EVALUATING THE EFFECTS OF MULTIPLE ENVIRONMENTAL STRESSORS ON THE BEHAVIOUR AND PHYSIOLOGY OF A FRESHWATER PREY FISH

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By

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ABSTRACT

The skin of many fishes contains large epidermal club cells (ECCs) that are known to release chemicals (alarm cues) that warn other fishes of danger. Initial research on ECCs focussed on their role in predator avoidance behaviour, however later research revealed that these cells might also have immune functions. Anthropogenic activities have dramatically increased over the past decades, with the consequence that many organisms simultaneously get exposed to multiple environmental stressors. We have seen considerable reductions in stratospheric ozone with a concomitant increase in global ultraviolet radiation (UVR). Metal pollution associated with industrial activity is also increasing on a global scale. Cadmium (Cd) is one such ubiquitous pollutant which is known to be toxic to organisms at extremely low concentrations. The main goal of my PhD research was to understand how multiple environmental stressors play a role in altering ECC investment and chemically-mediated predator-prey interactions by indirectly elucidating the evolutionary role of ECCs. The first experiment investigated the effects of *in vivo* ultraviolet radiation (UVR) exposure on ECC investment, physiological stress responses and potency of alarm cues in fathead minnows (*Pimephales promelas*). Subsequently, I investigated the interactive effects of UVR and/or waterborne cadmium (Cd) exposure using the same end points. I found that minnows exposed to UVR, either in the presence or absence of Cd, showed consistent decrease in ECC investment compared to non-exposed controls. There was a significant increase in cortisol levels of UVR exposed minnows compared to unexposed minnows. However, the combined exposure of UVR and Cd reduced cortisol levels relative to that in UVR only exposure. Surprisingly, there was no difference in the potency of the cues prepared from the skin of UVR and/or Cd exposed or non-exposed fish indicating that UVR and/or Cd exposure combined may have little influence on chemically-mediated predator-prey interactions. In aquatic systems, much

of the negative effects of UVR are minimized by dissolved organic carbon (DOC) which is known to attenuate rates of UVR across the water column. In my third study, I investigated if DOC played a role in ameliorating the effects of *in vivo* UVR exposure on physiological stress and ECC investment in fathead minnows. I used two sources of DOC, a commercial soil based DOC (Sigma Aldrich Humic Acid) and a terrigenous source of DOC (Luther Marsh Natural Organic Matter). I found that fish exposed to UVR, in the presence of either source of DOC, in the presence and absence of UV blocking filter, maintained high ECC investment and reduced cortisol levels compared to fish exposed to UVR only. Studies that have examined factors that influence ECC investment have often been hampered by large variation in baseline levels of ECC. The larger the baseline variation in ECC number, the more difficult it is to elucidate factors responsible for changes in ECC investment. While I did not find this problematic in my work with UVR and Cd, others have failed to find effects in manipulative experiments. Consequently, my fourth study examined between and within variation in ECC investment across multiple sites in Saskatchewan and tried to investigate if holding fish under controlled laboratory conditions for up to 28 days would help reduce variation in ECC investment between and within populations. I found some evidence that I could reduce within population variation in ECC investment through time, but could not reduce among-population variation in mean ECC investment.

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Abbreviations and Acronyms

ANCOVA- analysis of covariance

ANOVA – analysis of variance

AREB – Animal Research Ethics Board

$CaCO_3$ – Calcium carbonate

CCME – Canadian Council of Ministers of the Environment

Ca – calcium

Cd – cadmium

DOC – dissolved organic carbon

DOM – dissolved organic matter

DO – dissolved oxygen

°C – Degree Celsius

EPA – Environmental Protection Agency

HPI – Hypothalamic-Pituitary-Interrenal

µg/L – microgram per litre

mg/L – milligram per litre

MS222 – Tricaine methanesulfonate

M-1 – Marshy Creek-1

M-2 – Marshy Creek-2

M-3 – Marshy Creek-3

M-4 – Marshy Creek-4

ng/ml – nanogram per millilitre

NOM – natural organic matter

PAS – Periodic acid Schiff's reagent

PL – Pike Lake

ROS – reactive oxygen species

SE – standard error

SD – standard deviation

TOC – total organic carbon

UCAC – University of Saskatchewan Committee on Animal Care and Supply

UVR – ultraviolet radiation

FORMAT OF THE THESIS

This thesis has been organized in manuscript format for publication. As a result, there may be some repetition of information throughout the thesis.

Chapter 2 has been published in the Biological Journal of the Linnean Society, 2012, Volume 105: pages 832-841, under the joint authorship of Maud Ferrari, Jeff Sereda, Som Niyogi and Douglas Chivers (University of Saskatchewan).

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Epidermal club cells and their contents

The skin of a teleost fish comprises two main layers: (1) the epidermis, which is the outer region and includes mucous cells, epidermal club cells (ECCs), and filament cells; and (2) the dermis, which lies under the epidermis and contains scales and various pigment cells. The epidermis is the region that acts as the living interface between the fish and the external environment (Roberts and Bullock 1981). Ostariophysan fishes comprise for about 28% of known fish species and about 72% of freshwater species (Nelson 1984). A salient feature of fishes belonging to this superorder is the presence of specialized cells in the epidermal region called Epidermal Club Cells (ECCs) (Figure 1.1) with some exceptions (Pfeiffer 1977). Histologically, ECCs and mucous cells can be differentiated from each other by a differing reaction to periodic acid Schiff's (PAS) reagent (Smith & Murphy 1974). PAS stains specifically for the presence of polysaccharides, mucopolysaccarides and basement membranes in tissue samples. Mucous cells are normally PAS-positive (dark coloured due to the presence of mucopolysaccarides), are generally located more superficial in the epidermis than ECCs (Smith and Murphy 1974), and release their contents through a pore connected to the skin's surface (Smith 1982). ECCs, on the other hand, are PAS-negative (light coloured) and are characterized by a round, centrally located, haematoxylin stained nucleus.

Scientists have tried to characterize the chemical contents of ECCs. Lebedova et al. (1975) and Kasumyan and Ponomarev (1987) used gel chromatography to explain similarity in biochemical properties of fish skin belonging to the order Cypriniformes, to which fathead minnows belong. Their work suggested that ECC contents were likely a complex of active

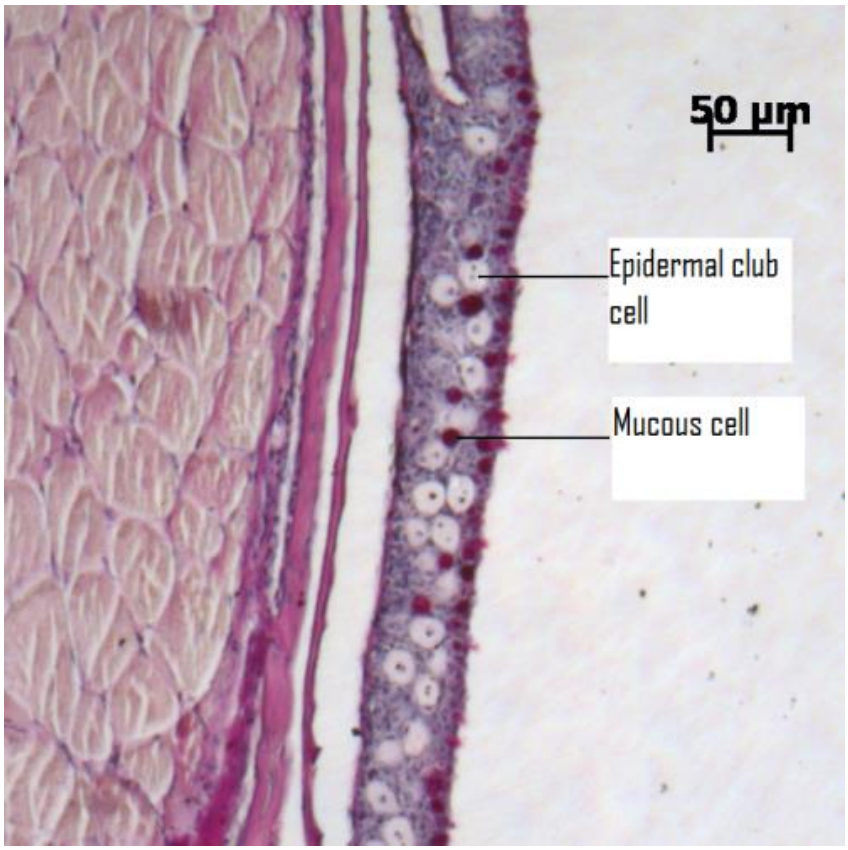

Figure 1-1 – Cross section (5 mm thick) of the minnow epidermis showing the mucous cells and epidermal club cells. This section was stained with periodic acid-Schiff's reagent (PAS) and then counterstained with haematoxylin (H). The mucous cells that are periodic acid Schiff's reagent with Harris' haematoxylin (PAS-H) positive appear dark and ECCs that are PAS-H negative were rendered colourless with a dark central nucleus (Manek et al. 2012).

compounds and a protein. The chemical that elicited the strongest fright reaction were molecules with a molecular weight ranging from 1100 dalton to more than 1500 dalton (Lebedova et al. 1975, Kasumyan and Ponomarev 1987). Pfeiffer et al. (1985) suggested that hypoxanthine-3-N-oxide is the putative component of ostariophysan alarm substance. However, the molecular weight of hypoxanthine-3-N-oxide is considerably smaller (350-500 dalton) as compared to the molecules proposed by Lebedova et al. (1975) and Kasumyan and Ponomarev (1987). Brown et al. (2000) carried out a study (laboratory and field based) to determine if nitrogen oxide functional group, purine skeleton or the combination of the two were a functional component in alarm substance. They showed that 3-N-oxide group particularly may be responsible for eliciting antipredator responses in ostariophysan fishes. Mathuru et al. (2012) using anion-exchange chromatography followed by high resolution gel filtration found a high molecular weight (30 kilodalton) and a low molecular weight (1 kilodalton) fraction supporting findings of Lebedova et al. (1975). ECCs contain glycosaminoglycan and thus may act as a source of chondroitin. They showed chondroitin is an active ingredient of alarm substance in zebrafish. These results indicate that alarm substance is a mixture that includes more than one component.

1.2. Evolution of epidermal club cells

The selection pressures leading to the evolution of ECCs in the context of predator-prey interactions in fishes has been a topic of great interest to evolutionary ecologists. Scientists have known for a long time that when damaged during an attack by a predator, these cells release a substance ‘alarm cue’ (previously called alarm pheromone and originally termed Schreckstoff) that elicits a fright response in nearby conspecifics (Chivers and Smith 1998, Ferrari et al. 2010). Alarm cues have been demonstrated in several groups of fishes including Ostariophysans (minnows, catfishes, sucker), salmonids (trout and salmon), and percids (darters and perch). By their nature,

these cues represent a reliable indicator of risk, and individuals responding to these cues with an antipredator response have been shown to have higher survival during encounters with predators (Mirza and Chivers, 2001, Brown 2003). Fishes often show graded behavioural responses to alarm cues, exhibiting higher intensity responses to increasing concentrations of alarm cues (Ferrari et al. 2005, 2006). Understanding the evolution of ECCs has been troublesome because it is unclear how these 'signals' could benefit the sender.

Only three of the 16 potential benefits to alarm cue senders proposed by Smith (1992) have received empirical testing. They include the kin selection hypothesis (Wisenden et al. 1995; Wisenden 1998), the attraction of secondary predator hypothesis (Mathis et al. 1995; Chivers *et al.* 1996) and the anti-parasite/anti-pathogen hypothesis (Chivers et al. 2007; James et al. 2009). The initial two hypotheses were predation centered and focussed on the chemical signalling role of alarm cues. The third hypothesis proposed by Chivers et al. (2007) provided the first strong support that ECCs evolved as part of the immune system, and that the alarm signalling function may have evolved secondarily. As a result of their strategic location, ECCs could provide a first line of defence against agents such as pathogens or parasites that penetrate through the skin, or promote the healing of damaged tissue as a result of agents such as UVR. (Al-Hassan et al. 1985, Smith 1992, Blazer et al. 1997, Chivers et al. 2007). Chivers et al. (2007) showed that an increase in ECC density was induced in fathead minnows (*Pimephales promelas*) after exposure to pathogenic water moulds (*Saprolegnia ferax* and *Saprolegnia parasitica*) and parasitic larval trematodes (*Uvulifer ambloplitis*). However, studies by James, Wisenden and Goater (2009) showed that ECC density was not affected by infection from cercariae of trematodes (*Ornithodiplostomum* sp). Chivers et al. (2007) reported that yellow perch (*Perca flavescens*) heavily infested with larval trematodes also exhibited a higher ECC density compared to control subjects. Halbgewachs et al. (2009) showed

that an intraperitoneal injection of cortisol resulted in reduced numbers of ECCs in fathead minnows. There was also a significant reduction in respiratory burst activity of kidney phagocytes indicating that there was suppression of the innate immune system. Similarly, minnows exposed to Cd had a reduced ability to increase ECCs upon exposure to pathogens (Chivers et al. 2007). Furthermore, Manek et al. (2012, 2013) showed that minnows exposed to UVR exhibited a rise in cortisol production and a corresponding reduction in ECC numbers. These studies support the immune function of ECCs and suggest that the function of alarm signalling may have evolved secondarily.

1.3. Effects of UV radiation in aquatic ecosystem

For much of the past century, we have witnessed dramatic increases in levels of ultraviolet radiation (hereafter UVR) hitting the surface of the earth as a result of reductions in stratospheric ozone (Newman et al. 2006). The implementation of the Montreal Protocol has ameliorated much of the ozone depletion, but it is difficult to ascertain how consistently ozone recovery will be due to factors such as changes in cloud cover, air pollutants and aerosols, all of which influence climate change (Mackenzie et al. 2011). UVR forms a part of the electromagnetic spectrum, which is divided into three groups: UV-A (320–400 nm), UV-B (280–320 nm), and UV-C (100– 280 nm). UV-A, despite being the main component of the solar UVR and having greater penetration power in the ecosystems, is far less harmful than UV-B. UV-C is absorbed by molecular oxygen (O_2) in the atmosphere and most of the UV-B is absorbed by the ozone layer (O_3) (Madronich et al. 1998).

An increase in UVR penetration has the potential to cause considerable stress to aquatic organisms. UVR damages DNA and impairs an organism's ability to reproduce, sense its environment, and resist disease (Blaustein et al.1994, 1997; Williamson and Rose 2010). In fishes, UVR causes cataracts and skin lesions (Mayer, 1992). It impairs immune function (Salo et al.

2000a) and induces physiological stress responses (Manek et al. 2012, 2014). A comparative study of the effects of UV-A and UV-B on roach (*Rutilus rutilus*) has shown that exposure to both UV-A and UV-B causes suppression in mitogenic proliferation of blood lymphocytes. UV-A radiation decreases haematocrit, plasma protein, and plasma immunoglobulin levels, and increases the proportion of blood cells (Salo et al. 2000b). UV-B affects the functioning of the head kidney and blood phagocytes, induces granulocytosis and lymphocytopaenia in the blood, and increases plasma cortisol concentrations (Salo et al. 2000a). UV-B also causes skin burns in brown trout (Noceda et al. 1997). These results indicate that ambient levels of UVR can act as a potential environmental stressor and an immunosuppressant in fishes.

1.4. Effects of cadmium in aquatic ecosystems

Cadmium (Cd) is a heavy metal that is considered to be a priority pollutant in aquatic systems because of its toxicity at very low concentrations (Campbell 2006). Cadmium mainly enters the aquatic environment from atmospheric deposition and effluents from smelting, metal-mining and refining (CCME 1996). Based on the criteria of the United States Environmental Protection Agency (U.S. EPA), at hardness of 120 mg/L, the Cd concentration that is believed to protect 95% of freshwater species in a 24-h exposure is 2.5 μg/L (U.S. EPA 2001). Cadmium has a myriad of effects on physiology and behaviour of fish. Cd is known to be a Calcium (Ca)-antagonist and causes toxicity by inducing disruption of Ca-homeostasis, particularly during acute exposure (Niyogi and Wood 2004a). It also acts as an immunosuppressant (Sanchez-Dardon et al. 1999) and an endocrine disruptor. Lacroix and Hontela (2004) showed that Cd inhibits adrenocorticotrophic hormone (ACTH) stimulated cortisol secretion from the interrenal cells in fish. Cd exposure alters shoaling behaviour in fathead minnows and renders minnows more vulnerable to predation by largemouth bass (*Micropterus salmoides)* (Sullivan et al. 1978). This

result may be explained by the deleterious effects of Cd on lateral line and olfactory perception. Cadmium exposure causes severe epithelial necrosis throughout the olfactory epithelium in fathead minnows (Stromberg et al. 1983) and accumulates in olfactory sensory neurons, the olfactory nerve and the anterior part of the olfactory bulb (Tjalve et al. 1986, Gottofrey and Tjalve 1991, Scott et al. 2003). Chronic exposure to environmentally relevant levels of waterborne Cd (2 μg/L) alters responses to alarm cues in embryonic and larval stages of zebrafish (*Danio rerio*) (Blechinger et al. 2007, Kusch et al. 2007) and rainbow trout *(Oncorhynchus mykiss)* (Scott et al. 2003).

Acute exposure to waterborne Cd at high concentrations results in gill epithelium hyperplasia (increased cell production in tissue or organ) and necrosis (cell death) (McGeer et al. 2012). At lower concentrations, the primary effects of chronic Cd exposure are disruption of ion homeostasis (particularly Ca^{2+} regulation) and generation of reactive oxygen species (ROS), which can be linked to multiple physiological impacts and performance impairments following Cd exposure (U.S. EPA 2001). As an immunotoxin to fish, Cd was reported to reduce the respiratory burst (a measure of immune activity) of kidney phagocytes (Hutchingson & Manning 1996). An exposure to 2 μg Cd/L has been shown to result in reduced macrophage-mediated immune function in fish, and this would translate into a reduced ability to fight off bacterial or fungal infections as well as other diseases (Zelikoff et al. 1995).

1.5. Role of dissolved organic carbon in aquatic ecosystems

Organisms residing in clear, shallow high elevation lakes, where fluctuations in UVR levels could be much higher, are more vulnerable to harmful effects of UVR due to shallow depth and higher absorption, reducing refuge from damaging levels of radiation (Williamson 1995). The level of UVR transmission varies across lakes and is greatly influenced by water chemistry. Dissolved organic matter (DOM), a component of natural organic matter (NOM), is an important

water chemistry parameter in aquatic ecosystems. DOM is measured as dissolved organic carbon (hereafter DOC measured in mg/L) (Steinberg et al. 2008). There have been extensive studies focussing on the impacts of NOM on aquatic organisms looking at physiological effects (Campbell et al. 1997, Wood et al. 2003, Matsuo et al. 2004, Glover et al. 2005a, Galvez et al. 2009), toxic effects (Matsuo et al. 2006, Meinelt et al. 2007), and the ability of NOM to alter the uptake and toxic effects of organic chemicals (Haitzer et al. 1998, Qiao and Farrell, 2002). DOC is the fraction of DOM that passes through a 0.45 µm membrane and is chemically made of humic and fulvic fractions or acids (Buffle, 1984, Thurman, 1985).The attenuation rate of visible light and UVR in the water column is largely regulated by the concentration and absorptivity of DOC rightfully terming it a "natural sunscreen" (Kirk et al. 1994, Morris et al. 1995, Porcal et al. 2009). Some studies have suggested that DOC may completely protect aquatic organisms like amphibians from damage caused by UV-B radiation (Adams et al. 2001, Palen et al. 2002). DOC affects other water quality parameters in aquatic systems, namely pH, and has the ability to impart colour (Porcal et al., 2009). Chromophoric dissolved organic matter (CDOM, the light absorbing component of DOC) strongly and selectively absorbs UVR radiation. In recent times, a variety of environmental changes namely increasing atmospheric CO_2 concentration, global warming, nitrogen deposition, decreased sulfate deposition are causes for changes in DOC concentrations across aquatic systems (Porcal et al. 2009).

1.6. Stress physiology - cortisol as an indicator of stress

The terms "stress" and "stressors" have been widely used by specialized group of scientists such as cell biologists, ethologists, ecologists, physiologists and toxicologists to describe the physical condition of an organism. Selye (1973) suggests that stress is "the nonspecific response of the body to any demand made upon it". Selye (1985) suggested that stress should be divided into

two phases: “eustress”/allostatic load or the healthy stress and “distress”/allostatic overload or bad stress. Eustress occurs as a response of the organism undergoing situations that provoke physiological changes that optimize its biological performance, for example exercise. Distress occurs when certain factors promote physiological changes on an organism that may compromise organism’s integrity. Wandelaar Bonga (1997) described stress as a condition that an organism experiences when a physical agent disrupts its dynamic equilibrium called homeostasis. This disruption in homeostasis can be primary, secondary or tertiary in level depending on the agent and duration of exposure (Wandelaar Bonga 1997). Some examples of primary stress responses include activation of specific brain centers, resulting in release of hormones such as catecholamines and corticosteroids (Wendelaar Bonga 1997). Some examples of secondary stress responses are the immediate actions and effects of these hormones at the blood and tissue level, causing changes in the blood and tissue chemistry, e.g. an increase of plasma glucose (Barton 1997, Begg and Pankhurst 2004).This could result in increases in cardiac output and oxygen uptake, mobilization of energy substrates and disturbance of hydromineral balance. The most detrimental form of response which is a tertiary response involves effects of the agent at the organism level. Some examples at the tertiary level include inhibition of reproduction, immune response, growth and reduction in capacity to tolerate multiple/additional stressors (Wendelaar Bonga 1997).

Throughout this thesis, I cautiously use the term stress to refer to a change in physiological responses by estimating levels of cortisol in the blood of fathead minnows. Cortisol is the principal glucocorticoid secreted by the interrenal tissue (steroidogenic cells) located in the head-kidney of teleost fish (Iwama et al. 1999). It is released by the activation of the Hypothalamic-Pituitary-Interrenal (HPI) axis. Cortisol activates glycogenolysis (breakdown of glycogen to glucose-1-phosphate and glycogen) and gluconeogenesis (synthesis of glucose from molecules that are not

carbohydrates, such as amino and fatty acids) processes in fish, but also causes chromaffin cells to increase the release of catecholamines which further increase glycogenolysis and modulate cardiovascular and respiratory function (Reid et al. 1992, Reid et al. 1998). The functions of cortisol in fish are highly diverse which include regulating hydromineral balance and energy metabolism, reducing growth rate and suppression of the reproductive system. Similarly, when present at higher than normal levels for a prolonged period of time, cortisol also causes generalized suppression of the immune system. The purpose of cortisol-induced immunosuppression is likely to ensure the redirection of energy stores to activities that are more critical to the immediate survival of the animal or to prevent a deleterious overshoot of the immune response (Sapolsky et al. 2000). There is a plethora of literature that uses cortisol levels to evaluate stress levels in fishes exposed to a variety of biotic and abiotic factors (Flik et al. 2006, Martinez-Porchal et al. 2009).

1.7. Study system: fathead minnows

Fathead minnows are small freshwater fish (adult length: 4-6 cm), commonly found in rivers, lakes or ponds throughout North America (Page and Burr 1991). Their small size makes them vulnerable to a variety of aquatic and terrestrial predators including fishes, birds, snakes and invertebrates. Minnows belong to the superorder Ostariophysi, which means that they possess ECCs containing the alarm cues described above. During the mating season (from April to August depending on the latitude), the males lose their ECCs. Breeding males can be easily differentiated from breeding females. Fathead minnows have been extensively used as test species for both toxicological and behavioural studies. Their relatively small size and easy maintenance make them a preferable species for laboratory-based studies. Moreover, fathead minnows have been a classic study system for chemical ecology of predator-prey interactions, including studies addressing the evolutionary role of ECCs.

1.8. Research hypotheses and objectives

It is rare that animals are exposed to single stressors and consequently research aimed at identifying the interactive effects of multiple stressors is needed (Boone et al. 2007). The overall objective of this thesis was to elucidate the evolutionary role of ECCs and understand how multiple environmental stressors play a role in altering ECC investment and chemically-mediated predator-prey interactions. In my thesis, I present a series of experiments divided in four data chapters designed to address the following questions.

Question 1: Does UVR have an effect on ECC investment, physiological stress, and potency of alarm cues prepared from the skin of UVR exposed fathead minnows?

Hypotheses: I hypothesize that physiological stress and ECC investment will vary in minnows depending on their exposure to UVR and that the potency of alarm cues will vary with an alteration in ECC investment.

Predictions: I predict that exposure to UVR will increase cortisol levels and consequently will increase ECC investment and will increase the level of anti-predator responses (potency of alarm cues) prepared from the skin of UVR-exposed minnows.

Question 2: Does UVR and/or Cd have an effect on ECC investment, physiological stress and potency of alarm cues prepared from the skin of fathead minnows exposed to UVR and/or Cd?

Hypotheses: I hypothesize that physiological stress and ECC investment will vary in minnows depending on their exposure to Cd and/or UVR and that the potency of alarm cues will vary with an alteration in ECC investment.

Predictions: I predict that UVR exposure will result in an elevation in cortisol. However, Cd will result in endocrine disruption and lower the characteristic elevation in cortisol typically observed upon exposure to UVR, as found in our previous study (Manek et al. 2012). I also predict

that elevated cortisol production in response to Cd and/or UVR exposure will result in lowered ECC investment in minnows exposed to UVR only compared to Cd and/or UVR exposed minnows. Finally I predict that Cd and/or UVR exposure will lower the level of anti-predator response (potency of alarm cues) prepared from the skin of Cd and/or UVR exposed minnows.

Question 3: Does DOC ameliorate the effects of UVR on ECC investment and physiological stress response in fathead minnows?

Hypotheses: I hypothesize that physiological stress and ECC investment will vary in minnows depending on their exposure to UVR under different sources and concentrations of DOC.

Predictions: I predict that exposure to UVR under increased DOC levels will help in maintaining low cortisol levels relative to those exposed to UVR only. I also predict that under the influence of increased DOC levels and UVR exposure, minnows will be able to maintain a high level of ECC investment.

Question 4: Is there a difference in ECC investment in fathead minnows between and within sites across Saskatchewan? If there is a difference, can controlled laboratory conditions help reduce the difference?

Hypotheses: I hypothesized that the ECC investment in fathead minnows will vary both between and within sites. I also hypothesize that variation in ECC investment within and between sites will be reduced by housing fish under controlled laboratory conditions.

Predictions: I predict that there will be greater variation in ECC investment between sites than within sites and that holding fish for up to 28 days will help reduce variation in ECC investment.

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CHAPTER 2[a]

THE EFFECTS OF ULTRAVIOLET RADATION ON A FRESHWATER PREY FISH: PHYSIOLOGICAL STRESS RESPONSE, CLUB CELL INVESTMENT AND ALARM CUE PRODUCTION

[a] This chapter examines the effects of UVR exposure on the physiological stress response and behaviour in fathead minnows. The purpose of chapter 2 was to investigate if stress mediated changes in ECC investment had any effect on chemically mediated predator-prey interactions in fathead minnows. The contents of chapter 2 have been published in the journal *Biological Journal of the Linnean Society*, 2012, Volume 105: pages 832-841, under joint authorship with, Maud Ferrari, Jeff Sereda, Som Niyogi and Doug Chivers (University of Saskatchewan)

2.1 INTRODUCTION

Recent anthropogenic activities have caused a considerable reduction in stratospheric ozone, with a corresponding increase in the amount of ultraviolet radiation (UVR) hitting the surface of the earth (Newman et al. 2006). Chromophoric dissolved organic matter (CDOM) functions to protect aquatic organisms from the detrimental effects of UVR by attenuating solar radiation selectively and strongly within the UVR range (Scully and Lean, 1994; Williamson and Zagarese 1994). An increase in UVR penetration has the potential to cause considerable stress to aquatic organisms. Aquatic species in general and fishes in particular, are vulnerable to stress-induced changes in their environment primarily through their skin and gills, which are constantly being exposed to the surrounding water. Fish skin lacks the keratinized outer layer, which acts as a protective layer against stressors for many vertebrates (Bullock 1982). Consequently, this multilayered assemblage of cells should serve as an integral part of its defence system and respond rapidly to external stimuli (Zaccone et al. 2001).

The skin of fish comprises two main layers: (1) the epidermis, which is the outer region and includes mucous cells, epidermal club cells (ECCs), and filament cells; and (2) the dermis, which lays under the epidermis and contains scales and various pigment cells. The epidermis is the region that acts as the living interface between the fish and the external environment (Roberts and Bullock, 1981). The selection pressures leading to the evolution of ECCs in fishes have been a topic of great interest to evolutionary ecologists. When damaged during an attack by a predator, these cells release a substance (‘alarm cue’) that elicits a fright response in nearby conspecifics (Chivers and Smith 1998, Ferrari et al. 2010). Fishes often show graded behavioural responses to alarm cues, exhibiting higher intensity responses to increasing concentrations of alarm cues. Alarm cues have been demonstrated in several groups of fishes including Ostariophysians (minnows,

catfishes, sucker), salmonids (trout and salmon), and percids (darters and perch). By their nature, these cues represent a reliable indicator of risk, and individuals responding to these cues with an antipredator response have been shown to increase survival during encounters with predators (Mirza and Chivers 2001, Brown 2003). Understanding the evolution of ECCs has been troublesome because it is unclear how these 'signals' could benefit the sender. Although many hypotheses have been proposed to explain the evolution of such cells (Smith 1992), Chivers et al. (2007) provided the first strong support that these cells evolved as part of the immune system, and that the alarm function may have evolved secondarily. As a result of their strategic location, ECCs could provide a first line of defence against agents such as pathogens or parasites that penetrate through the skin, or promote the healing of damaged tissue as a result of agents such as UVR. (Al-Hassen et al, 1985, Smith, 1992, Blazer et al. 1997, Chivers et al. 2007). Chivers et al. (2007) showed that an increase in ECC density was induced in fathead minnows (*Pimephales promelas*) after exposure to pathogenic water moulds (*Saprolegnia ferax* and *Saprolegnia parasitica*) and parasitic larval trematodes (*Uvulifer ambloplitis*). However, studies by James et al. (2009) showed that ECC density was not affected by infection from cercariae of the trematode, (*Ornithodiplostomum* sp). Chivers et al. (2007) reported that yellow perch (*Perca flavescens*) heavily infested with larval trematodes also exhibited a higher ECC density compared to control subjects. Furthermore, Halbgewachs et al. (2009) demonstrated that intraperitoneal injections of cortisol suppressed the innate immune system of minnows and reduced ECC investment. We do not know how alterations in ECC density mediated through immune responses would affect chemically-mediated predator–prey interactions.

UVR forms a part of the electromagnetic spectrum, which is divided into three groups: UV-A (320–400 nm), UV-B (280–320 nm), and UV-C (100– 280 nm). UV-A, despite being the main

component of the solar UVR and having greater penetration power in the ecosystems, is far less harmful than UV-B. UV-C is absorbed by molecular oxygen (O_2) in the atmosphere and most of the UV-B is absorbed by the ozone layer (O_3) (Madronich et al. 1998). Ambient levels of UV-B have been shown to cause mortality in embryonic and larval amphibians (Blaustein et al. 1997, 1998). A comparative study of the effects of UV-A and UV-B on roach (*Rutilus rutilus*) has shown that exposure to both UV-A and UV-B causes suppression in transiently mitogenic proliferation of blood lymphocytes. UV-A radiation decreases haematocrit, plasma protein, and plasma immunoglobulin levels, and increases the proportion of blood cells (Salo et al. 2000b). UV-B affects the functioning of the head kidney and blood phagocytes, induces granulocytosis and lymphocytopaenia in the blood, and increases plasma cortisol concentrations (Salo et al. 2000a). UV-B also causes skin burns in brown trout (Noceda et al. 1997). These results indicate that ambient levels of UVR can act as a potential stressor and an immunosuppressant in fish.

The present study aimed to investigate the responses of fathead minnow to UVR exposure. Previous studies have demonstrated a correlation between UVR exposure and ECC investment in two different groups of fishes (percids and ostariophysians). For example, johnny darters (*Etheostoma nigrum*) and yellow perch (*Perca flavescens*) both had significantly more ECCs on their dorsal surface than their flank, and more ECCs on their flank than on their ventral surface (Chivers et al. 2007). Similarly, fathead minnows have more ECCs on their dorsal surface than their ventral surface (Hugie 1990). Consequently, as a result of this indirect evidence, we propose that UVR exposure could increase ECC investment in fathead minnows. However, if UVR exposure acts as an immunosuppressant via increasing cortisol, the epidermal immune response might be inhibited. We also examined whether the number of mucous cells was reduced by exposure to UVR, as previously documented by Blazer et al. (1997) and Kaweewat and Hofer

(1997). Finally, we tested whether minnows respond differentially to skin extracts produced by High UVR and non-exposed minnows. Changes in ECC numbers associated with the UVR exposure could lead to a change in the behavioural response of the minnows to skin extracts. Ferrari et al. (2006) demonstrated that minnows displayed a greater intensity antipredator response when the amount of skin extract introduced into their tank was increased. In the present study, we control the volume of skin added to each tank and test for differential responses that arise from the UVR exposure treatments.

2.2 MATERIALS & METHODS

2.2.1 Fish collection and maintenance

Adult fathead minnows (mean ± S.D., standard length = 4.97 ± 0.08 cm; weight = 2.9 ± 0.7 g) were collected between April and May 2009, from the Feedlot pond located on the University of Saskatchewan campus using Gee's improved minnow traps. Fish were housed in a 1600-L flow-through pool containing dechlorinated tap water. The water was maintained at approximately 19 ± 2 °C under a 14:10 h light/dark cycle. Fish were fed commercial flake food *ad libitum* throughout the experiment and were acclimated for several months before the experimental procedure. The water used for the experiments originated from the Saskatoon, SK, Canada municipal water supply and was run through an active carbon filter. Water chemistry parameters (temperature, dissolved oxygen, pH, total alkalinity, and total hardness; Table 2.1) were monitored every alternate day during the acclimation and experiment phase.

Table 2-1 – Mean water quality parameters ± S.E. during acclimation phase and experiment phase. One way ANOVA indicate no significant differences between treatments at α=0.05.

Treatment	**Temperature (°C)**	**Dissolved oxygen (mg/L)**	**pH**	**Alkalinity (mg/L, $CaCO_3$)**	**Hardness mg/L, $CaCO_3$)**
low UVR	19 ± 2	7.01 ± 0.15	7.42 ± 0.03	94 ± 0.2	120 ± 1.3
high UVR	19 ± 2	6.16 ± 0.11	7.45 ± 0.02	94 ± 0.3	120 ± 2.5
Control (Aquaria)	18.5 ± 1.5	6.9 ± 0.12	7.6 ± 0.06	N.A	N.A

N.A: Not available

2.2.2 UV System

Fathead minnows were exposed to artificial UVR (250 W m-2) *in vivo* in an Atlas SUNTEST XLS + Solar Simulator with Xeon lamp with a Suprax Daylight Glass Filter – 290 nm cut-off (Atlas Material Testing Technology LLC, Chicago, USA). Minnows were maintained in quartz beakers (diameter 13.8 cm, height 16.8 cm; QSI Quartz Scientific, USA) placed in a constant flow water bath to maintain ambient water temperature (Figure 2.1). Water temperature throughout the experiment was maintained at 19 ± 2 °C, which is representative of ambient surface water temperatures in Saskatchewan lakes throughout July and August. The water quality parameters (Table 2.1) were checked daily during the exposure period. We set the periodicity to 8:16 h light/dark cycle. The 8-h exposure included UV-A (320–400 nm), UV-B (280–320 nm), and photosynthetically active radiation (PAR; 400–700 nm). The spectroscopic readings of UVR were measured every 0.5 nm through the UVR (280–400 nm) and PAR (400–700 nm) spectrum, using an Ocean Optics USB 2000 spectroscopic radiometer. The spectral characteristics of UVR were analyzed using SPECTRA SUITE software (Ocean Optics). The spectral output from the Suntest Solar Simulator was comparable to mean noon time solar irradiance measured in Saskatoon, SK (Sereda et al. unpublished data). Total UVR emitted by the solar simulator was 45 W m-2; mean natural solar irradiance was 43 W m-2. Although there was little difference in the total UVR emitted from the solar simulator and the natural noon time solar irradiance in Saskatoon, the 8-h periodicity that we choose would result in a cumulative dose somewhat higher than normal for minnows at our latitude. However, the cumulative dose that we choose would be lower for minnows living at different latitudes. Indeed, the amount of UV exposure that the minnows receive across their geographical range will vary by as much as two-fold depending on latitude (Goncalves et al. 2010).

Figure 2-1 – Atlas Suntest XLS+ Solar Simulator set up with 2 L quartz beaker housing fathead minnows placed in a water bath exposed to UVR for 8H a day. Attached airstones ensured constant supply of oxygen and prevented stratification of temperature across the beaker. Digital temperature probes provided a range of the temperature through the entire experiment.

2.2.3 Experimental design

The objective of this experiment was three-fold: (1) to observe the effects of UVR on ECC and mucous cell investment; (2) to observe the effects of UVR on physiological stress response; and (3) to observe the effect of UVR on potency of alarm cues prepared from the skin of minnows exposed to UVR.

Male minnows have suppressed ECC numbers due to high testosterone levels; consequently, the experiment was performed from September 2009 to March 2010, outside the breeding season of minnows. In the non-reproductive phase, male and female minnows are difficult to morphologically differentiate. Four randomly chosen minnows were introduced in each of the two quartz beakers in the solar simulator. We used a design, whereby the fish in two beakers were exposed to UVR (i.e. high UVR group) for 4 days. Subsequently, two beakers had their top and sides covered with a 2-mm thick Lexan polycarbonate sheet with the fish exposed to the treatment for the same 4-day period. The polycarbonate sheeted removed 76% of the UVB and UVA radiation; hence, this treatment is referred to as the low UVR group. We alternated having high UVR and low UVR fish in the solar simulator to control for order effects. Filtering efficiency of the polycarbonate sheets was monitored throughout the study to ensure that there was no change in radiation treatment. The fish were left to acclimate for 24 h before the start of the exposure. After this acclimation period, fish were exposed to artificial solar radiation of 250 W/m^2 for 8 h every day over a 4-day period. A preliminary trial indicated no effect of UVR on ECC numbers after only 2 or 3 days. We ran 12 blocks containing two high UVR group beakers, and 13 blocks containing two low UVR group beakers. The four minnows in each beaker were not independent, so that we used the ‘beaker’, and not the individual minnows, as our replicate unit.

Eight of 96 minnows in the high UVR group died, whereas no fish died in the low UVR group. Minnows that did not survive until the end of the exposure were excluded from further analysis. After 96 h of exposure, the fish were sacrificed with a blow to the head (University of Saskatchewan Animal Care Protocol Number 2009091) and blood was immediately extracted from eight blocks of each group for cortisol analysis. Two high UVR group minnows and two low UVR group minnows were randomly selected for histological analysis and were preserved in 10% neutral buffered formalin until further processing to obtain skin sections. The remaining two minnows from each beaker were used to obtain skin fillets for the behavioural bioassay. The skin was removed from the lateral sides of the fish and alarm substance was prepared and frozen in 20-mL aliquots until used for the assay. To control for the potential that holding conditions in the solar simulator were stressful, four untreated minnows, taken from our stock tank, were sacrificed at the same time as each of the experimental minnows. We refer to these as our control group for the cortisol and histological analyses.

2.2.4 Experimental protocol for blood extraction

Blood extraction for cortisol analysis was performed *sensu* Halbegewachs et al. (2009). Blood samples (25–50 μL) were extracted from the caudal vein near the anal fin region of euthanized minnows. To obtain sufficient blood for the analysis, we pooled blood from four fish from the same beaker (high UVR, low UVR, and control). This blood was placed on ice and allowed to clot for at least 1 h. Serum was extracted from the blood after centrifugation and then frozen at -20 °C until it was used for analysis. The cortisol level in the extracted serum was measured by the Endocrine Laboratory at Prairie Diagnostic Service (University of Saskatchewan) in a Coat-A-Count radioimmunoassay, which is designed for the quantitative measurement of cortisol in serum.

2.2.5 Histological analysis of the skin

Tissue preparation for the analysis of the minnow epidermis was performed *sensu* Hugie (1990) with specific modifications. The entire fish was initially fixed in 10% neutral buffered formalin until tissue processing could be performed. Epidermal samples were taken from the dorso/lateral surface just behind the operculum to the dorsal fin. An automatic tissue processor (MUP1, Modular Vacuum Processor) was used to dehydrate the fixed skin tissue in a series of ethanol grades and perfused with paraffin wax. Tissues were then manually embedded in paraffin wax and sectioned using a rotary microtome (HM330; Heidelberg) at 5-mm thickness. After sectioning, three to five sections were placed on a pre-cleaned suprafrost slide (VWR micro slides). After the slides were dried on a slide warmer, they were deparaffinised, rehydrated, and then stained with periodic acid Schiff's reagent with Harris' haematoxylin (PAS-H) to darken the mucous cells and the basement membrane (PAS) and the nucleus (haematoxylin), rendering ECCs colourless and easily recognizable (Fig. 1.1). Images of each epidermal cross section were captured with a Zeiss Axioplan Fluorescence Microscope in conjunction with an AxioCamICc1 (Colour, 1.4 MP) digital camera at 10 X magnification. For each slide, the parameters recorded were: epidermal thickness, number of mucous cells per mm of skin, number of ECCs per mm of skin, and ECC area, which were all quantified using the image processing and analysis software IMAGE J, version 1.32, (http://rsb.info.nih.gov/ij/). The observer was blind with respect to the treatment. The size of minnows used for the histological measurements had a mean ± SD fork length of 5.5 ± 0.5 cm and a mass of 2.1 ± 0.6 g. We tested a total of 12, 13, and eight blocks in the high UVR group, low UVR group and control groups, respectively.

2.2.6 Behavioural assay on potency of skin extract

The skin extract for the behavioural assay was produced from high UVR (N=20, mean ± S.E. standard length: 4.97 ± 0.08 cm) and low UVR fathead minnows (N=20, mean ± S.E. standard length: 5.35 ± 0.14 cm). Skin from the lateral epidermal layer on either side of the body was removed and placed in 40 mL of chilled distilled water. We collected a total of 50.1 cm^2 of skin from the high UVR group and 54.9 cm^2 from the low UVR group. The skin fillets were homogenized with a Polytron homogenizer and filtered through filter floss to remove large particles. Serial dilutions were used to obtain a final concentration of 1 cm^2 of skin per 40 litres, a concentration known to elicit overt antipredator response in fathead minnows (Ferrari et al. 2005, 2006). The alarm substance was frozen at -20 °C in 20-mL aliquots until used.

The behavioural bioassay was carried out to evaluate the difference in the potency of alarm cues prepared from the skin of high UVR and low UVR minnows on the antipredator response of control minnows. The assay was performed in 74-L aquaria (60 x 30 x 40 cm), which were wrapped in black plastic on three sides so that fish in adjacent aquaria were not visible to each other. Each aquarium was filled with dechlorinated water and equipped with a single air stone.

Three randomly selected minnows were acclimated in each aquarium for at least 24 h before the assay. Each day, one-third of the aquaria (randomly chosen) were exposed to alarm cues from the high UVR group, a third were exposed to alarm cues from the low UVR group, and the remaining third were exposed to water (control). We tested a total of 20 groups of fish in each of the three treatment groups. The experiment was divided into three phases: an 8-min pre-stimulus phase, a 1-min stimulus injection phase, and an 8-min post-stimulus phase (Pollock and Chivers, 2004). We used a well-established protocol for measuring the antipredator responses of minnows (Ferrari et al. 2005). This included recording an index of shoaling and an estimate of activity level,

as measured by line crossing. The shoaling index of three fish was measured every 15 s. Shoaling index was analyzed by evaluating the distance between the three fish per aquarium every 15 s during the pre- and post-stimulus time (1 = no fish within a body length of another; 2 = two fish within a body length of each other; 3 = all the fish within a body length of each other). As a measure of line crossing, the number of line crosses was also recorded for one of the three minnows during the first 10 s of the 15-s period. The same fish was randomly selected and observed until the end of the conditioning period. An increase in shoaling index and a decrease in activity level are two typical antipredator responses in minnows (Chivers and Smith, 1998).

2.2.7 Statistical analysis

For parameters relating to histology, blood cortisol, and behavioural responses, Levene's tests were performed to check for homoscedasticity and Kolmogorov–Smirnov tests were performed to check for normality. All statistical analyses were performed using SPSS 17, version 17 (SPSS Inc.).

We used one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to compare the physiological stress response [cortisol levels (in ng mL-1), and skin parameters (epidermal thickness, number of ECCs per mm of skin, area of ECCs (in mm^2) and number of mucous cells] among high UVR group minnows, low UVR group minnows, and control minnows.

For the behavioural responses, we used the differences in shoaling index and line crossing from the pre-stimulus baseline as our raw data. The effect of cues (skin extract from high UVR group, low UVR group and water) on the behavioural response of control minnows was tested using a one-way ANOVA followed by a post-hoc Tukey test.

2.3 RESULTS

2.3.1 Physiological stress response

The one-way ANOVA revealed a significant effect of treatment on the serum cortisol levels measured in minnows (N = 8 per treatment, $F_{2,21}$ = 25.1, P<0.001; Fig. 2.2). Post-hoc Tukey tests revealed no significant difference in the serum cortisol levels of low UVR group and control groups (P=0.709). However, the tests high UVR group compared to that in the low UVR group (P<0.001) and control group (P<0.001). Serum cortisol levels of high UVR group minnows were almost five-fold higher than those found in the blood of the low UVR group minnows.

2.3.2 Histological parameters

One-way ANOVA revealed no significant effect of treatment on epidermal thickness (N=12, 13, and 8 for the high UVR group, low UVR group and control groups respectively, $F_{2,30}$ = 0.300, P=0.743). Similarly, there was no effect of treatment on mean ECC area, although there was a trend for ECCs to be smaller in the high UVR group ($F_{2,30}$ = 3.1; P=0.059). By contrast, there was a significant effect of treatment on the mean number of ECCs ($F_{2,30}$ = 6.7, P=0.004; Fig. 2.3). Post-hoc Tukey tests revealed no difference in mean number of ECCs between the low UVR group and control group (P=0.988). However, there was a reduction in the number of ECCs to almost half in the high UVR group compared to the UV filtered group (P=0.008) and control group (P=0.015). Similarly, one-way ANOVA revealed a significant effect of treatment on the mean number of mucous cells ($F_{2,30}$ = 1.8, P<0.001; Fig. 2.4). Post-hoc Tukey tests revealed no difference in mean number of mucous cells between the low UVR group and control group (P=0.934). However, there was a three-fold reduction in the number of mucous cells in the high UVR group compared to the low UVR group (P<0.001) and control group (P=0.003).

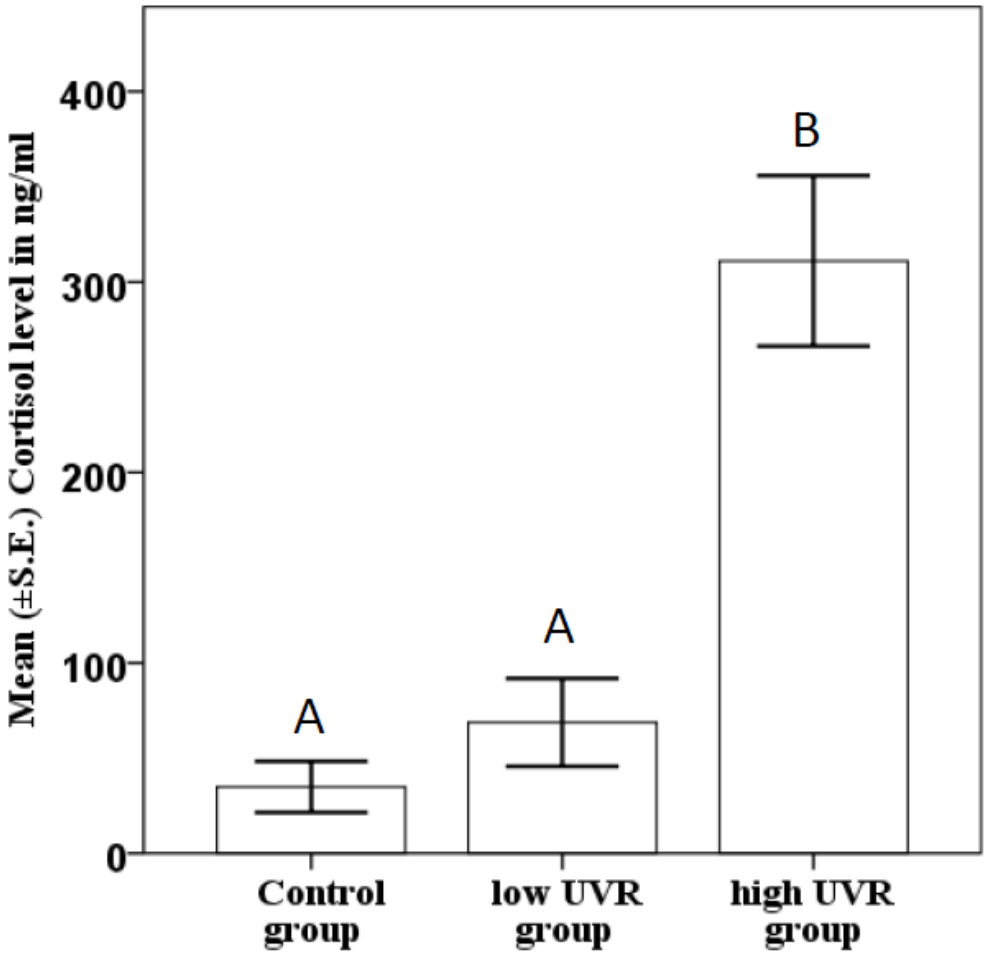

Figure 2-2 – Mean ± S.E. change in serum cortisol levels from blood of minnows exposed to UVR in the presence (low UVR group), absence of a UV blocking filter (high UVR group) and minnows housed in tanks for experimental purpose (control group) (N=group of four fish per unit). Different letters denote significant differences at $\alpha<0.05$.

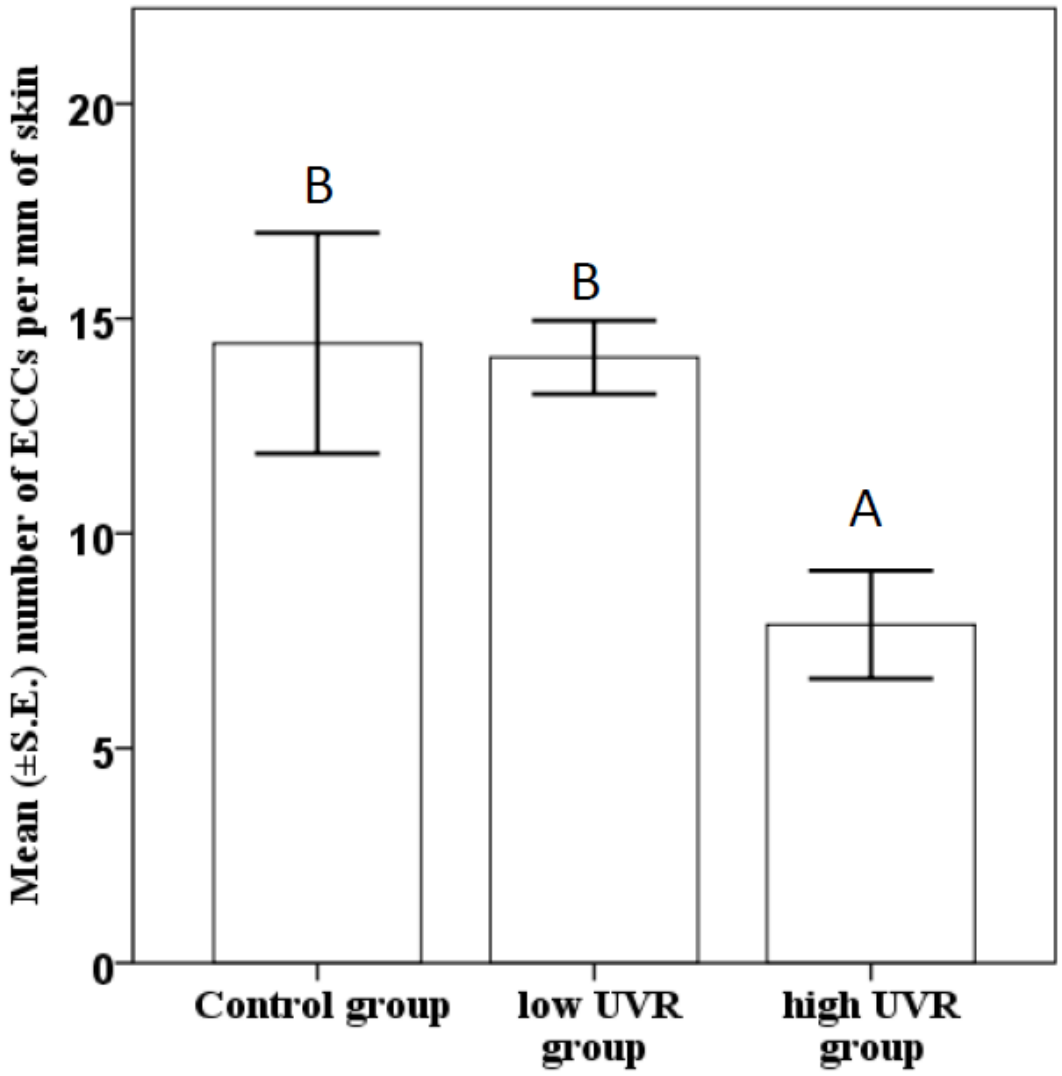

Figure 2-3 – Mean ± S.E. number of ECCs per mm of skin of minnows exposed to UVR in the presence (low UVR group), absence of a UV blocking filter (high UVR group) and minnows housed in tanks for experimental purpose (control group) (N=group of four fish per unit). Different letters denote significant differences at $\alpha<0.05$.

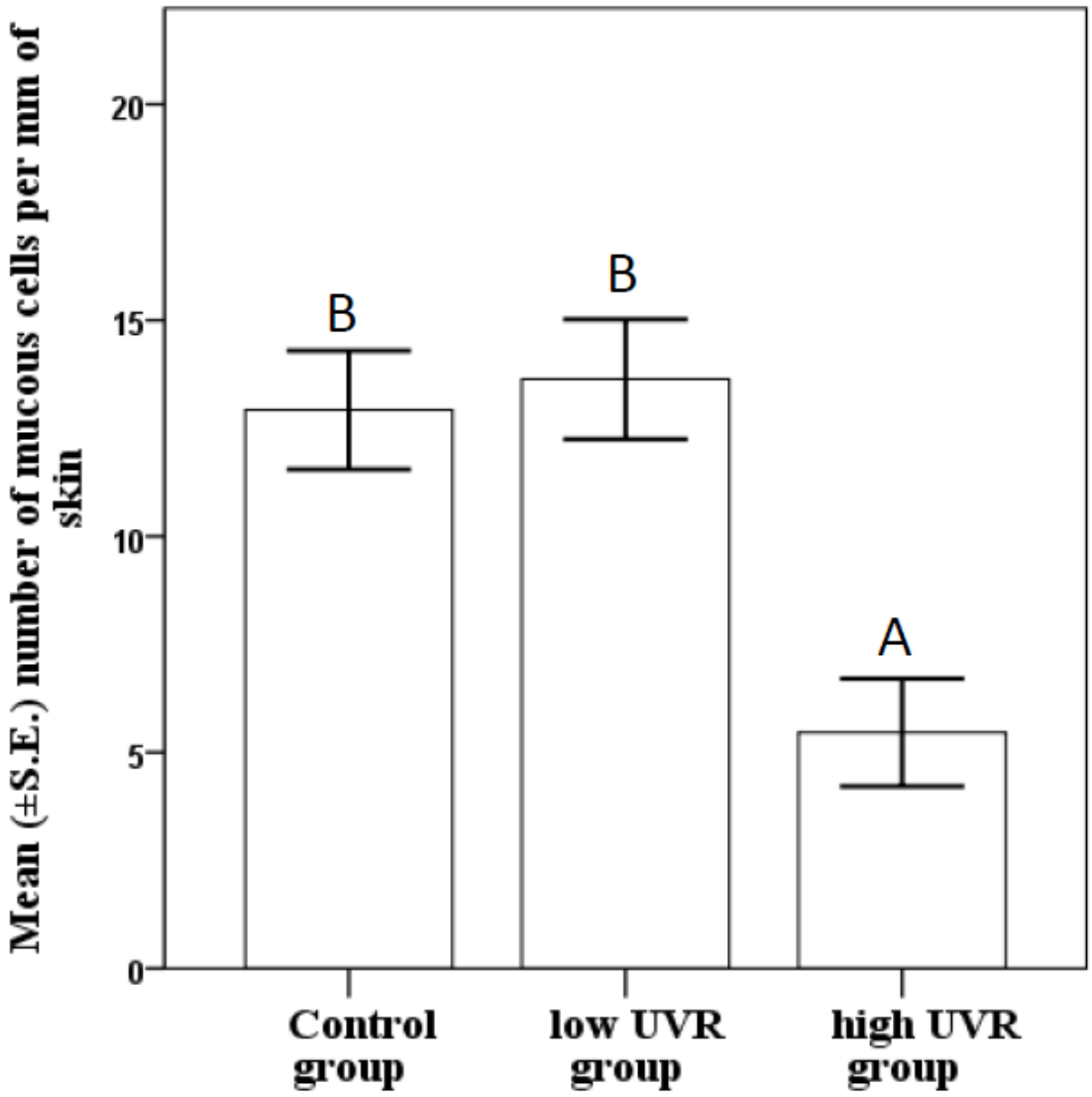

Figure 2-4 – Mean ± S.E. number of mucous cells per mm of skin of minnows exposed to UVR in the presence (low UVR group), absence of a UV blocking filter (high UVR group) and minnows housed in tanks for experimental purpose (control group) (N=group of four fish per unit). Different letters denote significant differences at $\alpha<0.05$.

2.3.3 Behavioural assay

One-way ANOVA revealed a significant effect of cue on the behavioural responses of minnows for both shoaling index (N = 20 per treatment, $F_{2,57}$ = 19.2, P<0.001; Fig. 2.5) and line crosses (N = 20 per treatment, $F_{2,57}$ = 17.1, P<0.001; Fig. 2.6). For both behavioural measures, post-hoc Tukey tests revealed a stronger antipredator response displayed by minnows exposed to alarm cues than minnows exposed to water (all P's<0.001). However, no difference were found between the responses of minnows to alarm cues from high UVR and low UVR minnows (shoaling index: P=0.99; line crosses: P=0.273).

2.4 DISCUSSION

Previous studies have suggested a link between exposure to UVR and ECC investment in both cyprinid (minnows; Hugie, 1990) and percid fishes (johnny darters, yellow perch; Chivers et al. 2007). Wild captured fish have more ECCs on their dorsal surface than their lateral surface and even fewer ECCs on their ventral surface. Consequently, we may expect that fish should increase ECC investment upon exposed to UVR. By contrast, minnows did not show an increase but rather a decrease in ECC investment when exposed to UVR. Interestingly, we did observe a five-fold increase in cortisol levels in the high UVR group, whereas low UVR minnows and control minnows did not differ in their levels of serum cortisol. Detection of increased cortisol levels has been used as an indicator of stress in fish (Barton 2002).This indicates that UVR exposure *per se*, and not holding conditions, elicited this five-fold increase in cortisol in the high UVR group. Other studies have documented similar UVR induced increases in plasma cortisol levels (Salo et al. 2000a, b). The observed cortisol levels in minnows from the low UVR group (mean ± S.E., 68.7 ± 13.5 $ng.mL^{-1}$) and control group (34.9 ± 23.1 $ng.mL^{-1}$) is comparable to the levels of cortisol (Dusan et al. 2006) reported in minnows exposed to handling stress (53 $ng.mL^{-1}$). These levels,

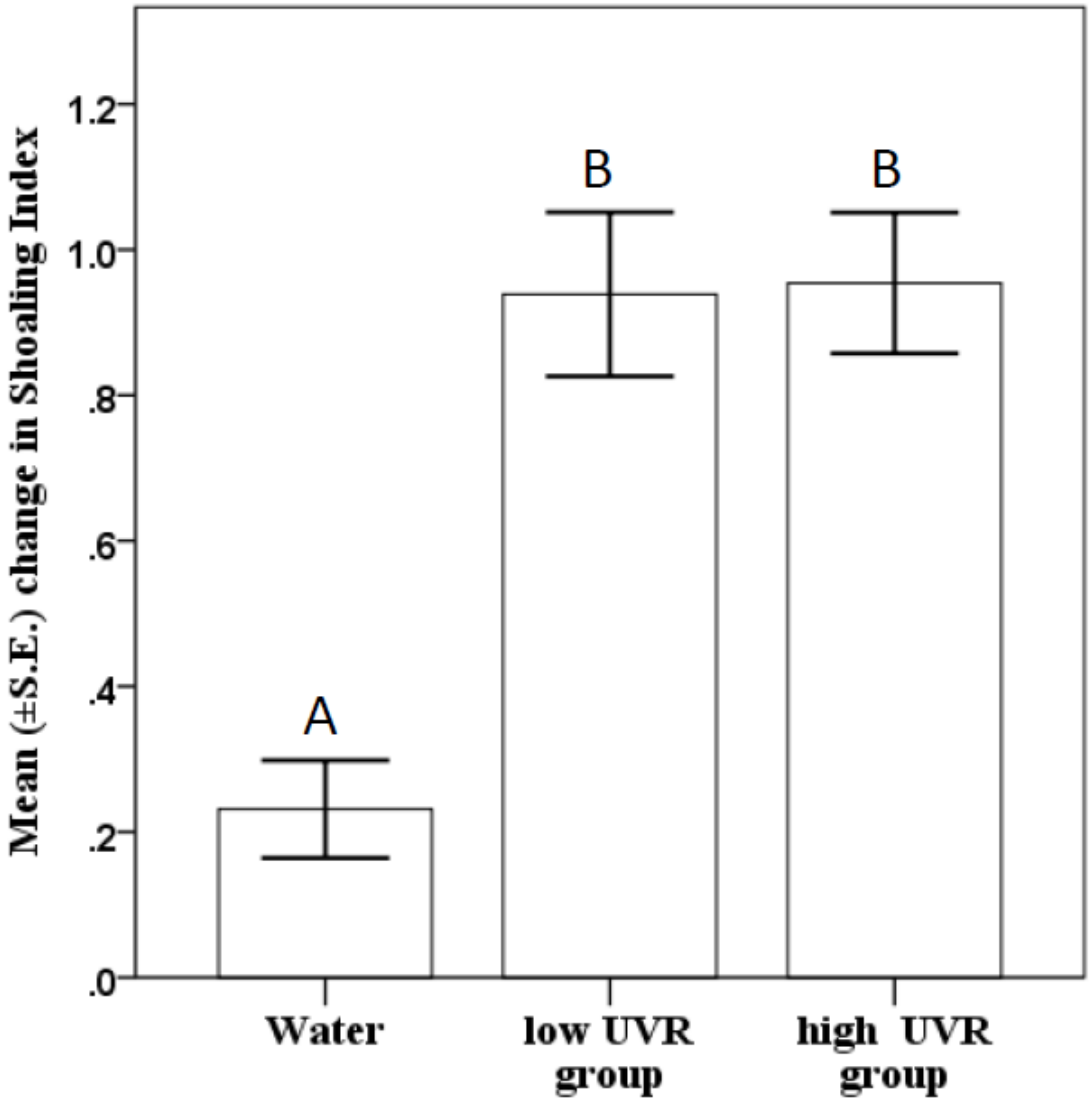

Figure 2-5 – Mean ± S.E. change in shoaling index for fathead minnows exposed to alarm cues prepared from skin of minnows exposed to UV radiation in the presence and absence of a UV blocking filter (N=20/treatment). Different letters denote significant differences at $\alpha<0.05$.

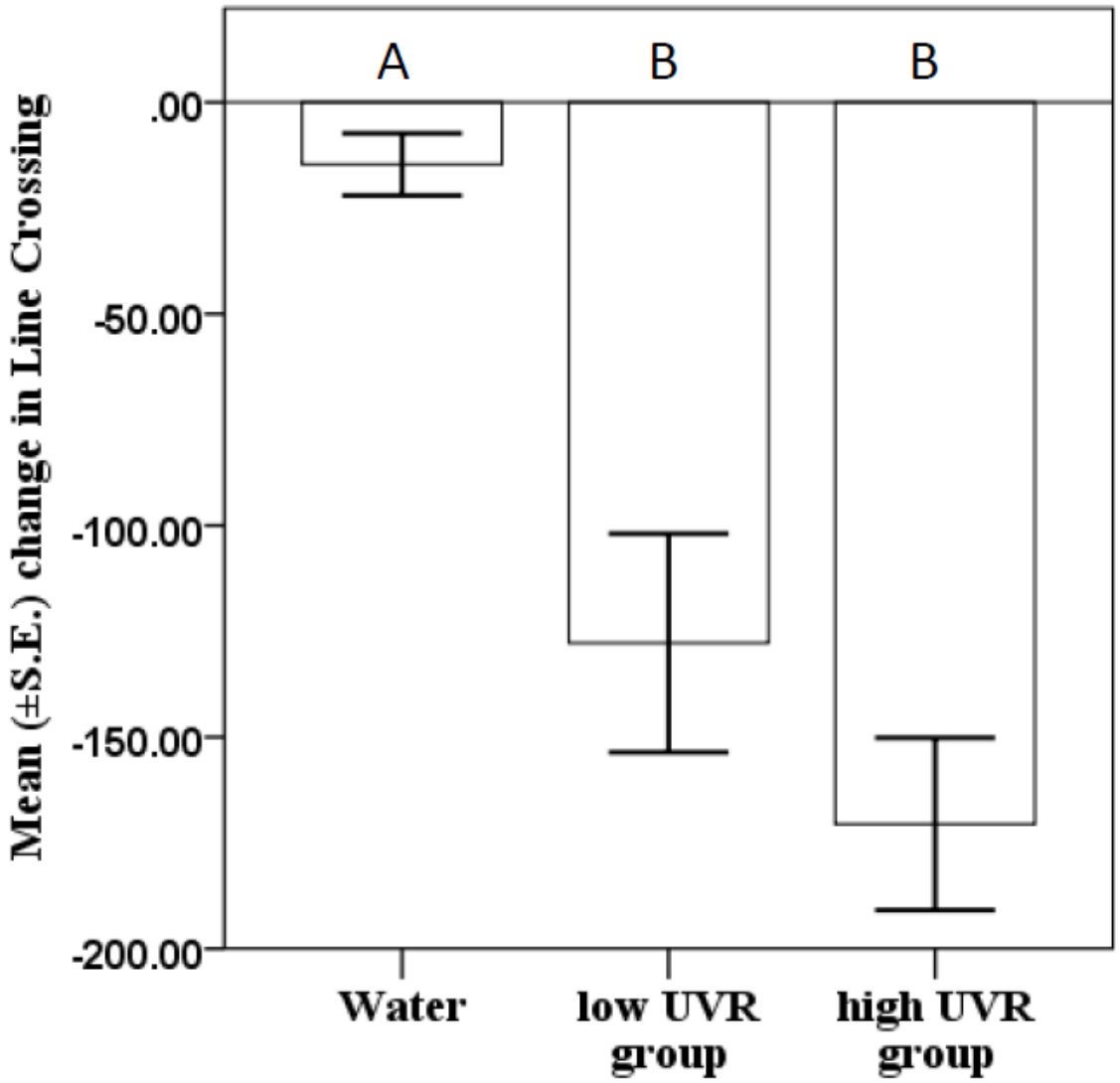

Figure 2-6 – Mean ± SE change in line crosses for fathead minnows exposed to alarm cues prepared from skin of minnows exposed to UV radiation in the presence and absence of a UV blocking filter. (N=20/treatment). Different letters denote significant differences at $\alpha<0.05$.

even though slightly higher than levels of unstressed minnows are five-fold lower than levels of cortisol from the blood of high UVR group (311.1 ± 44.7 $ng.mL^{-1}$). The elevation in cortisol levels probably prevented the adaptive epidermal responses that we predicted. Halbgewachs et al. (2009) showed that minnows exposed to cortisol had a suppressed immune system, as measured by a respiratory burst assay, and also a corresponding reduction in investment in ECCs.

The present study dictates that future research should focus on investigating long-term effects of UVR on the stress responses of fishes. Wild captured fishes often showed marked variation in ECC density (Manek et al. 2013). Consequently, in the present study, we held the minnows in the laboratory under standard conditions for several months in an attempt to reduce the variation in ECC number that we had at the beginning of the experiment. This means that, when we began our experiment, the minnows had been held for a long period in the absence of UVR. This is equivalent to minnows being held under the ice for several months with limited exposure to UVR. The stress response that we observed to UVR exposure may be a short-term response that reflects their limited UVR exposure over the past several months. If fish that are exposed to UVR for an extended period of time lose their stress response, then it is possible that UVR could indeed increase ECC investment, as suggested by the distribution of ECCs on the dorsal and ventral surface of darters, perch, and minnows. If UVR is not responsible for the difference in ECC distribution over the body of the fish, then we should consider other possibilities for these patterns, including the possibility that pathogenic agents differentially prefer to penetrate the dorsal surface than the ventral surface of the fish.

The results obtained in the present study also show that UVR exposure resulted in a reduction in number of mucous cells. This finding supports an earlier experiment on salmonids and cyprinids strengthening the hypothesis that UVR down-regulates mucous producing cells (Blazer et

al. 1997, Kaweewat and Hofer 1997). Despite the reduction in both ECCs and mucous cells, there was no reduction in the thickness of the epidermis between treatments. Changes in epidermal thickness are often associated with a reduction in body condition. We do not know whether there was a change in body condition for fish held in the different treatments because we did not weigh and measure the length of the fish before the experiment. Measuring the fish before the experiment would risk injury to the epidermis. It is possible that fish experiencing a reduction in body condition, perhaps as a stress response to UVR, could be responsible for the reduction in ECC and mucous cells, although this possibility remains to be investigated. Past evidence suggests that alarm cues are located in ECCs (Smith 1973). Indeed, laboratory and field experiments have shown that skin extract prepared from breeding male minnows, which lack ECCs, fails to elicit antipredator responses in conspecifics (Mathis and Smith 1992, Pollock et al. 2005). However, Carreau-Green et al. (2008) recently reported that adult minnows displayed an antipredator response to skin extracts from larval minnows that have not yet developed ECCs, suggesting that alarm cues may not just be produced in the ECCs but elsewhere in the skin of the minnows. In the present study, we did not observe any difference in the potency of the alarm cues prepared from the skin of high UVR group or low UVR group. There was an approximately 40% reduction in the number of ECCs in the high UVR group compared to the low UVR group. We know that minnows are known to exhibit a graded response to different concentrations of skin extracts (Ferrari et al. 2005); consequently, we would predict that there should have been a reduction in behavioural response of minnows exposed to skin extract from minnows in the high UVR group compared to the low UVR group. Our results appear consistent with the findings of Carreau-Green et al. (2008) suggesting that alarm cues may be produced outside of ECCs. However, it is also possible that minnows could up-regulate alarm cue production independent of ECC number. This explanation is not satisfying

given that the primary role of ECCs appears to be to act as immune cells and not as producers of alarm cues. A final alternative for the lack of behavioural difference between skin extract from High UVR and Low UVR group fish is that the behavioural sensitivity to different concentrations is not sufficiently fined tuned to detect a 40% reduction in ECC concentration. Ferrari et al. (2005) showed graded responses to alarm cues, although the magnitude of the differences. In concentrations that they used were greater than 40%.

The findings of the present study show that short-term exposure to UVR increases the physiological stress response in fishes, with the consequence that there is a reduction in ECC investment. Such a reduction in ECCs may lead to an increase in the vulnerability of the fish to secondary infections through suppression of the immune function.

CHAPTER 3[a]

THE INTERACTIVE EFFECTS OF MULTIPLE STRESSORS ON PHYSIOLOGICAL STRESS RESPONSE AND CLUB CELL INVESTMENT IN FATHEAD MINNOWS

[a] This chapter examines the effects of multiple environmental stressors such as UVR and/or Cd on the physiological stress response and behaviour in fathead minnows. Since I established in chapter 2 that UVR lowers ECC investment and increases physiological stress response in fathead minnows without altering chemically mediated predator prey interactions, the aim of chapter 3 was to investigate if UVR and/or Cd had any interactive effects on physiological stress response and ECC investment which could ultimately mitigate chemically mediated predator prey interactions. Chapter 3 has been published in Science of the Total Environment, 2014, Volume 467-477: pages 90-97, under the joint authorship with Maud Ferrari, Som Niyogi and Douglas Chivers (University of Saskatchewan).

3.1 INTRODUCTION

There is a rapid loss in global biodiversity as a result of anthropogenic changes occurring at local and global scales (Vitousek, 1994; Pereira et al., 2012). Identifying the reasons for such changes requires us to consider how different environmental stressors interact to affect the physiology, behaviour and ecology of individuals and how these changes subsequently affect species interactions. It is rare that animals are exposed to single stressors and consequently research aimed at identifying the interactive effects of multiple stressors is needed (Boone et al. 2007).

For much of the past century, we have witnessed increases in levels of ultraviolet radiation (hereafter UVR) hitting the surface of the earth as a result of reductions in stratospheric ozone (Newman et al. 2006). The implementation of the Montreal Protocol has ameliorated much of the ozone depletion, but it is difficult to ascertain how consistently ozone recovery would occur due to factors such as changes in cloud cover, air pollutants and aerosols, all of which influence climate change (Mackenzie et al. 2011). Increased levels of UVR have profound consequences for aquatic organisms. UVR damages DNA and impairs an organism's ability to reproduce by impairing egg production (Blaustein et al. 1994). It also impairs survival and growth in freshwater and marine aquatic systems (Blaustein et al. 1997), along with the ability of aquatic organisms to sense their environment, and resist disease (Williamson and Rose 2010). In fishes, UVR causes cataracts and skin lesions (Mayer 1992). It impairs immune function (Salo et al. 2000) and induces physiological stress responses (Manek et al., 2012). Much of the detrimental effects of solar radiation in aquatic ecosystems are negated by chromophoric dissolved organic matter (CDOM) which blocks UVR (Scully and Lean 1994, Williamson and Zagarese 1994).

Cadmium (Cd) is a heavy metal that is considered to be a priority pollutant in aquatic systems because of its toxicity at very low concentrations (Campbell 2006). It enters the aquatic

environment mainly from atmospheric deposition and effluents from smelting, metal-mining and refining (CCME 1996). Based on the criteria of the United States Environmental Protection Agency (U.S. EPA), at hardness of 120 mg/L, the Cd concentration that is believed to protect 95% of examined freshwater species in a 24-h exposure is 2.5 µg/L (U.S. EPA 2001).Cadmium has a myriad of effects on physiology and behaviour of fish. Cadmium is known to be a calcium antagonist and causes toxicity by inducing disruption of calcium homeostasis, particularly during acute exposure (Niyogi and Wood 2004a). It also acts as an immunosuppressant (Sanchez-Dardon et al. 1999) and an endocrine disruptor. Lacroix and Hontela (2004) reported that Cd inhibits adrenocorticotrophic hormone (ACTH) stimulating higher cortisol secretion from interrenal cells in rainbow trout (*Oncorhynchus mykiss*) – indicating that cadmium may disrupt normal stress response in fish. Cadmium exposure also alters shoaling behaviour in fathead minnows and renders minnows more vulnerable to predation by largemouth bass (*Micropterus salmoides)* (Sullivan et al. 1978). This result may be explained by the deleterious effects of Cd on lateral line and olfactory perception. Cadmium exposure causes severe epithelial necrosis throughout the olfactory epithelium in fathead minnows (Stromberg et al. 1983) and accumulates in olfactory sensory neurons, the olfactory nerve and the anterior part of the olfactory bulb (Tjalve et al. 1986, Gottofrey and Tjalve 1991, Scott et al. 2003). Chronic exposure to environmentally relevant levels of waterborne Cd (2 µg/L), has been shown to reduce responses to alarm cues in embryonic and larval stages of zebrafish (*Danio rerio*) (Blechinger et al. 2007, Kusch et al. 2007) and rainbow trout *(Oncorhynchus mykiss)* (Scott et al. 2003).

The goal of our work here was to examine the interactive effects of UVR and Cd exposure on stress physiology and epidermal club cell (hereafter ECC) investment in fathead minnows (*Pimephales promelas*). ECCs are ubiquitous among members of the Superorder Ostariophysi and

also occur in some other groups of fishes including percids (e.g. yellow perch, darters) (Ferrari et al. 2010). Evolutionary ecologists have long been interested in understanding the evolution of these cells. They are likely the primary site for production of chemical alarm cues. These chemical cues have shown to elicit typical anti-predator responses when released through damage to the skin, and detected by nearby conspecifics (Ferrari et al. 2010). Early work concentrated on predation-centered hypotheses (e.g. kin selection, Chivers et al. 2012), given that the contents of the cells serve to warn nearby shoal mates of danger. Chivers et al. (2007) provided an alternative hypothesis, arguing that ECCs provide a first line of defence against skin-penetrating pathogens. Exposure of ostariophysan fish such as fathead minnows to pathogenic watermoulds and larval trematodes causes an increase in ECC investment highlighting that the cells are part of the innate immune system. Halbgewachs et al. (2009) showed that an intraperitoneal injection of cortisol resulted in reduced numbers of ECCs in fathead minnows. There was also a significant reduction in respiratory burst activity of kidney phagocytes indicating that there was suppression of the innate immune system. Similarly, minnows exposed to Cd had a reduced ability to increase ECCs upon exposure to pathogens (Chivers et al. 2007). Furthermore, Manek et al. (2012) showed that minnows exposed to UVR exhibited a rise in cortisol production and a corresponding reduction in ECC numbers. Based on the finding of the studies described above, we infer that reduced ECC investment is linked with immunosuppression.

Understanding the combined effects of UVR and Cd exposure on ECC investment is fascinating because of the potential causal link with cortisol. Cadmium is an immunosuppressant (Sanchez-Dardon et al. 1999), but also reduces the physiological stress response in fish. For example, Scott et al. (2003) showed that rainbow trout exposed to Cd had a reduced ability to increase cortisol when they were exposed to risk. UVR appears to suppress the immune system but

it increases cortisol production in fish (Salo et al. 2000, Manek et al. 2012). However, it is not known how the physiological stress response (i.e. cortisol production) in fish is influenced by both UVR and Cd exposure, and whether the interactions of these two factors affect ECC investment. Similarly, it is also important to investigate whether changes in ECC investment impact chemically-mediated predator-prey interactions in fish. Specifically, if there is a reduction in ECC number, does that reduce the effectiveness of damaged fish skin to act as a cue that mediates predator–prey interactions? The main objectives of this experiment were three-fold: (i) to determine the effects of Cd exposure on physiological stress response and ECC investment in minnows, (ii) to determine if UVR in the presence and absence of Cd influences the physiological stress response and ECC investment of minnows and; (iii) to examine the effect of UVR and Cd on the potency of alarm cues prepared from the skin of minnows exposed to UVR and/or Cd. We use the term "potency" in this study to describe the capacity of alarm cues to elicit an antipredator response. We hypothesize that physiological stress and ECC investment will vary in minnows depending on their exposure to Cd and/or UVR and that the potency of alarm cues will vary with an alteration in ECC investment. Specifically, we predict that Cd and/or UVR exposure will result in an elevation in cortisol. However, Cd will result in endocrine disruption and lower the characteristic elevation in cortisol typically observed upon exposure to UVR, as found in our previous study (Manek et al. 2012). We also predict that the elevated cortisol production in response to Cd and/or UVR exposure will result in lowered ECC investment in minnows exposed to UVR only compared to Cd and/or UVR exposed minnows. Finally we predict that Cd and/or UVR exposure will lower the level of anti-predator response (potency of alarm cues) prepared from the skin of Cd and/or UVR exposed minnows.

3.2 MATERIALS & METHODS

3.2.1 Experimental fish

Approximately 300 adult fathead minnows (standard length ± S.D. = 5.27 ± 0.38 cm, weight ± S.D. = 2.05 ± 0.51 g) were collected between April and May 2010, from a pond located on the University of Saskatchewan campus, using Gee's improved minnow traps. Male minnows in the reproductive phase have suppressed ECC numbers due to high testosterone levels (Smith 1973). To ensure that minnows were in the non-reproductive phase, they were acclimated in the laboratory for at least one month prior to the experimental procedure. Maintaining the fish in the laboratory for an extended period can also reduce variation in baseline ECC production between minnows collected from the same site (Manek et al. 2013). Fish were housed in a 73-L aquaria containing dechlorinated tap water. The water was maintained at 18.4 ± 2.3°C and the photoperiod was set to 14:10 hr light:dark cycle. The water used for the experiments originated from the Saskatoon, SK, Canada municipal water supply and was tested on alternate days for water chemistry parameters (temperature, dissolved oxygen, pH, total alkalinity, nitrite, nitrate, chlorine, ammonia, dissolved oxygen and total hardness – table 3.1) during the acclimation and experimental phase.

3.2.2 Cadmium and UVR exposure

The experiment was divided into two phases, such that in phase one (hereafter referred as pre-UVR exposure phase), each of 10 groups of 4 minnows were exposed to either 5.4 μg/L of waterborne Cd or no Cd for 14 days. All Cd water came from a single stock solution produced with Cadmium nitrate (EM Science, USA) in de-ionized water. Minnows were held in groups of four in two litre beakers at 18.4 ± 2.3°C and were fed *ad libitum* daily with commercial flake food. Minnows in each beaker were not independent, so we considered the 'beaker', not the individual minnows, as our replicate unit in all analysis where we obtained more than one data point per

Table 3-1 – Mean ± S.E. water quality parameters for the pre-UVR exposure and UVR exposure phase. T-tests indicate no significant difference between treatments at alpha=0.05.

Water Quality Parameter	**Pre-UVR exposure phase**	**UVR exposure phase**
Temperature (^{o} C)	18.4 ± 2.1	18.5 ± 2.3
Dissolved Oxygen (mg/L)	7.1 ± 0.5	6.5 ± 0.4
pH	7.95 ± 0.23	8.01 ± 0.12
Hardness as $CaCO_3$	150.5 ± 0.5	150.5 ± 0.5
Ammonia in ppm (parts per million)	0.2 ± 0.1	0.2 ± 0.1

beaker. For phase two which involved exposing minnows to UVR and/or Cd, we could expose only 2 beakers at a time to UVR in the presence and absence of a UVR blocking filter. Thus, following phase one, two beakers housing four fish each were sacrificed for cortisol analysis and ECC analysis (see below) and the remaining two beakers housing four fish each were used in the second phase of the experiment. We had 8-10 replicates in each treatment.

The second phase (hereafter referred as the UVR exposure phase) started immediately after phase one (15-18 days after initiation of the experiment). In this phase, minnows from both the Cd and no Cd groups were exposed to solar radiation for 8 hours a day for four days in an Atlas SUNTEST XLS + Solar Simulator with Xeon lamp with a Suprax Daylight Glass Filter – 290 nm cut off (Atlas Material Testing Technology LLC, Chicago, USA) following the methods described by Manek et al. (2012). All of the minnows were exposed to UVR, half in the presence and half in the absence of a UV blocking filter. In the Low UVR group, the beakers had their top and sides covered with a 2 mm thick Lexan polycarbonate sheet. The polycarbonate sheet removed around 76% of the UVB and UVA radiation. This gave us a 2 x 2 design for the UVR exposure phase with the presence and absence of Cd (0 vs.5.4 μg/L) crossed with exposure to low or high levels of UVR. Eight groups with 4 minnows per group in each treatment were held in the solar simulator in quartz beakers (diameter 13.8 cm, height 16.8 cm, QSI Quartz Scientific, USA) with a complete water change being performed in each beaker each day.

At the end of the UVR exposure phase, fish were euthanized. Two of four minnows from each beaker were sacrificed to prepare alarm cues for the behavioral trials. These fish were euthanized with a blow to the head, in accordance with UCACS Animal Care protocol # 2009091. They could not be anesthetized prior to being sacrificed because the anesthetic could contaminate the alarm cue solution and compromise the behavioural assay. The remaining two fish were used

for histological analysis and were preserved in 10% neutral buffered formalin after they were euthanized with an overdose of MS222 or Aquacalm (Syndel Laboratories, Canada). The blood from all four minnows from each beaker was used in the cortisol analysis. We conducted between 8-10 replicates per treatment.

3.2.3 Cadmium analysis

Water samples (1 ml) from all treatment groups (pre-UVR exposure phase and UVR exposure phase) were collected in 1.5 ml polyethylene microfuge tubes every day during the experimental phase, and stored at 4^0 C until Cd analysis. To ensure that Cd remains in dissolved form, all water samples were acidified to a final strength of 0.2% nitric acid (trace metal grade, VWR, Canada), as suggested by the employed analytical protocol (Perkin Elmer, USA). Cadmium concentrations were estimated using a Graphite Furnace Atomic Absorption Spectrometer (AAnalyst 800, Perkin Elmer, USA). The mean concentration of Cd in the Cd spiked water samples was 5.40 ± 0.45 µg/L (mean ± S.D., N = 3 per day/per treatment). Cadmium levels in the remaining groups were below the detectable levels (detection limit=0.1µg/L).

3.2.4 Experimental protocol for blood extraction

Blood extraction for cortisol analysis followed the method described by Halbgewachs et al. (2009). Blood samples (25-50 µL) were extracted from the caudal vein near the anal fin region of euthanized minnows. In order to obtain enough blood for the analysis, we pooled blood from four fish from the same beaker. This blood was placed on ice and allowed to clot for at least one hour. Serum was extracted from the blood after centrifugation and then frozen at -20°C until it was used. The cortisol level in the extracted serum was measured by the Endocrine Laboratory at Prairie Diagnostic Service (University of Saskatchewan) in a Coat-A-Count radioimmunoassay (Immulite-

1000 Cortisol, Diagnostic Products Corporation, USA), which is designed for quantitative measurement of cortisol in serum.

3.2.5 Histological analysis of the skin

Tissue preparation for the analysis of the minnow epidermis followed the method described by Hugie (1990) with modifications (details can be found in Manek et al. 2012). The entire fish was initially fixed in 10% neutral buffered formalin until tissue processing could be performed. Epidermal samples were taken from the dorso/lateral surface just behind the operculum to the dorsal fin and placed in histocassettes and stored between two biopsy pads in formalin. An automatic tissue processor (MUP1, Modular Vacuum Processor) was used to dehydrate the fixed skin tissue in a series of ethanol grades and perfused with paraffin wax. Tissues were then manually embedded in paraffin wax and sectioned using a rotary microtome (HM330, Heidelberg) at 5μm thickness. Following sectioning, 3-5 sections were placed on a pre-cleaned suprafrost slide (VWR micro slides). After the slides were dried on a slide warmer, they were deparaffinised, rehydrated and then stained with periodic acid Schiff's reagent with Harris' haematoxylin (PAS-H) to darken the mucous cells and the basement membrane (PAS) and the nucleus (haematoxylin), rendering ECCs colourless and easily recognizable. Images of each epidermal cross section were captured with a Zeiss Axioplan Fluorescence Microscope with an AxioCamICc1 (Color, 1.4MP) digital camera at 10 X magnification. For each slide, we recorded the following parameters: epidermal thickness, number of ECCs per mm of skin, ECC density and ECC area, which were all quantified using Image J 1.32, an image processing and analysis program (available on the National Institute of Health's web page http://rsb.info.nih.gov/ij/). The main difference between number of ECCs per mm of skin and ECC density is that ECC density takes into account epidermal width. Two fish with the same number of ECCs per mm length of skin could have very different

ECC densities, given the differences in epidermal thickness (Manek et al. 2013). Thus we measured both parameters to dissect fine differences and select the stronger parameter, based on epidermal thickness results. Analyses by the observer were conducted blind to the treatment.

3.2.6 Behavioural assay on potency of skin extract

The skin extract for the behavioural assay was produced from fish following the UV exposure phase [Cd + high UVR (N=15, mean ± S.E. standard length: 5.15 ± 0.31cm), Cd + low UVR (N=15, 4.95 ± 0.22 cm), no Cd + high UVR (N=15, 4.79 ± 0.51 cm) and no Cd + low UVR (N=15, 5.35 ± 0.14 cm)]. Skin from the lateral epidermal layer on either side of the body was removed and placed in 40 ml of chilled distilled water. The skin fillet was homogenized with a Polytron homogenizer and filtered through filter floss to remove large particles. Serial dilutions were used to obtain a final concentration of 1 cm^2 of skin per 40-L, a concentration known to elicit overt antipredator response in fathead minnows (Ferrari et al. 2005, 2006). The alarm substance was frozen at -20°C in 20-ml aliquots until used.

The behavioural bioassay was carried out to evaluate the difference in the potency of alarm cues prepared from the skin of the above four groups and dechlorinated water as a control, on the antipredator response of minnows. None of the test minnows were exposed to UVR or Cd. The assay was performed in a total of seventy five, 37-L aquaria (60 x 30 x 40 cm) which were wrapped in black plastic on three sides so that fish in adjacent aquaria were not visible to each other. Each aquarium was filled with dechlorinated water and equipped with a single air stone. Each tank had a 3 x 3- grid pattern drawn on the side. Three randomly selected minnows from the stock tank were transferred for acclimation in each testing aquarium for at least 24 h prior to the assay. On the day of the trial, one hour prior to initiation of the trial, minnows were fed commercial flake food *ad libitum* to ensure consistency with the methodology applied by Manek et al. (2012).

On average, 10 replicates were tested each day. Each day, each of the four alarm cues were randomly assigned to one fifth of the aquaria, while the remaining one fifth of the aquaria received a no cue control (water). We tested a total of 15 groups of fish in each of the five treatment groups. The experiment was divided into three phases: an eight-minute pre-stimulus phase, a one-minute stimulus injection phase followed by an eight-minute post-stimulus phase (Pollock and Chivers 2004). Prior to the pre-stimulus phase, 60 ml of water from each tank was withdrawn and discarded using injection tubes (to remove any stagnant water). Following the pre-stimulus phase, we injected 10 ml of the alarm substance or water. We used a well-established protocol for measuring anti-predator responses of minnows (Ferrari et al. 2005). This included recording an index of shoaling and an estimate of activity level as measured by line crossing. The shoaling index of three fish was measured every 15 seconds by evaluating the distance between the 3 fish per aquarium every 15 seconds during the pre- and post-stimulus periods using a timer (VWR Scientific) (1: no fish within a body length of another; 2: two fish within a body length of each other; 3: all the fish within a body length of each other). As a measure of activity, the number of line crosses was also recorded for one of the 3 minnows (focal fish) during the pre-stimulus phase. The same focal fish was selected and observed until the end of the post-stimulus phase. An increase in shoaling index and a decrease in activity level are two typical antipredator responses observed in minnows (Chivers and Smith 1998).

3.2.7 Statistical analysis

All statistical analyses were performed using SPSS (ver. 19, SPSS Inc, USA).

3.2.7.1 Physiological response and histological data:

Dusan et al. (2006) showed that minnows euthanized with an overdose of Aquacalm (methomidate hydrochloride) had a lower cortisol elevation from the baseline than those

euthanized with MS222 (tricaine methanesulfonate). For this reason we included both euthanasia methods (MS222 vs. Aquacalm) in our analysis. For the pre-UVR exposure phase, we performed 2x2 ANOVAs looking at the effect of Cd (Cd vs. no Cd) and euthanasia method (MS222 vs. Aquacalm) on cortisol levels and histological parameters. Similarly for the UVR exposure phase, we performed three-way ANOVAs, investigating the effect of Cd (Cd vs. no Cd), UVR (low vs. high) and euthanasia method (MS222 vs. Aquacalm). Cortisol data were log-transformed to meet homoscedasticity assumptions. All other raw data met parametric assumptions.

3.2.7.2 Behavioural data

For the behavioural responses, we used the differences in shoaling index and line crossing from the pre-stimulus baseline as our raw data. The effects of cues on the behavioural response of control minnows were tested using one-way ANOVAs followed by *post hoc* Tukey tests.

3.3 RESULTS

3.3.1. Evaluation of mortality

During the 14 day pre-UVR exposure phase, no mortality was recorded in either the control group (no Cd) or the Cd exposed group. In the UVR exposure phase, 10% of minnows in the Cd + high UVR group (4 of 40) died while 5% of minnows in the no Cd + high UVR group died (2 of 40). In the Cd + low UVR group, 7.5% of minnows died (3 of 40) while no fish died in the no Cd + low UVR group. Minnows that did not survive until the end of the exposure were excluded from further analysis.

3.3.2. Cortisol levels

3.3.2.1 Pre-UVR exposure phase

The 2x2 ANOVA revealed a significant effect of Cd ($F_{1,11}$=30.9, P<0.001) and a significant effect of euthanasia method ($F_{1,11}$=18.3, P=0.001), but no interaction between the two ($F_{1,11}$=1.7,

P=0.21) on the mean cortisol levels of minnows. We found that fish euthanized via Aquacalm had a 5-fold lower concentration of cortisol in the no Cd group and a 2-fold lower concentration of cortisol in the Cd exposed group than those euthanized with MS222 (figure 3.1). In addition, fish exposed to Cd, had a significantly lower level of cortisol than those not exposed to Cd.

3.3.2.2 UVR exposure phase

The 3-way ANOVA revealed a significant effect of Cd ($F_{1,22}$=5.8, P=0.025), a significant effect of UVR ($F_{1,22}$=37.6, P<0.001), and a significant effect of euthanasia agent ($F_{1,22}$=24.5, P<0.001). However, none of the 2- or 3-way interactions were significant (all P's>0.35). Similar to the pre-UVR exposure phase results, our results indicate that fish euthanized with Aquacalm had a lower level of cortisol than those euthanized with MS222 and fish exposed to Cd had lower mean cortisol levels than those not exposed to Cd. In addition, we found that minnows exposed to UVR had a greater level of mean cortisol production than those not exposed to UVR (figure 3.2). Cortisol levels in our current study are consistent with our previous work, where we showed that exposure to UVR resulted in a significant increase in cortisol production in fish (Manek et al., 2012). However, the results of our 2x2 analysis in the pre-UVR exposure phase and 3-way analyses in the UVR exposure phase indicate that Cd played a significant role in disrupting elevation of cortisol production. This was irrespective of the type of euthanizing agent used or combined exposure to UVR and Cd.

3.3.3. Histological parameters

3.3.3.1 Epidermal thickness

3.3.3.1.1 Pre-UVR exposure phase

The 2x2 ANOVA revealed no significant effect of Cd ($F_{1,16}$=0.16.9, P=0.69) or euthanasia method ($F_{1,16}$=0.77, P=0.392) and did not reveal any interaction between the two ($F_{1,16}$=0.03,

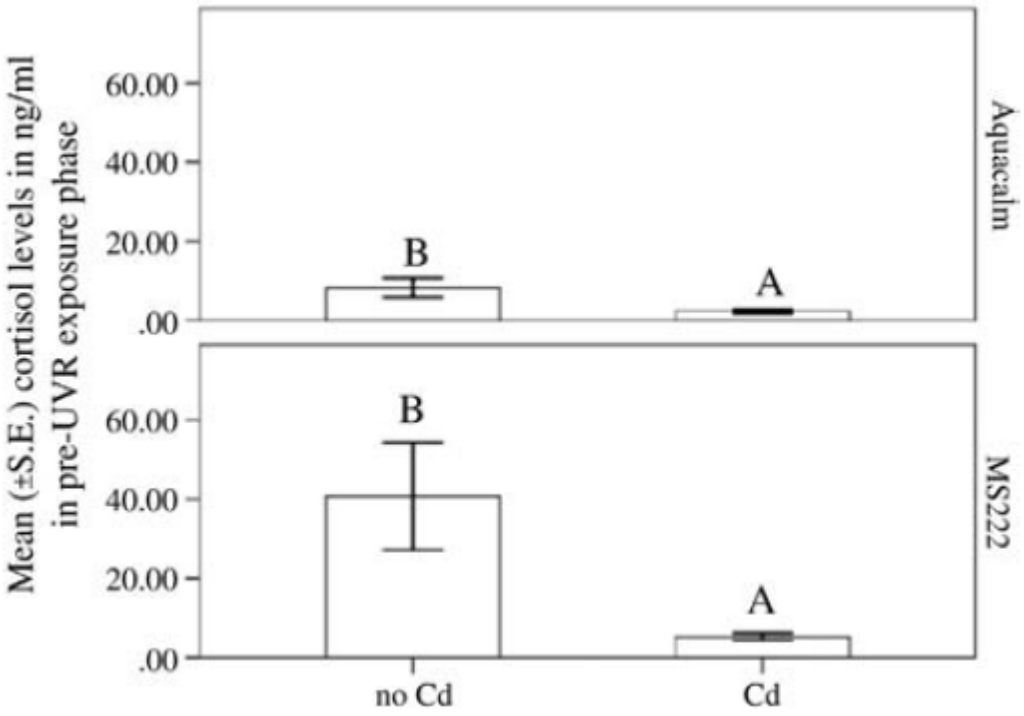

Figure 3-1 – Mean ± S.E. serum cortisol levels from blood of minnows exposed to Cd (no Cd vs. Cd) in the pre-UVR exposure phase. [N=4-5/euthanizing agent (MS222 vs. Aquacalm)]. Different letters indicate significant differences at $\alpha<0.05$.

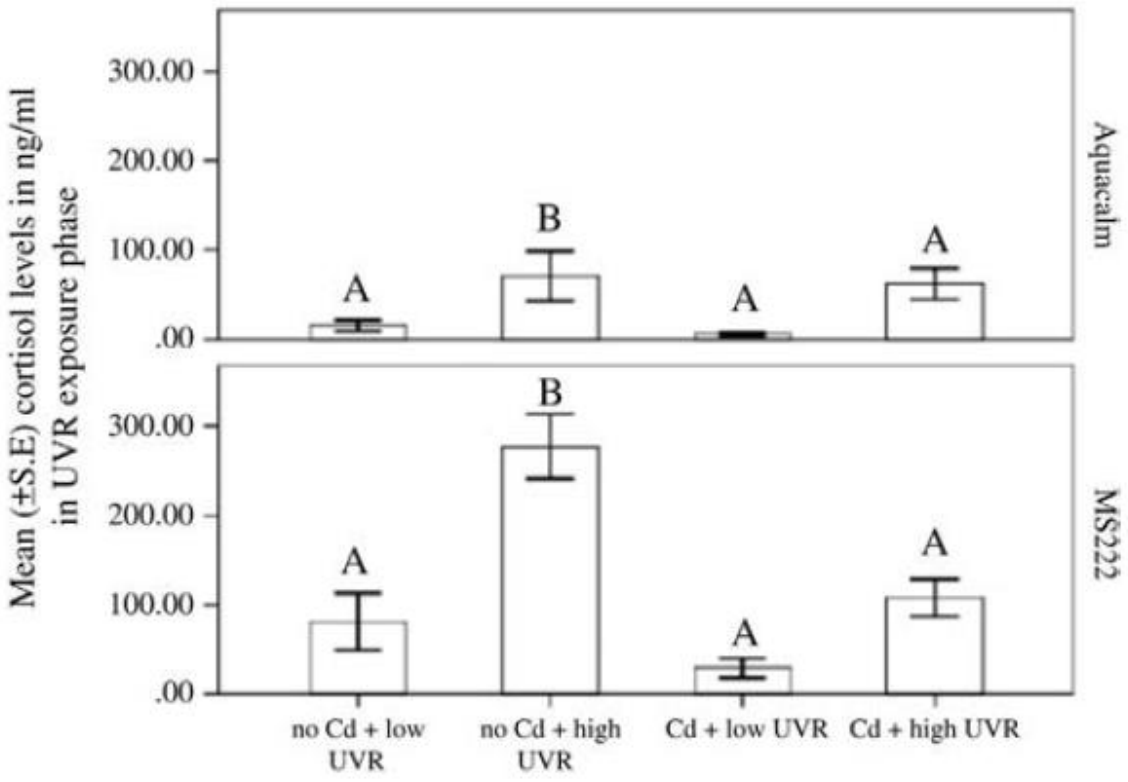

Figure 3-2 – Mean ± S.E. serum cortisol levels from blood of minnows exposed to UVR (high UVR vs. low UVR) and Cd (no Cd vs. Cd) in the UVR exposure phase [N=4-5/euthanizing agent (MS222 vs. Aquacalm)]. Different letters indicate significant differences at $\alpha<0.05$.

P=0.85) on mean epidermal thickness of minnows (No Cd+MS222 group mean epidermal thickness ± S.D. = 37.6 ± 6.6 µm; No Cd+Aquacalm group: 36.1 ± 3.6 µm; Cd+MS222: 38.8 ± 6.6 µm; Cd+Aquacalm group: 36.6 ± 2.1 µm).

3.3.3.1.2 UVR exposure phase

The 3-way ANOVA revealed a significant effect of Cd ($F_{1,25}$=14.14, P=0.001), but there was no effect of UVR ($F_{1,25}$=0.35, P=0.55), or euthanasia agent ($F_{1,25}$=0.08, P=0.775) or any significant 2- or 3-way interactions (all P's>0.32) on epidermal thickness. Fish exposed to Cd had lower mean epidermal thickness than those not exposed to Cd (no Cd + high UVR mean epidermal thickness ± S.D. = 29.03 ± 6.1 µm; no Cd + low UVR: 30.70 ± 5.1 µm; Cd + low UVR: 35.01 ± 2.2 µm; Cd + high UVR: 38.90 ± 6.0 µm). These results indicate that the Cd + high UVR group had an almost 25% thicker epidermis as compared to most of the other groups. Given that Cd influenced epidermal thickness it is imperative that this be accounted for in any analysis of ECC investment. Two fish with the same number of ECCs per mm length of skin could have very different ECC densities, given differences in thickness. Consequently, in our experiment we analyzed ECC density rather than number of ECCs per mm of skin (data not shown).

3.3.3.2 ECC Density

3.3.3.2.1 Pre-UVR exposure phase

The 2x2 ANOVA revealed no significant effect of Cd ($F_{1,16}$=0.35, P=0.56) or euthanasia method ($F_{1,16}$=0.23, P=1.0) on mean ECC density. Also, there was no interaction between the two ($F_{1,16}$=0.46, P=0.5) on the mean ECC density of minnows (figure 3.3).

3.3.3.2.2 UVR exposure phase

The 3-way ANOVA revealed no significant effect of Cd ($F_{1,25}$=0.32, P=0.571), or euthanasia agent ($F_{1,25}$=3.8, P=0.063), but a significant effect of UVR ($F_{1,25}$=27.94, P<0.001). However, none

of the 2- or 3-way interactions were significant (all P's>0.26). Similar to the pre-UVR exposure group results, our results indicate that fish euthanized with Aquacalm did not differ in mean ECC density than those euthanized with MS222. However, we found that minnows exposed to UVR had a 2-fold lower ECC density than those not exposed to UVR (figure 3.4).

3.3.3.4 ECC area

3.3.3.4.1 Pre-UVR exposure phase

The 2x2 ANOVA revealed no significant effect of Cd ($F_{1,16}$=0.14, P=0.713), or euthanasia method ($F_{1,16}$=0.31, P=0.58) on mean ECC area. Also it revealed no interaction between the two ($F_{1,16}$=0.20, P=0.65) on the mean ECC area of minnows (data not shown).

3.3.3.4.2 UVR exposure phase

The 3-way ANOVA revealed no significant effect of Cd ($F_{1,27}$=3.94, P=0.057), or UV ($F_{1,27}$=0.97, P=0.333), or euthanasia agent ($F_{1,27}$=0.77, P=0.388) on mean ECC area. Also, none of the 2- or 3-way interactions were significant (all P's>0.51) (data not shown).

3.3.3.4 Behavioural assay

The one-way ANOVAs revealed a significant effect of cue on the behavioural responses of minnows for both shoaling index ($F_{4,70}$=6.3, P<0.001, figure 3.5) and line crosses ($F_{4,70}$=4.1, P=0.005, figure 3.6). For both behavioural measures, post-hoc Tukey tests revealed a stronger antipredator response displayed by minnows exposed to alarm cues than minnows exposed to water (all P's<0.001). However, *post hoc* tests revealed no difference among treatments for minnows exposed to alarm cues (P>0.05).

3.4 DISCUSSION

Understanding the effects of multiple stressors on organisms is an emerging discipline in stress ecology (Altshuler et al. 2011). It is particularly interesting to determine the combined

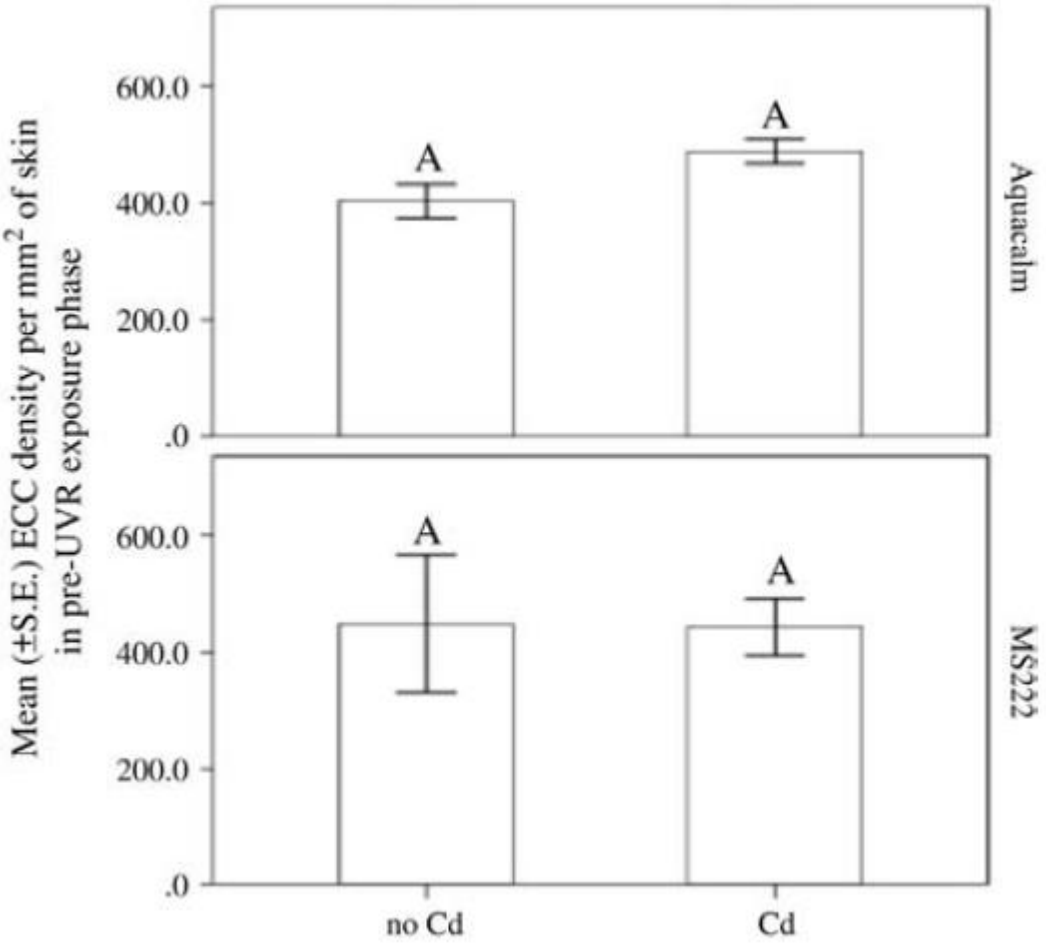

Figure 3-3 – Mean ± S.E. ECC density per mm² of skin of minnows exposed to Cd (no Cd vs. Cd) in the pre-UVR exposure phase. [N=4-5/euthanizing agent (MS222 vs. Aquacalm)]. Different letters indicate significant differences at $\alpha<0.05$.

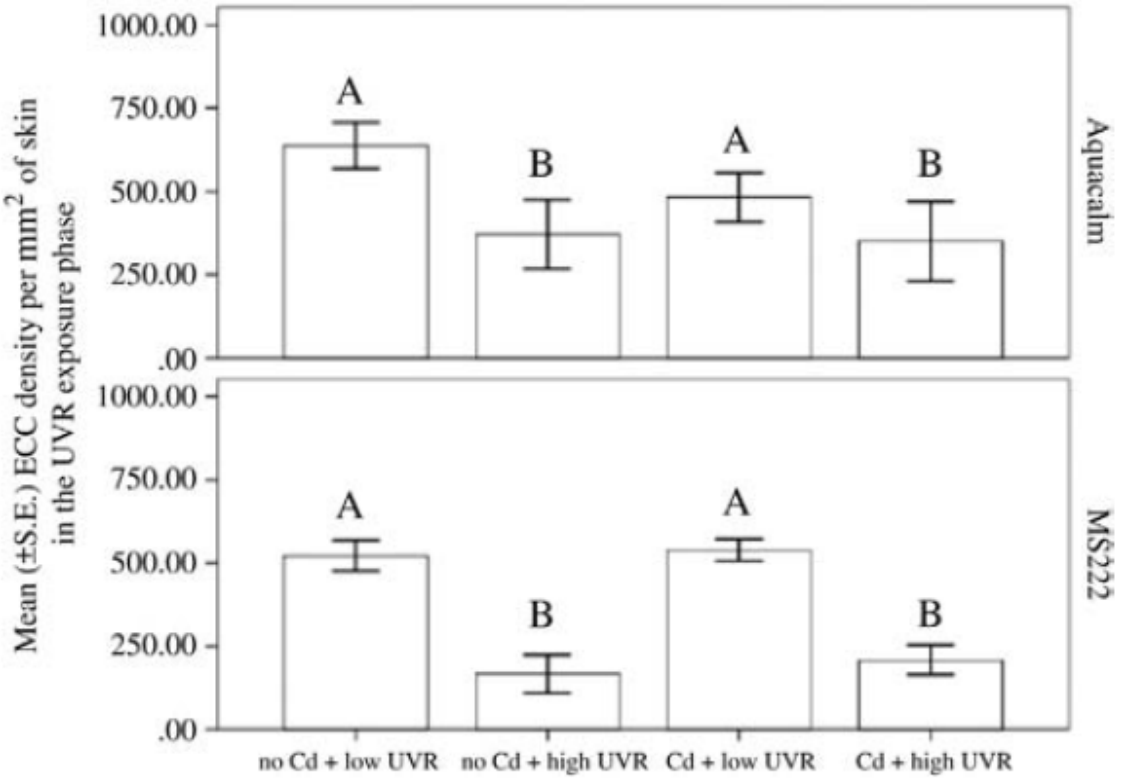

Figure 3-4 – Mean ± S.E. ECC density per mm² of skin of minnows exposed to UVR (high UVR vs. low UVR) and Cd (no Cd vs. Cd) in the UVR exposure phase [N=4-5/euthanizing agent (MS222 vs. Aquacalm)]. Different letters indicate significant differences at $\alpha<0.05$.

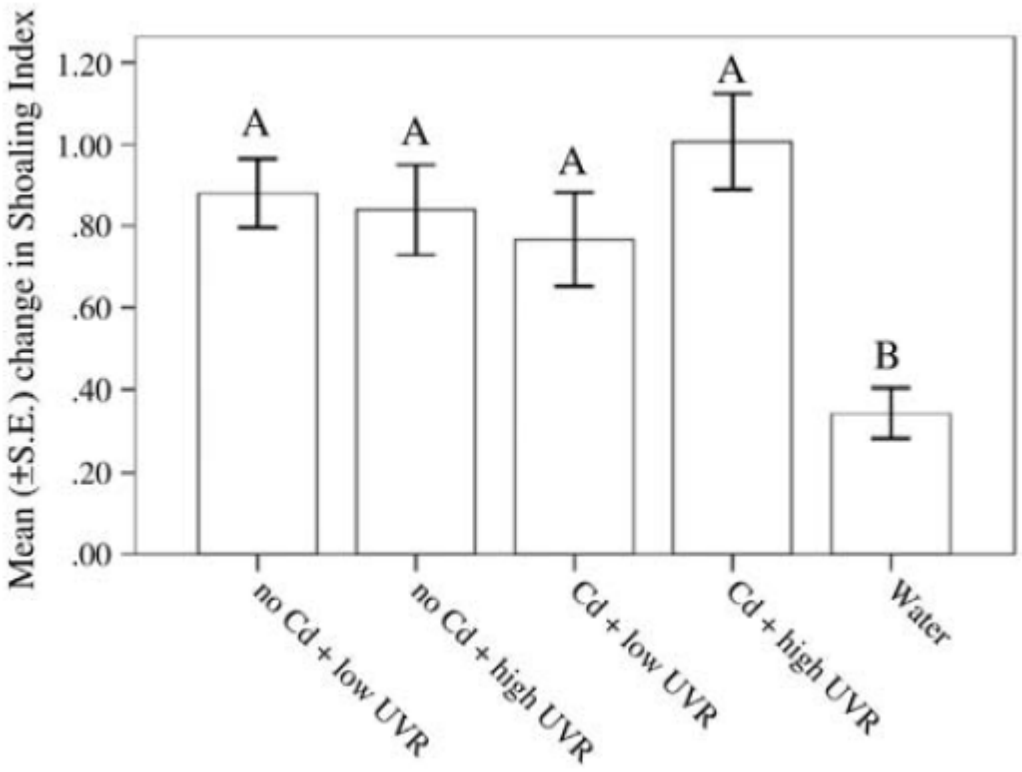

Figure 3-5 – Mean ± S.E. change in shoaling index for fathead minnows exposed to alarm cues prepared from skin of minnows exposed to UVR in the presence and absence of a UVR blocking filter (high UVR vs. low UVR) crossed with the presence and absence of cadmium (Cd vs. no Cd). N= 15/treatment. Different letters indicate significant differences at $\alpha<0.05$.

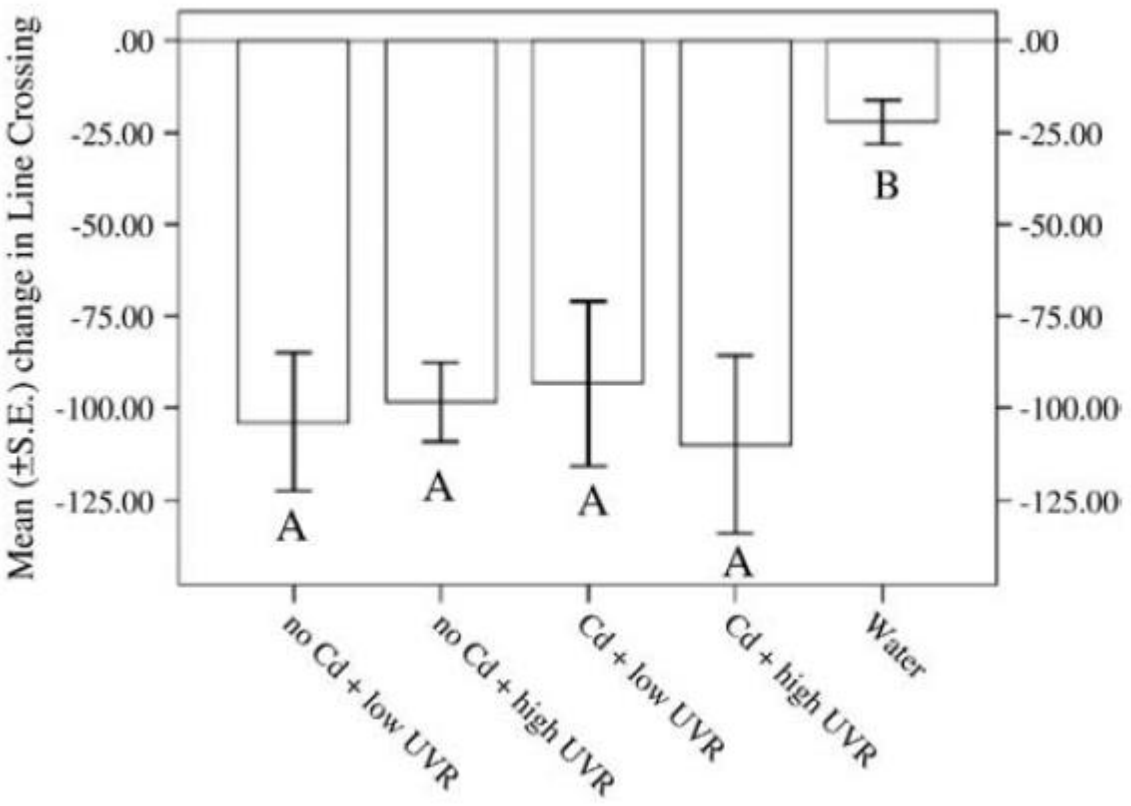

Figure 3-6 – Mean ± S.E. change in line crosses for fathead minnows exposed to alarm cues prepared from skin of minnows exposed to UVR in the presence and absence of a UVR blocking filter (high UVR vs. low UVR) crossed with the presence and absence of cadmium (Cd vs. no Cd). N= 15/treatment. Different letters indicate significant differences at $\alpha<0.05$.

effects of different stressors when we predict a priori that different stressors could lead to different outcomes. Our previous studies on ECC investment in fishes provide such a case. While trying to address the evolutionary role of ECCs (immune-function hypothesis), Chivers et al. (2007), showed that minnows exposed to 5.64µg/L of waterborne cadmium for 14 days were no longer able to increase production of ECCs in response to pathogenic challenge. Similarly, our previous work indicated that minnows exposed to 30 µg/L of waterborne cadmium for 14 days in the absence of pathogenic zoospores did not reduce ECC density relative to that observed in control fish (Kusch et al., unpublished data). It was also observed that a 14-day exposure to 5.64 and 30.00 µg/L of waterborne Cd resulted in immunosuppression, measured as a significant reduction in respiratory burst activity. Supporting the immune-function hypothesis, Halbgewachs et al. (2009) found an inverse relationship between cortisol and ECC investment in fathead minnows. They showed that intra-peritoneal injections of cortisol, and the associated suppression of the immune system as measured by a respiratory burst assay, resulted in lower numbers of ECCs in fathead minnows. Likewise, Manek et al. (2012) found that exposure to UVR led to an increase in cortisol production and lower ECC production in minnows.

Exposure to Cd likely has the opposite effect on cortisol production than exposure to UVR. Lacroix and Hontela (2004) have shown using an in vitro approach that short-term acute Cd exposure to adrenocortical cells of fish can cause endocrine disruption by lowering the characteristic elevation in cortisol production in response to stress. Similarly, Scott et al. (2003) found that in vivo exposure to waterborne Cd at a concentration of 2 µg/L for 7 days resulted in a decrease of the characteristic elevation of cortisol observed in rainbow trout exposed to alarm cues. The impaired elevation in cortisol production is likely due to the disruption of the hypothalamo-pituitary-interrenal axis, which is the primary pathway for cortisol production in fish (Wendelaar

Bonga, 1997). Based on the studies described above we can suggest that there may be a fascinating link between exposure to UVR, and immunosuppressants like Cd, which could be driven by changes in cortisol levels (Chivers et al. 2007, Manek et al. 2012). An elevation in cortisol production is associated with a reduction in ECC investment. However, it was not known what happens to cortisol production when fish are exposed to both Cd and UVR, and subsequently how that influences ECC investment and the potency of alarm cues produced by the fish. Normally expression of an elevated cortisol response under physiologically stressful conditions is necessary for an organism to maintain normal homeostasis (Sapolsky et al. 2000). If aquatic organisms that are stressed by exposure to elevated UVR are unable to express an elevated cortisol response due to factors like Cd exposure (endocrine disruption), their physiological state could be further deteriorated ultimately making it detrimental for the basic survival of that organism.

One of the key goals of our current study was to better understand the immune-function hypothesis with relation to cortisol production and ECC investment using multiple environmental stressors. Thus, we selected 5 µg/L waterborne Cd exposures for the current study based on the observations of the previous studies mentioned above. Moreover, it is also important to note that our chosen Cd exposure concentration is environmentally relevant since similar Cd concentrations have been reported in many contaminated natural aquatic ecosystems (CCME 1996). Similarly, the experimental set up and duration of UVR exposure in our current study is almost identical to the one described by Manek et al. (2012). The total level of UVR emitted by the solar simulator in the present study was 250 W/m^2 which is a cumulative dose that includes UVR and PAR (photochemically active radiation). If we dissect out the actual UVR exposure only, it was around 45 W/m^2, which is comparable to natural levels of UVR in mid-summer in Saskatchewan (43 W/m^2). The levels of UVR that fathead minnows are exposed to across their geographical range

can vary 2 fold depending on their latitude (Goncalves et al. 2010). The level of UVR exposure in our current study was a cumulative dose of exposure which is comparable to levels of UVR in mid-summer in Saskatchewan (Sereda et al. unpublished results).

Results of the histological parameters in our current study revealed an interesting link between ECC investment and cortisol. We found that minnows exposed to UVR had lowered ECC density irrespective of the type of euthanizing agent used. Despite the reduction in cortisol production due to Cd exposure in the Cd + high UVR group, we did not find a significant difference in mean ECC density or ECC area between the Cd + high UVR group and no Cd + high UVR group. This indicates that the reduction in cortisol production due to Cd was not enough to change ECC investment. An elevation in cortisol production may trigger the reduction in ECC investment, but the increased levels of cortisol in our current study was probably not sufficient enough to lead to greater reductions in ECC investment in fathead minnows relative to controls.

We did not find any significant difference in antipredator response to alarm cues from the skin of UVR and Cd exposed minnows. This behavioural observation is consistent with our previous observations (Manek et al. 2012). Previous research has shown that fish often exhibit a graded response to chemical alarm cues, such that a high concentration of alarm cues can elicit a high level of antipredator response in fishes as compared to medium and low concentrations (Ferrari et al. 2005). For our behavioural experiment, we used a high concentration of alarm cues (1 cm^2 in 40 L of water, as in Ferrari et al. 2005) to test antipredator responses (line crosses and shoaling index) in control minnows (not exposed to either UVR or Cd). It is possible that minnows were responding at their highest intensity to these cues, the result of which was that we could not detect any fine differences in antipredator response. One more factor complementing this observation could be that an almost 2-fold reduction in ECC production is not enough to alter

alarm cue potency. Future studies where minnows are exposed to a medium and low concentration of alarm cues could help reveal if there exists any modest difference in response to alarm cues from the skin of minnows exposed to UVR or Cd. A lack of significant difference in behavioural response to alarm cues despite a 2-fold reduction in ECC production also contributes to a growing body of evidence that suggests that alarm cues may not be exclusively produced in ECCs but elsewhere in the skin of cyprinid fish skin (Carreau-Green et al. 2008).

Overall, the key findings of our current study support most of our predictions. At the waterborne Cd exposure concentration used in our study, we found that: (i) Cd can influence UVR driven increases in cortisol elevation with no down regulation of ECC investment, (iia) the duration of UVR exposure in our study in the absence of Cd results in characteristic elevation of cortisol levels and down regulates ECC investment, (iib) the duration of UVR exposure in our study in the presence of Cd, disrupts characteristic elevation of cortisol production and down regulates ECC investment and; (iii) UVR and Cd did not elicit different behaviours at the concentration of alarm cue prepared from UVR and/or Cd exposed minnows despite reduction in ECC investment. Our work points to the fact that it is difficult to predict the physiological, behavioural and ecological effects of exposure to multiple stressors. Future studies could build upon this framework to identify the relative importance of different stressors under different conditions. For example, water chemistry can play a significant role in affecting bioavailability of heavy metals and levels of UVR exposure. Specifically, water hardness has been demonstrated to be the most significant modulator of waterborne Cd bioavailability to fish (Niyogi et al. 2008). It is well characterized that hardness cations, particularly Ca^{2+} ions, compete with free Cd ions (Cd^{2+}) for binding sites on the fish gill, and thereby decrease Cd bioavailability and toxicity (Niyogi et al. 2008). In our current experimental setup, we used dechlorinated Saskatoon city water, which has a moderate level of

hardness (150-160 mg/L as $CaCO_3$). This could have resulted in lowered bioavailability of Cd^{2+} ions to the fish in our study, which might have resulted in lack of effect on ECC investment and alarm cue production. Thus, it would be interesting to examine the effects of UVR and Cd in fish during exposure at a relatively lower hardness. The DOC concentration in dechlorinated Saskatoon city water varies between 2.6-3.2 mg/L (Manek et al. submitted). Likewise, it would be interesting to examine the effects of UVR in the presence of higher levels of DOC, which is known to attenuate UVR (Williamson et al. 2001). The DOC levels across lakes and wetlands in Saskatchewan range from 4.1 to 156.2 mg/L (Arts et al. 2000), so there is considerable scope for empirical testing. In addition to identifying the relative importance of multiple stressors, we need to understand time lag effects. There was no effect of Cd on skin thickness pre-UVR, but an effect after the UVR phase. Could this be due to duration of Cd exposure instead of UVR exposure? In our study minnows were exposed to Cd for 18 days prior to being euthanized. We know that Cd accumulates in various target tissues like liver, kidney, gill and ovary/testes in fish due to its long half-life and low rate of excretion (Norey et al. 1990, Sorensen 1991, Hollis et al. 2000). Perhaps the disrupted cortisol production would be much greater in magnitude with a longer exposure time. A depuration period following Cd exposure, prior to any exposure to UVR, could help understand if the disrupted cortisol production as a result of endocrine disruption can be reversed in these fish.

With ever increasing disturbance to aquatic and terrestrial ecosystems, we are seeing organisms exposed to multiple stressors. Our results highlight that identifying the effects of simultaneous exposure to these stressors will remain a challenge for environmental scientists.

CHAPTER 4[a]

THE EFFECTS OF ALTERED WATER CHEMISTRY (DOC CONCENTRATION AND SOURCE) ON PHYSIOLOGICAL STRESS RESPONSE AND CLUB CELL INVESTMENT IN FATHEAD MINNOWS

[a] This chapter examines the effects of DOC quantity and quality on ECC investment and physiological stress response in fathead minnows. Since I established in chapter 2 and 3 that UVR plays a role in increasing physiological stress response and lowers ECC investment without altering chemically mediated predator-prey interactions, the purpose of chapter 4 was to investigate if DOC plays a role in lowering physiological stress response and help maintain a high level of ECC investment in fathead minnows. The contents of chapter 4 have been submitted in the journal Science of the Total Environment under joint authorship with Maud Ferrari, Doug Chivers and Som Niyogi (University of Saskatchewan).

4.1 INTRODUCTION

Increasing levels of ultraviolet radiation (hereafter UVR) hitting the surface of the earth as a result of reductions in stratospheric ozone has been a topic of concern for several decades (Newman et al. 2006). The implementation of the Montreal Protocol has ameliorated much of the ozone depletion, but it is difficult to ascertain how persistence of ozone recovery due to factors such as changes in cloud cover, air pollutants and aerosols, all of which are influenced by climate change (McKenzie et al. 2011). Extensive studies provide warning about the potentially damaging effects of UVR on freshwater ecosystems ranging from bacteria and phytoplankton to zooplankton and fish (Siebeck et al. 1994, Williamson and Zagarese 1994, Williamson 1995). In aquatic ecosystems, the range of solar radiation, including UVR, penetrating water bodies varies due to changes in a variety of abiotic factors like solar zenith angle, ozone depleting chemicals, greenhouse gases, water vapour, density of cloud cover, elevation and absorption and scattering by dissolved and particulate matter (Sullivan et al. 1992).

Organisms residing in clear, shallow high elevation lakes, where fluctuations in UVR levels could be more exaggerated, are more vulnerable to harmful effects of UVR due to shallow depth and higher absorption, reducing refuge from damaging levels of radiation (Williamson 1995, Blaustein et al. 1997). The level of UVR transmission varies across lakes and is greatly influenced by water chemistry. Dissolved organic matter (DOM), a component of natural organic matter (NOM), is an important water chemistry parameter in aquatic ecosystems. DOM is measured as dissolved organic carbon (hereafter DOC measured in mg/L) (Steinberg et al. 2008). There have been extensive studies on the impacts of NOM on aquatic organisms focussing on its influence on physiological (Campbell et al. 1997, Wood et al. 2003, Matuso et al. 2004, Glover et al. 2005, Galvez et al. 2009) and toxicological effects (Matuso et al. 2006, Meinelt et al. 2007). DOC is

generally defined as the fraction of DOM that passes through a 0.45 µm membrane and is chemically made of humic and fulvic fractions or acids (Buffle 1984, Thurman 1985). The attenuation rate of visible light and UVR in the water column is largely regulated by the concentration and absorptivity of DOC, rightfully referring it a "natural sunscreen" (Kirk et al. 1994, Morris et al. 1995, Porcal et al. 2009). Some studies have suggested that DOC may completely protect aquatic organisms like amphibians from damage caused by UV-B radiation (Adams et al. 2001, Palen et al. 2002). DOC affects other water quality parameters in aquatic systems, namely pH and has the ability to impart colour (Porcal et al. 2009). Chromophoric dissolved organic matter (CDOM), is the light absorbing fraction of DOC and it selectively removes the shorter wavelengths of UVR and visible light (Williamson and Rose 2010). In recent times, a variety of environmental changes namely increasing atmospheric CO_2 concentration, global warming, nitrogen deposition, decreased sulfate deposition are considered to be causes for changes in DOC concentrations in many aquatic systems (Porcal et al. 2009).

For more than half a century, evolutionary ecologists have been trying to understand the evolutionary role of epidermal club cells (ECCs), which are specialized cells found in the skin of fishes belonging to superorder Ostariophysi, with a few exceptions (Ferrari et al. 2010). Initial research indicated that these cells are the primary site of production and maintenance of alarm cues, which are released when the cells are ruptured, as during a predator attack. Anti-predator responses to such alarm cues are found across a wide range of taxa including gastropods, echinoderms, amphibians and fishes (Ferrari et al. 2010). Initial research focussed on predation-centered hypothesis (kin selection hypothesis and attraction of secondary predator hypothesis) to explain the evolution of alarm cues. Chivers et al. (2007) deviated from the predation-centered hypothesis and proposed the immune function hypothesis (anti parasitic/anti pathogenic

hypothesis). They indicated by performing a series of experiments that ECCs have a role to play in innate immune responses. Specifically, they showed that exposure to pathogenic watermoulds and larval trematodes cause an increase in ECC investment highlighting that these cells are part of the innate immune system. Halbgewachs et al. (2009) suggested a link between exposure to an immunosuppressant (intraperitoneal injection of cortisol) and reduced ECC investment. Manek et al. (2012, 2014) showed that exposure to immunosuppressants like UVR or cadmium (Cd) resulted in increased cortisol production and lowered ECC investment, providing further evidence that ECCs have an immune function. Manek et al. (2012, 2014) also showed that despite a reduction in ECC investment due to exposure to UVR and/or Cd, there was no difference in the anti-predator response to alarm cues prepared from the skin of UVR and/or Cd exposed fathead minnows. These results suggest that ECCs have an important role to play in innate immune responses and that the alarm function may have evolved secondarily.

Based on the known effects of DOC on UVR attenuation rates and findings of our previous studies showing that UV radiation decreases ECC investment through altering cortisol production, the objective of this study was to examine the effects of increased DOC levels and UVR exposure on physiological stress responses and ECC investment in fathead minnows (*Pimephales promelas*). To understand the comparative effect of DOC from different sources on the aforementioned objectives, we studied effects of increased DOC levels using two sources, commercially available Sigma Aldrich Humic Acid (hereafter SAHA) and field collected Luther Marsh natural organic matter (hereafter LM NOM). In a fully factorial (2x3 design), we exposed fish to one of the two DOC treatments along with a water control (3 levels of DOC), and exposed them to UVR in the presence or absence of a UVR blocking filter (2 levels of UVR). We hypothesized that physiological stress and ECC investment will vary in minnows depending on their exposure to

UVR under different sources and concentrations of DOC. Specifically, we predicted that exposure to UVR under increased DOC levels would help in maintaining low cortisol levels relative to those exposed to UVR only. We also predicted that under the influence of increased DOC levels and UVR exposure, minnows would be able to maintain a high level of ECC investment.

4.2 MATERIALS & METHODS

4.2.1 Fish Collection and housing for the study

This study was carried out in strict accordance with the approved University of Saskatchewan Animal Care Protocol number 20090091. Adult fathead minnows (Standard length ± S.D. = 5.2 ± 0.38 cm, Weight ± S.D. = 2.05 ± 0.51 g) were collected between May and June 2011, from the Feedlot pond located on the University of Saskatchewan campus using Gee's improved minnow traps. Male minnows have suppressed ECC numbers due to high testosterone levels in the reproductive phase (Smith 1973). To ensure that minnows were in the non-reproductive phase, they were acclimated in the laboratory for at least one month prior to the experimental procedure. This could also help reduce any variation in baseline ECC production between individuals (Manek et al. 2013). Fish were housed in a 73-L aquaria containing dechlorinated tap water. The water was maintained at around 19 ± 2° C and the photoperiod was set to 14:10 hr light:dark cycle. The fish were fed commercial flake food *ad libitum* throughout the acclimation phase and during the experiment. The water used for the experiments originated from the Saskatoon, SK, Canada municipal water supply and was periodically tested for water chemistry parameters (temperature, dissolved oxygen, pH, chlorine, ammonia, dissolved oxygen and hardness – presented in Table 4.1) every alternate day during the acclimation and experimental phase.

Table 4-1 – Mean ± S.E. water quality parameters of all treatment groups exposed to UVR (in the presence and absence of a blocking filter), and under different sources of DOC (DCW vs. LM NOM vs. SAHA). Analysis of one-way ANOVA indicate no significant difference between treatments at P=0.05.

Treatment	**Temperature (° C)**	**Dissolved Oxygen (mg/L)**	**pH**	**Hardness (as mg/L $CaCO_3$)**	**Ammonia (in ppm)**
high UVR + DCW	18.4 ± 2.1	6.5 ± 0.5	7.95 ± 0.23	150.5 ± 0.5	0.2 ± 0.1
low UVR + DCW	18.5 ± 2.5	7.1 ± 0.4	8.11 ± 0.12	150.5 ± 0.5	0.2 ± 0.1
high UVR + LM NOM	18.3 ± 2.2	6.6 ± 0.4	8.23 ± 0.13	150.5 ± 0.5	0.2 ± 0.1
low UVR + LM NOM	18.1 ± 2.3	7.2 ± 0.5	8.27 ± 0.12	150.5 ± 0.5	0.2 ± 0.1
high UVR + SAHA	18.6 ± 2.2	6.7 ± 0.5	8.19 ± 0.24	150.5 ± 0.5	0.2 ± 0.1
low UVR + SAHA	18.2 ± 2.4	7.0 ± 0.4	8.13 ± 0.11	150.5 ± 0.5	0.2 ± 0.1

4.2.1 DOC and UVR exposure

Minnows were exposed *in vivo* to UVR for 8 hours a day for 4 days in an Atlas SUNTEST XLS + Solar Simulator with Xeon lamp with a Suprax Daylight Glass Filter – 290 nm cut off (Atlas Material Testing Technology LLC, Chicago, USA) in groups of 4 in quartz beakers (diameter 13.8 cm, height 16.8 cm, QSI Quartz Scientific, USA). Fish were exposed to UVR in the presence or absence of a UV blocking filter in dechlorinated water (hereafter DCW), SAHA or LM NOM spiked water. The UVR exposure procedure and duration described above was similar to the one described previously by Manek et al. (2012). Some beakers had their top and sides covered with a 2 mm thick Lexan polycarbonate sheet. The polycarbonate sheeted removed 76% of the UVB and UVA radiation. Hereafter, we refer to the group exposed to UVR in the presence of a blocking filter as the low UVR group, and the group without the blocking filter as the high UVR group. This gave us a 2 X 3 fully factorial design, where the type of UVR exposure (high UVR vs. low UVR) was crossed with the source of DOC (DCW vs. LM NOM vs. SAHA). A 100% water change was performed per beaker per day. The four minnows in each beaker were not independent, so we considered the 'beaker', not the individual minnows, as our replicate unit. We alternated the order of treatments and conducted between 6-8 replicates per treatment.

4.2.2 LM NOM and SAHA stock solution preparation

Organic matter of an aquatic system can be characterized by source or origin. One such source of origin is terrigenous (NOM produced on land and then transported into the water body). LM NOM is an example of a terrigenous source of DOC. LM NOM was collected from Luther Marsh, Ontario in September 2009, via a portable reverse-osmosis apparatus (Sun et al. 1995), resulting in a stock with a concentration of 502.87 mg of carbon per litre (mg/L) and pH of around

2.7. Since the DOC concentration of the stock solution was known, we spiked the stock solution to end up with a final concentration of around 5.5 mg/L in the LM NOM water.

SAHA is a commercial source of DOC derived from coal, and has been extensively used as a DOM analogue in various physiological and toxicological studies (Glover et al. 2005, Glover and Wood 2005). A stock solution of SAHA was prepared by mixing 1g of SAHA sodium salt (Sigma Aldrich, USA) in one litre of de-ionized water. The water was thoroughly stirred using a magnetic stirrer and then stored in a flask which was tin foiled and kept at 4ºC in the refrigerator until used for spiking in the exposure water. We spiked the stock solution to obtain a final concentration of around 4.5 mg/L in the SAHA water. We originally aimed for similar (but not exact) final concentrations of DOC in SAHA and LM NOM. We decided to use a slightly lower amount of SAHA than LM NOM due to the greater chromomorphic properties of SAHA and its lower solubility (Al-Reasi et al. 2012). Our pilot studies showed significant treatment effects even with a marginal increase in DOC concentration.

4.2.3 DOC level analysis

Water samples were collected from beakers (DCW, LM NOM spiked water and SAHA spiked water) pre and post exposure to UVR. DOC levels were analyzed at the Saskatchewan Research Council (SRC) using UV persulfate digestion with IR detection on a Shimadzu TOC-VWP Analyzer equipped with ASI-V autosampler (detection limit: 0.2 mg/L). Saskatchewan Research Council Analytical is accredited by the Standards Council of Canada, in cooperation with the Canadian Association for Environmental Analytical Laboratories.

4.2.4 Euthanization

Dusan et al. (2006) and Manek et al. (2014) showed that minnows euthanized with an overdose of Aquacalm (methomidate hydrochloride) had a lower cortisol elevation from the

baseline than those euthanized with MS222. Thus, at the end of the 96 hr UVR exposure, minnows were euthanized with an overdose of Aquacalm for blood extraction and cortisol analysis. After extracting blood from the caudal vein region, all minnows were preserved in 10% neutral buffered formalin until further processing to obtain skin sections for histological analysis.

4.2.5 Experimental protocol for blood extraction

Blood extraction for cortisol analysis followed the method described by Halbgewachs et al. (2009). Blood samples (25-50 µL) were extracted from the caudal vein near the anal fin region of euthanized minnows. In order to obtain enough blood for the analysis, we pooled blood from four fish in the same beaker. This blood was placed on ice and allowed to clot for at least one hour. Serum was extracted from the blood after centrifugation and then frozen at -20°C until it was used for analysis. The cortisol level in the extracted serum was measured by the Endocrine Laboratory at Prairie Diagnostic Service (University of Saskatchewan) in a Coat-A-Count radioimmunoassay (Immulite-1000 Cortisol, Diagnostic Products Corporation, USA), which is designed for quantitative measurement of cortisol in serum.

4.2.6 Histological analysis of the skin

Tissue preparation for the analysis of the minnow epidermis followed the method described by Manek et al. (2012). Skin sections were stained with per-iodic acid Schiff's reagent with Harris' haematoxylin (PAS-H) to darken the mucous cells and the basement membrane (PAS) and the nucleus (haematoxylin), rendering ECCs colourless and easily recognizable. Images of each epidermal cross section were captured with a Zeiss Axioplan Fluorescence Microscope with an AxioCamICc1 (Color, 1.4MP) digital camera at 10 X magnification. For each slide, we recorded the following parameters: epidermal thickness, number of ECCs, ECC density and ECC area, which were all quantified using Image J 1.32, an image processing and analysis program (available

on the National Institute of Health's web page http://rsb.info.nih.gov/ij/). The observer was blind with respect to the treatment.

4.2.7 Statistical analysis

Levene's tests were performed to check for homoscedasticity and Kolmogorov-Smirnov tests were performed to check for normality distribution. All statistical analyses were performed using SPSS (ver. 19, SPSS Inc, USA). We performed a series of 2x3 ANOVAs to assess the effect of DOC (DCW vs. SAHA vs. LM NOM) and UVR exposure (low UVR vs. high UVR) on DOC levels, cortisol levels and histological data (which included ECC density, epidermal width and ECC area).

4.3 RESULTS

4.3.1 Evaluation of mortality

In the experiment, 10% of minnows in the high UVR+DCW died and 5% minnows in the high UVR+SAHA group died. There was no mortality recorded in the low UVR+DCW group, high UVR+LM NOM, low UVR+LM NOM and low UVR+SAHA group. Minnows that did not survive until the end of the exposure were excluded from further analysis.

4.3.2 DOC levels

The 2x3 ANOVA revealed a significant effect of source ($F_{2,12}$=80.15, P<0.001), however, it did not reveal a significant effect of UVR exposure ($F_{1,12}$=1.22, P=0.291) or any interaction between the two ($F_{2,12}$=0.13, P=0.714) on mean DOC levels (Figure 4.1). Tukey post-hoc comparisons revealed that the 3 groups differed in DOC levels (all P's<0.001), with the water control having the lowest level and the LM NOM having the highest level of DOC.

4.3.3 Cortisol levels

The 2x3 ANOVA revealed a significant interaction between UVR and DOC on mean cortisol levels ($F_{2,37}$=26.91, P<0.001). Moreover, there was a significant effect of DOC ($F_{2,37}$=13.88, P<0.001), and a significant effect of UVR exposure ($F_{1,37}$=6.82, P=0.013), (Figure 4.2).

4.3.4 Histological parameters

4.3.4.1 ECC Density

The 2x3 ANOVA revealed a synergistic interaction between DOC source and UVR ($F_{2,38}$=10.19, P<0.001) on mean ECC density of minnows. There was also a significant effect of DOC source ($F_{2,38}$=4.2, P=0.021) and a significant effect of UVR exposure ($F_{1,38}$=11.09, P=0.002) (Figure 4.3).

4.3.4.2 Epidermal width

The 2x3 ANOVA revealed a significant effect of DOC ($F_{2,38}$=19.9, P<0.001), however, it did not show a significant effect of UVR exposure ($F_{1,38}$=0.34, P=0.562) or any interaction between the two ($F_{2,38}$=0.13, P=0.876) on mean epidermal width of minnows (Figure 4.4).Tukey post-hoc comparisons revealed that fish in LM NOM treatment had the thickest epidermis compared to fish in other treatment groups (both P's<0.001). However, we did not to find a thickness difference between fish in DCW and SAHA treatments (p=0.242).

4.3.4.3 ECC area

The 2x3 ANOVA revealed a significant effect of DOC ($F_{2,38}$=20.07, P<0.001), however, did not show a significant effect of UVR exposure ($F_{1,38}$=2.99, P=0.092) or any interaction between the two ($F_{2,38}$=0.52, P=0.598) on mean ECC area of minnows (Figure 4.5). Post-hoc tests revealed

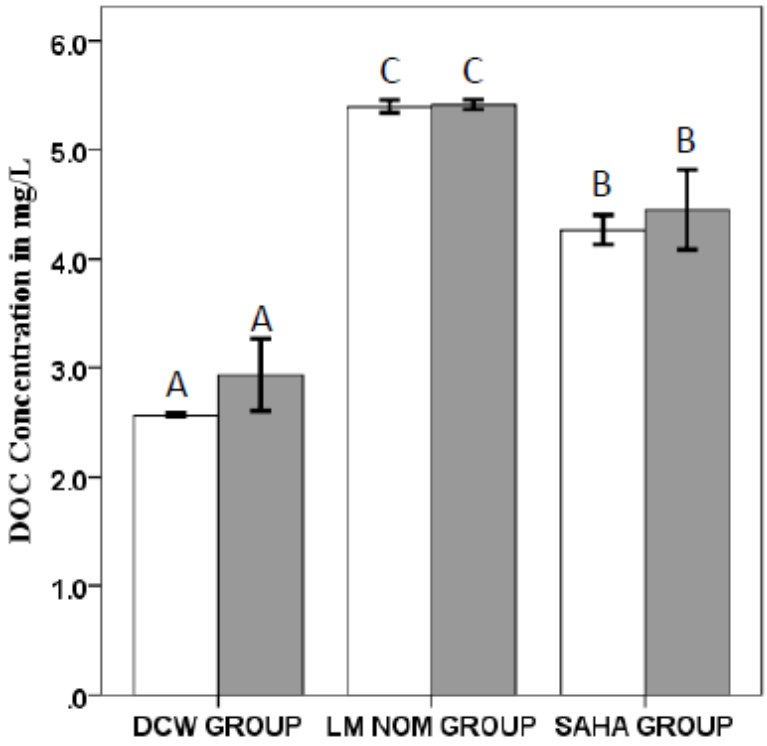

Figure 4.1 Mean ± S.E. DOC levels from water samples from the DCW, LM NOM or SAHA groups in the presence and absence of a UV blocking filter (white bar graph denotes the high UVR group and grey bar graph denotes low UVR group; n=6-8/treatment). Different letters denote significant difference at $\alpha<0.05$.

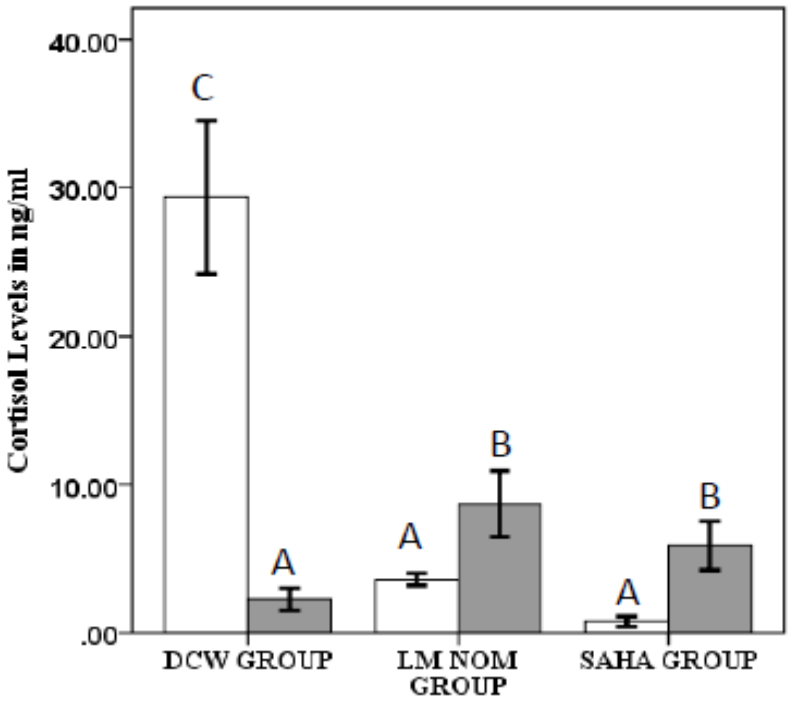

Figure 4-2 – Mean ± S.E. cortisol levels from blood of minnows exposed to DCW, LM NOM or SAHA in the presence and absence of a UV blocking filter (white bar graph denotes the high UVR group and grey bar graph denotes low UVR group; n=6-8/treatment). Different letters denote significant difference at $\alpha<0.05$

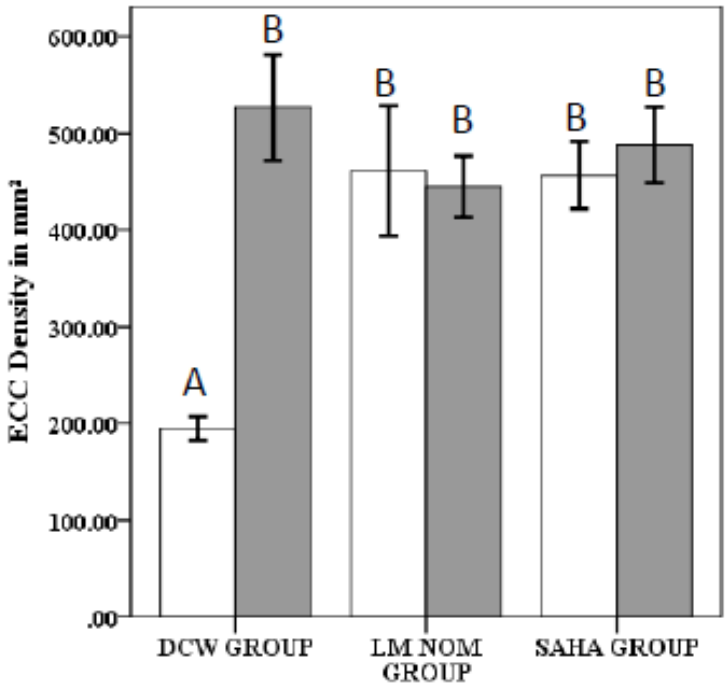

Figure 4-3 – Mean ± S.E. ECC density from the skin of minnows exposed to DCW, LM NOM or SAHA in the presence and absence of a UV blocking filter (white bar graph denotes the high UVR group and grey bar graph denotes low UVR group; n=6-8/treatment). Different letters denote significant difference at $\alpha<0.05$.

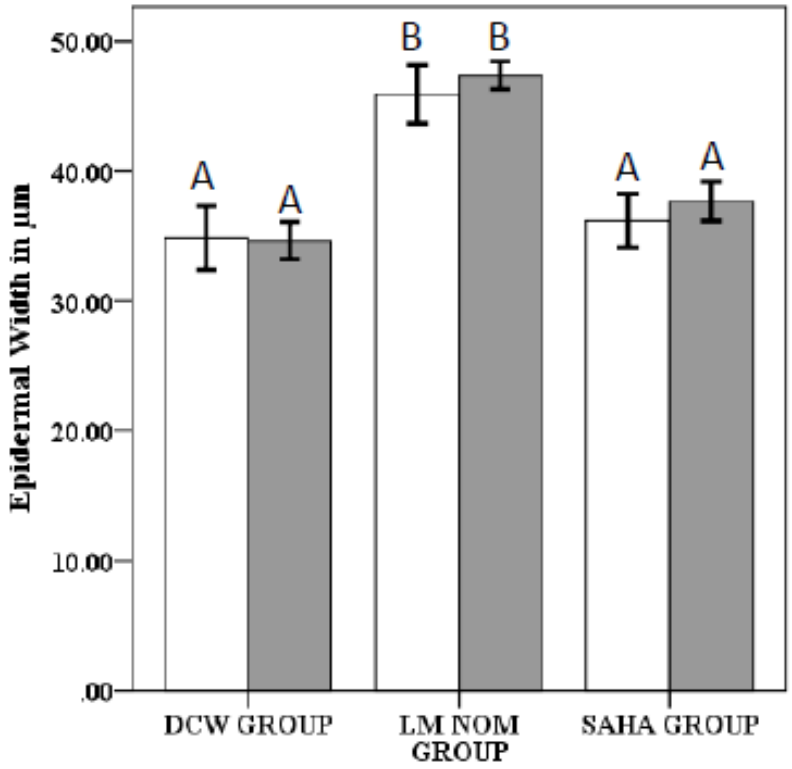

Figure 4-4 – Mean ± S.E. epidermal width from the skin of minnows exposed to DCW, LM NOM or SAHA in the presence and absence of a UV blocking filter (white bar graph denotes the high UVR group and grey bar graph denotes low UVR group; n=6-8/treatment). Different letters denote significant difference at $\alpha<0.05$.

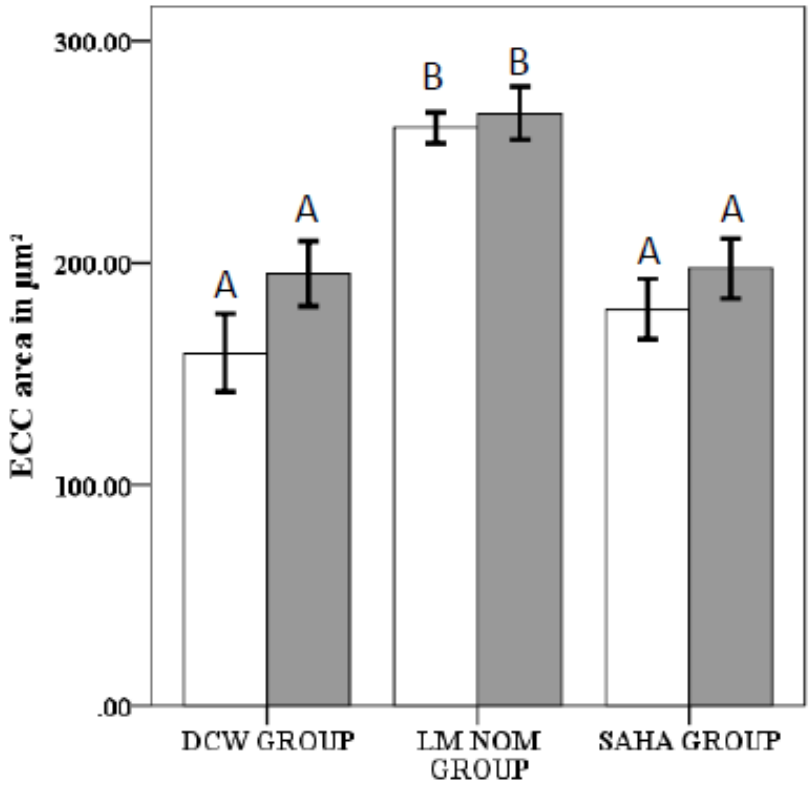

Figure 4-5 – Mean ± S.E. ECC area from the skin of minnows exposed to DCW, LM NOM or SAHA in the presence and absence of a UV blocking filter (white bar graph denotes the high UVR group and grey bar graph denotes low UVR group; n=6-8/treatment). Different letters denote significant difference at $\alpha<0.05$.

that fish in LM NOM treatment had the largest cells ($P<0.001$ when compared to the other groups), but fish in DCW and SAHA treatment groups did not differ in their ECC areas ($P=0.419$).

4.4 DISCUSSION

The attenuation rate of UVR in water bodies is highly regulated by DOC, the concentration of which is known to be highly variable across the aquatic ecosystems (Steinberg et al. 2008, Arts et al. 2000). Since it is well established that DOC acts as a natural sunscreen, it seemed logical to evaluate if altered DOC concentrations played a role in ameliorating physiological stress responses and upregulating ECC investment in fathead minnows. Our work provides clear evidence that this is the case.

Previous studies focused on understanding how DOC influences toxicity of various metals have shown that different source of NOM can have substantially different protective effects (Al-Reasi et al. 2011). This work cautions us against considering only the quantity of DOC, and suggests that we should also consider the sources of the DOC. To emphasize this aspect, we used two different sources of DOC: (i) a natural terrigenous source of DOC in the form of LM NOM, and (ii) a coal based readily available commercial source of DOC in the form of SAHA. Given that SAHA is a commercial humic substance, we wanted to compare the protective effects of SAHA on physiological stress response and ECC investment to a real-world aquatic humic substance LM NOM. Both sources of DOC have different specific UV absorption coefficients (measured in cm^2 mg^{-1}). The specific absorption coefficient of SAHA is 79.98 cm^2 mg^{-1} (Al-Reasi et al. 2012). LM NOM is primarily humic acid-like material (74%) and its specific absorption coefficient is 37.8-39.3 cm^2 mg^{-1} (Al-Reasi et al. 2012, Gheorghiu et al. 2010). A higher specific UVR absorption coefficient of SAHA compared to LM NOM indicated that the former has greater chromomorphic attributes (potentially greater UVR absorption capacity) than the latter (Al-Reasi et al. 2011).

DOC by itself has some beneficial effects on fish physiology such as facilitating ion uptake and regulation and amelioration of low pH associated stress (Wood et al. 2011). We found similar results where the presence of DOC from either sources (LM NOM vs. SAHA) helped lower cortisol levels and increase ECC investment. Since ECCs are linked with innate immune responses, we can suggest that exposure to DOC by itself may have been beneficial to fathead minnows. The concentration of DOC in DCW that minnows were housed in prior to and during UVR exposure was around 2.7 mg/L. We initially aimed at nearly doubling the concentration of DOC by spiking LM NOM or SAHA to obtain a final concentration of around 5-5.4 mg/L. These are levels that typically occur in aquatic ecosystems across the Canadian Prairies; however, there is extreme variation in DOC levels, with some lakes and wetlands in the Great Plains exceeding 150 mg/L (Arts et al. 2000). Despite the significant differences in DOC levels obtained by spiking LM NOM vs. SAHA vs. DCW, and irrespective of the type of UVR exposure (high UVR vs. low UVR), we found that the presence of either LM NOM or SAHA helped to maintain low mean cortisol levels and a high mean ECC density in fathead minnows. Indeed, we found that a marginal increase (45-55%) in DOC levels (in mg/L) lowered cortisol levels and correspondingly increased ECC density. Based on the results of cortisol levels and ECC investment in our study, we decided not to use a higher concentration of LM NOM or SAHA. It is particularly important to note here that LM NOM, which is a natural aquatic DOC with lesser chromomorphic properties, was as effective as SAHA in ameliorating stress response and ECC investment in fathead minnows used in the present study. Previous studies have shown that increased exposure to UVR and altered pH (acidification) are known to have a negative effect on DOC by resulting in loss of DOC via photolysis (CDOM absorbance loss) in aquatic systems (Gennings et al. 2001, Molot et al. 2005). We found no significant difference in pH levels across groups exposed to UVR with DCW, SAHA or LM NOM

(Table 4.1). In fact, the pH of Feedlot pond was around 8.4 at the time the minnows were collected. Preliminary analysis of DOC concentration also revealed no significant difference in DOC levels in DCW, LM NOM or SAHA treatment water pre and post UVR exposure, in the presence or absence of a UVR blocking filter. Since our study was laboratory based and under controlled conditions, the duration of UVR exposure and concentration of DOC that we selected for this study possibly was not enough to result in loss of DOC or drastically alter the pH.

The total level of UVR emitted by the solar simulator in the present study was around 250 W/m^2 (UVR and Photosynthetically Active Radiation combined). The levels of UVR that fathead minnows are exposed to across their geographical range can vary 2 fold depending on their latitude (Goncalves et al. 2010). If we dissect out the actual UVR exposure only, it was around 45 W/m^2, which is comparable to natural levels of UVR in mid-summer in Saskatchewan (43 W/m^2) (Sereda et al., unpublished results).

Results of our current study supports previous findings related to the immune-function of ECCs (Chivers et al. 2007, Halbgewachs et al. 2009, Manek et al. 2012, Manek et al. 2014). As previously mentioned, ECCs were originally linked with production and maintenance of "alarm cues" which are chemical cues that elicit anti-predator response in conspecifics (Ferrari et al. 2010). In our previous work, we evaluated if any changes in ECC numbers as a result of immunosuppression (UVR and/or Cd exposure) would have any effect on the level of anti-predator response to cues prepared from the skin of UVR and or/Cd exposed fathead minnows. We found no significant difference in anti-predator response to alarm cues prepared from the skin of UVR and/or Cd exposed or filtered fathead minnow, as a result of which we concluded that focussing on the immune function of ECCs was of prime importance (Manek et al. 2012, Manek et al 2014). Thus

the main focus of this study was to ascertain if DOC played a role in affecting the immune-function of ECCs (cortisol production and ECC investment).

Most studies examining the effects of stressors on ECC investment have found effects on ECC numbers. There are a few cases where differences in epidermal thickness and in area of ECC were reported (Wisenden and Smith 1997, Iger et al. 1994). Here we found that minnows in LM NOM treatment had a thicker epidermis and a larger mean cell size (measured as cross sectional area of the cells) compared to those in SAHA spiked water or DCW group. Additional manipulative experiments are needed to explain these patterns.

Our current study reveals interesting ameliorative effects of increased DOC concentration on stress response and ECC investment in fish during a short term exposure to UVR under controlled laboratory conditions. Future research should evaluate the long term exposure effects and determine if the chemical properties of DOC we selected for this study are altered over a prolonged period of exposure to UVR, and what effect this could have on physiological stress response and ECC investment in fathead minnows. It would also be interesting to examine the effects of lower DOC levels as a result of photolysis observed in natural systems due to various anthropogenic activities and climate change. With recent changes to aquatic systems resulting from anthropogenic disturbances, it is becoming critical to understand how changing water quality is affecting fish health. Since fathead minnows represent a widely distributed freshwater fish belonging to superorder Ostariophysi, our current results could be extrapolated to similar aquatic freshwater systems.

CHAPTER 5[a]

WITHIN AND BETWEEN POPULATION VARIATION IN EPIDERMAL CLUB CELL INVESTMENT IN A FRESHWATER PREY FISH: A CAUTIONARY TALE FOR EVOLUTIONARY ECOLOGISTS

[a] This chapter targets a problem faced by multiple studies that reveal a huge variation in levels of baseline ECC investment. This chapter has two main objectives. The initial objective of this chapter is to examine ECC investment in wild caught fathead minnows across multiple sites in Saskatchewan. The second objective of this chapter is to examine if controlled laboratory conditions for up to 28 days plays a role in lowering variation in ECC investment in fathead minnows collected from different sites across Saskatchewan. The contents of Chapter 5 have been published in PLoS ONE, 2013 8(3): e56689. doi:10.1371/journal. pone.0056689, under joint authorship with Maud Ferrari, Robyn Pollock, Dan Vicente, Lynn Weber and Doug Chivers (University of Saskatchewan).

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5.1 INTRODUCTION

Many species of prey fishes, particularly those members of the superorder Ostariophysi, possess large epidermal club cells (hereafter ECCs) in their skin (Pfeiffer 1977, Smith 1992). Understanding the selection pressure leading to the evolution of those cells has been somewhat elusive. Following from the pioneering work of Von Frisch (1938, 1941), initial experiments focussed on predation-centered hypotheses for the evolution of the cells, but more recently much more emphasis has been placed on immune-centered hypotheses (Chivers et al. 2012).

When the skin of the fish is damaged and the ECCs are ruptured, as would occur during a predator attack, chemicals initiating anti-predator responses in nearby conspecifics are released in the water column. Not surprisingly, these chemicals are often referred to as chemical alarm cues. In a pioneering experiment, Smith (1973) established that, during the breeding season, male minnows lose their ECCs and skin extracts made from breeding minnows do not evoke anti-predator behaviour in conspecifics. This finding lead to the conclusion that ECCs are the source of the alarm cues and has been supported by numerous studies (Smith 1976, Smith and Smith 1983, Pollock et al. 2005, Pfeiffer et al. 2006). However, a recent study by Carreau-Green et al. (2008) suggested that the skin of juveniles of one species of fish may evoke an alarm response in conspecifics even before the cells appear. If this finding is supported by additional experiments, it would provide strong evidence against the role of the cells as the source of alarm cues. Moreover, a recent paper by Mathuru et al. (2012) indicates that GAG chondroitin may be a major component of alarm cues in ostariophysan fishes. There is no known link between chondroitins and ECCs, further weakening the conclusion that ECCs may be responsible for evoking the alarm reactions. Alarm cues may also be mixtures of chemicals with different constituents in different parts of the epidermis including the ECCs.

Understanding the evolution of ECCs as production and/or storage areas for alarm cues has been problematic because the sender of the cue needs to be captured in order for the cues to be released. The critical question that needs to be addressed is: what is the benefit to the sender of this signal? Early research has focussed on the potential for kin associations to explain the existence of ECCs (Wisenden et al. 1995, Wisenden and Smith 1998). However, there is limited evidence that most fishes shoal with kin or that kin selection could explain ECC evolution (Chivers et al. 2012).

Other predation-centered hypotheses for the evolution of alarm cues suggest that the chemicals may have evolved as predator attractants (Mathis et al. 1995). Secondary predators attracted to the location of damaged prey may fight over the prey, giving a chance to the captured prey to escape (Chivers et al. 1996). There is some evidence for the secondary predator attraction hypothesis, but the frequency with which predators would interfere with each other may be rare and hence this explanation is somewhat unsatisfying.

Chivers et al. (2007) provided an alternative to the predation-centered hypotheses for the evolution of alarm cues. They suggested that ECCs may act as a first line of defence against pathogens and parasites that penetrate through the skin. Indeed, they showed that exposure to both skin-penetrating pathogens (water moulds) and parasites (larval trematodes) lead to increases in ECC numbers, suggesting that these cells are part of the immune system (Chivers et al. 2007). Skin infections do not always lead to an increase in ECCs. A study by James et al. (2009) showed that minnows exposed to cercariae of a highly specialized minnow trematode *Ornithodiplostomum ptychocheilus*, where able to infect the host without eliciting an increase in ECC investment. Halbgewachs et al. (2009) tested the immune system hypothesis by suppressing the immune system of fishes with cortisol and showing that consequently, the number of ECCs

dramatically decreased. In a similar experiment, Chivers et al. (2007) showed that fish that had their immune systems suppressed with heavy metals (Cd) lost their ability to increase ECC investment upon exposure to pathogens. In this case evolutionary selection to produce ECCs was driven by disease/pathogen dynamics and the anti-predator function of the cells evolved secondarily, because they represent a reliable signal that a conspecific in the vicinity was recently captured by a predator.

A number of experimental studies have identified factors that may be important in determining ECC investment in fishes. For example, Wisenden and Smith (1997) showed that fathead minnows fed higher food rations had higher numbers of ECCs than those fed lower rations. Moreover, individuals raised with unfamiliar conspecifics had more ECCs than individuals raised in familiar groups (Wisenden and Smith 1998). Environmental stressors including UV radiation, rapid temperature changes and poor water quality have been shown to result in elevated cortisol levels which are indicators of stress levels and are strongly correlated with reduced ECC investment in fish (Halbgewachs et al.2009, Pickering 1994, Manek et al. 2012). Epidermal injury induced by handling and transportation can also result in changes in cortisol levels and hence could likewise influence ECC numbers (Barton and Iwama 1991).

Despite the wealth of studies showing that specific factors influence ECC investment, there are some notable inconsistencies with researchers being able to document changes in ECC investment. For example, Pollock (2011) found inconsistent ECC responses of minnows to pathogenic water moulds. This finding weakens the immune-function hypothesis. It is clear that we still have much to learn about what drives ECC density in fishes. One finding that is immediately apparent from Pollock's work is that the baseline level of ECCs in control treatments was extremely variable. Fish were collected from different populations and were held in the

laboratory for different periods of time prior to experimentation. Each of these factors could have led to variation in the baseline level of cells and hence could have compromised her ability to provide strong experimental tests of factors influencing ECC investment. In another study, Michalak (2006) completed two different pathogen experiments using minnows caught from the same shoal. Again, she found a large discrepancy in the baseline number of ECCs in the two experiments despite the fact that her control conditions were identical. An obvious source of variation could have been differences in the time the minnows were held in the laboratory.

If we are to develop a comprehensive understanding of factors that drive ECC investment and explain the evolution of these cells, we need to step back and begin to understand the source and magnitude of variation in ECC numbers. Hence the goal of our current work was threefold. First, we tested for differences in ECC investment among four local populations of wild-caught fathead minnows collected at the same time of year. Between-population differences in ECC numbers has been documented by Hugie (1990). Unfortunately, his data presentation does not allow us to understand the magnitude of the differences he observed. The second goal of our study was to test for within-population differences in ECC investment among four sites within a single waterbody. The final goal of our work was to understand if raising fish under standard laboratory conditions could reduce differences in ECC investment and hence be used as a technique for researchers that want to conduct manipulative experiments to test factors influencing ECC investment. This technique could not only be used to reduce differences in ECC number for fish caught from a single population, but could also reduce between population differences and may be a valuable technique to allow tests of how populations with different predation or pathogen exposure respond to experimental manipulations.

5.2 MATERIALS & METHODS

5.2.1 Fish collection for field survey

Non-breeding adult fathead minnows were collected from four different populations in and near Saskatoon, Saskatchewan, Canada in late fall 2009 using seine nets and minnow traps. We caught the fish outside the breeding season because male minnows in reproductive condition have reduced numbers of ECCs (Smith 1973). Feedlot Pond is a 1-ha pond located on the University of Saskatchewan campus in Saskatoon. The pond was originally filled from the South Saskatchewan River in 1959 to provide water for agricultural purposes. Historically, water (and potentially fish) from the river were pumped into the pond on an annual basis, but no water has been pumped into the pond for at least 15 years and consequently it can be considered a closed system. Pike Lake is an oxbow lake of the South Saskatchewan River, located approximately 33 km south of Saskatoon. Water (and possibly fish) are pumped from the river occasionally to maintain water levels in the lake. Both Marshy Creek and Oscar Creek drain into Redberry Lake, a large saline lake within an enclosed evaporation basin approximately 73 km northwest of Saskatoon. Both Marshy Creek and Oscar Creek contain numerous beaver dams and culverts that impede the movement of fish in the creek.

For the field survey we collected 50 minnows from each of Feedlot Pond, Pike Lake and Oscar Creek populations. In Marshy Creek we collected 50 minnows from each of four locations (hereafter referred as M-1,M-2,M-3 and M-4) separated by approximately 6 to 12 km. Table 5.1 provides a summary of water quality parameters (water temperature, dissolved oxygen, pH, salinity and conductivity) that were recorded at each site at the time the fish were captured. Immediately after capture, the fish were euthanized with an overdose of MS222 in accordance with the Animal Care Protocol Number 20050067. After being weighed and having their

Table 5-1 – Mean ± S.E. water quality parameters for field survey.

Group	Temperature (°C)	Dissolved oxygen (mg/L)	pH	Salinity (in ppt)	Conductivity (µS)/cm
Pike Lake	16.7 ± 0.5	9.97 ± 0.11	7.8 ± 0.1	0.2 ± 0.01	929 ± 56
Oscar Creek	11.2 ± 0.5	7.2 ± 0.12	7.7 ± 0.2	0.6 ± 0.02	903 ± 62
Feedlot Pond	15.4 ± 0.4	10.7 ± 0.13	8.9 ± 0.1	0.3 ± 0.01	N.A.*
Marshy Creek - 1 (M-1)	8.8 ± 0.2	9.68 ± 0.11	7.5 ± 0.2	0.6 ± 0.01	764 ± 88
Marshy Creek-2 (M-2)	9.5 ± 0.6	6.6 ± 0.12	7.8 ± 0.1	0.7 ± 0.03	943 ± 45
Marshy Creek-3 (M-3)	8.4 ± 0.3	7.3 ± 0.11	7.5 ± 0.2	0.5 ± 0.04	668 ± 66
Marshy Creek-4 (MS-4)	7.3 ± 0.4	6.38 ± 0.12	7.3 ± 0.1	0.5 ± 0.02	700 ± 23

* - not available

standard lengths measured for analysis of Body Condition Index (hereafter BCI), these minnows were fixed in 10% neutral buffered formalin (3.7 % formaldehyde w/v) until processed for histological analysis. Table 5.2 provides a summary of the body condition parameters as well as the mean number of blackspots on each fish. Blackspot disease is commonly observed in freshwater fish as pinhead sized spots located on the fins and body of infected fish (Pickering 1994, Hunter and Hunter 1938). It is caused by a trematode parasite (*Ornithodiplostomum sp*.) having a three-host life cycle, where fish is the second intermediate host (Steedman 1991). Exposure to trematodes is known to influence ECC investment (Chivers et al. 2007).

5.2.2 Laboratory maintenance study:

Minnows from the Pike Lake site and two Marshy Creek sites (M-1 and M-3) were randomly selected for the laboratory study. We collected adult minnows from each of the three sites and transported them to the laboratory. The containers housing minnows were aerated until they were brought to the lab and gradually transferred to tanks ensuring a ± 1º C variation in temperature between their containers and aquaria water. Minnows were divided in groups of 10 and placed in 74-L aquaria (60 x 30 x 40 cm) each of which was equipped with an airstone. We had a total of 10 tanks of 10 fish from each of the three locations. For statistical purposes, we considered the tank not the individual fish as our replicative unit. The minnows were reared under standard laboratory conditions for up to 28 days. They were maintained on a 14:10 hr light:dark cycle and fed Nutrafin© tropical flake food *ad libitum* (guaranteed 46% minimum crude protein, 5% minimum crude fat, 2% maximum crude fibre, 8% maximum moisture) twice daily. We conducted a 10% water change each week and measured water quality parameters every other day to check for temperature, pH, nitrate, nitrite, hardness and chlorine levels.

On day 14, minnows from five randomly chosen aquaria from each population were euthanized with an overdose of MS222 in accordance with the Animal Care Protocol Number 20050067. After being weighed and having their standard lengths measured for analysis of BCI, these minnows were fixed in 10% neutral buffered formalin until they were processed for histological analysis. Minnows from the remaining five aquaria from each population were euthanized on day 28 for histological analysis.

5.2.3 Histological preparation:

Tissue preparation for the analysis of the minnow epidermis followed the methods described by Hugie (1990) with modifications (Manek et al. 2012). Epidermal samples were taken from the dorso-lateral surface just behind the operculum to the dorsal fin and placed between two biopsy pads in histocassettes and stored in formalin. An automatic tissue processor (MUP1, Modular Vacuum Processor) was used to process the fixed skin tissue in a series of ethanol grades and perfused with paraffin wax. Tissues were then manually embedded in paraffin wax. The resulting tissue, embedded in a paraffin block was sectioned using a rotary microtome (HM330, Heidelberg) at 5µm thickness. Following sectioning, 3-5 sections were placed on a pre-cleaned suprafrost slide (VWR micro slides). After the slides were dried on a slide warmer, they were deparaffinised, rehydrated and then stained with periodic acid Schiff's reagent with Harris' haematoxylin (PAS-H) to darken the mucous cells and the basement membrane (PAS) and the nucleus (haematoxylin), rendering ECCs colourless and easily recognizable [16] . Images of each epidermal cross section were captured with a Zeiss Axioplan Fluorescence Microscope with an AxioCamICc1 (Color, 1.4MP) digital camera at 10 X magnification. For each slide, we recorded the following parameters: mean number of ECCs per mm of epidermis, mean epidermal thickness and mean ECC density (number of ECC's per area of epidermis in mm² taking epidermal thickness

into account) and using Image J 1.32, an image processing and analysis program (available on the National Institute of Health's web page http://rsb.info.nih.gov/ij/). The observer was blind with respect to the treatment.

5.2.4 Statistical analysis

Previous studies looking at ECC parameters in fish have used different methods to report ECC parameters. Often researchers provide data on the average number of cells in a given length of epidermis tissue [most often ECCs/mm of skin, (Von Frisch 1938, Chivers et al. 2007)]. However, given that the epidermis of fishes can vary considerably in thickness, other researchers have instead reported the density of ECCs in the skin (Pfeiffer et al. 2006, James et al. 2009). Indeed, two fish with the same number of cells per mm length of skin could have very different ECC densities. We wanted to compare these approaches by asking whether we would reach the same conclusions using both measures. Consequently, to determine whether the mean number of ECCs per mm or the mean density of ECCs differed among the four populations in our first experiment we used a one-way ANCOVA with blackspot load and body condition as covariables. Body condition was calculated as the Studentized residuals of the regression between ln (length) and ln (mass) of the fish. The values were logged to linearize the relationship between length and weight. For the within population test, we likewise compared the mean number of ECCs per mm and the mean density of ECCs among the four Marshy Creek collection locations with an ANCOVA.

For the laboratory experiment, we were interested in knowing whether holding fish under standard laboratory conditions for an extended period of time would reduce the within- and between- population difference in ECC parameters. We did not know how fast the populations could converge; consequently we collected data at both 14 and 28 days. However, visual inspection of the data made it obvious that the populations did not converge by day

14. Consequently, to keep the analysis simple, we restricted our analysis to only comparisons between days 1 and 28. For day 1 we compared whether the three test groups differed in either mean number of ECCs per mm or the mean density of ECCs. We repeated the same analysis on day 28. We also used a Levene's test to compare whether the coefficient of variation for the populations changed through time.

5.3 RESULTS

An examination of the skin sections revealed striking differences among populations in the mean thickness of the epidermis. Pike Lake fish had a much thinner epidermal layer than those from the other populations (Table 5.2). Given that the width of the epidermis did not overlap between populations we could not include it as a covariable in our analysis.

Our results for ECC investment varied depending on whether the data were analysed as ECC/mm or as ECC density. We found an overall significant difference in mean number of ECCs per mm of skin among fish from each of the four populations (ANCOVA: $F_{3,250} = 36.6$, $P<0.001$, figure 5.1A), but neither body condition ($F_{1,250} = 0.29$, $P=0.59$) nor parasite burden ($F_{1,250} = 1.4$, $P=0.23$) accounted for a significant amount of the variance. Tukey post- hoc tests reveal that all populations differed from each other with the exception of Marshy Creek and Oscar Creek. There were nearly three times as many ECCs per mm of epidermis in the Oscar Creek population as compared to the Pike Lake population. Our conclusions about differences among populations in ECCs are very different if we consider ECC density as opposed to number of ECCs per mm. Again, there was an overall difference among populations (ANCOVA: $F_{3,249} = 5.26$, $P = 0.002$. figure 5.1B). Neither body condition ($F_{1,249} = 0.29$, $P=0.59$) nor blackspot burden ($F_{1,249} = 1.43$, $P=0.23$) accounted for a significant amount of the variance. The Pike Lake population did not differ from any of the others with regards to ECC density. Indeed, there were no differences in any of the

Table 5-2 – Mean ± S.E. body condition index parameters and blackspot burden for fish used in the field survey and lab study.

Group	Days in the Lab	Mass M (g)	Standard length L (cm)	Body Condition Index: M/L^3*100	Epidermal thickness (µm)	Blackspot burden
Pike Lake	Day 0	1.65 ± 0.11	4.48 ± 0.12	1.9 ± 0.01	26.23 ± 1.76	0.71 ± 0.23
	Day 14	1.65 ± 0.08	4.78 ± 0.07	2.0 ± 0.01	38.40 ± 1.28	0.87 ± 0.21
	Day 28	1.88 ± 0.07	4.64 ± 0.06	2.0 ± 0.01	45.18 ± 1.45	0.51 ± 0.19
Oscar Creek		1.84 ± 0.10	4.56 ± 0.11	1.8 ± 0.01	59.79 ± 1.94	1.73 ± 0.19
Feedlot Pond		1.40 ± 0.11	4.79 ± 0.12	1.2 ± 0.01	56.25 ± 2.81	0.00 ± 0.00
Marshy Creek – 1	Day 0	1.49 ± 0.08	4.20 ± 0.09	2.0 ± 0.01	62.35 ± 5.61	1.50 ± 0.17
	Day 14	1.71 ± 0.08	4.62 ± 0.08	1.8 ± 0.01	45.36 ± 3.35	1.28 ± 0.19
	Day 28	1.48 ± 0.12	4.56 ± 0.12	1.6 ± 0.01	37.52 ± 3.00	1.11 ± 0.29
Marshy Creek – 2		1.15 ± 0.12	3.71 ± 0.13	2.0 ± 0.02	58.47 ± 2.32	0.30 ± 0.23
Marshy Creek – 3	Day 0	0.84 ± 0.08	3.45 ± 0.09	2.0 ± 0.01	60.11 ± 1.73	0.66 ± 0.16
	Day 14	0.83 ± 0.08	3.55 ± 0.09	1.8 ± 0.01	35.72 ± 1.56	1.50 ± 0.23
	Day 28	1.22 ± 0.13	4.01 ± 0.16	1.8 ± 0.01	36.20 ± 2.57	1.00 ± 0.37
Marshy Creek – 4		1.74 ± 0.09	4.64 ± 0.10	1.8 ± 0.01	72.59 ± 1.85	0.89 ± 0.18

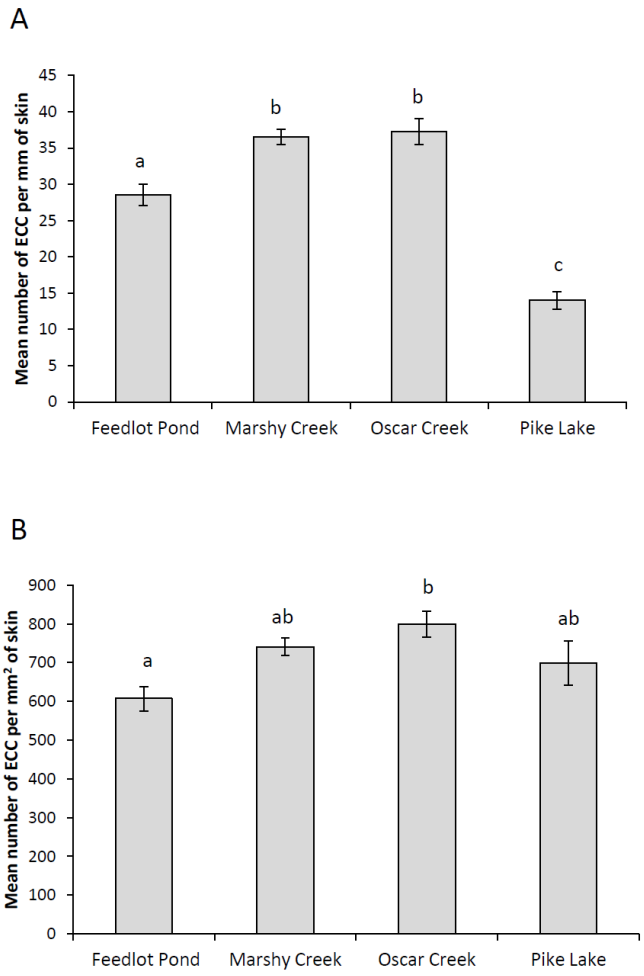

Figure 5.1 Mean ± S.E. number of EEC per mm of fish skin (A) and density of ECCs (B) for fathead minnows collected from each of the four populations (N=32-50 per population). Different letters indicate significant differences at $\alpha<0.05$.

post-hoc comparisons, except that the Feedlot population had a lower density of ECCs than Oscar Creek and tended to have fewer ECCs than Marshy Creek (P=0.054).

Our within-population comparison revealed an overall significant difference in ECCs per mm of epidermis among the four sample locations within Marshy Creek (ANCOVA: $F_{3,146}$ = 32.0,P<0.001, figure 5.2A) but neither body condition ($F_{1,146}$ = 0.29, P=0.59) nor parasite burden ($F_{1,146}$ = 0.01, P=0.93) accounted for a significant amount of the variance. All sample locations differed from each other with the exception of M-2 and M-3 (Tukey tests: P>0.9). Unlike between populations, there is very little difference in epidermal thickness within the Marshy Creek population. This meant that the results we found for difference in ECC density among the 4 sample locations in Marshy Creek matched those looking at number of ECCs per mm of epidermis. Again there was an overall significant difference between sites (ANCOVA: $F_{3,146}$ = 66.9, P<0.001, figure 5.2B), but neither body condition ($F_{1,146}$ = 0.67, P =0.42) nor parasite burden ($F_{1,146}$ = 3.6, P=0.06) accounted for s significant amount of the variance. Each of the sites had an ECC density different than the others except site M-2 and M-3 (P>0.9).

In our laboratory test we found that all three groups [M-1, M-3 and Pike Lake (PL)] of minnows differed in number of ECCs per mm of epidermis at the beginning of the experiment (ANCOVA, $F_{2,117}$ = 65.9, P<0.001, all post hoc tests P<0.001, figure 5.3) . There was no effect of body condition ($F_{1,117}$ = 0.03, P=0.87) or parasite burden ($F_{1,117}$ = 0.60, P=0.44). In contrast, when we consider ECC density, there was an overall difference between the three groups ($F_{2,12}$ = 32.7, P<0.001, figure 5.3B). Again, body condition ($F_{1,117}$ = 0.54, P=0.46) and parasite burden ($F_{1,117}$ = 0.24, P=0.63) did not explain any of the variation. M-1 and PL were both different from M-3 (P<0.001), but similar to each other (P = 0.46). If we consider number of ECCs per mm of epidermis, there was an overall reduction in the difference among populations through time

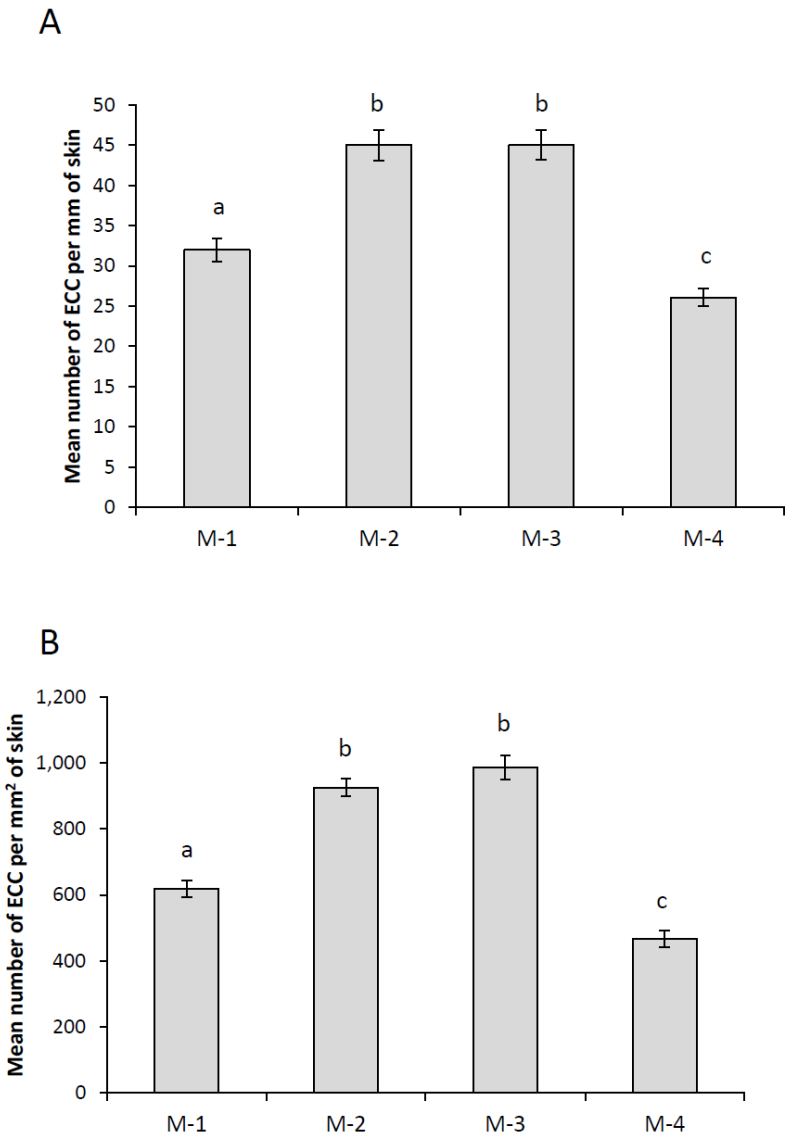

Figure 5-2 – Mean ± S.E. number of EEC per mm of fish skin (A) and density of ECCs (B) for fathead minnows collected from each of the four Marshy Creek locations (N=40-50 per location). Different letters indicate significant differences at $\alpha<0.05$.

(Levene's test: $F_{1,193} = 18.9$, $P<0.001$, Total $Var_{Day\ 1} = 185$; Total $Var_{Day\ 28} = 46$). By the end of the experiment, we found that there were still significant differences among the three groups ($F_{2,9} = 8.4$, $P=0.009$). The two Marshy Creek populations converged to a similar number of ECCs, but the PL group still had fewer ECCs (Tukey tests: $P=0.009$). If we consider ECC density, we find an overall difference at the end of the study ($F_{2,9} = 8.6$, $P=0.008$). Again both Marshy Creek sample locations showed similar ECC densities, but the Pike Lake population differed from both of the Marshy Creek sites ($P=0.011$). It is very interesting to observe that there is less difference between the three groups at the end of the experiment than at the beginning of the experiment when we compare the number of ECCs per mm of epidermis. Looking at the range on figure 5.3, we see that three groups appear to be converging towards a similar value. In stark contrast, when we compare the density of cells between the three groups at the beginning and the end of the experiment, we find that the groups appear to be diverging rather than converging (Levene's test: $F_{1,193} = 6.06$, $P = 0.015$, Total $Var_{Day\ 1} = 31188$; Total $Var_{Day\ 28} = 82714$).

5.4 DISCUSSION

The results of our field survey revealed surprising differences in ECC numbers both within and between populations of minnows. Minnows captured from four different populations showed a threefold difference in mean number of ECCs per mm of skin between Oscar Creek and Pike Lake. We need to be clear that our goal here was not to investigate potential factors that could influence ECC investment between populations, but there were obvious physico-chemical differences between the waterbodies that could contribute to the variation we observed. There was a

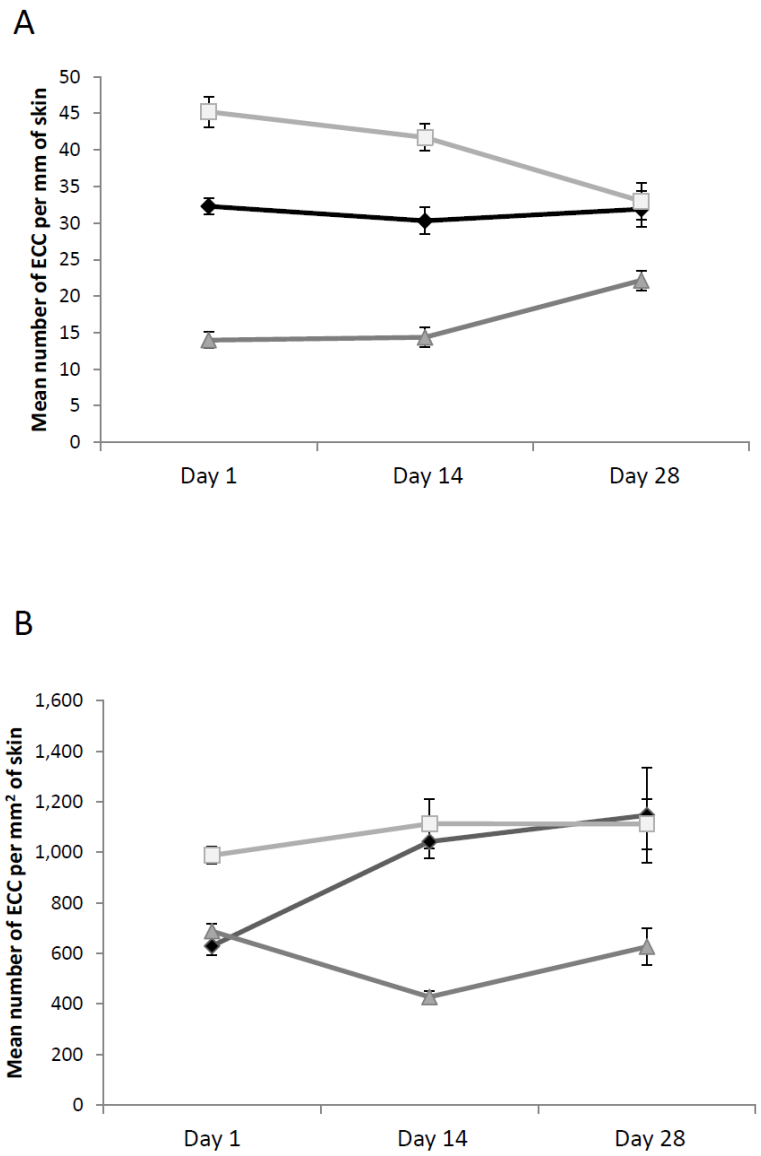

Figure 5-3 – Mean ± S.E. number of EEC per mm of fish skin (A) and density of ECCs (B) for fathead minnows collected from Pike Lake (dark gray triangles) and Marshy Creek-1 (black diamonds) and Marshy Creek-3 (grey squares). The graph shows number of ECCs at three points in time (days 1, 14 and 28) after the initiation of the laboratory experiment (N=10 fish per tank x 5 tanks for each location).

considerable difference in temperature, but this variable is likely related to the specific weather conditions on the day of collection. We also noted a rather large variation in salinity and conductivity. Other factors that we did not quantify are also likely of considerable importance. For example, in wetlands and lakes in Saskatchewan, dissolved organic carbon (DOC) is known to range from 4.1 to156.2 mg/L (Arts et al. 2000). DOC reduces penetration of UV radiation, and according to the results of Manek et al. (2012), this differential UV exposure should lead to differences in ECC investment. The other notable factor that could contribute to differential ECC investment is differential levels of pathogens and parasites. We observed both within- and between-population differences in levels of blackspot disease. Minnows are known to increase ECC number in response to trematode infection (Chivers et al. 2007, James et al. 2009), hence this could contribute to the variation we observed. Likewise, different levels of food resources could lead to variation (Wisenden and Smith 1997).

More surprising than the between population differences in ECC numbers was the considerable within-population differences. We observed approximately twice the number of ECCs in one of the Marshy Creek sites than in one of the other Marshy Creek sites. This seems like a large difference given that different shoals of minnows were collected within a 12 km stretch of the creek. Physico-chemical characteristics within the creek may be less variable than between the creek and the other waterbodies. Likewise, there is likely less variation in resource levels within a given site than between sites; hence we should expect to find fewer differences in ECCs within populations than between them. Fishes are known to shoal with individuals of similar size, body condition and parasite load (Krause and Godin 1996). This preferential shoaling may contribute to the considerable variation that we saw between shoals. The site-to-site differences at Marshy

Creek could also reflect variation in snail habitat (source of trematode infection) or availability of perches used by hunting kingfishers (source of parasite eggs).

The results from our laboratory experiment showed that fish from different populations responded differentially to our standard laboratory conditions. We observed that minnows from Pike Lake showed no change in number of ECCs per mm through time while some of the Marshy Creek fish increased their ECCs more than others. Our ability to use standard laboratory conditions as a tool to collapse the differences in ECC parameters gave somewhat mixed results. We found that we could converge ECC number/mm and density within a population (Marshy Creek) but not between populations (Marshy Creek vs. Pike Lake). Through time we observed a substantial convergence in the mean number of ECC/mm and the ECC density between the two Marshy Creek sites. This likely indicates that fish from different sites within Marshy Creek show considerable variation in ECCs when they are subject to different local conditions but they start to converge to the same number and density of ECCs when raised in a common environment. The convergence in ECC density within Marshy Creek meant that both the Marshy Creek sites actually diverged from the Pike Lake fish. It remains unknown whether more time in the laboratory would lead to convergence, however, most studies acclimate fish for less than 28 days prior to initiating experiments. Taken together, our results indicate that future researchers need to use extreme caution when attempting to conduct experiments to elucidate factors responsible for driving variation in ECC numbers. If the fish were to have converged to a similar mean value with little variation, then we could expect to be able to find subtle effects of various experimental manipulations. Given the large differences we observed between populations, we may only expect to identify factors that have large effect sizes.

Previous studies examining ECC parameters have reported ECC density while others have reported differences in numbers of ECCs per mm (Von Frisch 1941, Chivers et al. 2007, James et al. 2009, Wisenden and Smith 1997). Surprisingly, we found a large discrepancy in the conclusions we would draw based on these measurements. For example, if we consider number of ECCs per mm, all four of the populations in our field survey differed from each other, whereas if we considered density of ECCs, then only Feedlot Pond was different from Oscar Creek. Likewise, if we look at the differences between populations through time, there was convergence in mean number of ECC per mm of epidermis while there was divergence in terms of ECC density. Is one of these measures better than the other? This may depend on the research question that is being asked. Perhaps studies done from a predation perspective may want to use one variable while those done from disease perspective may want to report the other. Our work points to the fact that future researchers need to justify their choice of variables. We suggest that epidermal thickness should be of prime importance when justifying whether mean number of ECCs per mm or mean ECC density should be selected. The mean number of ECCs per mm stands strong under conditions where there is no significant difference in epidermal thickness between treatments. Hundreds of studies have examined the importance of chemical alarm cues in mediating behavioural, morphological and life history defences in prey animals (Chivers and Smith 1998, Ferrari et al. 2010). Our results have important implications for this work. We suggest that researchers may be inadvertently introducing more variation into their experiments than they realize. For example, many studies have reported that alarm cues are collected by making several vertical cuts along the flank of the fish and then flushing the skin with water to collect the cues (Ferrari et al. 2011, Commens and Mathis 1999, Lönnstedt et al. 2011). In most of these studies, the researchers use a different donor fish for each replicate. This technique could introduce

considerable variation in the amount of alarm cues used in the experiment. This is critical given that several species of fishes like other prey animals, show threat-sensitive responses to variation in alarm cue concentration (Ferrari et al. 2005, Ferrari et al. 2006). Fish exposed to high levels of alarm cues show very strong responses while fish exposed to lower concentrations show weaker responses. Moreover, fish learn the threat level of predators based on alarm cue concentration present during conditioning (Ferrari et al. 2005, Zhao et al. 2006). Fish exposed to high concentrations of alarm cues paired with unknown predator odour learn the predator as a high risk, while those exposed to low concentrations of alarm cues, learn the predator as a mild threat. Behavioural ecology is ripe with experiments showing that prey animals show sophisticated behavioural responses to slight variation in risk. Our results indicate that the experimental techniques used to induce variation in risk may be substantial. Many other studies use a single solution of homogenized skin to produce alarm cues (Wisenden et al. 2003, Brown et al. 2011). This reduces the variation in alarm cue concentration between replicates in the same experiment. However, we caution that making comparisons between studies is still problematic given the variation that we observed. Another source of variation that was not addressed in our study relates to seasonal variation in ECC investment. Breeding male minnows are known to lose their alarm cues during the breeding season (Smith 1973), but whether other seasonal variation exists remains unknown. Pathogens, parasites and UV radiation, as well as food level, all vary seasonally and hence could drive seasonal differences in ECC number.

Our work has implications not only for those studying behaviour, but also those that examine predator-induced changes in morphology. Such changes are often triggered by exposure to chemical alarm cues; the investment in morphological defences may be directly linked to perceived threat level (Stabell and Lwin 1997, Chivers et al. 2008). We suggest that the large

variation in ECC numbers that we observed provides a source of variation that may be under appreciated by researchers studying morphological change. A similar argument can be made for those researchers that study life history changes induced by alarm cues.

Fathead minnows belong to a large superorder of fish, the Ostariophysi, a group that includes the minnows, characins, catfishes, loaches and suckers. Similar alarm cue systems are also known in other groups of fishes, including the salmonids, cichlids, poeciliids and percids (Mathis 2009, Ferrari et al. 2010). Moreover, alarm cues are known in larval amphibians and numerous taxa of invertebrates. Our cautionary note about within and between population variation in alarm cue concentration likely applies to all of these taxa.

CHAPTER 6

GENERAL DISCUSSION

6.1. Significance of assessing multiple environmental stressors

As a result of anthropogenic activities, there has been extensive loss in biodiversity across various ecosystems (Vitousek 1994, Pereira et al. 2012). Research aimed at examining the combined effects of environmental stressors have started to emerge in order to understand the underlying causes for the loss in biodiversity (Altshuler et al. 2011). It is very unlikely that organisms are exposed to a single environmental stressor and consequently research aimed at identifying the interactive effects of multiple stressors is extremely essential (Boone et al. 2007). It is particularly interesting to determine the combined effects of different stressors when we predict a priori that different stressors could lead to different outcomes. This was the case when I considered the effects of UVR combined with Cd on aquatic organisms.

6.2 Cortisol as an indicator of stress across experiments

A main component of this thesis was to evaluate physiological stress by assessing cortisol levels from the blood of minnows exposed to UVR, waterborne Cd or altered DOC quality (source) and quantity (concentration). I found significant effects of UVR on physiological stress responses (in chapter 2) where minnows exposed to UVR (in the absence of a UVR blocking filter) showed a significant increase in cortisol levels. I also found that an increase in quantity of DOC (with or without UVR) irrespective of the source (LM NOM vs. SAHA), helped maintain low cortisol levels in minnows (in chapter 4). Interestingly, I also found that the presence of Cd (with or without UVR) disrupted the characteristic elevation of cortisol production (endocrine disruption) typically found when minnows were exposed to UVR only (in chapter 3). Even though I found very interesting changes in cortisol levels based on the type of environmental stressor and

type of exposure, the baseline levels of cortisol that I found across all these studies were highly variable (Table 6.1). Experiment 2 has been sub- divided into 2A and 2B to provide cortisol values obtained by using two euthanizing agents (2A-MS222, 2B-Aquacalm).

I attribute the variation in baseline cortisol levels across these experiments to a variety of causes. The primary cause is likely the type of euthanizing agent I used. Initially I used MS222 to euthanize minnows for assessing cortisol and ECC investment in experiment 1. However, when I used Aquacalm in experiment 2, I did find that Aquacalm played a significant role in lowering baseline cortisol levels in minnows exposed to UVR and/or Cd. This observation is strongly supported by the work of Dusan et al. (2006). Interestingly, in experiment 3 (Chapter 4), I found that the baseline cortisol levels were further lowered in minnows exposed to LM NOM, SAHA or DCW despite of using the same euthanizing agent- Aquacalm.

I have to take into account that each experiment was performed in a different year (one experiment per year from 2009-2012) using fathead minnows that were wild caught from the same collection site (Feedlot Pond). Even though minnows were acclimated for at least a month in the laboratory under controlled laboratory conditions to lower intra-population variation in ECC investment (Manek et al. 2013), I could not control for factors such as water chemistry at the collection site, time of catch, pre-existing pathogenicity and/or age of fish prior to collection. Extensive studies evaluating effects of environmental stressors across species ontogeny have shown that life stage is a very significant factor, where early life stages such as embryos, larvae and early juveniles can be relatively more sensitive to environmental stressors compared to adults (Wendelaar Bonga 1997). In the three studies where I measured cortisol (chapter 2, 3 and 4), I used wild caught fathead

Table 6-1 – Mean ± S.E. cortisol levels (in ng/ml) found in fathead minnows exposed to multiple environmental stressors in the laboratory.

Treatment	**Experiment 1 (MS222)**	**Experiment 2A (MS222)**	**Experiment 2B (Aquacalm)**	**Experiment 3 (Aquacalm)**
high UVR	311.046 ± 44.7	267.118 ± 32.61	70.60 ± 13.01	29.36 ± 2.74
low UVR	68.74 ± 13.5	81.08 ± 28.24	15.36 ± 11.25	2.25 ± 0.43
Control group (Aquaria minnows)	34.89 ± 23.1	N/A*	N/A*	N/A*
high UVR + Cd	N/A*	108.036 ± 32.61	48.49 ± 13.01	N/A*
low UVR + Cd	N/A*	29.14 ± 12.61	6.95 ± 13.01	N/A*
14 day control group	N/A*	40.66 ± 12.61	10.89 ± 13.00	N/A*
14 day Cd exposed group	N/A*	5.14 ± 2.61	2.02 ± 1.31	N/A*
high UVR + SAHA	----------	----------	----------	0.76 ± 0.24
low UVR + SAHA	----------	----------	----------	5.86 ± 2.73
high UVR + LM NOM	----------	----------	----------	3.60 ± 0.32
low UVR + LM NOM	----------	----------	----------	7.40 ± 2.73

*- not available

minnows for my research. A typical life span of minnows ranges from 1-3 years. I did not know the exact age of fish prior to collection.

I did not assess any difference in baseline cortisol levels before and after exposure to UVR, Cd or DOC in any of the above experiments because the quantity of blood required precluded this approach. Moreover, I needed to avoid any epidermal damage and potential handling associated stress which could have masked the effects of the environmental stressors that I was ultimately interested in evaluating.

6.3. Significance of the laboratory study

The primary goal of this research was to understand how multiple environmental stressors play a role in altering ECC investment associated with immune function and chemical signalling. Karl Von Frisch came up with the term "Schreckstoff" in late 1930's to describe a chemical we currently refer to "alarm substance" which is known to play a significant role in eliciting anti-predator responses across a wide range of taxa including gastropods, echinoderms, amphibians and fishes (Ferrari et al. 2010). These cues also alter life history and change morphology (Ferrari et al. 2010). I wanted to test if environmental stressors alter the potency of alarm cues by studying changes in ECC investment. My research strongly supports the idea that the primary goal of ECCs is not to elicit anti-predator responses through the production and maintenance of alarm cues. A message to all evolutionary ecologists is that multiple environmental stressors such as UVR and/or Cd do not change the potency of high concentration (1 cm^2 in 40 L) of chemical alarm cues from the skin of fathead minnows. I did not find any significant difference in antipredator response to alarm cues from the skin of UVR and/or Cd exposed minnows.

6.4. Significance of the field study

Numerous studies that have examined factors that influence ECC investment have often been hampered by large variation in baseline levels of ECCs. The larger the baseline variation in ECC number, the more difficult it is to elucidate factors responsible for changes in ECC investment. While I did not find this problematic in my work with UVR, Cd and DOC, others have failed to find effects in manipulative experiments. I found some evidence that I could reduce within population variation in ECC investment through time, but could not reduce among-population variation in mean ECC investment. Given the large variation I observed in wild fish and my limited ability to converge mean ECC numbers by holding fathead minnows under standard laboratory conditions, I caution that future studies may be hard pressed to find subtle effects of various experimental manipulations.

Another significant point highlighted through this study was the significance of appropriate usage of ECC parameters. Previous studies examining ECC parameters have reported ECC density while others have reported differences in numbers of ECCs per mm (Von Frisch 1941, Chivers et al. 2007, James et al. 2009, Wisenden and Smith 1997). Surprisingly, I found a large discrepancy in the conclusions one would draw based on these measurements. For example, if I consider the number of ECCs per mm, all four of the populations in our field survey differed from each other, whereas if I considered the density of ECCs, then only Feedlot Pond was different from Oscar Creek. Likewise, if I look at the differences between populations through time, there was convergence in mean number of ECC per mm of epidermis while there was divergence in terms of ECC density. Is one of these measures better than the other? This may primarily depend on type of research question that is being asked. Perhaps studies done from a predation perspective may want to use one variable while those done from disease perspective may want to report the other. My

work points to the fact that future researchers need to justify their choice of variables. My work has implications not only for those studying behaviour, but also those that examine predator-induced changes in morphology. Such changes are often triggered by exposure to chemical alarm cues; the investment in morphological defences may be directly linked to perceived threat level (Stabell and Lwin 1997, Chivers et al. 2008). I suggest that the large variation in ECC numbers that was observed provides a source of variation that may be under appreciated by researchers studying morphological change. A similar argument can be made for those researchers that study life history changes induced by alarm cues.

6.5. Future directions

Fish often seek shade. Such behaviour in salmonids has been thought to be principally a predator avoidance response (Kelly and Bothwell 2002). Likewise, juvenile perch (*Percha fluviatilis L.)*, prefer structured instead of open water habitats when exposed to predation (Snickars et al. 2004). Could such shade seeking be driven by only by predation? Given the negative effects of UVR that I documented, UVR avoidance seems like a logical choice for fish. This may be a particularly interesting line of research given that UVR is known to influence habitat preference under predation risk in the three spined stickleback (*Gasterosteus aculeatus*) (Rick and Bakker 2010).

My research has evaluated the effects of multiple environmental stressors on one aquatic prey species-fathead minnows. It would be of great importance and considerable relevance if a keystone predator or alternate prey species is used to evaluate the aforementioned endpoints addressing a causal link between environmental stressors and chemical ecology of predator-prey interactions. It is unknown if the effects of UVR, Cd and altered DOC source and concentration that I found in the laboratory under controlled environmental conditions would be strongly

reproducible in the field. A comparative field study would give a realistic comparison of the effects of UVR, Cd and DOC on ECC investment, physiological stress response and anti-predator response in fathead minnows.

For more than 70 years, ecologists have been focussed on the alarm function of the Ostariophysan ECC. There are numerous studies showing that fishes change their behaviour and morphology in response to cues released from injured conspecifics (Smith 1992, Chivers and Smith 1998). Recent research provided evidence that the immune function of ECCs. Chivers et al. (2007) tested the anti-parasitic/anti-pathogen hypothesis suggesting that ECCs play a role in immune responses. James et al. (2009) provided contradicting evidence for this hypothesis. However, they suggested a number of possibilities that may be the reason for their observations. Their work underscored the significance of causes such as exposure to novel pathogens or parasites, duration of exposure, pre-existing body condition of fish and host age which may play an important role in altering the immune response to an immunosuppressant such as pathogenicity. It would be appropriate to suggest that ECCs of ostariophysan fishes such as fathead minnows are an integral part of a complex response system that counteracts a variety of biotic (pathogenicity) and abiotic (UVR, Cd) immunosuppressants. I can conclude that ECCs play a primary role in immune responses and the production of alarm cues for anti-predator responses is a secondary function.

CHAPTER 7

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APPENDICES

Appendix A - Periodic Acid Schiff's Reagent (PAS) Staining Protocol

Prepare solutions and stains prior to stain procedure (see Appendix B). Each step is conducted in a separate bath in an individual container. All steps should be conducted in a fumehood.

Step	Duration	Contents of bath
1.	3 minutes	Xylene
2.	3 minutes	Xylene
3.	1 minute	Xylene/Absolute ethanol (50%/50%)
4.	1 minute	100% ethanol
5.	1 minute	100% ethanol
6.	1 minute	95% ethanol
7.	1 minute	Tap water
8.	Rinse	Distilled water
9.	5 minutes	1% periodic acid (3g in 600 mL distilled water)
10.	5 minutes	Running tap water (gentle)
11.	Rinse	Distilled water (really well)
12.	15 minutes	Schiff's Reagent (light sensitive- dish tin foiled on all sides)
13.	10 minutes	Running tap water
14.	Rinse	Distilled water

15.	45 seconds	Harris haematoxylin
16.	Wash	Tap water (several changes, repeatedly, until clear)
17.	2 -3 dips	Acid alcohol (18 mL 1N HCl in 582 mL 95% ethanol)
		(time will vary depending on how blue you want the nuclear material to stain – acid alcohol pulls the blue out)
18.	Wash	Tap water in dish (repeatedly, several changes)
19.	15 seconds	Saturated aqueous lithium carbonate
20.	1 minute	Running tap water
21.	Rinse	Distilled water
22.	1 minute	95% ethanol and 100 % (50 % / 50 %)
23.	1 minute	100% ethanol
24.	1 minute	100% ethanol
25.	1 minute	100% ethanol / xylene (50% / 50%)
26.	1 minute	Xylene
27.	1 minute	Xylene

Remove slides from xylene bath and let dry in fume hood for several hours.

Appendix B – Stain Recipes

Harris Haematoxylin (500 mL)

50 g	Potassium alum
500 mL	Distilled water
2.5 g	Haematoxylin
25 mL	100% ethanol
0.5 g	Mercuric oxide
20 mL	Glacial acetic acid

1. Dissolve the alum in warm (47°C) distilled water in a 2 L glass flask.
2. Dissolve the haematoxylin in the 100% ethanol then add it to the alum solution.
3. Rapidly bring the mixture to a boil and slowly and carefully add the mercuric oxide.
4. Rapidly cool the stain by plunging it into an ice water bath.
5. Once cool, add the acetic acid and the stain is ready for immediate use.

This stain will last a few months, but the best results if it is made fresh every month.

Schiff's Reagent (600 mL)

3 g	Basic fuchsin (Pararosaniline hydrochloride)
600 mL	Distilled water
90 mL	Hydrochloric acid (1N)
9 g	Potassium metabisulphite
3 g	Activated powdered charcoal.

1. Dissolve 3 g of basic fuchsin in 600 mL of boiling distilled water in a 2 L glass flask.
2. Remove heat, and stir for 5 minutes.
3. Cool to 50° C, filter and add 90 mL of 1 N hydrochloric acid.
4. Cool to 25° C, and add 9 g of potassium metabisulphite and stir.
5. Store in the dark at room temperature (21° C in tin foiled bottle) for 24 hours.
6. Add 3 g of activated powdered charcoal. Shake for 1 minute.
7. Filter to remove the charcoal and store the solution in the dark (tin foiled bottle) at 1-4°C.
8. Filtrate should be colourless. Use at room temperature. As long as the solution remains colourless it can be used. Discard when a pinkish colour develops.

Periodic Acid (600 mL)

Periodic acid	3 g
Distilled water	600 mL

Discard if it goes brown.

Acid Alcohol

95% alcohol	582 mL
Hydrochloric acid	18 mL

Saturated Lithium Carbonate

Lithium Carbonate	1.2 g
Distilled water	600 mL

1N Hydrochloric Acid (dilute)

Distilled water	92 mL
Concentrated HCl	80 m