**Is the epidermal club cell part of the innate immune system in fathead minnows?**

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## Abstract

Fishes in the superorder Ostariophysi, including fathead minnows (*Pimephales promelas*), possess specialized epidermal club cells that contain an alarm substance. Damage to these cells, as would occur during a predator attack, causes the release of the alarm substance and can indicate the presence of actively foraging predators to nearby conspecifics. For nearly 70 years, research involving epidermal club cells has focused on the alarm substance and the role it plays in predator/prey interactions. However, recent studies have indicated that there may be a connection between epidermal club cells and the fish immune system. Fish increase investment in epidermal club cells upon exposure to skin penetrating pathogens and parasites. In this study I tested for differences in epidermal club cell investment by fathead minnows exposed to the immunosuppressive effects of the glucocorticoid hormone cortisol. In experiment 1, fathead minnows were exposed to either a single intraperitoneal injection of corn oil or no injection at all. The purpose of this experiment was to determine whether corn oil, the vehicle for cortisol injections in later experiments, had an effect on epidermal club cell density. The treatments had no effect on epidermal club cell size, cell area, or epidermal thickness. In experiment 2, skin extract was prepared from the skin of corn oil injected and non injected fathead minnows as in experiment 1 to determine whether corn oil had an effect on the epidermal club cell alarm substance concentration. The treatments showed no significant differences in observed anti-predator behaviour, including change in shelter use, dashing and freezing. In experiment 3, fathead minnows were exposed to either a single intraperitoneal injection of cortisol or corn oil. The purpose of this experiment was to determine whether cortisol, a known immunosuppressant, had an effect on epidermal

club cell investment. Fathead minnows exposed to a single cortisol injection had significantly reduced respiratory burst activity of kidney phagocytes indicating that there was suppression of the innate immune system. Furthermore, cortisol treated fathead minnows showed significantly lower numbers of epidermal club cells. The treatments had no effect on individual epidermal club cell area, epidermal thickness and serum cortisol levels after 12 days. The results from this experiment suggest that pharmacological cortisol injections in fathead minnows have a suppressive effect on the fish innate immune system. Furthermore, the findings that cortisol induced immunosuppression also influences epidermal club cell investment provides support for the hypothesis that epidermal club cells may function as part of the fish immune system.

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# Chapter 1: Introduction

## 1.1 Background Information:

Ostariophysian fish make up the second largest superorder of fish. This diverse group contains nearly 75% of all freshwater fish species and over 25% of all known fish species in this world (Moyle and Cech 2004). The major characteristic of all Ostariophysian fish is that they contain specialized cells in their epidermis which differ from the epidermal cells found in most fish species (Pfeiffer 1977). Damage to the cells during an attack by a predator causes the release of the cell contents. Nearby conspecifics (fish of the same species) which recognise the content of these cells often exhibit anti-predator behaviours (Smith 1982). For this reason, researchers (Smith 1982, Chivers and Smith 1998, Pollock and Chivers 2004, and Ferrari et al. 2005) have often referred to the contents of epidermal club cell as alarm cues (or alarm substance, fear substance, Schreckstoff and damage release cue) and the cells releasing them as alarm cells (or alarm substance cells and epidermal club cells). The superorder Ostariophysi includes groups of fish such as minnows, catfish, knifefish and carp.

Considerable research has investigated the role of chemical alarm cues in mediating predator/prey interactions (Smith 1982, Chivers and Smith 1998, and Ferrari et al. 2005). For example, the recognition of the alarm cue reduces the risk of predation by eliciting an anti-predator fright response, thus increasing the fitness. This fright response may include behavioural changes such as freezing, dashing, area avoidance, increased shelter use and/or increased shoaling which decrease the chances of falling victim to a potential predator attack (Chivers and Smith 1998). During experimental predation trials evidence suggests that prey survival time does, in fact, increase

significantly when subjects are pre-warned with alarm substance (Mathis and Smith 1993b, and Mirza and Chivers 2000). Similarly, studies have shown that sympatric heterospecifics (different species co-occuring in the same area) can learn to recognize alarm cues and exhibit anti-predator behaviour as well (Pollock and Chivers, 2004). Recent experiments demonstrate that Ostariophysians show varying intensities of anti-predator response depending on the concentration of the conspecific alarm cues (Ferrari et al. 2005, and Zhao and Chivers 2005). Ferrari et al. (2005) showed that higher alarm cue concentrations elicited a greater anti-predator response than lower alarm cue concentrations suggesting that concentration was indicative of predation threat. These results provide evidence of the usefulness of behavioural bioassays in determining potential changes in epidermal club cell (ECC) alarm substance concentration. In addition to warning prey of a predation event, alarm cues also function in predator recognition by labelling the predators and their territories as a threat (Mathis and Smith 1993a).

Although mucous cells are found in conjunction with club cells in the epidermis of Ostariophysian fish (Figure 1.1) they can be distinguished histologically from each other by a differing reaction to periodic acid Schiff's (PAS) reagent (Smith & Murphy 1974). PAS stains for the presence of polysaccharides, mucopolysaccharides and basement membranes in tissue samples. Mucous cells are normally PAS-positive (dark coloured due to the presence of mucopolysaccarides), are located more superficial in the epidermis than the club cells (Smith & Murphy 1974), and release their contents through a pore connected to the skin's surface (Smith 1982). Epidermal club cells, on the other hand, are PAS-negative (light coloured) and are characterized by a round, centrally

located, hematoxylin stained nucleus (Figure 1.1). Epidermal club cells are located outside the dermal scales and tend to be localized towards the basal zone of the epidermis; they have not been observed to reach the outer layer of the epidermis, unless mechanical damage has occurred.

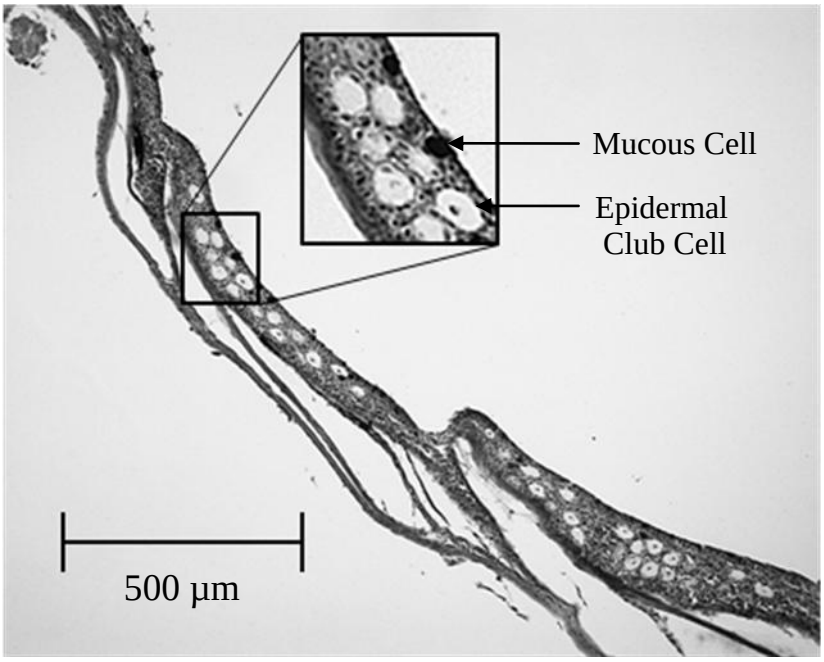


**Figure 1.1:** A cross section of fathead minnow skin showing the epidermal club cell (ECC) with a stained nucleus and mucous cell indicated by arrows. This section was stained with periodic acid-Schiff's reagent (PAS), and then counterstained with haematoxylin (H). Club cells are PAS-H negative and appear white with dark central nuclei, while mucous cells are generally PAS-H positive and appear dark.

### 1.2 Evolution of Epidermal Club Cells and Content:

The evolution of the ECC content in predator-prey systems remains ambiguous in behavioural ecology. The ECC chemical content is clearly beneficial to the recipient as it warns prey of a potential predation threat, but is produced at a cost to the sender as damage or possibly death has occurred. How could a signalling system that requires damage, in many cases death, be maintained through the evolutionary history of the Ostariophysians? Smith (1986) proposed kin selection as a possible hypothesis for the

evolution of Ostariophysian alarm cues, where the sender increases its inclusive fitness by warning kin of nearby predation threats. Another evolutionary hypothesis that has been postulated is that the chemical substance acts as a secondary predator attractant, as studies have shown that predators can cue in on these alarm cues attracting them to the sender (Mathis et al. 1995). Chivers (1996) showed that the presence of a second predator acting on the same prey can create competition, thus indirectly increasing the chances of survival for the targeted prey.

Both of the above hypotheses place primary emphasis on the involvement of alarm cues as a function of predation risk reduction. If the ECC content was selected for in Ostariophysian fish due to its alarm functioning capabilities, manipulation of the apparent risk of predation should result in variation in club cell investment. Hugie (1990) observed that different natural populations of fathead minnows (*Pimephales promelas*) showed varying degrees of alarm cell investment and he postulated that the disparity may be attributed to differing degrees of predation experienced by the various populations. However, Hugie (1990) did not directly quantify predation pressure on the different populations tested. More recently, Hindman (2002) showed that variation in potential predation risk was unable to cause a significant change in ECC investment. The variables of cue concentration, frequency and duration were all addressed in Hindman's experimental investigations. Variation in predator odour and heterospecific alarm cues concentrations also resulted in no significant change in ECC investment (Hindman 2002). Overall, the failure to correlate ECC activity with predation risk in the study by Hindman (2002) raises serious doubts about the strengths of earlier hypotheses (Chivers and Smith 1998) linking evolution of the ECC to the anti-predator signalling function of these cells.

Another hypothesis explaining the evolution of the ECC in fishes is the potential function of ECC contents as an anti-pathogenic/ anti-parasitic agent which protects individuals from infection following damage to the epidermal skin layer (Smith 1982, Magurran et al. 1996). Michalak (2006) and Chivers et al. (2007) directly investigated other possible selective forces behind the natural variation in club cell investment by exposing fish to various pathogen and parasite treatments. Specifically, fathead minnows where exposed to infection via cysts of common water molds (*Saprolegnia ferax* and *Saprolegnia parasitica*) and infection via larval trematode (*Uvulifer ambloplites).* Skin samples from infected individual fish were examined histologically for changes in ECC investment. The results showed that there was a significant increase in ECC numbers for fathead minnows exposed to pathogenic molds. Furthermore, the change in investment was not graded, but rather was triggered when a specific zoospore threshold had been reached. Similar increases in ECC density were observed when the fish were exposed to the larval trematode parasites (Chivers et al. 2007).

Effects of ECC content on pathogenic mold proliferation was also examined in which *S. ferax* colonies were cultured in the presence of various fish skin extracts (with or without ECC) (Michalak 2006). There was a reduction in the density of *S. ferax* colonies on plates when exposed to the skin extracts from fathead minnows, providing evidence towards the anti-pathogenic function of the ECC. The findings of the study by Michalak (2006) suggest that ECC may participate in immune responses following skin damage.

If ECC act as a part of the immune system of fish then ECC investment should be hindered in the presence of an immunosuppressant. An experiment conducted by Chivers

et al. (2007) exposed fathead minnows to the heavy metal cadmium, a potential immunosuppressant in fishes (Sanchez-Dardon et al. 1999), at biologically relevant levels (0.564 µg/L and 5.64 µg/L; Jensen and Bro-Rasmussen 1992) in conjunction with an infection threat by of *S. ferax.* The presence of cadmium resulted in a reduction in ECC investment. These results and those of Michalak (2006) support the proposed hypothesis that ECC content originally evolved in fishes as a part of the immune system to provide an anti-pathogenic/anti-parasitic response to infectious organisms. This suggests that the ECC contents ability to function as an alarm cue in regards to predation pressure, could have arisen as a secondary function over the extensive evolutionary history of these aquatic organisms (Wisenden 2003, Wisenden and Stacey 2005). This would provide the best explanation for the ECC evolutionary paradox, suggesting that ECC's may actually have evolved as part of the fish's innate immune system.

**1.3 Other Factors Affecting Epidermal Club Cell Investment:**

Wisenden and Smith (1998) showed that food availability and shoalmate familiarity influenced ECC investment. After 16 days, fathead minnows fed high rations displayed a higher ECC number than fathead minnows fed low rations. This indicates that club cell investment is energetically expensive and could be highly influenced by accessibility to food. Fathead minnows housed with familiar shoalmates displayed a decrease in club cell number; conversely, being housed with unfamiliar shoalmates resulted in a significant increase in ECC investment when compared to singly housed minnow controls. This was speculated at the time to be a reflection of the anti-predator defence mechanism mediated by school composition. However, another explanation may

be that ECC investment was altered in order to protect the fish from potential exposure to unfamiliar pathogens present on the foreign fish.

Even earlier there was indirect evidence that ECC may be part of the immune system as seen by Smith (1973). During the breeding season male fathead minnows stop shoaling, become extremely territorial and exhibit vigorous contact with abrasive surfaces during spawning site preparation (Smith, 1973). However, this epidermal damaging behaviour does not seem to trigger an alarm reaction and frighten away prospective female mates and/or attract unwanted predators. Smith (1973) showed that there is a temporary seasonal loss of ECC in males related to the increase in androgen production during the breeding season, thus allowing the male fathead minnows to carry out abrasive breeding behaviour with seemingly no adverse affects. Furthermore, Smith (1974) showed that intraperitoneal injection of 17α- methyltestosterone into both male and female fathead minnows caused a significant decrease in ECC number. More recent field experiments by Pollock et al. (2005) also supports Smith's results, showing that male and female fathead minnows avoided skin extracts prepared from non-breeding male and female minnows; however, they failed to avoid skin extracts from breeding male minnows, and the unknown heterospecific swordtail (*Xiphophorus helleri*) control.

These results suggest that ECC proliferation in fish may be under hormonal control. However, the mechanism by which testosterone inhibits ECC activity in fathead minnows was not studied by Smith (1974) and Pollock et al. (2005). Testosterone is well known to have a general immunosuppressive function in vertebrates, including fish (Cuesta et al. 2007). The observations by Smith (1974) and Pollock et al. (2005) of reduced ECC activity during periods of high testosterone levels in male fatheads might be

explained as a generalized suppression of the immune system during the breeding period. This also leads to the possibility that other hormonal modulators of the vertebrate immune system may alter ECC activity in fish. One of the well known modulators of the immune system in vertebrates is the stress hormone cortisol (or corticosterone, depending on the species) secreted by the adrenal gland (Sapolsky et al. 2000). However, the effect of cortisol on the ECC has not been studied and further research is needed to confirm the possibility that hormone regulators of the immune system also alter ECC activity in teleosts.

**1.4 Stress and Endogenous Cortisol Production in Fish:**

Stress in an unavoidable aspect for all living vertebrates and is defined as a condition that disrupts the dynamic equilibrium of an organism's normal homeostasis due to the actions of some intrinsic or extrinsic stimuli (Wendelaar Bonga 1997, and Chrousos and Gold 1992). As a result of stress, these organisms elicit a select set of compensatory behaviours and physiological responses to overcome the potential threat/harm of the stressor. One such physiological response is the release of cortisol. Cortisol is a glucocorticoid hormone responsible for the primary response to stress in teleost fish. Flik et al. (2006) exposed carp (*Cyprinus carpio)* to a sudden drop in water temperature as a stressor and were the first to visualize the activation of the endocrine stress axis in fish with the aid of magnetic resonance imaging (MRI). Initially, stressor-sensing neurons stimulate the hypothalamic nucleus preopticus (NPO), which contain corticotropin releasing hormone (CRH) producing cells. These cells are neuroendocrine cells that act directly on the pituitary *pars distalis* of the anterior pituitary, in a paracrine fashion, through the localized release of CRH. CRH interacts with adrenocorticotropic

hormone (ACTH) producing cells that are located in the *pars distalis*. Upon stimulation of the *pars distalis* by CRH, ACTH is released into the blood stream where it then travels to the headkidney and stimulates the interrenal tissue to secrete cortisol into the circulatory system (Donaldson, 1981). Cortisol's function in fish is highly diverse. Such activities 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 (Sapolskey et al. 2000).

**1.5 The Fish Immune System:**

A functioning immune system is essential to combat disease and infection and maintain the survival of all living vertebrates. The immune system in teleost fishes is composed of both specific and innate anti-pathogenic mechanisms (Ellis, 1989). Non-specific, innate immune mechanisms include the skin, scales, and mucus which provide a protective outer barrier. The innate immune system is also composed of polymorphonuclear leukocytes (neutrophils, basophils, and eosinophils), monocytes and macrophages which provide internal non-specific protection. Pathogenic agents that breech these barriers and enter the organism initiate an innate immune response. However, if the innate immune system is unsuccessful in preventing the establishment of the pathogenic agent, then the host relies on specific immune mechanisms to eliminate the invasive agent. Such specific immune mechanisms include antibodies and

lymphocytes (T and B cells) which function in recognizing the specific harmful foreign bodies within the organism and eliminating them.

Phagocytes are part of the innate or non-specific immune system and function in limiting dissemination of pathogens (Ellis, 1989). They lack extreme specificity and are generally the first cells involved in a cell-mediated host immunoresponse to pathogens breeching the protective epidermal layer. When a phagocytic cell comes into contact with a foreign body via receptor mediation or hydrophobic membrane interactions, the cell engulfs the particle through endocytosis forming an internalized vesicle called a phagosome. These phagosomes are acted upon by internal cellular lysosomes which fuse to the phagosome forming a phagolysosome. After formation of the phagolysosome there is a noticeable increase in oxygen consumption by the phagocyte. The increased oxygen within the phagocyte is acted upon by respiratory burst oxidase forming superoxide anions ($O_2^-$). These superoxide anions are then converted into a number of different reactive oxygen species (ROS), such as, hydrogen peroxide, hydrogen radical, hypochlorous acid and peroxynitrite (Neumann et al., 2001). All of these products are extremely reactive with organic molecules and result in a catabolic effect on the engulfed particle. This is commonly referred to as respiratory burst activity (Scott and Klesius 1981). ROS in the presence of luminol initiates a chemical reaction that produces a chemiluminescence response as light photons are emitted from luminol. These light photons are quantifiable and allow for an indirect means of assessing immune system and phagocytic activity in vertebrates (Scott and Klesius 1981).

## 1.6 Research Objectives

The overall goal of my research was to test the hypothesis that the ECC may have a primary role in the innate immune system of teleost fish. In my thesis, fathead minnows were exposed to elevated levels of cortisol, via an intraperitoneal injection, for a prolonged period of time which should suppress their immune system activity. In teleosts, cortisol has been shown to specifically decrease the phagocytic activity of leukocytes which are cells responsible for defending the fish against infectious disease and foreign materials (Ainsworth et al. 1991, Wendelaar Bonga 1997, Hironobu et al. 2002, Esteban et al. 2004). Immunosuppression following cortisol injection in fathead minnows was quantified by measuring the amount of chemiluminescence released from the respiratory bursts of active phagocytes from both kidneys as outlined in Hutchinson and Manning (1996) and Scott and Klesius (1981). After quantifying immune system suppression, a small skin section was removed from behind the fish operculum for histological analysis of ECC number. It is predicted that prolonged exposure to cortisol should decrease the respiratory burst activity of the phagocytes in fathead minnows. Given this reduced innate immune function, it was hypothesized that ECC density will also decrease in the presence of prolonged exposure to cortisol. Significant results from these experiments showing that cortisol does decrease the immune system activity and ECC number would provide strong evidence that the ECC play a primary role in the immune system of the Ostariophysians and that the function of the ECC in releasing an alarm substance may have evolved secondarily.

## Chapter 2: Materials and Methods:

My MSc. research consists of three experiments. Firstly, I exposed fathead minnows (*Pimephales promelas*) to either an intraperitoneal injection of corn oil or a control non-injection in order to determine if corn oil has any effect on ECC investment. Corn oil was used as a cortisol injection vehicle in experiment 3 and there is currently no published studies indicating whether or not corn oil has an effect on ECC investment in fathead minnows. After fish had been exposed to single corn oil injection for 12 days, skin extracts from the fish were removed for histological analysis and the density of ECC was determined. The second experiment was a behavioural bioassay which required skin extract from the treated and non-treated fish in experiment one. The skin extract from each treatment was diluted to three different concentrations and exposed to individual test fish. The test fish were observed for changes in behavioural responses. These results allowed a determination of whether there were any changes in ECC substance concentration upon injection with corn oil. In the third experiment, I attempted to suppress the immune system of fathead minnows with an intraperitoneal injection of a pharmacological dose of cortisol, a well known immunosuppressant in fish. After the test subjects had received a single cortisol injection, kidney phagocytes were isolated 12 days later and tested for respiratory burst activity. Furthermore, blood samples were taken at the end of 12 days and the final blood cortisol levels were determined. Following the immune assay and blood removal, skin extracts from the fish were removed for histological analysis and the ECC density was calculated. These results will allow a determination of whether or not ECC change with respect to immunosuppression.

## 2.1. Basic Experimental Protocol

### 2.1.1 Fish Collection and Maintenance:

Fathead minnows used in all experiments were collected using Gee's improved minnow traps from Feedlot Pond, located just north of the University of Saskatchewan campus. Feedlot Pond is a water reservoir used for the irrigation of crops and is used as a water source for research cattle. The fathead minnows used in experiment 1 were captured in December 2007 and were housed in a 360 L flow through stream aquarium containing dechlorinated tap water. The minnows used for experiments 2 and 3 were captured in May 2007 but all breeding males were not used in the experiments. Breeding males were easily identified by tubercles on their snouts and thickened dorsal mucous secreting pads behind their head (McMillian and Smith 1974). The fish from experiments 2 and 3 were housed in together in a 1600 L flow through stream aquarium containing decholrinated tap water. Both aquariums were maintained at approximately 17°C. A 10L:14D photoperiod in the laboratory was used to prevent the onset of breeding condition. Fish were fed commercial flake food *ad libitum* until the start of the experiments.

### 2.1.2 Kidney Phagocyte Isolation:

The lymphatic system is a major component of the immune system in all vertebrates. The pronephros or anterior head kidney of teleost fish is considered to contain the majority of lymphatic tissue and was therefore the target tissue for immunological studies in teleost fish (Scott and Klesius 1981). Due to the relatively small size (2.3 g average) of fathead minnows used in the present study, it was not possible to isolate the anterior kidney accurately in fathead minnows. Therefore, the entire fathead

minnow kidney was used in subsequent experiments to ensure sufficient lymphatic tissue was obtained.

Techniques used to isolate fathead minnow phagocytes were modified from those described by Hutchinson and Manning (1996). Individual whole kidneys were removed by dissection and pooled with kidneys from other fathead minnows housed in the same aquarium. Generally, kidney tissue from 3-7 minnows were required to produce sufficient material for experiments. Each pool of tissue was considered to be an independent sample (i.e. n=1). Tissue was suspended in 5 mL of Hanks Balanced Salt Solution complete without phenol red (Appendix 1). The kidney tissue was place in a manual glass Broek homogenizer, gently oscillated up and down 9 times and then filtered through a large diameter nylon mesh (200 µm). The resulting cell solution was then transferred to 50 mL plastic tubes and centrifuged on an IEC countertop centrifuge at setting #2 (~ 450 x g) for 8 minutes. The supernatant was discarded. Kidneys cells were washed twice with HBSS without phenol red and then filtered through a second finer nylon mesh (100 µm). Cells collected after these washes were resuspended to a density of 1.0 $x10^6$/mL. Cell viability was determined via trypan blue dye exclusion test using a hemocytometer (Hudson and Hay 1989) and was consistently above 95% viability (Appendix 2). Cells obtained with this technique are considered to be mainly leucocytes as kidney epithelial cells are generally destroyed during homogenization (Hutchinson and Manning 1996).

**2.1.3 Measurement of Respiratory Burst Activity (ROS) in Phagocytes:**

The respiratory burst activity of the fathead minnows phagocytes were assessed using the chemiluminescence (CL) assay method of Hutchinson and Manning (1996). The CL assay method measures the light emitted when luminol is activated by the release

of ROS from phagocytes activated by exposure to serum opsonins (Scott and Klesius 1981). Luminol is added as it will react with the ROS produced during the respiratory burst of the phagocytic event eliciting the release of photons. Luminol is chemically converted from a stable electron state to an excited electron state in the presence of ROS. It is when the excited luminol state returns to the stable electron state that there is an emission of radiation in the form of chemiluminescence. The opsonized zymosan represents the organic foreign particle that will be recognized and destroyed, thus is required to initiate the phagocytic event.

A number of experiments were conducted to determine an appropriate source of serum opsonins for the CL. During preliminary method development, serum from a number of different species were collected, stored at -20°C and tested with fathead minnow phagocytes to determine which serum produced the best results (Appendix 3). The reason for choosing opsonins from another species for use with fathead minnows immune phagocytes was due to the limited size of fathead minnows. The amount of blood required to obtain enough opsonized serum from fathead minnows would have required sacrificing a large numbers of minnows. It was determined that carp *(Cyprinus carpio)* opsonins provide the appropriate phagocytic compatibility with fathead minnow leukocytes (Fig 2.1).

Sexually mature male and female common carp (5 of each sex;body mass greater than 5 kg) were obtained in early June 2006 from local anglers fishing the Long River near the hamlet of Holmfield in southern Manitoba, Canada. After landing, the anglers euthanized the carp by a sharp blow to the head; blood samples were taken almost immediately after euthanasia. Approximately 30 mL of whole blood was obtained from

the caudal vasculature of each fish, placed on wet ice and allowed to clot at 4°C overnight. Serum was separated by low speed centrifugation (1000 x g) of the clotted blood and subsequently stored at -20° C for use in the phagocyte respiratory burst assay.

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) was developed into a stock solution containing 11.1 mg/mL. From this stock solution a 1:100 dilution was produced resulting in a 0.11 mg/mL working solution which was used for the immunological bioassays (Appendix 4). Luminol was stored in the dark at 4°C when not in use.

All CL assays were conducted at room temperature using a liquid scintillation counter (Packard, Tri-Carb 2200CA) functioning as a photon detector. Assays were conducted in 5ml plastic liquid scintillation vials and consisted of $1.0 \times 10^6$ kidney cells suspended in HBSS complete without phenol red, 60 µL of 0.01 mg/mL luminol solution and 240 µL of carp opsonized zymosan suspension.

Initially the kidney cell suspension was added to the plastic liquid scintillation vial, followed by the luminol solution and finally the opsoninzed zymosan suspension. As the liquid scintillation counter counting chamber only holds one vial at a time, a 30 second delay was introduced between the addition of the oponized zymosan to individual vials to ensure activity duration is controlled for. Each pooled sample was tested in quadruplicate along with a control vial to allow for the measurement of background activity. The control vial had 260 µL of Hanks balanced salt solution complete without phenol red (HBSS) substituted in place of the opsoninzed zymosan. The liquid scintillation plastic vials were then immediately placed into the counting chamber of the LSC, the lid closed to prevent any light exposure and counts were taken every 30 seconds for 120 minutes. All CL assays was conducted within one hour following collection of

the minnow kidney tissue. There is a proportional relationship between the number of recorded photon counts and the activity of the isolated immune phagocytes (Scott and Klesius 1981).

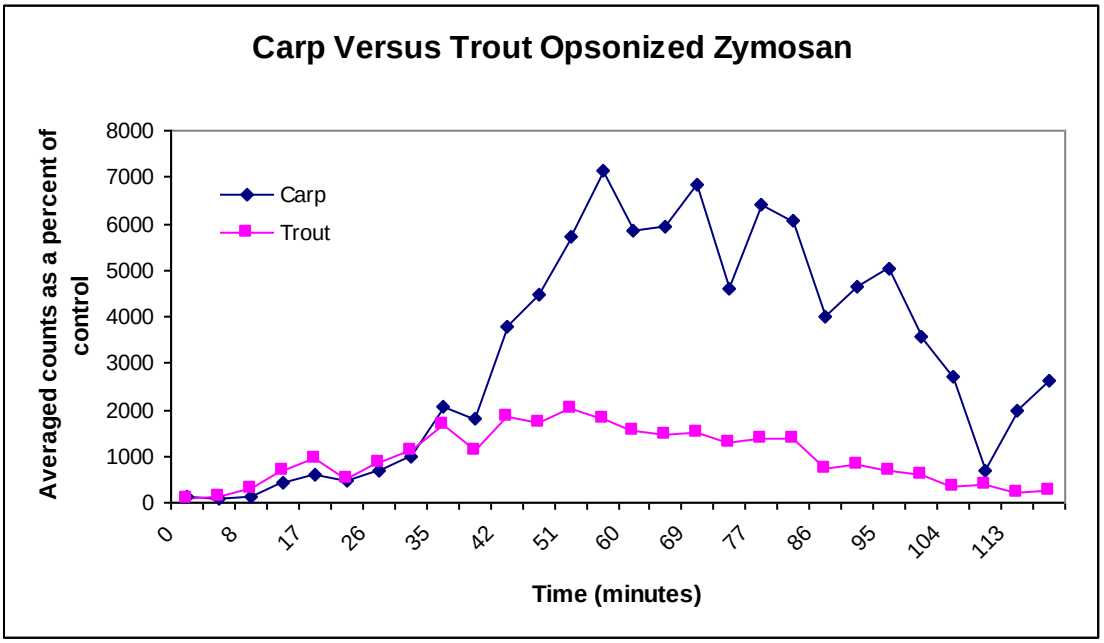


**Figure 2.1:** Averaged duplicated photon counts per 30 seconds of both rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) opsonized zymosan measured as a percent of the control over 2 hours. The control substituted PBS for opsonized zymosan.

### 2.1.4 Preparation of Fish Skin Extract:

Skin extract for the behavioural bioassays was produced from 5-7 fathead minnows. For each skin extract, donor fish were killed with a blow to the head (in accordance with the Canadian Council of Animal Care) and skin fillets were removed, from either side of the body, measured and placed in 40 mL of distilled water. The skin extract was then corrected to 1cm$^2$ of skin per 10 mL and frozen in 15 mL aliquots at -20°C. On the day of the behavioural experiments the frozen skin extract was thawed and diluted to a 1 cm$^2$ of skin per 20 mL stock solution. From the stock solution three

experimental solutions were developed: low (1 $cm^2$ of skin per 240 mL), medium (1 $cm^2$ of skin per 120 mL) and high (1 $cm^2$ of skin per 40 mL) concentration of alarm cues.

### 2.1.5 Histological Preparation of Fish Skin

Fathead minnows were euthanized with MS-222 (methane tricaine sulfonate) at the end of the experiments. The fish were then measured (fork length in mm) and then weighed (in grams). Fish from the same aquarium were placed together in histological jars containing 10% formalin for at least 24 hours before being processed into slides. During preparation, 5 x 8 mm skin sections were taken from each fish posterior to the operculum (Figure 2.2) and stored in histological cassettes in 10 % formalin. Individual skin samples were then embedded into paraffin blocks and sectioned into 5-6 µm thick portions with a microtome blade. Five to seven skin sections from an individual fish sample were placed on a slide and allowed to dry for 24 hours. The sectioned tissues were then stained with periodic acid Schiff's reagent with a Harris's haematoxylin (PAS-H) to darken the mucous cells and the basement membrane (PAS) and nucleus (haematoxylin), leaving the epidermal club cells colourless and easily recognized (Hugie 1990).

Images of each epidermal cross section were captured with an Olympus BX41 microscope with a DVC 1300C colour digital camera at 10 times magnification. Epidermal thickness, number of ECC, area of ECC, number of ECC per mm and number of ECC per $mm^2$ were determined 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/ ). Epidermal thickness was determined by measuring the basement membrane to the outer edge of the epidermis at three different locations on the

image and averaged for the statistical analysis. All quantification and analysis of the skin samples were preformed blind with respect to the experimental treatment.


**Figure 2.2:** Location of epidermal skin section removed from the fathead minnow and used for histological processing.

### 2.1.6 Blood Cortisol Analysis

On day 12 following injection of corn oil or corn oil containing cortisol, fathead minnows were euthanized with MS-222. Blood samples collected from the caudal vasculature with a disposable 26 gauge needle and 1 mL syringe. Blood was allowed to clot on ice for approximately 1 hour before centrifugation at 15,000 x g. Serum was separated from the blood cells and equal amounts of serum (10 µL) from 5 to 7 fish were pooled for subsequent measurement of serum cortisol levels. Analysis of the serum cortisol was performed by the Endocrine Lab at the Western College of Veterinary Medicine (U of S) in a commercial Seimens Immulite 1000 automated chemiluminescent immunoassay system. The Immunlite cortisol assay is a self automated machine in which 10 uL of pooled fathead minnow blood was added to an Immulite test unit containing a polystyrene bead coated with polyclonal rabbit antibody specific for cortisol along with cortisol conjugated to alkaline phosphate (Reimers et al 1996). The unconjugated fathead serum and enzyme labelled cortisol compete for a limited number of antibody binding sites during an incubation period of 30 minutes at 37°C with intermittent shaking. After

the incubation period, the unbound conjugated cortisol is removed via 5 centrifugal wash cycles. Finally, the substrate AMPPD (1,2-dioxetane 3-(2'-spiroadamantane) -4- methoxy - 4 - (3' phosphoryloxy) phenyl - 1,2- dioxetane disodium salt) is added to the test unit and further incubated for 10 minutes. The reaction between and the AMPPD and the alkaline phosphatase on the conjugated cortisol bound to the antibody coated bead that elicits photon emissions. An internal luminometer takes 12 one second readings of the photon emissions and calculates hormone levels relative to a standard displacement curve from known cortisol concentrations provided by Siemens Inc. Serum cortisol values were expressed as ng hormone per mL of pooled serum.

**2.2 Experimental Outline:**

**2.2.1 Experiment 1: The Effect of Corn Oil on Epidermal Club Cell Density**

The objective of this experiment was to determine if intraperitoneal corn oil injections had an effect on epidermal club cell density. One hundred and forty fathead minnows were collected from a 360 L flow through stream aquaria containing dechlorinated tap water and transferred into 74 L glass aquaria and allowed to acclimate for 48 hours. The glass aquaria were equipped with a gravel bottom, an airstone and opaque barriers preventing visual stimuli from neighbouring aquaria. These aquaria were held under the same holding conditions as previously stated (section 2.2.1) and the fish were fed commercial flake food *ad libitum* once a day.

Test subjects were housed in groups of 7 fathead minnows per aquarium with a total of 10 aquaria per treatment with the sample unit being the aquarium itself. On the day of the experiment, aquariums were randomly chosen to receive one of two treatments: corn oil injected treatment (sham) and a non-injected control treatment. In the

injected treatment each of the 7 housed fathead minnows were anesthetised with MS-222 and received a single intraperitoneal injection of 25 uL of corn oil. In the control treatment the 7 housed fathead minnows were anesthetised with MS-222 and returned to their designated aquarium without an intraperitoneal injection. Fish were housed for an additional 12 days under the above mentioned conditions, fed *ad libitum* once a day and daily mortality was recorded.

At the end of the experiment all subjects were euthanized with an overdose of MS-222 and placed in a vial containing 10% formalin. Skin sections (8x5 mm) from each subject was obtained from behind the operculum and stored in 10% formalin for histological preparation. Following the removal of the epidermal skin section histological preparation occurred via embedding, cross sectioning and staining of the tissue samples with PAS-H as described in section 1.1. Microscopic images were captured and analyzed for any significant changes in ECC number, ECC area, epidermal thickness, epidermal area, number of ECC per mm and number of ECC per $mm^2$.

### 2.2.2 Experiment 2: The Effect of Corn Oil on Alarm Substance Concentration

The objective of this experiment was to determine if intraperitoneal corn oil injections had an effect on alarm cue concentration in the ECC via the use of a well-establish behavioural bioassay (Ferrari et al. 2005). Sixty fathead minnows were collected from a 1600 L flow through stream aquarium containing dechlorinated tap water in the R.J.F Smith Centre for Aquatic Research. Behavioural observations were completed in 37 L glass aquaria containing dechlorinated water. Each aquarium was equipped with an airstone and a gravel substrate and maintained under the same conditions as stated above (section 2.2.1). Fathead minnows were acclimated individually in their observation

aquarium for 24 hours prior to testing. The test subjects were visually isolated from neighbouring subjects via an opaque barrier between each aquarium. In the center of the aquarium there was a 4.5 cm² ceramic tile mounted on 2.0 cm high glass legs. Attached to the air hose, there was also a 2 m long injection tube functioning for the introduction of the stimuli from a distance that will allow for a reduction of a potential observer effect (Figure 2.3).

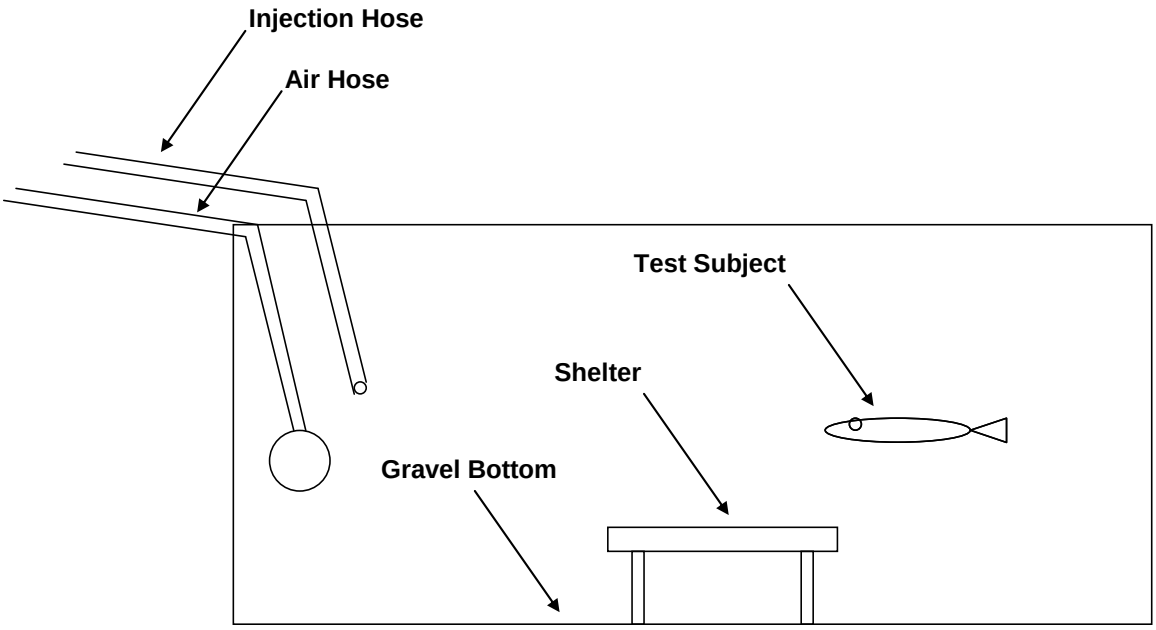


**Figure 2.3:** Schematic diagram of behaviour trial test aquarium for experiment 2

Prior to the start of this experiment, skin extracts were prepared from 4 groups of 7 fathead minnows. Two groups of fathead minnows were anesthetised with MS-222 and injected intraperitoneally with 25 uL of corn oil. The other two groups of fathead minnows were anesthetised but received no intraperitoneal injections. Each group was allowed to recover from the anaesthetic and returned to an individual aquarium. After 12 days, the fish were euthanized with a blunt force to the head and skin extract was

prepared, pooled and place in 15 mL aliquots and frozen at -20°*C* until the start of the bioassay as described above (section 2.1.4).

In order to determine if there is a difference in ECC concentration between the two treatments, the number of ECC per $mm^2$ of skin used to prepare the extract must be equal between the two treatments. Cell number in the two groups was determined by histological analysis (see section 2.1.5). There was no significant difference between the number of ECC per $mm^2$ in either treatment (Sham mean 3.779 ± 0.246 $mm^2$, Control mean 3.451 ± 0.296 $mm^2$). Therefore, the number of cell per $mm^2$ in the skin extract did not have to be controlled for.

On the day of the behavioural assay 15 ml frozen samples of the corn oil injected skin extract and control skin extract were thawed at room temperature. The corn oil injected and control samples were further diluted to a standardized stock solution of 1 $cm^2$ of skin per 20 mL of distilled water. From this stock solution three diluted experiment solutions were developed: low (1 $cm^2$ of skin per 240 L), medium (1 $cm^2$ of skin per 120 L), and high (1 $cm^2$ of skin per 40 L). Ferrari et al. (2005) showed that these concentrations of skin extract cause fathead minnows to display a graded behavioural response.

At the beginning of the behavioural trial, each aquarium was assigned to randomly receive 10 mL of skin extract from one of the 6 treatments. Each trial consisted of an 8 minute pre-stimulus observation period and an 8 minute post-stimulus observation period (Pollock and Chivers, 2004). During each observation period the time spent under the shelter, the occurrence of dashing and freezing were all recorded. Dashing is defined as a rapid burst of aberrant swimming, while freezing is the act of remaining motionless

for a minimum time period of 30 seconds. These three behaviours are known as anti-predator responses and are characteristic of fathead minnows exposed to conspecific or sympatric heterospecific ECC substance (Chivers and Smith, 1998). Prior to the injection of the stimulus, 60 ml of aquarium water was removed and used to flush the injected stimulus from the hose into the aquarium. To take into account any discrepancy in time it took to complete the injection, I defined time zero at completion of the 60 mL flush that followed the stimulus. The difference between these three behavioural characteristics during the pre and post observation periods was accredited to the recognition and reaction to the injected stimulus. The mean change in behaviour elicited by each response was then compared to determine if the corn oil treatment altered the fright response behaviour of the test minnows.

### 2.2.3 Experiment 3: The Effects of Cortisol on Epidermal Club Cell Investment and Immune System Physiology

The objective of this experiment was to pharmacologically suppress the fish immune system with an intraperitoneal cortisol injection and correlate it with a change in ECC density. Several hundred fathead minnows were obtained from a 1600 L flow through stream aquarium containing dechlorinated tap water in the R.J.F. Smith Centre for Aquatic Ecology. Prior to the start of the experiments, fathead minnow were transferred to 74 L aquaria and allowed to acclimate for 48 hours. The glass aquaria were equipped with gravel on the bottom, an airstone and opaque barriers preventing visual stimuli from neighbouring aquariums. These aquariums were held under the same holding conditions as previously stated and the fish were fed commercial flake food *ad libitum* once a day.

Test subjects were housed in groups of 7/8 fathead minnows per aquarium with a total of 21 aquaria per treatment with the sample unit being the aquarium itself. On the first day of the experiment two aquaria were randomly chosen to receive one of two treatments: cortisol injected treatment and corn oil injected control treatment. In the cortisol injected treatment each of the fathead minnows were anesthetised with MS-222, weighed and received an intraperitoneal injection of 10 µL of 0.1µg of cortisol per gram of fish. Preliminary mortality tests showed that 0.2 µg/g of fish resulted in a high mortality rate, whereas, 0.05 µg/g resulted in no mortality. Therefore, 0.1µg/g of fish was chosen as an intermediate test concentration. In the control treatment the fathead minnows were anesthetised with MS-222 then injected intraperitoneally with 10 µL of corn oil per gram of fish and returned to their designated aquaria. On the second day, two more aquaria were randomly chosen to receive one of the two treatments. This was repeated for a total of 9 days. Due to time constraints, the daily treatment of two aquaria over 9 days allowed for the completion of two respiratory burst assay per day at the end of the 12 day treatment period. On the $10^{th}$ and $11^{th}$ days, the remaining aquaria were randomly chosen to receive one of the two injection treatments, 12 aquaria on one day and 12 on the other. The treated subjects were housed for a total of 12 days under the above mentioned conditions, fed *ad libitum* once a day and daily mortality was recorded.

At the end of the experiment, the treated fathead minnows from the first 18 treated aquaria were euthanized, weighed and measured (fork length) on the appropriate days in accordance with U of S animal care protocol (20060100). An abdominal incision posterior to the head was made so the gastrointestinal tract, gonads and swim bladder could be removed. With the aid of fine forceps, the whole kidney was removed and place

in Hank's Balanced Salt Solution (HBSS) complete without phenol red and used for the CL immunoassay. The remaining treated fish were euthanized with an overdose of MS-222, had their blood collected for cortisol analysis (see section 2.1.6) and then were stored in a vial containing 10% formalin at the end of the 12 days. Skin sections (8x5 mm) from each of these subjects was also obtained from behind the operculum and stored in 10% formalin for histological preparation. After all the epidermal sections were prepared, microscopic images were captured and analyzed for any significant changes in ECC number, ECC area, epidermal thickness, epidermal area, number of cells per mm and number of cell per $mm^2$ (Figure 2.4)

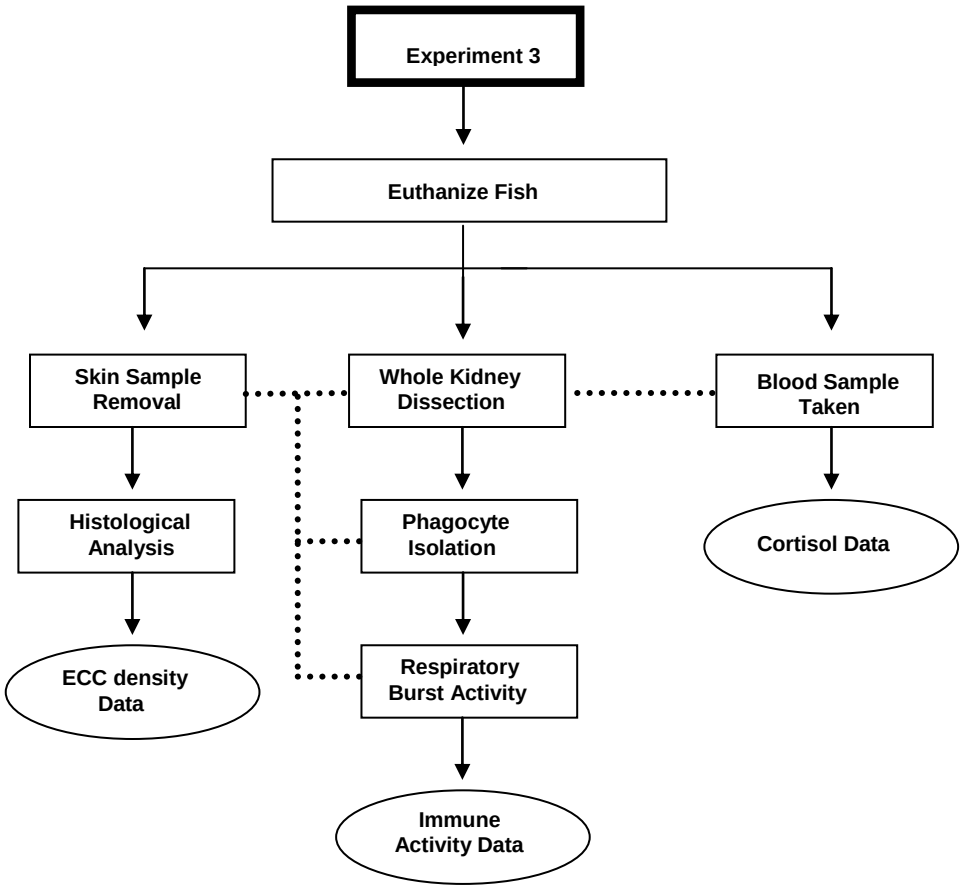


**Figure 2.4:** Flow chart depicting experimental outline. Experiment 3 consists of rearing fish with normal and elevated levels of cortisol, after 12 days data was collected on ECC density, immune activity and serum cortisol levels. Dotted lines indicate procedures that occur immediately after the fish is sacrificed and are under a time constraint.

## 2.3 Statistical analysis:

### 2.3.1 General Statistical analysis

For experiment one and three, all data parameters relating to the epidermis, fish body mass, body condition index, blood cortisol and respiratory burst activity, a Kolmogorov-Smirnov test was conducted to determine whether data were normal. All data was normally distributed and homoscedastic (equal variance), were therefore analyzed using an independent t-test (Zar 1999). All data using these parameters were presented as means ± standard error. For all aquariums experiencing fish mortality a Fisher's exact test was conducted (Zar 1999). Body condition index (BMI) for each minnow as an indication of body condition was calculated using the formula:

$$B.C.I = \frac{\text{mass (g)}}{\text{length (cm)}^3}$$

In experiment two, behavioural data was collected for three different behaviours, change in shelter use (continuous data) and occurrences of dashing and freezing behaviour (binomial data). Each behaviour was analyzed independently of each other. The shelter used data was analyzed using a 2x3 ANOVA comparing treatment, concentration and interaction (Zar 1999). Dashing and freezing behaviour data was analyzed using the Fisher's exact probability test (Zar 1999). Data analysis for all three experiments was carried out using SPSS 15 software.

# Chapter 3: Results

## 3.1 Experiment one: The Effect of Corn Oil on Epidermal Club Cell Investment and Fish Mortality

Statistical analyses were based on the mean histological data obtained from all fathead minnows within each individual aquarium. The treatments were corn oil injected (n=10 aquaria) where individual fathead minnows received a 25 µL intraperitoneal injection of corn oil and the control treatment which fathead minnows recieved received no injection (n=10).

### 3.1.1 Epidermal Club Cell Density and Cell Size

A Kolmogorov-Smirnov test established that the number of ECC per mm of epidermis data was normally distributed (Z=0.508, P=0.958). The variance between all treatments were equal (F=1.355, P=0.111). The results of an independent t-test showed there was no effect of treatment on the number of epidermal club cells per mm from skin of fathead minnows ($t_{18}$=2.811, P=0.111) (Figure 3.1). In order to calculate the average number of club cells per millimetre, I determined the mean number of club cells per millimetre of epidermis for each fish then averaged the values for all fish within each individual aquarium. A Kolmogorov-Smirnov test showed that the mean number of club cells per $mm^2$ of epidermis were normally distributed in both groups (Z=0.568, P=0.904). The variance between all treatments were equal (F=0.401, P=0.535) Furthermore, data from an independent t-test indicated that there was no significant difference in mean number of club cells per $mm^2$ due to treatment ($t_{18}$=0.610, P=0.550) (Figure 3.2). A Kolmogorov-Smirnov test established that the mean individual club cell area ($\mu m^2$) data was distributed normally (Z=0.527, P=0.944). The variance between all treatments were

equal (F=1.058, P=0.317). The mean individual club cell area for the corn oil treatment (16.70 ± 0.49 μm²) and the non injected treatment (17.63 ± 0.75 μm²) was determined from a sample size of n =10 per treatment. A follow up independent t-test showed no significant difference due to treatment ($t_{18}$=1.053, P=0.306).

### 3.1.2 Epidermal Thickness

A Kolmogorov-Smirnov test established that the data for epidermal thickness was normally distributed (Z=0.687, P=0.732). The variance between all treatments were equal (F=0.426, P=0.522). The mean epidermal thickness for the corn oil treatment (20.21 ± 0.69 μm) and the non injected treatment (20.25 ± 0.52 μm) was determined from a sample size of n =10 per treatment. The results of an independent t-test showed no effect of treatment on epidermal thickness ($t_{18}$=-0.037, P=0.971).

### 3.1.3 Mortality

Statistical analysis for mortality was based on individual aquaria that experienced fish mortality, regardless of the number of individual fish dying per aquaria. After 12 days the corn oil treatment had 3 aquaria that experienced fish mortality and the non injected treatment experienced 5. These data were collected from a sample size of n=10 per treatment. The results from a Fisher's Exact Probability Test showed that there was no significant difference in the number of aquaria experiencing fish mortality due to treatment (Two-tail P=0.659)

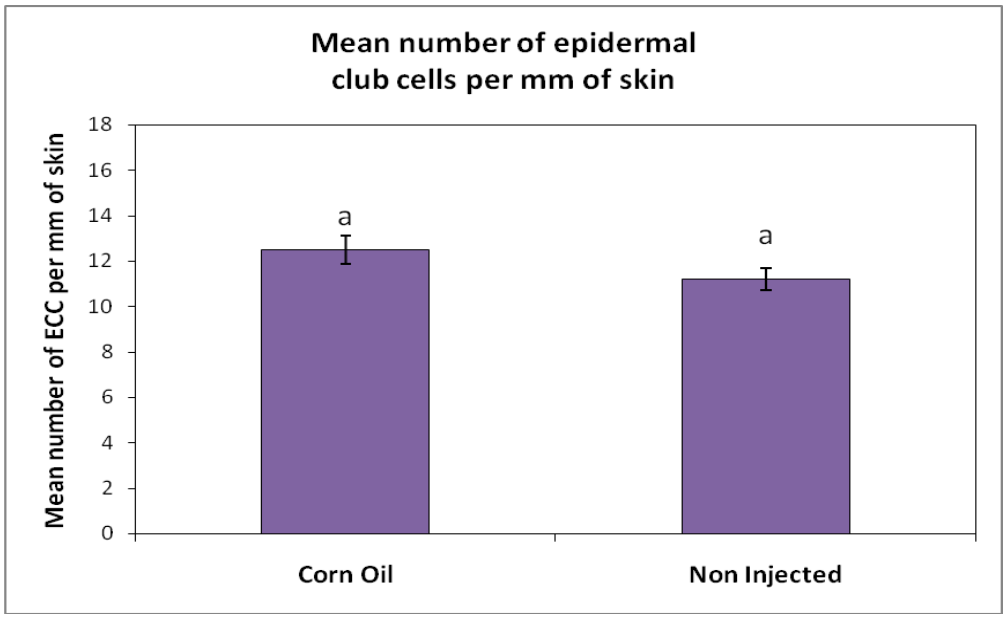


**Figure 3.1:** Mean (± SE) number of epidermal club cells (ECC) measured along the length of skin collected from fathead minnows 12 days after treatment with a single injection of 25 µL of corn oil or with no injection at all. Statistical analysis was conducted on a sample size of n=10 in each treatment. There was no statistical difference (t-test P= 0.111) between the two groups as indicated by the similar letters above each bar.

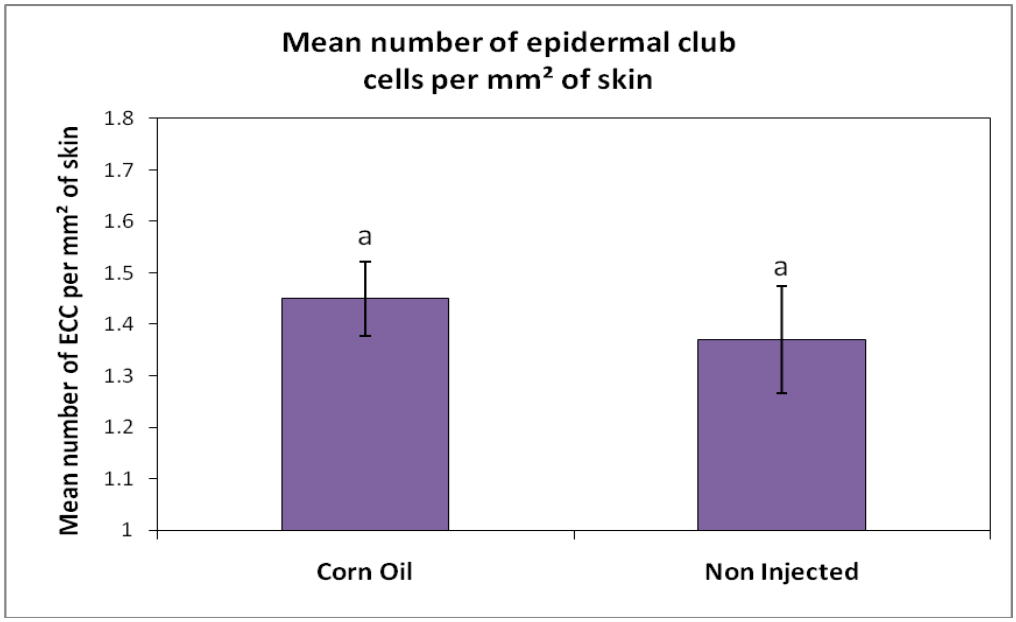


**Figure 3.2:** Mean (± SE) number of epidermal club cells (ECC) per mm² measured from skin collected from fathead minnows 12 days after treatment with a single injection of 25 µL of corn oil or with no injection at all. Statistical analysis was conducted on a sample size of n=10 in eachtreatment. There was no statistical difference (t-test P=0.904) between the two groups as indicated by the similar letters above each bar.

### 3.2 Experiment 2: The Effect of Corn Oil on Alarm Substance Concentration

Statistical analysis for fish exhibiting dashing or freezing fright responses were based on the proportion of fish displaying the behaviours in the post-injection time period only. The sample size for the experiment was 10.

#### 3.2.1 Change in shelter use

Statistical analyses for shelter use were based on the change in time spent under the shelter between 8 minute pre-stimulus period and 8 minute post-stimulus period immediately after injection of skin extract prepared from fathead minnows 12 days after treatment with 25 uL of corn oil or with no injection. The sample size was 10 for each treatment and is based on 10 aquaria housing one test subject. A 2x3 ANOVA (two types of treatment by three stimulus concentrations) was performed on these data. There was a significant effect of stimulus concentration ($F=7.098$, $P= 0.02$) on shelter use in this experiment but no significant differences were found between the two treatments ($F=1.046$, $P=0.311$) or the interaction between treatment and stimulus concentration ($F=0.240$, $P=0.787$) (Figure 3.3)

#### 3.2.2 Dashing behaviour

Dashing behaviour in this experiment was recorded as binomial data if the individual fish did or did not display a dashing response in the post-injection time period. The results from a Fisher's Exact Probability Test showed that there was no significant difference in effect due to treatment at the high, medium or low concentrations of the skin extract stimulus (two tailed $P= 0.582$, $P=1.00$, and $P=1.00$ respectively) (Figure 3.4).

### 3.2.3 Freezing behaviour

Freezing behaviour in this experiment was recorded as binomial data if the individual fish did or did not display a freezing response in the post-injection time period. The results from a Fisher's Exact Probability Test showed that there was no significant difference in effect due to treatment at the high, medium or low concentrations of skin extract (two tailed P= 0.303, P=0.628, and P=1.00 respectively) (Figure 3.5).

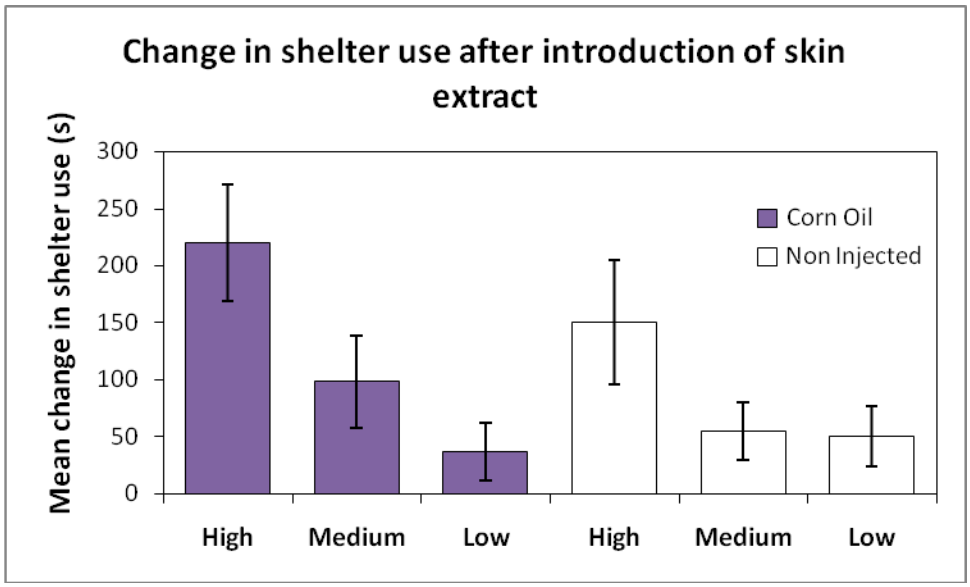


**Figure 3.3:** Mean (± SE) change in shelter use (seconds) between 8 minute pre-stimulus and 8 minute post-stimulus skin extract injection with skin developed from fathead minnows treated with a single injection of 25 µL of corn oil (Sham) or with no injection for 12 days (n=10). Cell number per mm skin and cell area is equal between the two treatments. High skin extract concentration is equivalent to1 mm² of skin per 40 L distilled water, medium concentration is equivalent to 1 mm² per 120 L distilled water and low concentration is equivalent to 1 mm² per 240 L distilled water. There were no significant differences between treatments at any concentration.

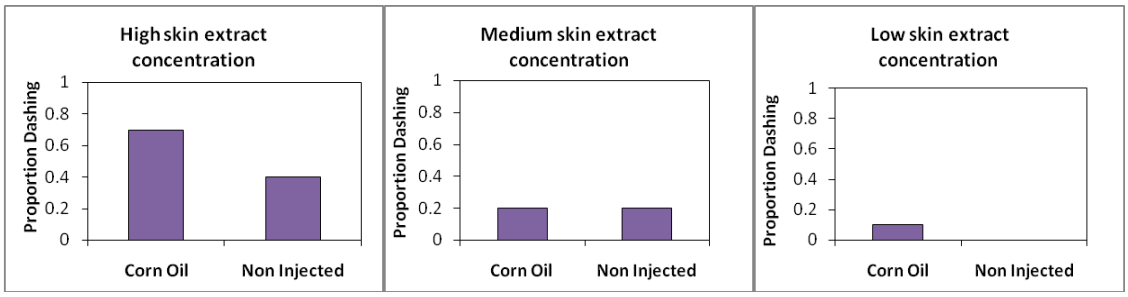


**Figure 3.4:** Proportion of fish that exhibited a dashing fright response after skin extract injection from fathead minnows treated with a single injection of 25 µL of corn oil or with no injection for 12 days (n=10). Cell number per mm skin and cell area is equal between the two treatments. High skin extract concentration is 1 mm² per 40 L distilled water, medium concentration is 1 mm² per 120 L distilled water and low concentration is 1 mm² per 240 L distilled water. There were no significant differences between treatments at any concentration.

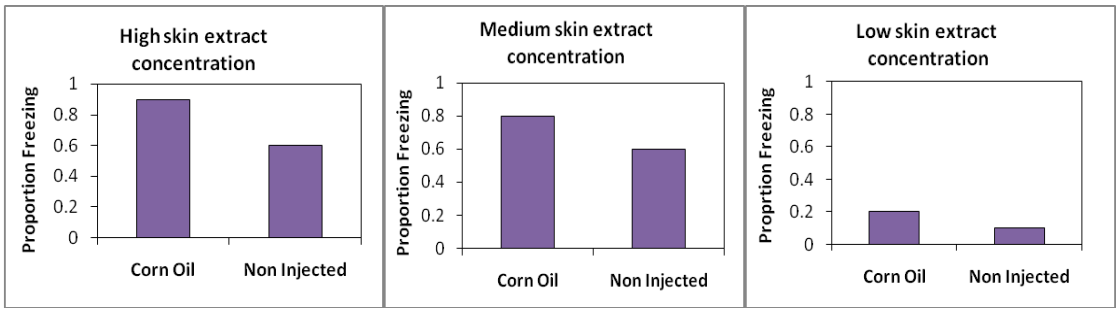


**Figure 3.5:** Proportion of fish that exhibited a freezing fright response after skin extract injection from fathead minnows treated with a single injection of 25 µL of corn oil or with no injection for 12 days (n=10). Cell number per mm skin and cell area is equal between the two treatments. High skin extract concentration is 1 mm² per 40 L distilled water, medium concentration is 1 mm² per 120 L distilled water and low concentration is 1 mm² per 240 L distilled water. There were no significant differences between treatments at any concentration.

## 3.3 Experiment 3: The Effects of Cortisol on Epidermal Club Cell Investment and Immune System Physiology

Statistical analyses for this experiment were based on the mean histological data obtained from the fathead minnows per individual aquarium. In order to measure ECC density, I determined the mean number of club cells per millimetre and millimeter$^2$ of epidermis for each fish and the mean area of all cells in the field of view. I then averaged the values for all fish within each individual aquarium. The treatments were cortisol

injected (n=21 aquaria) where fathead minnows received an intraperitoneal injection of 10 µL of 0.1µg cortisol per gram of fish and a sham treatment (n=18) which received 10µl of corn oil per gram of fish.

### 3.3.1 Epidermal Club Cell Density and Cell Size

A Kolmogorov-Smirnov test established that the ECC per mm of epidermis data was normally distributed (Z=0.656, P=0.782). The variance between all treatments was equal (F=0.01, P=0.920). The results of an independent t-test revealed that there was a significant effect of treatment on the number of ECC per mm of epidermis ($t_{37}$= -2.546, P=0.015) (Figure 3.6), with the cortisol treated fish having a significantly lower cell number than the sham treatment (13.2 and 20.0 cells per mm, respectively).

A Kolmogorov-Smirnov test showed that the mean number of club cells per area $mm^2$ of epidermis was normal (Z=0.424, P=0.994). The variance between all treatments was equal (F=2.760, P=0.105).The data from an independent t-test indicated that there was a significant difference in mean number of ECC per $mm^2$ due to treatment ($t_{37}$= -2.08, P=0.045 (Figure 3.7), with the cortisol treatment having a lower cells density per $mm^2$ than the sham treatment (mean groups of 0.44 and 0.66 cells per $mm^2$, respectively).

A Kolmogorov-Smirnov test established that the mean individual club cell area data was distributed normally (Z=0.437, P=0.991). The variance between all treatments were equal (F=2.094, P=0.156). The mean individual club cell area for the cortisol treatment (118.11 ± 5.28 $\mu m^2$) and the sham treatment (115.42 ± 4.41 $\mu m^2$) was determined by a sample size of n =21 for the cortisol treatment and n=18 for the sham treatment. A follow up independent t-test showed no significant difference due to treatment ($t_{37}$= 0.306, P=0.736).

### 3.3.2 Epidermal Thickness

A Kolmogorov-Smirnov test established that the data for epidermal thickness was normally distributed (Z=0.754, P=0.621). The variance between all treatments were equal (F=3.749, P=0.061). The mean epidermal thickness for the cortisol treatment (33.82 ± 1/27 µm) and the sham treatment (34.60 ± 0.79 µm) was determined by a sample size of n =21 for the cortisol treatment and n=18 for the sham treatment. The results of an independent t-test showed no effect of treatment on epidermal thickness ($t_{37}$= -0.555, P=0.834).

### 3.3.3 Kidney Phagocyte Respiratory Burst Activity

Statistical analysis for kidney phagocyte respiratory burst activity was determined by pooling fathead minnow kidney phagocytes from all the surviving fish housed in individual aquaria (n=7 or 8 aquaria per treatment). The phagocytes were collected 12 days after the fish received a single intraperitoneal injection of corn oil (sham) or cortisol (10 µL of 0.1µg of cortisol per gram of fish). Chemiluminescence (measured as photon counts) produced by the phagocytes in each treatment was measured at time intervals over a 60 minute period and total photon emission was calculated as the area under the curve from this 60 minute time period. A Kolmogorov-Smirnov test determined that the data for the area under the chemiluminescence curve were normally distributed in the two treatment groups (Z=0.569, P=0.902). The variance between all treatments were equal (ANOVA, F=.295, P=0.596).The results from an independent t-test showed that where was an effect due to treatment ($t_{13}$= -2.246, P=0.043) (Figure 3.8) with phagocytes from cortisol treated fish displaying a significantly lower total chemiluminescence over 60

minutes compared to fish treated with corn oil only (43115 ± 7917 and 66064 ± 6592 photon counts over 60 minutes, respectively).

### 3.3.4 Serum Cortisol Measurements

Statistical analysis for the serum cortisol levels were based on a sample size of n=8 aquaria and were produced by pooling blood from 2-4 surviving fathead minnows housed in the same aquaria. A Kolmogorov-Smirnov test established that the mean serum cortisol data was normally distributed (Z=0.569, P=0.902). The variance between all treatments were equal (F=0.944, P=0.348). The mean serum cortisol levels for the cortisol treatment (22.28 ± 3.13 ng/mL) and the sham treatment (23.93 ± 3.94 ng/mL) was determined from a sample size of n =8 per treatment. A follow up independent t-test showed no significant difference due to treatment ($t_{14}$= -0.328, P=0.748).

### 3.3.5 Effect of Cortisol Treatment on Body Mass and Condition

A Kolmogorov-Smirnov test determined that the data for mean pre-experimental body mass, mean post-experiment body mass, the difference between pre- and post-treatment body mass and post-experimental body condition were normally distributed (Z=0.629 P=0.824, Z=0.674 P=0.753, Z=0.447 P=0.998, Z=0.583 P=0.886, and Z=0.637 P=0.812, respectively). The variance between all treatments were equal (F=0.675 P=0.416, F=0.102 P=0.752, F=1.852 P=0.181, F=0.052 P=0.823, and F=0.439 P=0.511). The results from an independent t-test showed that there was no significant difference due to treatment in mean pre-experimental weight ($t_{40}$= 0.921, P=0.362) (Table 3.1), mean post-experimental weight ($t_{40}$= 0.432, P=0.668) (Table 3.1) or post-experimental body condition ($t_{40}$= -1.180, P=0.255) (Table 3.1). Furthermore, independent t-test showed that there was no significant difference within treatment before and after the 12 day treatment

period for both the cortisol treatment ($t_{40}$= 0.251, P=0.803) (Table 3.2) and the sham treatment ($t_{40}$= -0.576, P=0.568) (Table 3.2)

### 3.3.6 Effect of Cortisol on Fish Mortality

Statistical analysis for mortality was based on individual aquaria that experienced fish mortality, regardless of the number of fish deaths per aquaria. After 12 days, the cortisol treatment had 15 aquaria that experienced fish mortality and the sham treatment experienced 13. These data were collected from a sample size of n=21 per treatment. The results from a Fisher's Exact Probability Test showed that there was no significant difference in mortality due to treatment (Two-tail P=0.744)

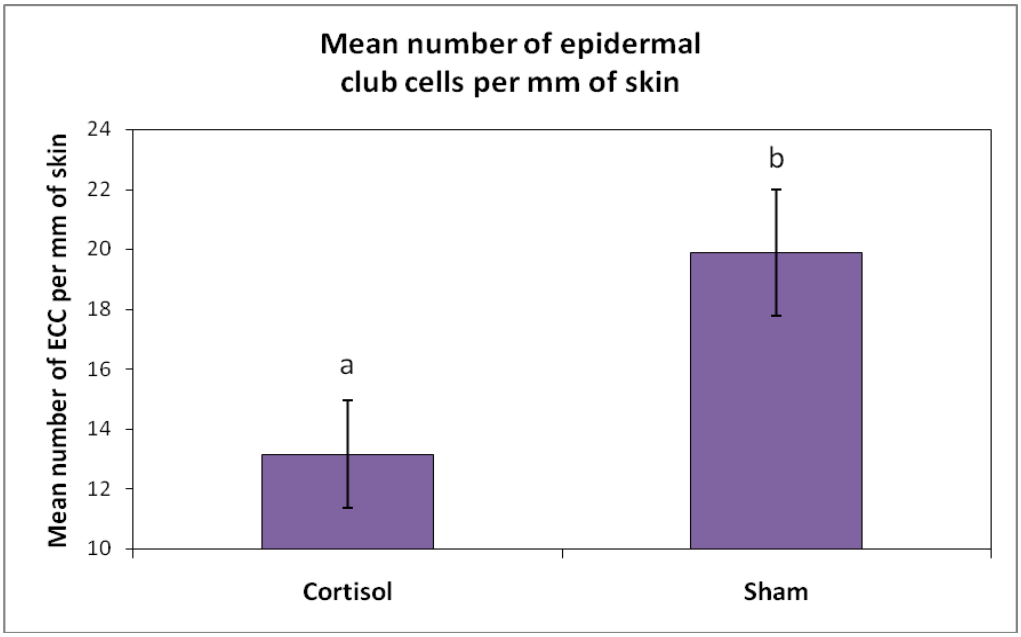


**Figure 3.6:** Mean (± SE) number of epidermal club cells (ECC) per mm measured from skin collected from fathead minnows 12 days after treatment with a single injection of 10 µL of 0.1µg of cortisol per gram of fish (Cortisol) or injected with the equivalent volume of corn oil (Sham). Statistical analysis was conducted on a sample size of n=21 for cortisol and n= 18 for sham. There was a statistical difference between the two groups (t-test; P=0.015). Different letters denote significant differences at P< 0.05.

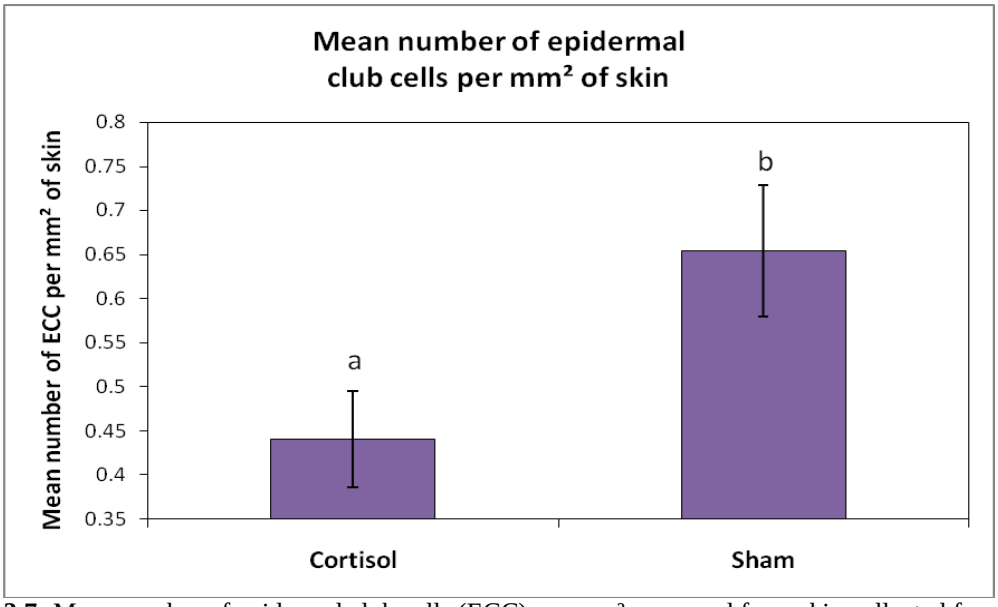


**Figure 3.7:** Mean number of epidermal club cells (ECC) per mm² measured from skin collected from fathead minnows 12 days after treatment with a single injection of 10 µL of 0.1µg of cortisol per gram of fish (Cortisol) or injected with the equivalent volume of corn oil (Sham). Statistical analysis was conducted on a sample size of n=21 for cortisol and n= 18 for sham. There was a statistical difference between the two treatments (t-test; P=0.045). Different letters denote significant differences at P< 0.05

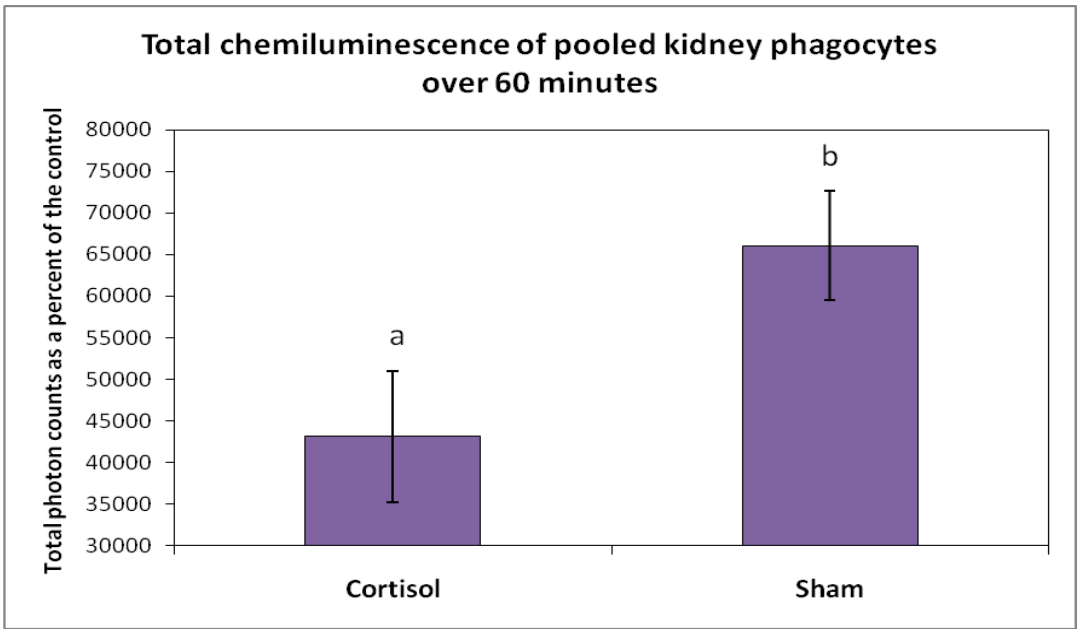


**Figure 3.8:** Total chemiluminescence over 60 minutes (± SE) by pooled kidney phagocytes collected from fathead minnows 12 days after treatment with a single injection of 10 µL of 0.1µg of cortisol per gram of fish (Cortisol) or injected with the equivalent volume of corn oil (Sham). Statistical analysis was conducted on a sample size of n=8 for sham and n=7 for cortisol. Different letters denote significant differences between treatments (t-test; P=0.043).

**Table 3.1:** Mean body mass and body condition of fathead minnows used in experiment 3. There was no statistical difference in mean body parameters do to or between treatments before or after injections for 12 days.

| | Cortisol Treatment | Sham Treatment |
|---|---|---|
| Mean body mass before treatment | 2.38 ± 0.06 g | 2.31 ± 0.05 g |
| Mean body mass after 12 day treatment | 2.36 ± 0.07 g | 2.32 ± 0.08 g |
| Mean body condition after 12 day treatment | 0.0160 ± 0.0006 $g/cm^3$ | 0.0172 ± 0.0008 $g/cm^3$ |

## Chapter 4: Discussion

My research and that of Michalak (2006) provides the first direct evidence that ECC of fathead minnows may function as part of the innate immune system in Ostariophysian fishes. The purpose of the first two experiments was to determine if the control injection of corn oil had an effect on either ECC investment or on the alarm function of the ECC contents. There currently is no published data indicating whether or not corn oil itself has any effect on epidermal club cells investment. Corn oil was chosen to be the vehicle for the cortisol injection due to its lipophilic nature, ability to dissolve cortisol and relatively low viscosity for ease of injection. Intraperitoneal corn oil injections showed no effect on any measure associated ECC number per mm of epidermis, ECC number per area ($mm^2$) of epidermis, mean epidermal thickness, or mean ECC area ($\mu m^2$). Furthermore, skin extract prepared from corn oil injected fathead minnows had the same capacity to induce anti-predator behaviour as skin extract prepared from control subjects. Thus, I concluded that corn oil had no effect on any aspect of the ECC investment or the alarm substance's ability to function as a predation cue and that it was an appropriate vehicle for intraperitoneal injections in subsequent experiments.

The purpose of the third experiment was to determine if pharmacological cortisol-induced immunosuppression of the innate immune system is correlated with a change in ECC density. The first part of the experiment was to test whether the selected dose of cortisol (10 µL of 0.1µg per gram of body weight) did have an immunosuppressive effect on the innate immune system of fathead minnows. The phagocyte respiratory burst assay confirmed that fish treated with intraperitoneal cortisol injection did, in fact, exhibit a

reduction in kidney leukocyte activity 12 days later. These results combined with numerous other studies show that elevated levels of cortisol over a prolonged period of time does have a suppressive effect on the phagocytic component of the innate immune system (Ainsworth et al. 1991, Wendelaar Bonga 1997, Hironobu et al. 2002, Esteban bet al. 2004, and Wang et al 2005). Furthermore, a recent study by Vizzini et al. (2007) using isolated peritoneal leukocytes from sea bass (*Dicentrarchus labrax*) and incubated *in vitro* with various concentrations of cortisol showed a dose dependant cortisol inhibitory effect on the chemiluminescent activity of peritoneal leukocytes.

After immunosuppression of the kidney phagocytes was confirmed, the epidermal skin samples of cortisol treated fish were analyzed for ECC changes. The results indicated a significant decrease in the number of ECC per mm of epidermis, as well as, in the number of ECC per $mm^2$ of epidermis. Until recently, the only published data demonstrating a reduction in ECC number in fathead minnows was Smith (1974) where the loss of ECC was illustrated in breeding males and Smith (1973) where ECC loss was induced by intraperitoneal injections of methyltestosterone. During the breeding season, in association with elevated levels of androgen, male fathead minnows develop tubercles on their snouts and soft dorsal mucous secreting pads. Both the tubercles and dorsal pad function in male reproductive behaviour (McMillian and Smith 1974).

The immunocompetence handicap hypothesis suggests that hormones which enhance male ornamental expression can be detrimental to the immune system and thus provide an honest signal of male quality (Folstad and Karter 1992). Applying this hypothesis may explain why fathead minnows show a reduction in ECC during the breeding season. The increased investment in secondary sexual characteristics may

present a trade-off between reproductive success and immune function. In support of this hypothesis, studies by Kurtz et al. (2007) showed that three-spined stickleback (*Gasterosteus aculeatus*), when treated with testosterone, illustrated an increase in colouration, as well as, a reduction in 3 out of 4 innate immune system assays. This indicates that the immunocompetence handicap hypothesis can be applied to small, shoaling freshwater fish. Thus, androgen production during the breeding season by male fathead minnows may divert energy away from the immune system (such as ECC) and redirect it towards the development of secondary sex characteristics. As a result, the higher quality fish would be able to withstand this reduction in immune function and survive to reproduce, providing an honest signal of male quality.

This proposed connection between ECC and immune function is further supported by Michalak (2006) who showed that an increase in club cell density was induced in fathead minnows following the exposure to the pathogens *Saprolegnia ferax* and *Saprolegnia parasitica* and the parasitic larval trematode *Uvulifer ambloplite.* These results suggest that these fish may be up-regulating their immune defences in response to an increased risk of infection by investing in ECC proliferation. Furthermore, Chivers et al. (2007) showed that 14 days of continuous exposure to relatively low levels of cadmium had the capacity to suppress the immune system as illustrated by the inhibition of increase in ECC investment exposed in conjunction *Saprolegnia ferax.*

ECC are ubiquitous among the members of the superorder Ostariophysi (Pfeiffer, 1977). However, some non-ostariophysian fishes have ECC with similar histological characteristics, including members from the percidae family. Chivers et al. (2007) reported that a yellow perch (*Perca flavescens*) heavily infested with larval trematodes

also exhibited higher ECC density when compared to control subjects. Collectively, these results provide compelling evidence that the 'so-called' alarm cells of fishes are likely maintained by natural selection owing to the benefit they confer against infection following epidermal injury by invading pathogens and parasites.

At the beginning of experiment 3, fish were injected with 10 µL of 0.1 µg of cortisol per gram of body weight, the mean weight of the cortisol injected fish was 2.38 g (± 0 .0553 g), therefore on average the fathead minnows were each injected intraperitoneally with 237 µg (± 5.53 µg) of cortisol. At the end of the 12 day experiment the pooled blood samples indicated that there was no significant difference in mean blood cortisol levels between the cortisol and sham injected fish (22.3 ng/mL ± 3.123 and 23.9 ng/mL ± 3.938, respectively). The most likely explanation for this result is that the injected cortisol was metabolized and cleared over the 12 day period returning plasma cortisol levels back to normal homeostatic levels. These post experimental plasma cortisol levels are similar to those reported in previous studies which measured baseline plasma cortisol level in fathead minnows. Palic et al. (2006) exposed fathead minnows to handling and crowding stress for 20 minutes, collected blood samples and compared plasma cortisol levels to a control non-stressed group. The results indicated that after a stressful event fathead minnow cortisol levels rose from a baseline (non-stressed) level of 14 ng/mL to a maxium stress cortisol level of 53 ng/mL after 30 minutes and returned to near baseline levels after 90 minutes. These results support the hypothesis that the cortisol levels obtained at the end of experiment 3 reflect those of a baseline cortisol level found in fathead minnows.

As stated earlier, the fish in experiment 3 received, on average, 237 ug of cortisol injected into their peritoneal cavity. These levels are reported to be pharmacological levels (higher than naturally produced serum cortisol levels) because they exceed those of any currently reported biological plasma cortisol levels in fathead minnows. For example, Palic et al. (2006) reported stressed level of plasma cortisol as high as 53 µg/mL, 30 minutes after 20 minute of handling and crowding. Furthermore, Richards et al. (2007) attempted to stress fathead minnows with high frequency strobe lights at either a 1 hour or a 7 hour time interval and compare stressed cortisol levels to a non-stressed group. They were unsuccessful at increasing cortisol levels with the strobe light, but obtained average plasma cortisol levels of 147.9 ng/mL in the 1 hour control and 99.3 ng/mL in the 1 hour stressed group, as well as, 153.4 ng/mL for the 7 hour control and 97.5 ng/mL for the 7 hour stressed treatment. These authors were unclear as to why the control cortisol levels were higher than the stressed treatment but hypothesized it was due to stress from maintaining the fish in complete darkness for 24 hours prior to the experiment, thus attributing the high levels of reported plasma cortisol levels to that which would be found in stressed fish. Regardless, I am confident that the fish in experiment 3 received cortisol injections which exceed those of any currently reported biological stress induce level, although, it is unclear as to how much and how long the injected cortisol was maintained in the blood stream of the injected fathead minnows as plasma cortisol levels were not monitored continuously throughout the duration of the experiment. It is, however, assumed that the levels were higher than normal and for a prolonged period of time as Wojtaszek et al. (2002) showed that pharmacological levels

of intraperitoneal injected cortisol remain elevated in carp (*Cyprinus carpio*) up to 9 days post injection.

Overall, my results provide convincing evidence linking the ECC with the immune system activity and provide a good foundation for further work on this intriguing hypothesis. For example, future studies could examine the time course of ECC reduction when exposed to high levels of cortisol, as well as how long it takes to re-establish baseline ECC density after cortisol levels have reached normal homeostatic levels. The results of Experiment 3 showed that there was a reduction in ECC density 12 days after the fish were exposed to a high dose of cortisol; however, at the end of the experiment there was no significant difference in plasma cortisol levels between the treated and controlled groups. This indicates that there is likely a lag between the acquisition of baseline cortisol levels and the reestablishment of normal ECC density. Likewise, it should be addressed whether there is a gradation in suppression in ECC when the fish are exposed to varying levels of immunosuppressants. Vizzini et al. (2007) showed a dose dependant cortisol inhibitory effect on the respiratory burst activity by isolated peritoneal leukocytes from sea bass incubated *in vitro* with various concentrations of cortisol. These results indicate that there is a gradation in suppression in the innate immune system when exposed to varying concentrations of cortisol. It would be interesting to investigate if ECC exhibit a similar reductive gradation response to that of other innate immune system components.

It will also be important to investigate whether or not physiological relevant prolonged elevation of cortisol levels under physiologically relevant conditions have an impact on ECC investment in fish. The threat of predation on prey fish is a very stressful

event which elicits a physiological stress response. Rehnberg and Smith (1986) and Rehnberg and Schreck (1987) showed that both coho salmon (*Oncorhynchus kisutch*) and pearl dace (*Semotilus margarita*) exhibited heightened plasma cortisol levels after exposure to predator odour and conspecific skin extract, respectively. However, Hindman (2002) showed that variation in potential predation risk was incapable of causing a significant change in ECC investment even though the variables of cue concentration, frequency and duration were all addressed in her experiments. The most likely explanation for Hindman's (2002) could be due to the frequency of the cue administration. Although Hindman (2002) did vary the frequency of predator threat, the most frequent administration was biweekly for 7 weeks. These exposures to predator cue would have likely elicited a physiological stress response increasing plasma cortisol, but may not have been frequent enough to maintain sufficiently high plasma cortisol levels to induce immunosuppression. Palic et al. (2006) showed that plasma cortisol levels of fathead minnows after a stressful event returned to near baseline levels 90 minutes post-stressor. Repeating these experiments with frequent daily predation threats would not only make the experiment more ecologically relevant but possibly maintain a prolonged level of cortisol in which the effects on ECC could be investigated. Furthermore, other stressful life encounters such a competition, reproduction, food availability, temperature, toxicity and pH could be investigated.

Current investigation into the effects that physiologically relevant levels of cortisol have on the fish innate immune system show that duration of the stressor plays a crucial role. Pulsford et al. (1994) physically stressed dab (*Limanda limanda*) by mimicking transportation stress for 1 hour, and then several innate immune parameters

were measured and compared to a control group of unstressed fish. The results indicated an increased number of phagocytic cells in the peripheral blood, as well as, an increase in the phagocytic activity and induced chemiluminescence of kidney cells in the stressed fish. They concluded that stress induced catecholamine release caused the expulsion of stored splenic leucocytes into the peripheral blood flow and that the increased phagocytic and chemiluminescent activity of the kidney phagocytes was due to the stimulatory effect of cortisol. Conversely, Yin et al. (1995) exposed carp (*Cyprinus carpio*) to chronic stress by crowding them for 14 days and found significantly higher levels of plasma cortisol on days 1, 7 and 14. Examination of the innate immune system found that there was a reduction in phagocytic respiratory burst activity and a reduction in total plasma protein, lysozyme activity, and bactericidal complement activity. Furthermore, they investigated the susceptibility of the stressed fish to infection via a disease challenge test. Carp exposed to crowding stress for both 7 and 30 days showed a significant decrease in the $LD_{50}$ when exposed to three different concentrations of *Aeromonas hydrophila* intramuscular challenges. Ndong et al. (2007) exposed tilapia (*Oreochromis mossambicus*) acclimated at 27 ºC to varying temperatures between 19 and 35 ºC for 12-96 hours time periods. The examination of the innate immune system showed that there was a significant reduction in respiratory burst index, phagocytic activity, phagocytic index, lysozyme activity and alternative complement activity when exposed to the stress of high and low temperature changes. Furthermore, they found that tilapia exposed to 19 and 35ºC for over 48 hours showed a significant decrease in resistance against *Streptococcus iniae* challenges. These results indicate that stress induced cortisol can

suppress the immune system of fishes but that the duration of the stress likely plays an important role.

For nearly 70 years, ecologists have been focussed on the alarm function of the Ostariophysian ECC. There are abundant examples of how fishes change their behaviour and morphology in response to cues released from injured conspecifics (Smith 1992, Chivers and Smith 1998). However, my results and those of Chivers et al. (2007) provide strong support for the hypothesis that the primary function of ECC is associated with an immune function and that the alarm signalling component evolved secondarily. The contents of the cells, although immunological in nature, also act to warn nearby conspecifics of danger. This warning needs to be viewed in the context of public information and not alarm signalling by the prey. My results also indicate that a break from the traditional terminology applied to these skin cells is in order. I propose that it would be more appropriate for future researchers to refrain from describing theses cells as 'alarm cells'. My evidence supporting a role for these cells in the innate immune system suggests that the term 'epidermal club cell' is more appropriate.

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**Appendix 1**

**Hanks balanced salt solution complete without phenol red:**

| | **10x stock solution (200 mL)** |
|---|---|
| $CaCl_2$ x $2H_2O$ | 0.37 g |
| Potassium Chloride (KCL) | 0.80 g |
| Potassium Phosphate, Monobasic ($KH_2PO_4$) | 0.12 g |
| $MgSO_4$ x $7H_2O$ | 0.40 g |
| Sodium Chloride (NaCl) | 16.0 g |
| Sodium Phosphate Dibasic ($Na_2HPO_4$**)** | 0.12 g |
| D-Glucose | 2.0 g |

Dissolve $CaCl_2$ x $2H_2O$ separately in 10% of the final volume
Dissolve the rest of the reagents in 80% of the final volume
Combine both dissolved forms for total volume
Store at room temperature

**Appendix 2**

**Trypan blue exclusion and cell viability test**

80 uL of cell solution mixed with 80 uL of sterile trypan blue, dead cells stain dark blue and viable cells do not stain at all

Used hemocytometer to count cells, by averaging 4 square counts 4 times

Number of living cells:

average number of cells between 4 four averaged counts x 160,000 x 2 = Number of living cells/mL

Cell number was corrected to 1.0 x $10^6$ by dividing 1,000,000 by the number of living cells/mL

**Appendix 3**

**Zymosan opsonization preparation:**

20 mg Zymosan

Add 2 mL of phosphate buffered saline (PBS) (Appendix 5)

Boil for 30 minutes

Centrifuge at 600 x g for 5 minutes

Remove supernatant with pasture pipette

Resuspend in 4 mL of complete HBSS without phenol red to obtain a suspension of 5 mg/mL

Separate 5 mL serum into 1 mL aliquot and store in freezer

**Appendix 4**

**Luminol solution preparation:**

0.068 g of luminol dissolved in 6.1mL of distilled water

Stock solution: 11.1mg/mL

Working solution: dilute 1:100 with HBSS complete without phenol red (appendix 1) resulting in a 0.11 mg/mL solution

Store in plastic tubes in black box at 4 degrees Celsius (make 4-12 hours prior to use)

**Appendix 5**

**Phosphate Buffered Saline (PBS):**

Dissolve the following in 800 mL distilled $H_2O$:
- 8 g of Sodium Chloride (NaCl)
- 0.2 g of Potassium Chloride (KCl)
- 1.44 g of Sodium Phosphate Dibasic ($Na_2HPO_4$)
- 0.24 g of Potassium Phosphate, Monobasic ($KH_2PO_4$)

Adjust pH to 7.4 with HCl or NaOH
Adjust volume to 1L with additional distilled $H_2O$
Sterilize by autoclaving