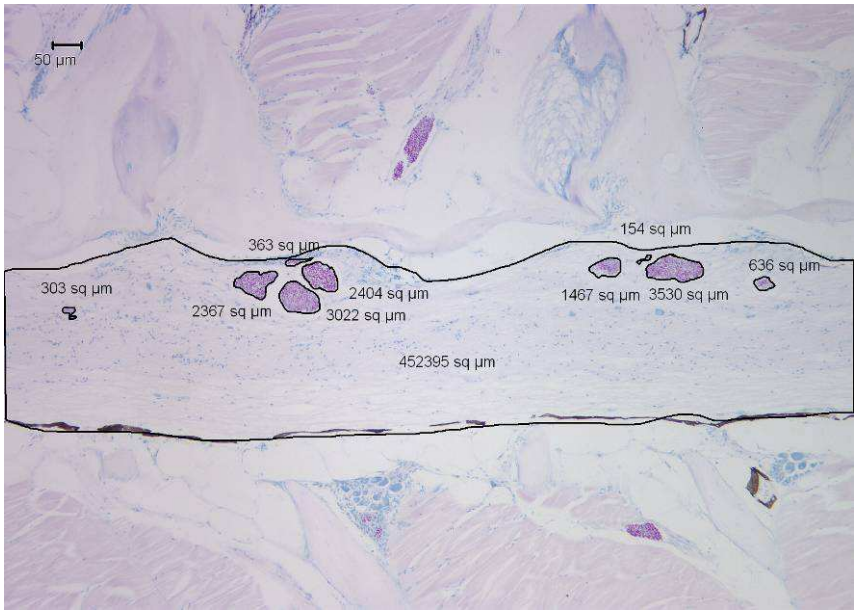
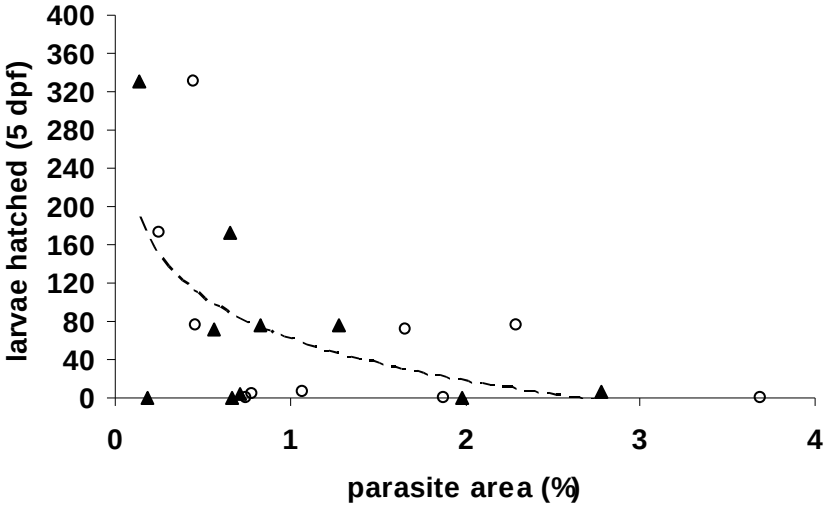


Figure A5.2: Sagittal section of the zebrafish spinal cord infected with *Pseudoloma neurophilia* (acid-fast staining). Numerous xenomas occupy the spinal tissue and surrounding meninges. A) Prior to measurement using SPOT software B) after measurement using SPOT software to measure the % area of parasite occupying spinal tissue (sum of areas with parasite/total spinal area).

A)



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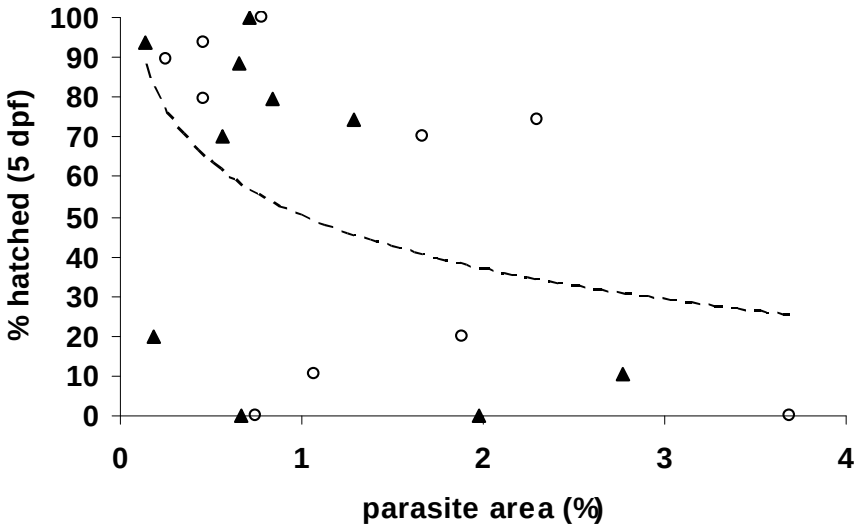
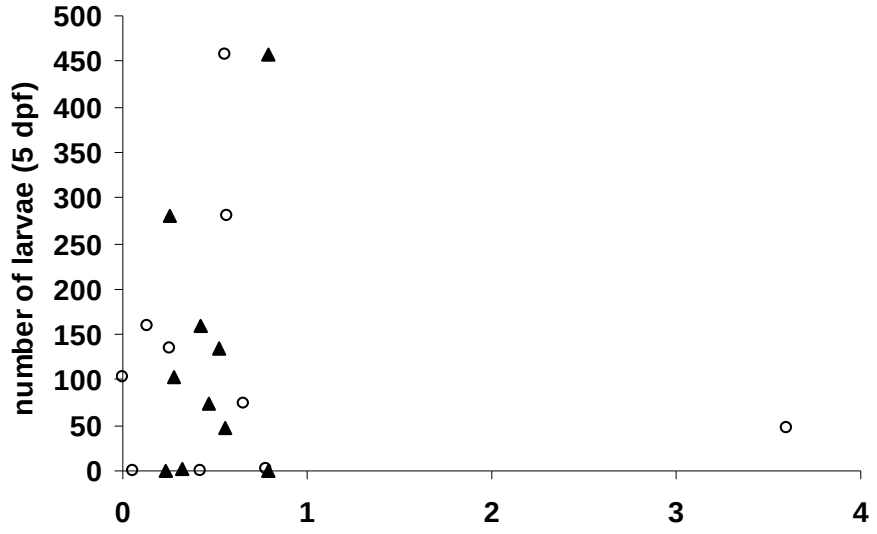


Figure A5.3: Zebrafish exposed to *Pseudoloma* and stressed. Number and percent of larvae hatched at 5 days post-fertilization (dpf) vs. parasite (xenoma) area (%) at week 20 post-exposure to *Pseudoloma*. A) The relationship between the number of larvae and parasite area was described by the following equation: number of larvae hatched = 61 – 63 ln (xenoma area); $R^2 = 0.3$; p=0.01. B) The relationship between the percent hatched larvae and parasite area was not significant ($R^2 = 0.18$; p=0.06). Male fish are indicated by an open circle (○). Female fish are indicated by a closed triangle (▲).

A)



B)

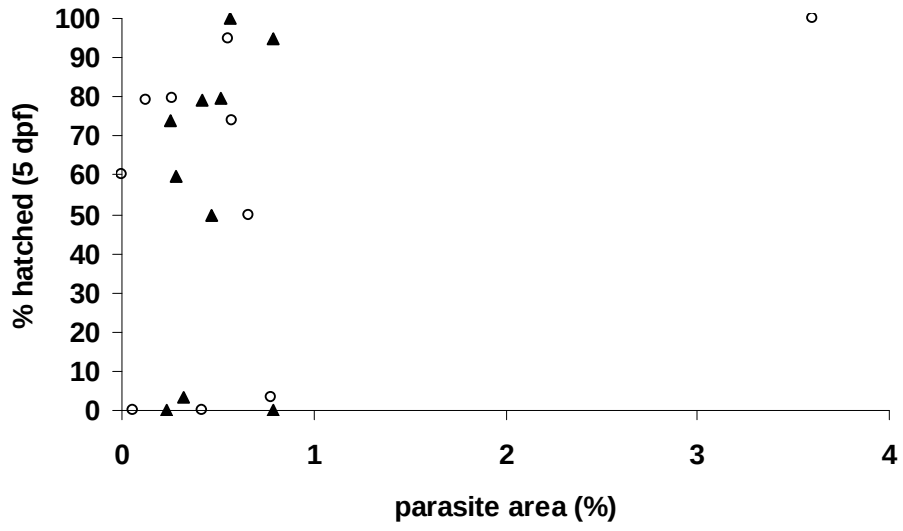


Figure A5.4: Zebrafish exposed to *Pseudoloma*. There was no relationship between the A) number of larvae hatched at 5 dpf (p=0.8), or B) the percent of larvae hatched at 5dpf (p=0.2) and parasite area (%).Male fish are indicated by an open circle (○). Female fish are indicated by a closed triangle (▲).