THE EFFECTS OF PATHOGENS ON CLUB CELL INVESTMENT IN FATHEAD MINNOWS, *PIMEPHALES PROMELAS*

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By

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ABSTRACT

Fish skin is a metabolically active tissue that responds quickly to stressors and is the first line of defence against physical damage. Club cells, characteristic components of Ostariophysian fish skin, release their contents into the surrounding water upon rupture (e.g. during predation). These chemical cues act as public information of predation risk. Despite the assumption that club cells evolved under the selective force of predation, studies demonstrated that predation has no effect on club cell investment. Rather, club cell production is stimulated by skin-penetrating pathogens and parasites. The experiments in this thesis investigate the responses of fish skin to manipulated pathogen risk. In the first experiment, fathead minnows (*Pimephales promelas*) were exposed to varying infective risk from two pathogen species that differ in pathogenicity, *Saprolegnia ferax* and *S. parasitica.* Although there was no difference in club cell density between fish exposed to the two *Saprolegnia* species, fish exposed to high concentrations of the pathogens had smaller club cells than those exposed to low concentrations. These results are the first to demonstrate a pathogen effect on the size of club cells. The second experiment investigated whether the physical presence of the pathogen was necessary for an alteration in epidermal parameters or whether *Saprolegnia parastitica* conditioned water was the only stimulus necessary to evoke a change. Results indicated a lack of treatment effect on club cell density, club cell size or epidermal thickness. The third experiment investigated the timing of club cell changes following a pathogen challenge. Although fish exposed to the *Saprolegnia ferax* treatment had higher club cell density than fish exposed to the control, there was no difference in club cell density between fish sacrificed on day 3, 6, 9 or 12. A portion of the test population for the third experiment was infected with black spot disease. When analyzed separately, trematode infected fish had smaller club cells than those that were uninfected. In light of inconsistent epidermal responses to pathogen challenges, and comparison with other studies, assessment of environmental stressors and population differences that may affect experimental outcomes and potentially interact with infectious agents is advised.

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1. INTRODUCTION

Considerable attention has been given in recent years to the effects of stress from various sources on fishes (Iger and Abraham 1990; Iger *et al*. 1994a-f; Iger and Wendelaar Bonga 1994; Wendelaar Bonga 1997; Nolan *et al*. 2000; Van der Salm *et al*. 2000). Stress is a condition in which a fish is unable to maintain homeostasis, or internal equilibrium, due to factors adversely affecting its well being. These factors include, but are not limited to, chemical stressors (e.g. accumulation of ammonia or nitrite, changes in pH or salinity, low dissolved oxygen, water pollution), physical stressors (e.g. sudden or extreme changes in temperature, ultraviolet radiation), biological stressors (e.g. crowding, predation, parasites, pathogens), and aquaculture procedural stressors (e.g. handling, transport, disease treatment). Fishes are especially vulnerable to such disturbances in their environment due to the intimate contact of their skin and gills with the surrounding water. Fish skin is a multilayered assemblage of biologically active cells that responds rapidly to stimuli and is an integral part of the fish's immune system (Zaccone *et al*. 2001).

1.1 FISH IMMUNE SYSTEM

The immune system plays an important role in homeostasis of a fish by maintaining health in both non-specific and specific ways. Non-specific defences include components that prevent invasion by potential pathogens. The first line of nonspecific defence is the mucus layer, secreted by the epithelial cells, external to the skin. In addition to the sticky, viscous consistency of the mucus that traps microorganisms and inhibits their movement, mucus also contains immunoreactive molecules (i.e. lysozyme, complement, and immunoglobulin), which inhibit the growth of pathogens and kill invading organisms such as bacteria (Ellis 2001). The second line of non-specific defence is the skin and scales. These provide mechanical protection, unless broken by injury or parasite, allowing bacteria or other pathogens to enter the fish's body. If pathogens breach these initial lines of defence, a local inflammatory response may occur,

increasing blood flow and delivering various types of white blood cells to the area (Roberts 1989).

The detection of an invading compound (antigen) and the subsequent creation of specialized response mechanisms to identify and destroy the invading compound comprise the specific immune response (Ellis 2001). When an antigen invades the fish body, it is recognized as being foreign by two types of white blood cells (T cells and B cells). T cells originate in the thymus gland of the fish located at the back of the gill plate. T cells multiply rapidly and make their way to the site of invasion by the foreign body and can attach themselves to the foreign body, and kill it. The B cell's primary function is to create specific antibodies on the surface of the cell, tagging the antigen for destruction by other components of the immune system including macrophages, which engulf and digest the tagged antigens (Roberts 1989).

While the non-specific and specific immune system are both important in the health of a fish, the experiments discussed in this thesis focus on the role of skin in the non-specific immune system of fish.

1.2 SKIN OF FISH

The major structural components of fish skin are consistent between species, however, the range and abundance of cell types may vary considerably. Each skin cell type has a different position, morphology and function. The skin of fish is composed of two main cellular layers: 1) the dermis, which contains the scales and various pigment cells, and 2) the epidermis, which acts as the interface between the fish and the external environment (Roberts and Bullock 1980) (Figure 1.1). Fish kept under unstressed conditions have the following general sequence of epidermal cell types from the base of the skin (basal lamina) to the exterior of the fish. Filament cells, the main structural component of the epidermis (Henrikson and Matoltsy 1968), appear as cuboidal or columnar cells in the basal layer of epidermis (Figure 1.1) (Whitear 1986). Although mitotic activity is detectable throughout the epidermis, it is most common in this basal layer. As filament cells mature, they migrate towards the surface of the epidermis, becoming more flattened. These flattened or squamous epithelial cells are referred to as

pavement cells and form the outermost cellular layer of the epidermis. The outer membrane of each pavement cell is folded into fine microridges producing fingerprint-like patterns on the cell surface (Buchmann and Bresciani 1998; Buchmann 1999). These microridges provide mechanical defence to trauma and aid in holding mucus secretions to the skin surface (Hawkes 1974). The external layers of the fish epidermis are continually sloughed off into the water and replaced by cells originating from the deeper layers of epidermis. It has been suggested that the filament cells are phagocytic (Peleteiro and Richards 1990; Iger and Abraham 1990; Åsbakk and Dalmo 1998) and the continual sloughing of these cells is an efficient process to eliminate foreign material from fish skin (Åsbakk and Dalmo 1998).

In addition to the filament cells, which make up the bulk of the epidermal tissue, a variety of other cell types exist (see Whitear 1986 for review), including mucous cells and club cells (Figure 1.1). Mucous cells, located within the upper epidermis, are goblet or flask shaped and able to secrete their contents onto the epidermal surface via a small pore (Mittal and Banarjee 1980; Whitear 1986). Note that the word mucus is a noun which represents the viscous, slippery substance that consists chiefly of mucin, water, cells, and inorganic salts, and the word mucous is used as an adjective to describe the cells which secrete the mucus (American Heritage Dictionary of the English Language). In fish, the layer of mucus serves many roles including reduction of friction in water and ease of locomotion, disease resistance, and protection against mechanical damage (Mittal and Banarjee 1980).

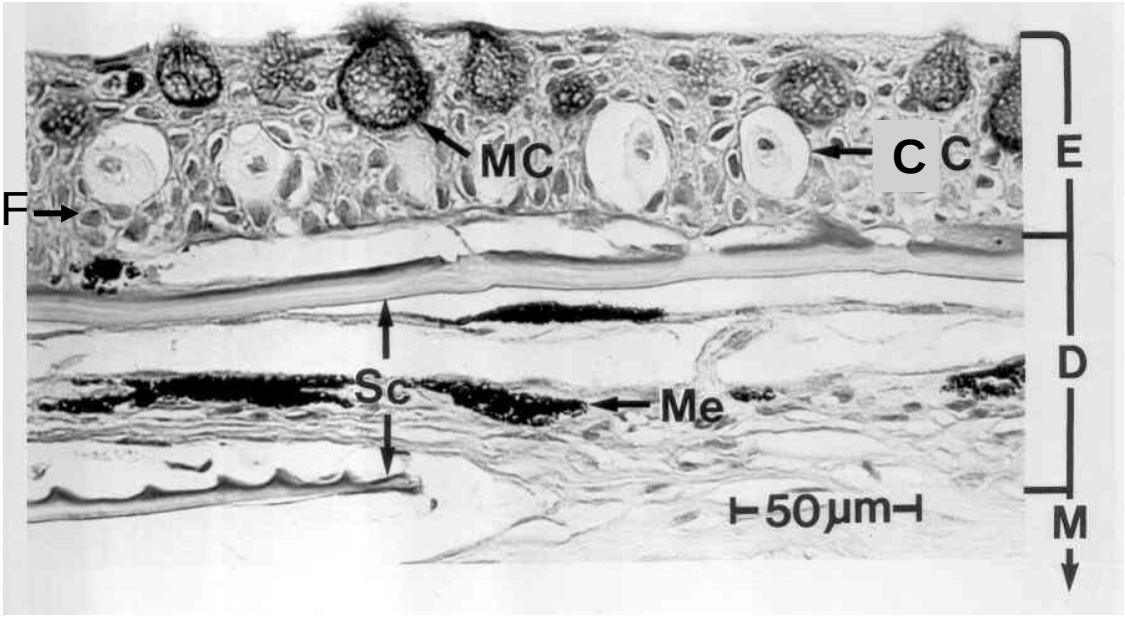

Figure 1.1. A cross section of fathead minnow skin showing the epidermal (E), dermal (D), and muscle (M) layers. A filament cell (F), club cell (CC), mucous cell (MC), melanophore (Me) and dermal scale (Sc) are indicated with arrows. Photo from Hugie (1990).

In contrast, club cells, distributed within the mid-epidermal layers in some groups of fish (Superorder Ostariophysi), are ovoid cells that do not have a direct link to the exterior of the fish (Pfeiffer 1977; Smith 1992). Evidence is mounting that club cells are a storehouse of biologically active compounds (Zaccone *et al*. 1994) and have multiple functions. Club cells of fathead minnows (*Pimephales promelas*), a member of the Superorder Ostariophysi, are the focus of this research.

1.3 CLUB CELLS AND THEIR CONTENTS

Club cells are ovoid or club-shaped epidermal cells characteristic of freshwater fishes in the Superorder Ostariophysi (e.g. minnows, characins, and catfish) (Pfeiffer 1977; Smith 1992). Several non-ostariophysan fish (e.g. Percidae - perch, walleye, sauger, darters; Cottidae - sculpin; and Anguilliformes - conger eel) also possess cells with similar histological characteristics (Whitear and Zaccone 1984; Smith 1992; Nakamura *et al*. 2001). Club cells lay external to protective dermal scales, and are easily

distinguished from mucous cells by 1) their lack of opening onto the epidermal surface, 2) the central position of their nucleus, and 3) the lack of reaction of the cells to the periodic acid – Schiff (PAS) stain. Since club cells lack a duct to the exterior, club cell contents may be expelled via active or passive transport or when the cells are ruptured, such as when skin is damaged. Club cells are often ruptured during predation events (von Frisch 1941; Smith 1992; Chivers and Smith 1998; Wisenden and Chivers 2006). Once released, the contents of the club cells are easily dispersed in water, and can act as public information indicating a predation event has taken place. The contents of the club cell can evoke context-dependent anti-predator behaviours (e.g. schooling, dashing, freezing, shelter use) among conspecifics (same species) and/or heterospecifics (different species) co-occurring in the same area (Chivers and Smith 1998; Tremaine *et al*. 2005; Wisenden and Chivers 2006; Pollock *et al*. 2003). Appropriate reaction to these chemical cues can increase prey survival in predator/prey interactions (Mathis and Smith 1993; Chivers *et al*. 2002). It is because of these anti-predator behaviours that researchers have used the terms pheromones, alarm substances, fear substances (*Schreckstoff*) and damage release cues to refer to the contents of the club cells (Smith 1982; Chivers and Smith 1998; Pollock and Chivers 2004; Ferrari *et al*. 2005).

Despite the many terms used to describe the contents of club cells, chemical characterization of the substance is currently insufficient. Lebedeva *et al*. (1975) and Kasumyan and Ponomarev (1987) used gel chromatography to demonstrate similarity in biochemical properties of fish skin in the order Cypriniformes, to which fathead minnows belong. They suggested that the biochemical nature of club cell contents was likely a complex of an active compound and a protein. The chemicals that elicited the strongest fright reactions were large molecules with molecular weights of approximately 1100 Da or greater than 1500 Da (Lebedeva *et al*. 1975; and Kasumyan and Ponomarev 1987).

Pfeiffer *et al*. (1985) suggested that hypoxanthine-3-*N*-oxide (H3NO) is the component of the Ostariophysan alarm substance system that elicits the anti-predator behaviour. Hypoxanthine-3-N-oxide, however, is considerably smaller than the molecules proposed by Lebedeva *et al*. (1975) and Kasumyan and Ponomarev (1987), with a molecular weight in the range of 350-500 Da. Hypoxanthine-3-*N*-oxide is a molecule

consisting of a purine skeleton with a nitrogen oxide functional group at the three position (Figure 1.2). Brown *et al*. (2000) designed an experiment, involving both field and laboratory components, to determine whether the nitrogen oxide functional group, purine skeleton, or the combination of the two together acted as the functional component in the alarm substance. Brown *et al*. (2000) demonstrated that the 3-*N*-oxide group in particular may be responsible for instigating the anti-predator behaviour in Ostariophysan fish. Hypoxanthine-3-*N*-oxide may be one of several possible molecules that make up a much more complex chemical substance. More research is required to understand the entire chemical make-up of the club cell contents, and to determine the extent of functions it may serve.

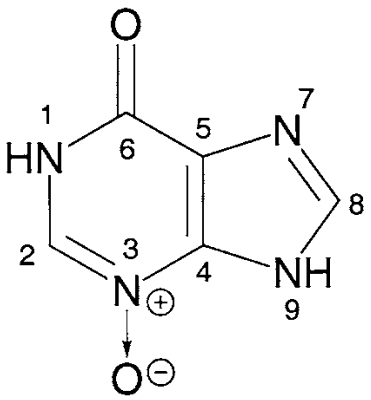

Figure 1.2. Hypoxanthine-3-N-oxide, with standard purine numbering scheme (from Brown et al. 2000).

1.4 EVOLUTION OF CLUB CELLS

Evolutionary ecologists have debated for decades over the selection pressures that could have lead to the evolution of club cells. While the release of club cell contents benefits individuals that receive the information regarding the current predation risk level, it is energetically costly for fish to produce and maintain club cells (Wisenden and Smith 1997). This energetic cost was demonstrated by Wisenden and Smith (1997) in an experiment where club cell counts were higher for fish on a high food ration, and thus higher physical body condition index (calculated by weight/standard length^3), than those on a low food ration.

Since club cells are costly to produce, there must be a benefit to fish that possess and maintain production of club cells over generations. Many hypotheses have been put forth to explain their origins, although only three have received any empirical testing. These include the kin selection hypothesis (Wisenden *et al*. 1995; Wisenden and Smith 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 production of club cells may be maintained by natural selection if the prey fish's kin are among the shoal under attack by a predator. If the fish within the shoal respond to the chemicals released from the ruptured club cells with an anti-predator response and escape, they could increase the inclusive fitness of the sender (Wisenden *et al*. 1995). However, studies are inconclusive regarding the genetic homogeneity of minnow shoals (Ferguson and Noakes 1981; Dowling and Moore 1986; Naish *et al*. 1993). Although it may be possible for kin selection to maintain club cells even if only a few members of the shoal are related (Wisenden *et al*. 1995), further research into the genetic makeup of a shoal is required.

Pike (*Esox lucius*) and predatory diving beetles (*Colymbetes sculptilis*), both predators of fathead minnows, have demonstrated an attraction to the chemical cues released by club cells (Mathis *et al*. 1995). The potential attraction of a second predator, such as a second pike, could disrupt the original predation event, increasing the prey-handling time of the first predator, and increasing the probability of escape for the prey (Chivers *et al*. 1996). This hypothesis argues that club cells are a feeding signal to secondary predators, however, the frequency with which predators exploit alarm cues may be rare.

Club cell contents in the two previous models are postulated to warn conspecifics or heterospecifics of nearby predation risk or to entice secondary predators to allow for potential escape. If predation is the selective force that maintains club cell production, then club cell density should change with predation risk. Hindman (2002) tested this hypothesis by conducting three predation-based experiments.

The first experiment examined fathead minnows exposed to different levels of predation threat including: 1) fathead minnow skin extracts containing club cells (indication of high risk), 2) skin extracts from brook stickleback (*Culaea inconstans*) which co-occur with minnows and share the same suite of predators (indication of medium-high risk), 3) skin extracts from swordtails (*Xiphophorus helleri*) which do not co-occur with minnows, do not possess club cells, and acted as a control for general injured fish cues (indication of low/no risk), and 4) distilled water (low/no risk) (Hindman 2002; Chivers *et al*. 2007). There was no significant effect on the epidermal thickness, number of club cells or number of mucous cells, although there was a visible trend of decreasing numbers of mucous cells in minnows exposed to the minnow skin extract compared to distilled water (Hindman 2002).

A second predation experiment conducted by Hindman (2002) exposed fathead minnows to chemical cues from either a familiar predator (northern pike, *Esox lucius*) or an unfamiliar predator (oscar cichlid, *Astronotus ocellatus*) (both of which had been fed either fathead minnows or swordtails), or a control of distilled water (Chivers *et al*. 2007). Diet-based predatory cues commonly elicit anti-predator responses (reviewed by Chivers and Mirza 2001); however, similar to the first study by Hindman (2002), diet-based predator odour had no effect on the epidermal thickness, number of mucous cells or club cells of the test minnows.

In a third predation experiment, fathead minnows were exposed to 0.4 nM concentration of hypoxanthine-3-*N*-oxide (H3NO, a chemical suggested to be involved in eliciting anti-predator responses (Pfeiffer *et al.* 1985; Brown *et al*. 2000)), the odour of pike fed minnows or a control of distilled water. No significant differences between treatments were noted in the epidermal thickness, number of mucous cells or club cells (Hindman 2002; Chivers *et al*. 2007).

Surprisingly, the number of club cells and mucous cells were not affected by the odours of predators feeding on conspecifics or heterospecifics, or by treatments of conspecific alarm substance, all of which are established indications of predation threat (Chivers and Smith 1998; Kats and Dill 1998). These results suggest that perceived

predation threat does not influence club cell investment in fathead minnows (Hindman 2002; Chivers *et al*. 2007).

If predation is not a factor in club cell investment, there must be other, less obvious, benefits to generating and maintaining club cells. Smith (1982) noted the similarity in chemical structure between club cell contents and certain antibiotics, and suggested that club cell function may include defence against parasites or pathogens. Abrasions and punctures break open the epidermis and the opportunity for infections to occur is much greater until the wound heals. Club cells are in an ideal location to release anti-pathogenic substances at the precise site of highest infection risk.

In an effort to test this hypothesis, Chivers *et al*. (2007) exposed fathead minnows to varying concentrations of a pathogenic water mould (*Saprolegnia ferax*). An appropriate response to pathogens may be to 1) increase club cell density, or 2) increase the activity of the club cells (e.g. have a higher turnover rate because the cell contents are depleted fighting the pathogen). Chivers *et al*. (2007) discovered that those fish challenged with the pathogen dramatically increased their number of club cells compared to the control after eleven days. However, pathogen exposure did not have an effect on other epidermal parameters such as club cell size, mucous cell density or size, or epidermal thickness. In another experiment, Chivers *et al*. (2007) found similar results when minnows were treated with varying numbers of skin burrowing trematodes. Those minnows that were treated with either low or high numbers of trematode cercariae (the fish-infective stage of the life cycle) had significantly more club cells than the control. Again, club cell size, mucous cell size and density, and epidermal thickness were not affected. An additional experiment conducted by Chivers *et al*. (2007) determined that fathead minnow skin homogenate inhibited the growth of a pathogen, *Saprolegnia ferax* hyphae, compared to controls of distilled water and swordtail skin extract (a fish that does not have club cells). Hence, minnows appear to increase their club cell investment in response to both pathogens and parasites, and some component of fathead minnow skin inhibits the growth of *S. ferax* hyphae, supporting the model that club cells have an anti-pathogen/anti-parasite function and may be associated with immune responses to skin damage.

If club cells are mediated by the immune system, club cell investment should diminish in the presence of an immunosuppressant. An experiment by Chivers *et al*. (2007) exposed fathead minnows to environmentally relevant levels of cadmium (Cd), a known immunosuppressant (Sanchez-Dardon *et al*. 1999), in conjunction with *S. ferax*. Those fish exposed to the *S. ferax* cysts (1900 cysts/L) in non-contaminated water had higher numbers of club cells per area of epidermis than those that were not exposed to *S. ferax*. Fish that were exposed to water with high levels of cadmium (5.64 µg/L) were no longer able to increase club cell numbers in response to a *S. ferax* challenge. Therefore, the connection between exposure to an immunosuppressant and the reduction in club cell investment suggests that club cells may be involved in the fish innate immune system.

Research by Halbgewachs (2008) tested for differences in club cell investment in fish exposed to another immunosuppressant, the hormone cortisol. Halbgewachs (2008) determined that minnows treated with a single intraperitoneal injection of cortisol had reduced respiratory burst activity of kidney phagocytes, indicating a suppressive effect on the innate immune system, and in turn had lower club cell densities. The conclusion that cortisol mediated immunosuppression affects club cell density is further evidence that club cells function as part of the fish immune system.

1.5 STUDY SYSTEM

The series of experiments outlined in this thesis continue to use the fathead minnow (*Pimephales promelas*) as the model fish species and the water mould *Saprolegnia* spp. as the model pathogen to build upon the previous research examining the effects of pathogens on club cell investment, and make advances towards understanding the role of pathogens in the evolution of club cells.

The fathead minnow is a small-bodied freshwater fish (3-6 cm adult standard length) that inhabits lakes, ponds, rivers and creeks throughout much of central North America (Scott and Crossman 1973). Due to their tolerance of a wide variety of conditions, small size, ease of spawning and short life span (2-3 years) they are model test species for laboratory studies and thus are used extensively in both behavioural and

toxicological research. Minnows belong to the Superorder Ostariophysi, a group characterized by the epidermal club cells that were described above.

Saprolegnia spp., a water mould (Oomycetes) that is nearly ubiquitous in freshwater ecosystems, may cause skin infections resulting in drastic declines in natural populations of many freshwater fish (Beakes *et al*. 1994; Bruno and Wood 1999), as well as affecting fish farms and other commercial fisheries operations (Van West 2006). *Saprolegnia* spp. hyphae invade epidermal tissues, often on the head or fins, and may eventually spread over the entire surface of the body causing cellular necrosis. In some cases, infection takes place very rapidly, and if left untreated, can lead to death by osmoregulatory failure (Hatai and Hoshiai 1994; Pickering and Willoughby 1982).

The life cycle of *Saprolegnia* spp., as well as its morphology, aid in its success as a pathogen. *Saprolegnia* spp. reproduces both sexually and asexually. In the asexual phase, *Saprolegnia* spp. releases motile zoospores that are formed within zoosporangia (Figure 1.3). Primary zoospores have their flagella inserted at the anterior of the cell, but are rather poor swimmers. These zoospores soon lose their flagella and encyst (primary cyst). Primary cysts then germinate to produce secondary zoospores with laterally positioned flagella, which are much more efficient at movement and dispersion. If they do not find a suitable host, they will again encyst, and then produce a tertiary zoospore. Tertiary zoospores are morphologically similar to secondary zoospores. The number of cyst-zoospore cycles an individual is capable of is unknown.

Once firmly attached to a suitable host, sexual reproduction takes place with the production of male (antheridia) and female (oogonium) gametangium. These unite, and the zygote produce is called an oospore.

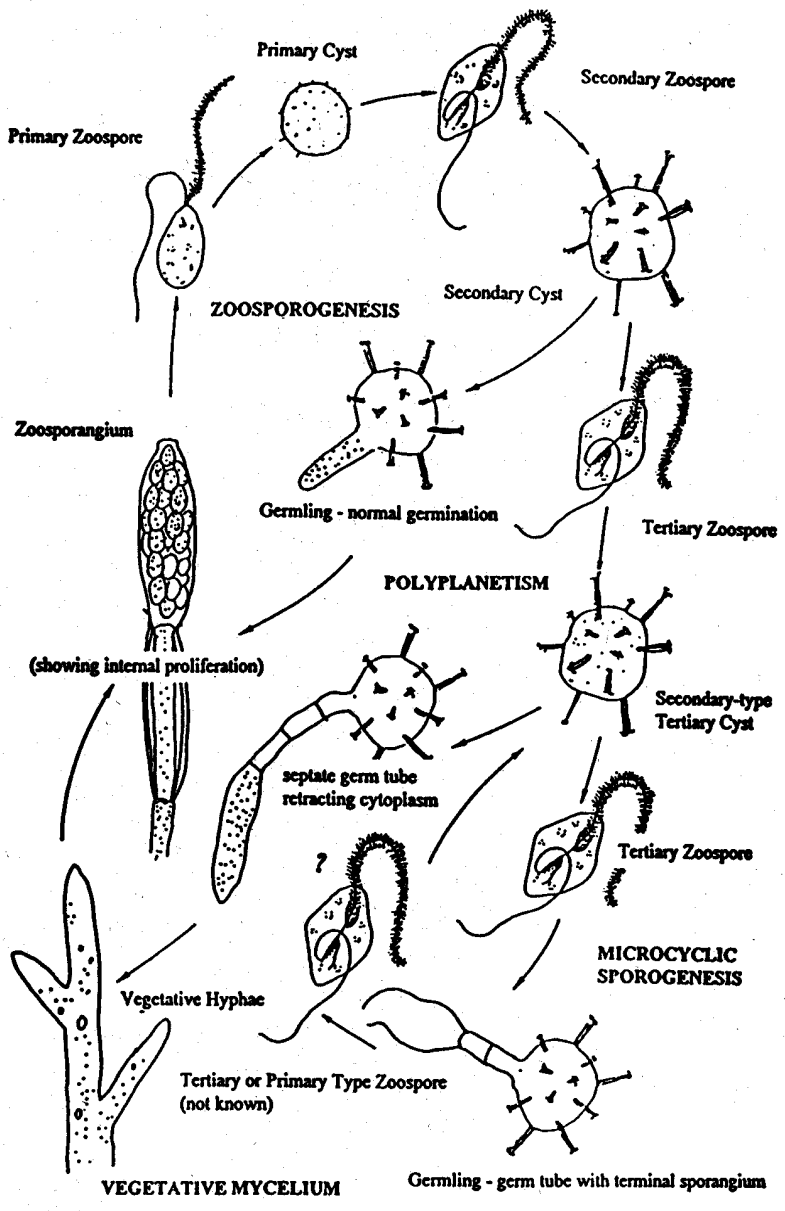

Figure 1.3. Asexual life cycle of *Saprolegnia parasitica*. Taken from Beakes et al. (1994).

1.6 RESEARCH OBJECTIVES

The overall objective of this thesis was to further examine the effect of the pathogen *Saprolegnia* spp. on the epidermal parameters of the fathead minnow and to answer the following questions:

1.6.1 Experiment 1: Can fathead minnows modify their club cell density or size, or epidermal thickness relative to their risk of infection?

In order to infect a fish, *Saprolegnia* spp. relies upon an effective passive attachment mechanism (Beakes *et al*. 1994) to attach to its host. Numerous spines on the cyst surface increase the contact area with the potential host, and aid in the physical entanglement with the fish's surface. Species with spiny cysts may also decrease the rate of spore sedimentation, keeping the spores suspended in the water column for longer than those with unornamented cysts (Beakes *et al*. 1994), giving them more opportunity to attach to a host. Isolates of *S. parasitica* have been found to be ornamented by bundles of spines (4-16) that are 2.5-14 µm in length whereas *S. ferax* isolates possess spines that are shorter (0.5-1.0 µm in length) and normally occur singly or very occasionally in groups of no more than three spines (Beakes *et al*. 1994) (Figure 1.4). It is for these reasons that *S. parasitica* is considered more pathogenic than *S. ferax*, although its aggression has never been fully tested. The objective of the first experiment was to test the following hypotheses: 1) that fathead minnows treated with *S. parasitica*, a reportedly more aggressive pathogen, would demonstrate increased club cell density and/or a high turnover rate of club cells compared to those treated with *S. ferax*; and 2) that fathead minnows treated with a higher concentration of a pathogen would display increased club cell densities and/or a high turnover rate of club cells compared to those treated with a lower concentration of pathogen.

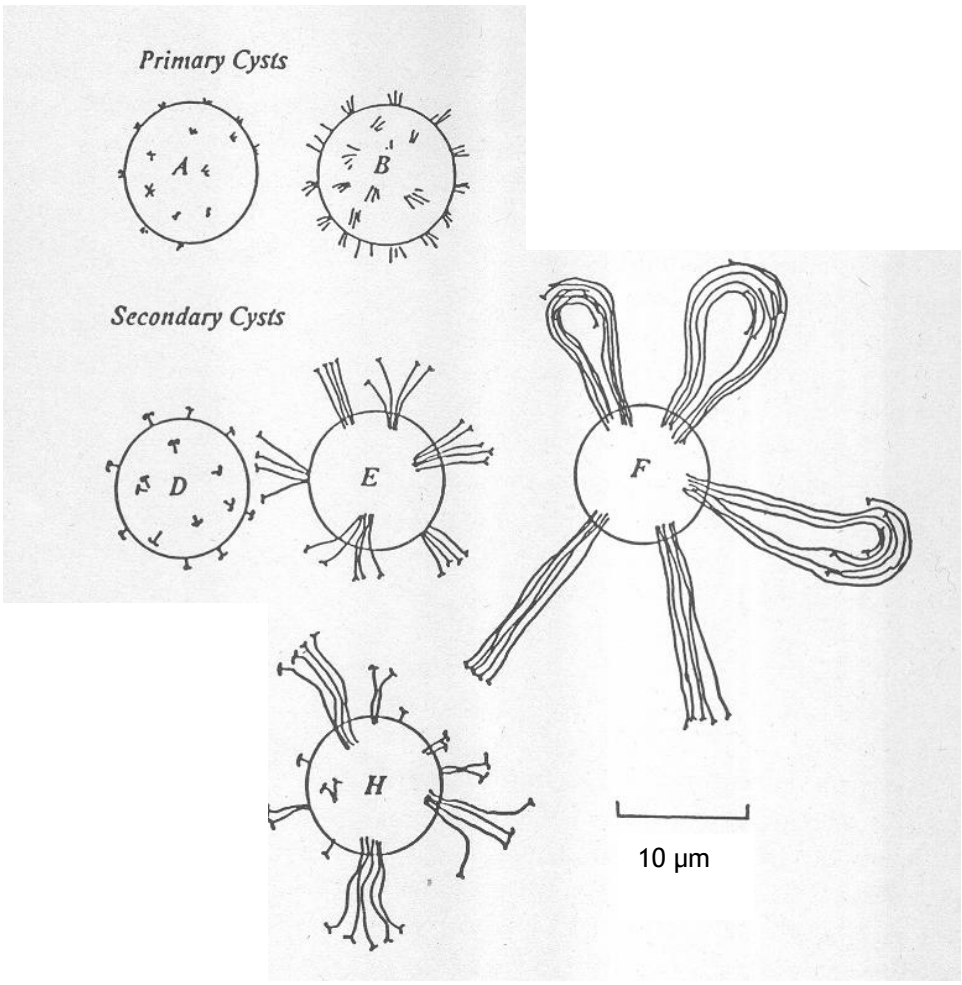

Figure 1.4. Schematic diagrams of primary cyst ornamentation of *S. ferax* (A) and *S. parasitica* (B) and secondary cyst ornamentation of short-spined *S. ferax* (D) and long-spined *S. parasitica* (E, F, H). From Beakes et al. (1994).

1.6.2 Experiment 2: Do fathead minnows exhibit a change in club cell density or size, or epidermal thickness in response to the physical presence of pathogens or to chemical cues of pathogens alone?

It has been demonstrated that club cell densities can increase in fathead minnows exposed to levels of *Saprolegnia* spp. as low as 20 cysts/L (Chivers *et al*. 2007). It seems that there would be a relatively small chance of attachment and physical penetration of the hyphae at such low concentrations. Perhaps other forms of stimuli are responsible for the increase in club cell density, thus the objective of Experiment 2 was to test the hypothesis that *Saprolegnia parasitica* conditioned water is all that is necessary to evoke an increase in club cell density and/or a higher turnover rate of club cells.

1.6.3 Experiment 3: How quickly can minnows alter their club cell density or size, or epidermal thickness?

The aforementioned experiments, as well as others in the literature, ended after 11-16 days of exposure. While it is evident that this period has allowed enough time for cellular changes to take place, the objective of Experiment 3 was to determine at what point during the exposure period a significant difference in the club cell density could be detected.

2. MATERIALS AND METHODS

2.1 FISH COLLECTION AND MAINTENANCE

Non-breeding fathead minnows were collected from Pike Lake, Saskatchewan, Canada (51° 53' 35" N, 106° 48' 40" W) using a beach seine in July 2005, and in May and October 2006. They were transported back to the University of Saskatchewan and housed for one week in a 6000 L flow-through pool filled with filtered dechlorinated tap water. Fish were maintained on a 14:10h light:dark cycle at 21 ºC, and were fed Nutrafin® tropical fish flakes (guaranteed 46% minimum crude protein, 5% minimum crude fat, 2% maximum crude fibre, 8% maximum moisture) *ad libitum*.

Prior to the start of the experiments, minnows were transferred into individual glass aquaria containing 9.5 L of filtered dechlorinated water and equipped with a single 2.5 cm cylindrical airstone. Fish were left to acclimate in these tanks for 8 to 12 d to ensure that their epidermis had healed if it had been damaged in the transfer. The day prior to exposure, a 50% water change was conducted in each tank by siphoning out 50% of the tank water and replacing it with filtered dechlorinated water at room temperature. Fish were maintained under the same light and food regime throughout the experiments.

2.2 *SAPROLEGNIA* CULTURES

Zoospores were prepared from cultures of *Saprolegnia ferax* (Gruithuisen) Thuret, 1850 (ATCC 36051) and *S. parasitica* Coker, 1923 (ATCC200013). *Saprolegnia* spp. colonies were grown in liquid YG media (ATCC medium 1994: 2.5 g yeast extract, 10.0 g glucose, 1.0 L distilled water) from 1 mm^3 cubes cut from a vigorously growing colony on YG agar (15.0 g granulated agar added to liquid YG medium above). Colonies were grown 8 – 16 h at room temperature (~ 21 ºC). Zoosporogenesis was induced by aspirating the liquid medium and replacing it with dilute salt solution (DSS) (Dill and Fuller 1971). This was repeated four times in the first hour, then once an hour for 3 h. Zoospore release typically began at 6 h. The following day, the zoospore/dilute salt suspensions were transferred to sterile 50 mL centrifuge tubes and vortexed to induce

encystment. Mixtures were stirred for 10 s to resuspend the cysts immediately prior to each sampling event. Ten samples of 15 µL each were counted on a haemocytometer and an average cyst count was calculated. The suspensions were diluted with DSS to achieve appropriate concentrations.

2.3 EXPERIMENTAL PROTOCOL

2.3.1 Experiment 1: The effects of varying pathogen risk on fathead minnow club cell investment

In Experiment 1, fish were assigned to a treatment using a random number table and treated with 1 mL of either a low, medium or high concentration of *S. ferax* or *S. parasitica* cyst suspension on the first, fourth and tenth days of the experiment. Due to a contamination problem, no zoospores/cysts were produced on the seventh day and therefore the treatment was skipped for that day. Final concentrations were ~ 2 cysts/L, 20 cysts/L and 200 cysts/L for low, medium and high concentration treatments, respectively. Due to the 2 x 3 structure of the statistical analysis, a control treatment was not included.

On day 12 of the experiment, fish were euthanized with an overdose of tricaine methane sulphonate (MS-222) (in accordance with the University of Saskatchewan Committee on Animal Care and Supply Protocol 20050067), weighed (g) and then the standard length of the fish was measured (length from tip of snout to last vertebra in cm). A coefficient of body condition for each minnow was calculated according to the following equation:

$$K = \frac{\text{weight (g)}}{\text{standard length}^3 \text{ (cm)}} \times 100$$

Fish were then fixed in 10% neutral buffered formalin (3.7% formaldehyde w/v) in individual plastic containers.

2.3.2 Experiment 2: The effects of *S. parasitica* conditioned water on fathead minnow club cell investment

Treatments consisted of 1 mL of *S. parasitica* cyst suspension (approximately 200 cysts/L), 1 mL of *S. parasitica* cyst suspension that had been previously filtered (passed through a double layer of 8 µm Whatman Filter Paper Grade No. 2 and then examined using a haemocytometer to ensure no zoospores/cysts were present) (0 cysts/L), or 1 mL of dilute salt solution (a control for the media in which the cyst suspensions were made). Fish were treated every three days, on the first, fourth, seventh and tenth days of the experiment. On day 12 of the experiment, fish were euthanized, measured and preserved as in Experiment 1.

2.3.3 Experiment 3: The effects of exposure duration on fathead minnow club cell investment

Minnows were treated with 1 mL of either *S. ferax* cyst suspension (approximately 200 cysts/L) or dilute salt solution on day one, and then were randomly selected to be sacrificed on day three, six, nine or twelve. Once sacrificed, fish were measured and preserved as in experiments 1 and 2.

2.4 HISTOLOGY PREPARATION

A section of epidermis (8 x 5 mm) was excised posterior to the operculum on one side of each minnow (Figure 2.1), placed in a histocassette between two biopsy pads, and preserved in 10% neutral buffered formalin (3.7% formaldehyde w/v). The tissue was dehydrated using a series of ethanol soaks of increasing strengths (20 min in each of 70%, 90%, 95% and 100%), followed by 20 min in a 50:50 100% ethanol:xylene bath and then immersed in xylene for an additional 20 minutes. Tissue was then impregnated with liquid Polyfin® embedding and infiltration wax (Triangle Biomedical Sciences, Durham, NC, USA) that was melted at 60 ºC for 12-16 h. A temperature higher than 60 ºC can burn the wax, and a melting period of at least 6-8 hours was required for best infiltration (modified from Kiernan 1999; Presnell and Schreibman 1997). Tissue samples were immersed for 20 min each in two consecutive changes of liquid paraffin. Samples were sectioned (5-7 µm thick) on a rotary microtome. The tissue ribbons were floated on a warm water bath (50 ºC) and then 4-5 sections were placed onto labelled

Superfrost Plus® glass slides (VWR International, USA). The sections were allowed to dry on a warm plate (37° C) for 2-4 h. The tissue was rehydrated in a series of steps consisting of soaks in xylene and ethanol solutions of decreasing strengths (Appendix B). Slides were stained with periodic acid Schiff's reagent and counterstained with Harris' haematoxylin (PAS-H) as described in Bancroft and Stevens (1996) (Appendices B and C). Club cells are PAS-H negative and appear white with dark central nuclei, whereas mucous cells are PAS-positive and appear dark with basal nuclei (Figures 2.2 and 2.3).

Skin sections were imaged using a Zeiss META 510 laser scanning confocal microscope with a helium-neon laser illumination (543nm) and 25 X Plan-Neofluar (N.A. 0.8) objective lens, at a resolution of 1024 x 1024 pixels. The system simultaneously collects fluorescence and transmitted light images. Fluorescence was not important to the study in question, but rather this system was used for ease of image collection for basic image analysis tools. Therefore, only the transmitted light images were analyzed using Image J software (available on the National Institution of health's webpage http://rsb.info.nih.gov/ij).

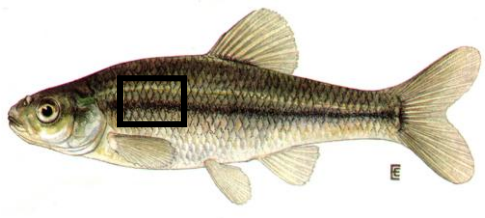

Figure 2.1. Section of epidermal tissue (5 x 8 mm) excised from the fathead minnow for histological processing. (Diagram modified from Kraft et al. 2006).

Three images were collected of a single skin thin section from each fish (typically 40 fish per treatment) in order to assess the number and area of club cells and epidermal thickness. Measurements of epidermal thickness were taken from the basement membrane to the outer edge of the epidermal cell layer at three different locations on the image (left, middle, and right) and averaged for statistical analyses (mean ± SE) (Figures 2.2 and 2.3). An image tool within Image J was utilized to outline and calculate the area

of skin in the field of view as well as the area of each club cell (as a proxy for cell volume), after which the percent area of cross sectional skin covered by club cells could also be calculated. Each fish was assigned a number at the beginning of the experiment to ensure that sample preparation, measurements and cell counts were done blind with respect to the experimental treatments.

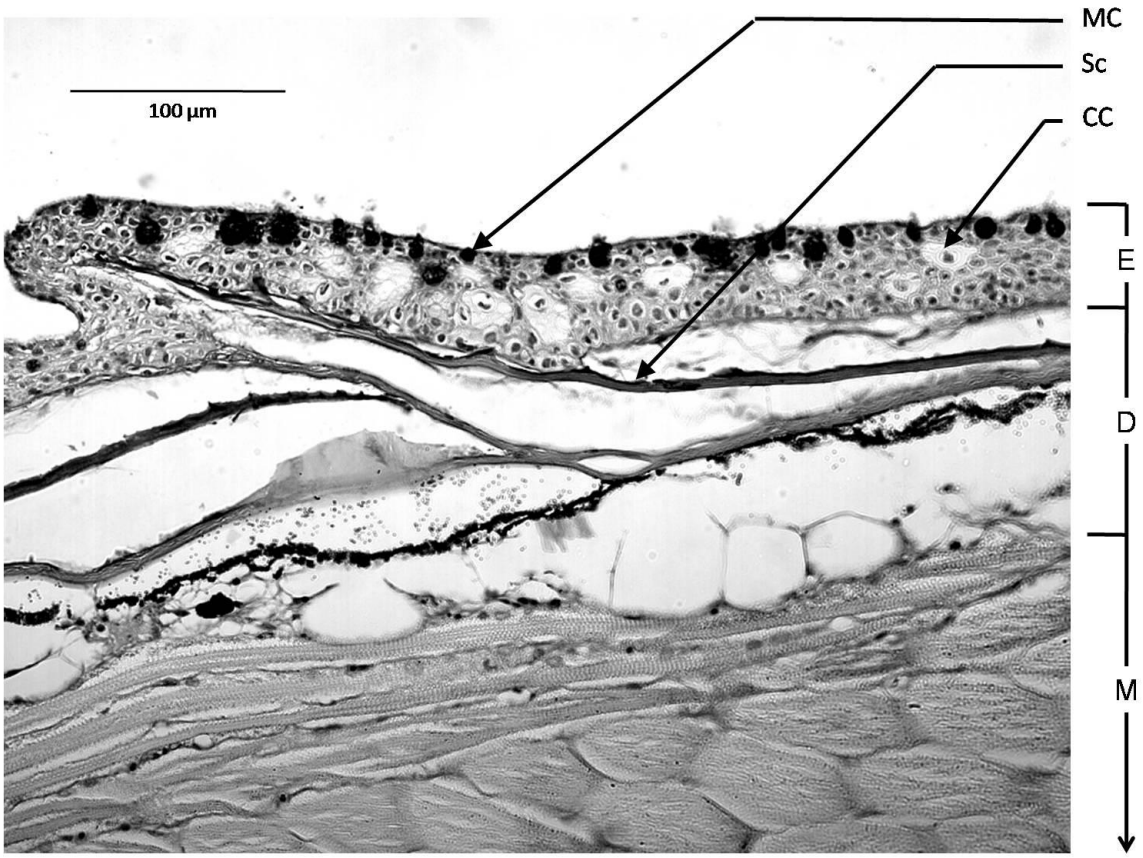

Figure 2.2. A cross section of fathead minnow skin showing the epidermal (E), dermal (D), and muscle (M) layers. Club cells (CC), mucous cells (MC), and dermal scales (Sc) are indicated with arrows. This section was stained with periodic acid-Schiff's reagent, and then counterstained with haematoxylin. Club cells are PAS-H negative and appear white with dark central nuclei, while mucous cells are generally PAS-H positive and appear dark. Photo by R. Pollock.

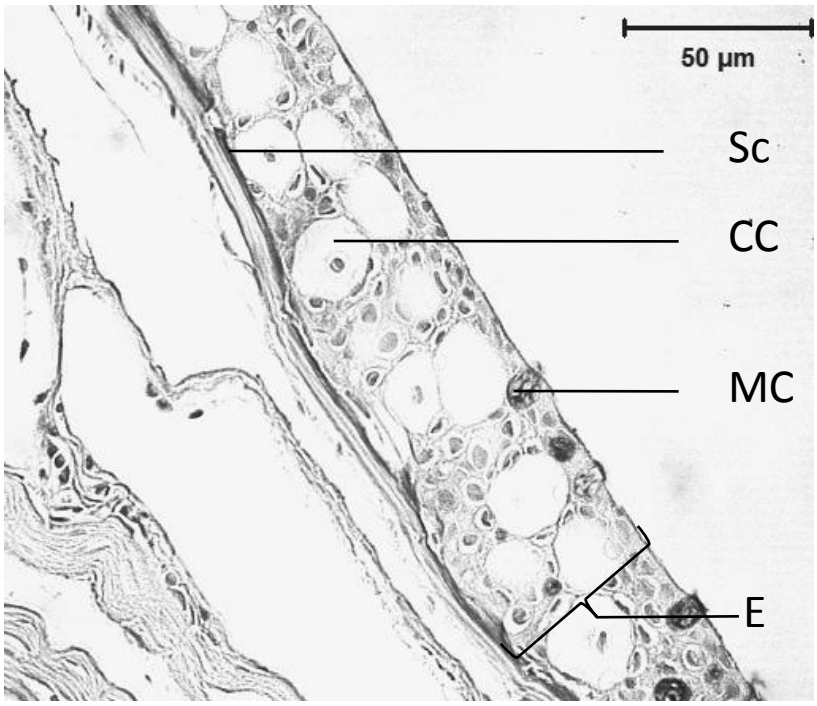

Figure 2.3. A higher magnification (1.7X zoom) image of a cross section of fathead minnow epidermal (E) layer including club cells (CC) and mucous cells (MC). Dermal scales (Sc) are also indicated. Photo by R. Pollock.

2.5 GENERAL STATISTICAL ANALYSES

Statistical analyses were conducted on the mean histological data obtained from three images of a single skin cross section from each fish. For all experimental data, assumptions of normality were tested using a Kolmogorov-Smirnov test that compares the cumulative distribution of the data to the expected cumulative normal (Gaussian) distribution. Levene's test of homogeneity was used to test for equality of variance across treatments. For those data that displayed normal distribution and equality of variance, a two-tailed ANOVA was used to test for significant differences between treatment groups for parameters such as epidermal thickness or individual club cell area. A non-parametric equivalent (Kruskal-Wallis test) was applied to the data if they were not normally distributed or if the variances were not equal. Due to histological inconsistencies (e.g. tissue torn during histological preparation) the area of *continuous* epidermal tissue within the microscope field sometimes differed between samples. For

this reason, the variance associated with differing epidermal area was accounted for by using epidermal area as a covariable when conducting an analysis of variance (ANCOVA). ANCOVAs using skin area as a covariable met the assumptions of an ANCOVA including linearity of response, homogeneity of slopes, independence of data, equality of variance, and normality of data. Using the Bray Curtis index of similarity, outliers were removed that were greater than 2.5 standard deviations (SD) away from the mean. All statistical analyses were conducted using SPSS version 16.

3. RESULTS

3.1 EXPERIMENT 1: THE EFFECTS OF VARYING PATHOGEN RISK ON FATHEAD MINNOW CLUB CELL INVESTMENT

Statistical analyses were based on sample sizes ranging from 31-44 skin thin sections, each from an individual fish (see Table 3.1). Kolmogorov-Smirnov tests determined that the data were distributed normally and Levene's test of homogeneity showed that the variances were equal between treatments for all variables.

3.1.1 Mean body condition index

Body condition index values ranged from 1.2 to 3.5. A two-way ANOVA determined there were no significant effects of pathogen species or pathogen concentration on the body condition of the fish (ANOVA: pathogen $F_{[1]} = 0.025$, $P = 0.874$; ANOVA: concentration $F_{[2]} = 0.600$, $P = 0.549$), nor was there an interaction (ANOVA: pathogen*concentration $F_{[2]} = 1.407$, $P = 0.247$) (Table 3.1).

3.1.2 Mean epidermal thickness

Three measurements of epidermal thickness were taken for each photograph of skin (range: 20.8 µm to 61.4 µm), and statistical analyses were based on the mean of these three measurements. There was no effect of pathogen species, or of pathogen concentration, on the mean epidermal thickness (ANOVA: pathogen $F_{[1]} = 0.723$, $P = 0.396$; ANOVA: concentration $F_{[2]} = 0.490$, $P = 0.613$), nor was there was an interaction between the pathogen species and the concentration of cysts (ANOVA: pathogen*concentration $F_{[2]} = 2.644$, $P = 0.073$) (Figure 3.1, Table 3.1).

Table 3.1. Statistical results for Experiment 1.

Experiment 1		Sample size (n)	Mean (± SE) body condition index	Test	Results
S. ferax	2 cysts/L	40	1.66 ± 0.03	ANOVA	Pathogen $F_{[1]} = 0.025$, $P = 0.874$ Concentration $F_{[2]} = 0.600$, $P = 0.549$ Pathogen*Concentration $F_{[2]} = 1.407$, $P = 0.247$
	20 cysts/L	44	1.66 ± 0.02		
	200 cysts/L	31	1.71 ± 0.02		
S. parasitica	2 cysts/L	42	1.72 ± 0.02		
	20 cysts/L	41	1.65 ± 0.02		
	200 cysts/L	42	1.69 ± 0.02		
			Mean (± SE) epidermal thickness (µm)		
S. ferax	2 cysts/L	40	35.2 ± 1.2	ANOVA	Pathogen $F_{[1]} = 0.723$, $P = 0.396$ Concentration $F_{[2]} = 0.490$, $P = 0.613$ Pathogen*Concentration $F_{[2]} = 2.644$, $P = 0.073$
	20 cysts/L	44	33.8 ± 1.0		
	200 cysts/L	31	36.3 ± 1.4		
S. parasitica	2 cysts/L	42	35.5 ± 1.2		
	20 cysts/L	41	35.0 ± 1.2		
	200 cysts/L	42	32.4 ± 1.0		
			Mean (± SE) number of club cells		
S. ferax	2 cysts/L	40	14.9 ± 0.7	ANCOVA (skin area as covariable)	Pathogen $F_{[1]} = 0.048$, $P = 0.826$ Concentration $F_{[2]} = 1.540$, $P = 0.216$ Pathogen*Concentration $F_{[2]} = 0.091$, $P = 0.913$
	20 cysts/L	44	15.6 ± 0.7		
	200 cysts/L	31	17.3 ± 1.2		
S. parasitica	2 cysts/L	42	15.3 ± 1.0		
	20 cysts/L	41	15.9 ± 0.9		
	200 cysts/L	42	16.1 ± 1.0		

Table 3.1 continued. Statistical results for Experiment 1.

Experiment 1		Sample size (n)	Mean (± SE) area of club cell (μm^2)	Test	Results
S. ferax	2 cysts/L	40	156 ± 5.0	ANOVA	Pathogen $F_{[1]} = 1.957$, $P = 0.163$ Concentration $F_{[2]} = 4.002$, ***P* = 0.020**** Pathogen*Concentration $F_{[2]} = 0.095$, $P = 0.910$ Post hoc LSD test: area of club cell in 200 cysts/L < than area of club cell in 2 cysts/L
	20 cysts/L	44	146 ± 4.4		
	200 cysts/L	31	140 ± 5.9		
	20 cysts/L	41	142 ± 5.8		
	200 cysts/L	42	133 ± 5.4		
			Mean (± SE) percentage of skin cross section covered by club cells		
S. ferax	2 cysts/L	40	14.0 ± 0.7	ANOVA	Pathogen $F_{[1]} = 1.608$, $P = 0.206$ Concentration $F_{[2]} = 0.330$, $P = 0.719$ Pathogen*Concentration $F_{[2]} = 0.646$, $P = 0.525$
	20 cysts/L	44	14.2 ± 0.7		
	200 cysts/L	31	13.5 ± 1.0		
S. parasitica	2 cysts/L	42	13.2 ± 0.8		
	20 cysts/L	41	13.0 ± 0.7		
	200 cysts/L	42	13.6 ± 0.8		

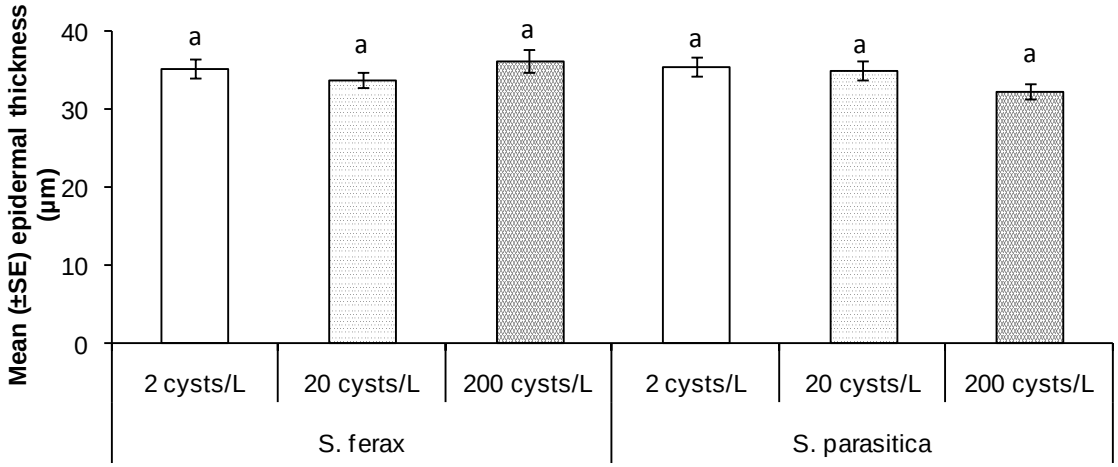

Figure 3.1. Mean (± SE) epidermal thickness (µm) of fathead minnows treated with *Saprolegnia ferax* or *S. parasitica* (2 cysts/L, 20 cysts/L or 200 cysts/L). Different letters denote significant differences at P ≤ 0.05; therfore, there were no significant differences between treatments.

3.1.3 Mean number of club cells

A two-way ANCOVA was performed to determine whether the species of pathogen, *S. parasitica* or *S. ferax*, or the concentration of pathogen (2, 20 or 200 cysts/L) had an effect on the number of club cells in the minnow skin. The mean number of club cells was calculated for each skin section photograph (range: 1 to 40), and then a mean was calculated for each treatment (Table 3.1). There was no difference in the number of club cells between fish treated with *S. parasitica* versus *S. ferax* (ANCOVA with skin area as covariable: pathogen $F_{[1]} = 0.048$, $P = 0.826$), nor was there a significant effect of the concentration of pathogen on the number of club cells (ANCOVA with skin area as covariable: concentration $F_{[2]} = 1.540$, $P = 0.216$) (Figure 3.2). No interaction between species of *Saprolegnia* and concentration was detected (ANCOVA with skin area as covariable: pathogen*concentration $F_{[2]} = 0.091$, $P = 0.913$). These results are graphically represented with the mean number of club cells on one axis and the mean area of epidermis (mm^2) on the other axis for ease of interpretation (Figure 3.2).

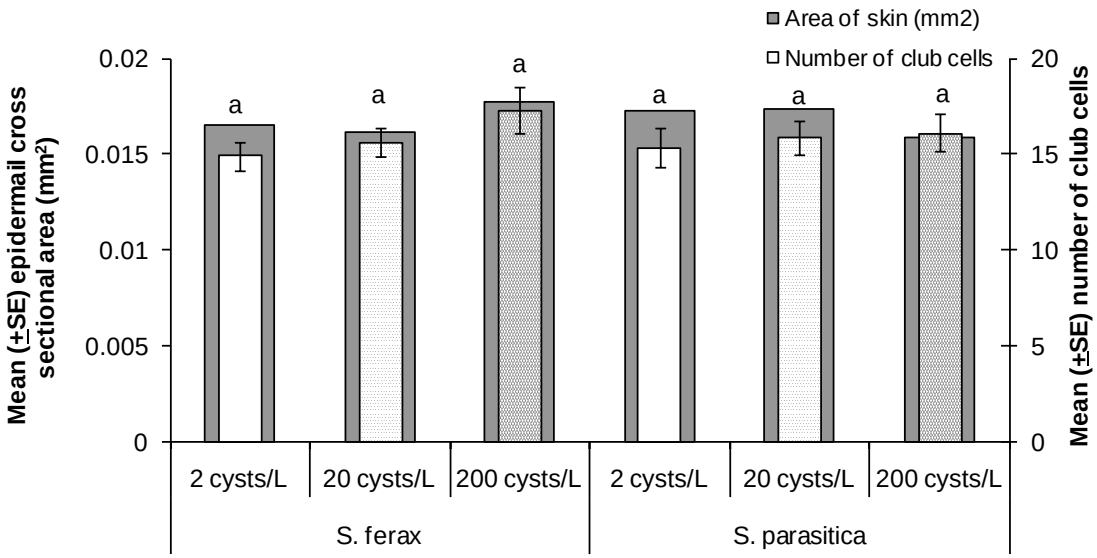

Figure 3.2. Mean (± SE) number of club cells with corresponding epidermal area (mm^2) in fathead minnows treated with *Saprolegnia ferax* or *S. parasitica* (2 cysts/L, 20 cysts/L or 200 cysts/L). Different letters denote significant differences at $P \leq 0.05$; therfore, there were no significant differences between treatments.

3.1.4 Mean area of individual club cells

In order to determine whether pathogen species and/or pathogen concentration had an effect on the size of individual club cells, an ANOVA was performed on the mean area of individual club cells within each treatment (mean area of individual club cells for fish treated with: low concentration of *S. ferax* treatment = 156 μm^2, medium concentration of *S. ferax* treatment = 145 μm^2, high concentration of *S. ferax* treatment = 140 μm^2, low concentration of *S. parasitica* treatment = 148 μm^2, medium concentration of *S. parasitica* treatment = 142 μm^2, high concentration of *S. parasitica* treatment = 133 μm^2). The area of an individual club cell ranged from 51 to 256 μm^2. There was no effect of pathogen species on the mean area of individual club cells (ANOVA: pathogen $F_{[1]} = 1.957$, $P = 0.163$) nor was there an interaction between pathogen species and concentration (ANOVA: pathogen*concentration $F_{[2]} = 0.095$, $P = 0.910$). However, the concentration of cysts significantly affected the mean area of individual club cells (ANOVA: concentration $F_{[2]} = 4.002$, $P = 0.020$) (Figure 3.3). Least significant difference *post hoc* tests indicate that the club cells in the high concentration treatments were significantly smaller than those in the low concentration treatments ($P = 0.005$).

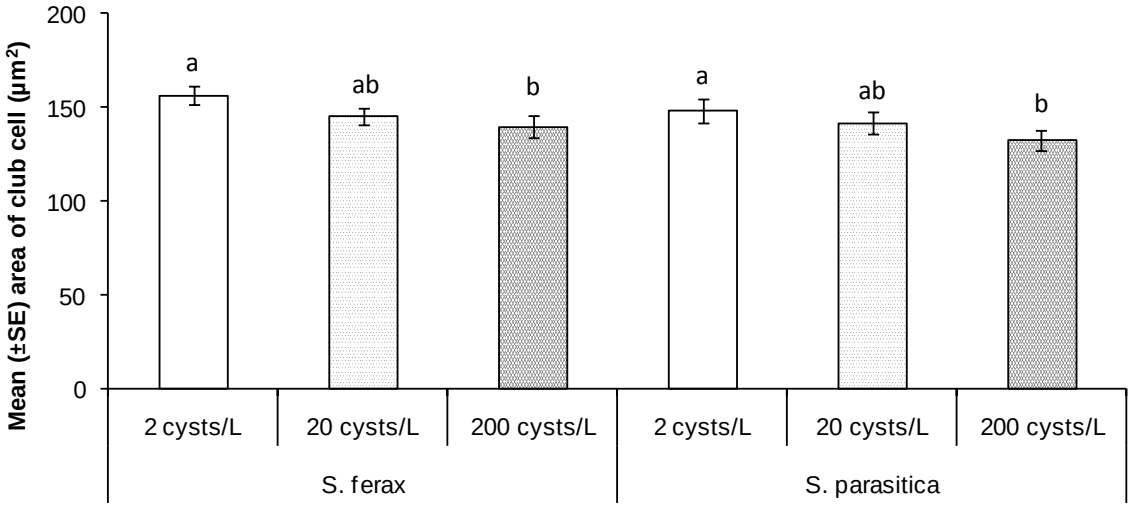

Figure 3.3. Mean (± SE) area of epidermal club cells in fathead minnows treated with *Saprolegnia ferax* or *S. parasitica* (2 cysts/L, 20 cysts/L or 200 cysts/L). Different letters denote significant differences at P ≤ 0.05.

3.1.5 Mean percentage of epidermal cross section area covered by club cells

The percentage of cross section area covered by club cells was calculated by summing the area of all club cells in an image and dividing by the total skin cross section area in the image. The percentage of cross section area covered by club cells was then averaged over the three images for each fish sample (range: 1% to 28%) (Table 3.1). A two-way ANOVA determined that there was no effect of pathogen (ANOVA: pathogen $F_{[1]} = 0.923$, $P = 0.338$), or concentration (ANOVA: $F_{[2]} = 0.008$, $P = 0.993$) on the percentage of skin area covered by club cells, nor was there an interaction (ANOVA: pathogen*concentration $F_{[2]} = 0.306$, $P = 0.736$) (Figure 3.4).

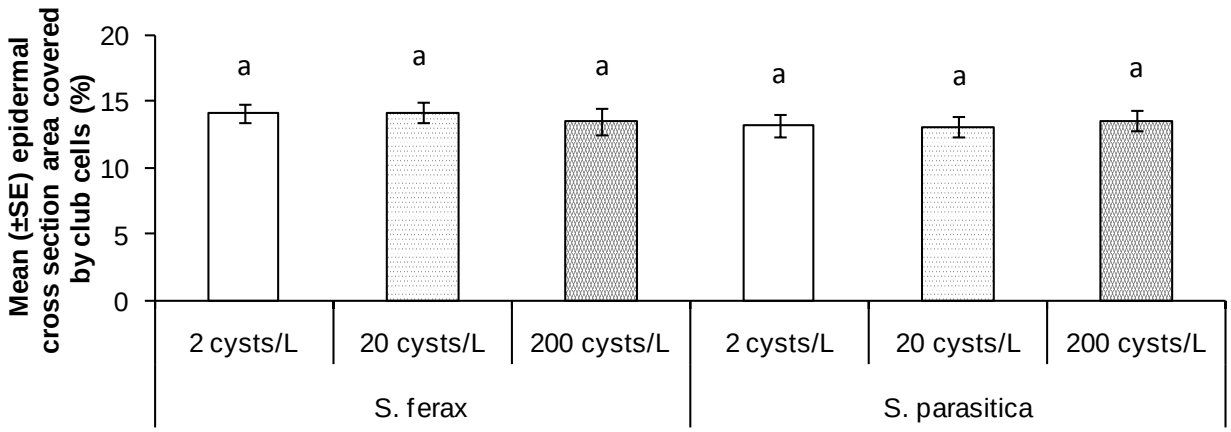

Figure 3.4. Mean (± SE) percentage of epidermal cross section area covered by club cells in fathead minnows treated with *Saprolegnia ferax* or *S. parasitica* (2 cysts/L, 20 cysts/L or 200 cysts/L). Different letters denote significant differences at $P \leq 0.05$; therfore, there were no significant differences between treatments.

3.2 EXPERIMENT 2: THE EFFECTS OF *S. PARASITICA* CONDITIONED WATER ON FATHEAD MINNOW CLUB CELL INVESTMENT

Due to fish mortality and problems with histological preparation, statistical analyses were based on the following sample sizes: cyst treatment (n= 47), cyst conditioned treatment (n = 49), control (n = 38). Kolmogorov-Smirnov tests determined the data were distributed normally and Levene's test of homogeneity showed the variances were equal between treatments for all variables.

3.2.1 Mean body condition index

Body condition index values ranged from 1.2 to 2.1. A one-way ANOVA found no differences in body condition index between treatments (ANOVA: treatment $F_{[2]} = 0.086$, $P = 0.918$) (Table 3.2).

3.2.2 Mean epidermal thickness

Three measurements of epidermal thickness were taken for each image of skin (range: 24 µm to 65 µm), and statistical analyses were based on the mean of those three measurements. There was no effect of treatment on the mean epidermal thickness (ANOVA: treatment $F_{[2]} = 1.840$, $P = 0.163$) (Figure 3.5, Table 3.2).

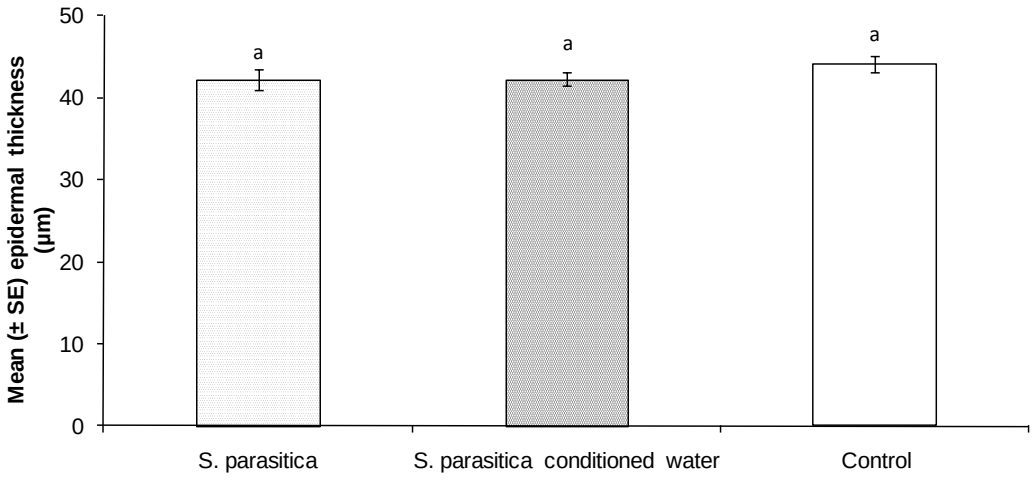

Figure 3.5. Mean (± SE) epidermal thickness in fathead minnows treated with *S. parasitica* (200 cysts/L) (n = 47), water conditioned with *S. parasitica* cysts (0 cysts/L) (n = 49), or a control (dilute salt solution)(n = 38). Different letters denote significant differences a $P \leq 0.05$; therfore, there were no significant differences between treatments.

Table 3.2. Statistical results for Experiment 2.

Experiment 2	Sample size (n)	Mean (± SE) body condition index	Test	Results
*S. parasitica (*200 cysts/L)	47	1.74 ± 0.02	ANOVA	Treatment $F_{[2]} = 0.086$, $P = 0.918$
S. parasitica conditioned water (0 cysts/L)	49	1.74 ± 0.02		
Dilute salt solution	38	1.72 ± 0.02		
		Mean (± SE) epidermal thickness (µm)		
*S. parasitica (*200 cysts/L)	47	42.2 ± 1.3	ANOVA	Treatment $F_{[2]} = 1.840$, $P = 0.163$
S. parasitica conditioned water (0 cysts/L)	49	42.3 ± 0.2		
Dilute salt solution	38	44.1 ± 1.0		
		Mean (± SE) number of club cells		
*S. parasitica (*200 cysts/L)	47	9.6 ± 0.6	ANCOVA (skin area as covariable)	Treatment $F_{[2]} = 0.890$, $P = 0.413$
S. parasitica conditioned water (0 cysts/L)	49	9.2 ± 0.4		
Dilute salt solution	38	10.3 ± 0.6		
		Mean (± SE) area of club cell (µm^2)		
*S. parasitica (*200 cysts/L)	47	181 ± 5.9	ANOVA	Treatment $F_{[2]} = 0.222$, $P = 0.801$
S. parasitica conditioned water (0 cysts/L)	49	177 ± 4.6		
Dilute salt solution	38	178 ± 6.4		

Table 3.2 continued. Statistical results for Experiment 2.

Experiment 2 (continued)	Sample size (n)	Mean (± SE) percentage of epidermal cross section covered by club cells	Test	Results
*S. parasitica (*200 cysts/L)	47	7.5 ± 0.4	ANOVA	Treatment $F_{[2]} = 1.609$, $P = 0.204$
S. parasitica conditioned water (0 cysts/L)	49	7.1 ± 0.3		
Dilute salt solution	38	8.1 ± 0.5		

3.2.3 Mean number of club cells

Differences in area of epidermal cross section between samples could potentially affect the number of club cells counted in the skin section, and thus an ANCOVA was performed with cross section area as a covariable. The number of club cells ranged from 1 to 19. There was no significant effect of treatment on the number of club cells (ANCOVA $F_{[2]} = 0.890$, P = 0.413) (Figure 3.6, Table 3.2). These results are graphically represented as mean number of club cells per on one axis and mean epidermal area (mm^2) on another axis for ease of interpretation (Figure 3.6).

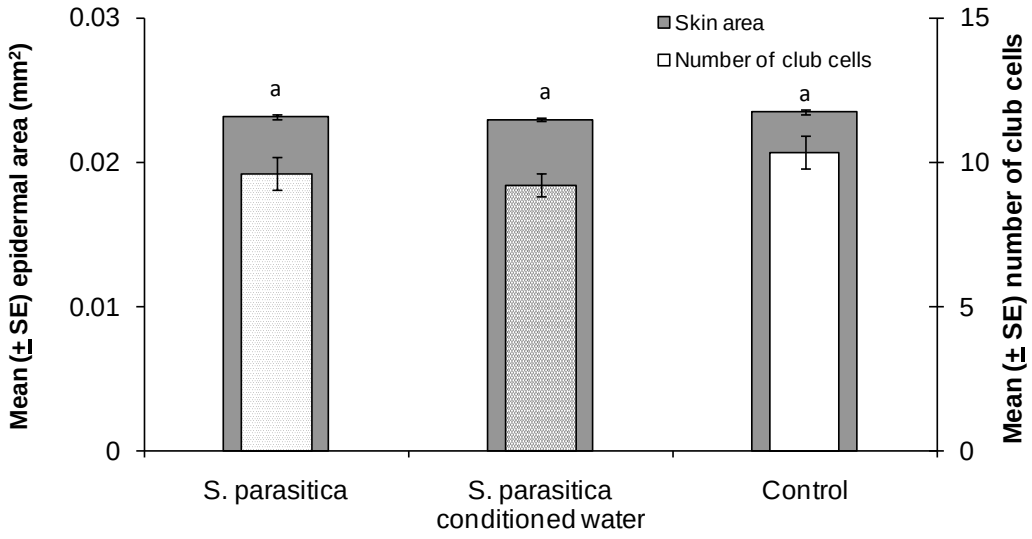

Figure 3.6. Mean (± SE) number of club cells with corresponding skin area (mm^2) of epidermal cross section in fathead minnows treated with *Saprolegnia parasitica* (200 cysts/L) (n = 47), water conditioned with *S. parasitica* cysts (0 cysts/L) (n = 49), or a control (dilute salt solution)(n = 38). Different letters denote significant differences a $P \leq 0.05$; therfore, there were no significant differences between treatments.

3.2.4 Mean area of individual club cells

In order to determine whether treatment had an effect on individual club cell area, the size of club cells were averaged per image (mean area of cells for: cyst treatment = 181 μm^2, cyst conditioned treatment = 176 μm^2, control treatment = 181 μm^2). Club cell area ranged from 50 μm^2 to 260 μm^2. An ANOVA determined there was no effect of treatment on mean individual club cell area (ANOVA: treatment $F_{[2]} = 0.222$, $P = 0.801$) (Figure 3.7, Table 3.2).

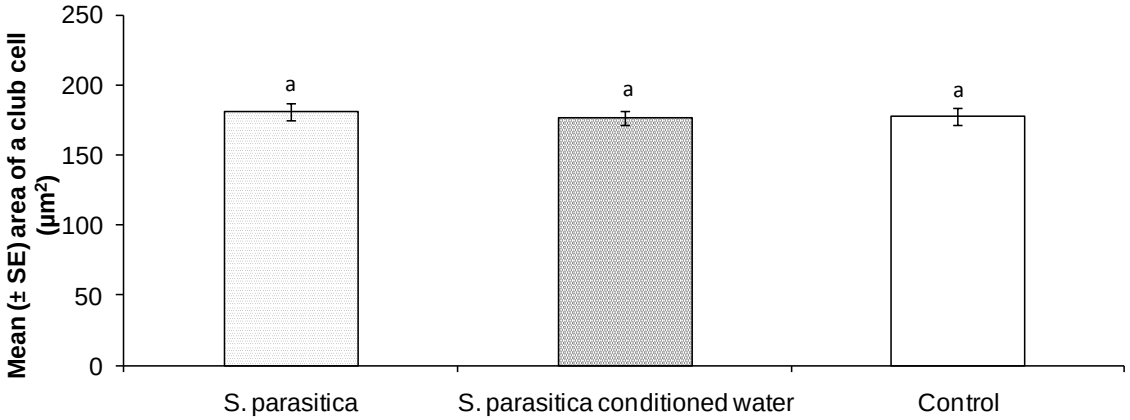

Figure 3.7. Mean (± SE) area of a club cell (μm^2) in fathead minnows treated with *S. parasitica* (200 cysts/L) (n = 47), water conditioned with *S. parasitica* cysts (0 cysts/L) (n = 49), or a control (dilute salt solution)(n = 38). Different letters denote significant differences at $P \leq 0.05$; therfore, there were no significant differences between treatments.

3.2.5 Mean percentage of epidermal cross section area covered by club cells

The percentage of skin area covered by club cells was calculated by summing the area of all club cells in an image and dividing by the total skin area in the image. The percentage of skin area covered by club cells was then averaged over the three images for each fish sample (mean percent area covered by club cells for: cyst treatment = 7.49%, cyst conditioned water treatment = 7.07%, control treatment = 8.13%). The percentage of skin area covered by club cells ranged from 1% to 15%. An ANOVA determined that

there was no effect of treatment (ANOVA: treatment $F_{[2]} = 1.609$, $P = 0.204$) (Figure 3.8, Table 3.2) on the percentage of epidermal cross section area coveres by club cells.

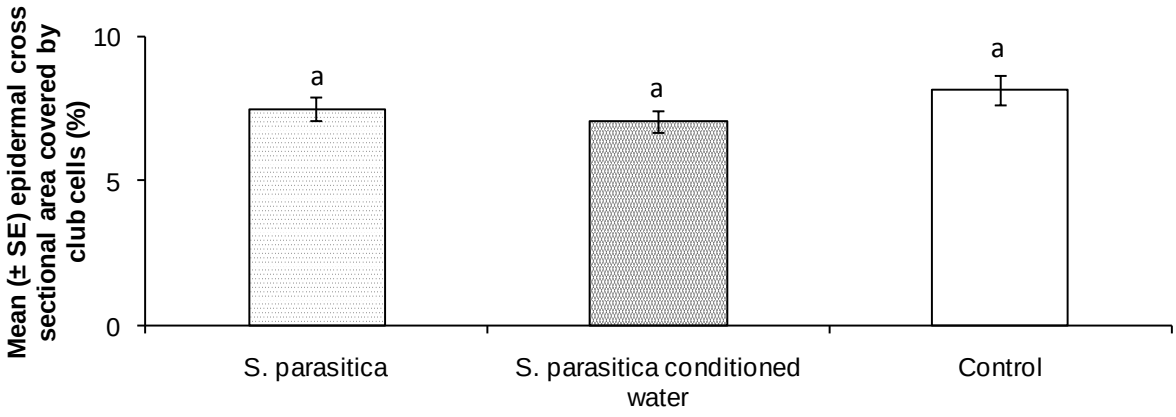

Figure 3.8. Mean (± SE) percentage of epidermal cross section area covered by club cells in fathead minnows treated with *Saprolegnia parasitica* (200 cysts/L) (n = 47), water conditioned with *S. parasitica* cysts (200 cysts/L) (n = 49), or a control (dilute salt solution) (n = 38). Different letters denote significant differences at $P \leq 0.05$; therfore, there were no significant differences between treatments.

3.3 EXPERIMENT 3: THE EFFECT OF EXPOSURE DURATION ON FATHEAD MINNOW CLUB CELL INVESTMENT

Statistical analyses were based on the following sample sizes: *S. ferax* cyst treatment (n = 120) and control (n = 121). Fish were exposed to the treatment for three (n = 71), six (n = 67), nine (n = 46) or twelve (n = 57) days. Upon completion of the exposure period, it was discovered that a portion of the fish exhibited signs of blackspot disease, a trematode infection that may have affected epidermal parameters. Therefore, fish were further grouped according to their health status (trematode infected, n = 58, or uninfected, n = 183). Kolmogorov-Smirnov tests determined that the data were distributed normally and Levene's test of homogeneity showed that the variances were equal between treatments for all variables.

3.3.1 Mean body condition index

Body condition index values ranged from 1.1 to 1.9. An ANOVA determined that there was no significant effect of treatment of cysts or DSS (ANOVA: treatment $F_{[1]} = 1.375$, $P = 0.242$) on the body condition index of the fish. Whether or not the fish was trematode infected also did not have an effect on the body condition index (ANOVA: health $F_{[1]} = 0.064$, $P = 0.800$). However, the duration for which the fish was exposed to the treatment did have an effect on the body condition index (ANOVA: day $F_{[3]} = 3.360$, $P = 0.020$), and subsequent post-hoc LSD tests indicated that those fish exposed to the treatment for twelve days were in poorer body condition than those fish exposed for nine or fewer days. Also, there was a significant difference between those fish exposed for three days and nine days, with the latter having better body condition (Table 3.3, Figure 3.9).

Table 3.3. Statistical results for Experiment 3.

Experiment 3			Sample size (n)	Mean (± SE) body condition index	Test	Results
		Day 6	29	1.55 ± 0.02		
		Day 9	17	1.58 ± 0.03		
		Day 12	21	1.44 ± 0.03		
	Dilute salt solution	Day 3	23	1.45 ± 0.07		
		Day 6	26	1.52 ± 0.05		
		Day 9	20	1.52 ± 0.07		
		Day 12	15	1.45 ± 0.03		
Trematode infected fish	*S. ferax* (200 cysts/L)	Day 3	5	1.00 ± 0.03		
		Day 6	3	1.51 ± 0.02		
		Day 9	3	1.58 ± 0.03		
		Day 12	10	1.50 ± 0.05		
	Dilute salt solution	Day 3	11	1.58 ± 0.03		
		Day 6	9	1.52 ± 0.05		
		Day 9	6	1.60 ± 0.06		
		Day 12	11	1.50 ± 0.04		

Table 3.3 continued. Statistical results for Experiment 3.

Experiment 3 (continued)			Sample size (n)	Mean (± SE) epidermal thickness (µm)	Test	Results
Uninfected fish	*S. ferax* (200 cysts/L)	Day 3	32	41.5 ± 1.7	ANOVA	Health $F_{[1]} = 0.418$, $P = 0.518$
		Day 6	29	41.8 ± 2.2		
		Day 9	17	46.9 ± 3.3		Treatment $F_{[1]} = 0.254$, $P = 0.615$
		Day 12	21	44.5 ± 2.6		
	Dilute salt solution	Day 3	23	39.2 ± 3.0		Day $F_{[3]} = 0.696$, $P = 0.555$
		Day 6	26	45.5 ± 2.1		
		Day 9	20	44.2 ± 6.7		Health*Treatment*Day $F_{[3]} = 0.901$, $P = 0.441$
		Day 12	15	39.3 ± 3.7		
Trematode infected fish	*S. ferax* (200 cysts/L)	Day 3	5	47.8 ± 3.1		
		Day 6	3	43.9 ± 2.4		
		Day 9	3	44.3 ± 2.1		
		Day 12	10	41.2 ± 2.4		
	Dilute salt solution	Day 3	11	40.5 ± 2.9		
		Day 6	9	42.7 ± 3.6		
		Day 9	6	47.7 ± 3.6		
		Day 12	11	43.6 ± 4.3		

Table 3.3 continued. Statistical results for Experiment 3.

Experiment 3 (continued)			Sample size (n)	Mean (± SE) number of club cells	Test	Results
Uninfected fish	*S. ferax* (200 cysts/L)	Day 3	32	12.9 ± 1.3	ANCOVA (skin area as covariate)	Health $F_{[1]} = 0.325$, $P = 0.569$ Treatment $F_{[1]} = 4.606$, ***P* =0.033**** Day $F_{[3]} = 0.635$, $P = 0.593$ Health*Treatment*Day = $F_{[3]} =$ 0.043, $P = 0.988$ Result: fish exposed to *S. ferax* treatment had more club cells than those exposed to dilute salt solution
		Day 6	29	13.7 ± 1.3		
		Day 9	17	13.8 ± 1.4		
		Day 12	21	13.8 ± 1.4		
	Dilute salt solution	Day 3	23	13.2 ± 2.9		
		Day 6	26	17.3 ± 5.8		
		Day 9	20	15.7 ± 7.2		
		Day 12	15	12.3 ± 1.9		
Trematode infected fish	*S. ferax* (200 cysts/L)	Day 3	5	13.7 ± 1.6		
		Day 6	3	12.3 ± 1.6		
		Day 9	3	11.8 ± 1.3		
		Day 12	10	11.4 ± 2.0		
	Dilute salt solution	Day 3	11	12.4 ± 1.4		
		Day 6	9	11.6 ± 2.2		
		Day 9	6	13.5 ± 1.8		
		Day 12	11	9.0 ± 1.4		

Table 3.3 continued. Statistical results for Experiment 3.

Experiment 3 (continued)			Sample size (n)	Mean (± SE) area of club cell (μm^2)	Test	Results
Uninfected fish	*S. ferax* (200 cysts/L)	Day 3	32	185 ± 10.1	ANOVA	Health $F_{[1]} = 3.921$, ***P* = 0.049****
		Day 6	29	188 ± 9.7		
		Day 9	17	219 ± 14.6		Treatment $F_{[1]} = 0.207$, $P = 0.649$
		Day 12	21	200 ± 13.8		
	Dilute salt solution	Day 3	23	217 ± 12.6		Day $F_{[3]} = 0.362$, $P = 0.780$
		Day 6	26	213 ± 13.6		
		Day 9	20	189 ± 12.5		Health*Treatment*Day $F_{[3]} = 1.176$, $P = 0.320$
		Day 12	15	201 ± 14.2		
Trematode infected fish	*S. ferax* (200 cysts/L)	Day 3	5	191 ± 16.3		
		Day 6	3	159 ± 22.0		
		Day 9	3	196 ± 14.0		
		Day 12	10	179 ± 22.2		
	Dilute salt solution	Day 3	11	160 ± 8.2		
		Day 6	9	204 ± 20.2		
		Day 9	6	201 ± 9.7		
		Day 12	11	168 ± 22.3		

Table 3.3 continued. Statistical results for Experiment 3.

Experiment 3 (continued)			Sample size (n)	Mean (± SE) percentage of epidermal cross section covered by club cells	Test	Results
Uninfected fish	*S. ferax* (200 cysts/L)	Day 3	32	6.9 ± 0.7	ANOVA	Health $F_{[1]} = 0.308$, $P = 0.580$
		Day 6	29	8.2 ± 0.8		
		Day 9	17	8.1 ± 0.8		Treatment $F_{[1]} = 2.231$, $P = 0.137$
		Day 12	21	7.5 ± 0.8		
	Dilute salt solution	Day 3	23	7.3 ± 0.7		Day $F_{[3]} = 0.767$, $P = 0.514$
		Day 6	26	7.7 ± 1.0		
		Day 9	20	6.7 ± 0.8		Health*Treatment*Day $F_{[3]} =$ 0.296, P = 0.828
		Day 12	15	7.1 ± 1.1		
Trematode infected fish	*S. ferax* (200 cysts/L)	Day 3	5	8.0 ± 1.7		
		Day 6	3	7.5 ± 2.6		
		Day 9	3	8.9 ± 1.0		
		Day 12	10	7.0 ± 1.3		
	Dilute salt solution	Day 3	11	5.8 ± 1.5		
		Day 6	9	7.5 ± 1.5		
		Day 9	6	7.2 ± 1.1		
		Day 12	11	4.9 ± 0.7		

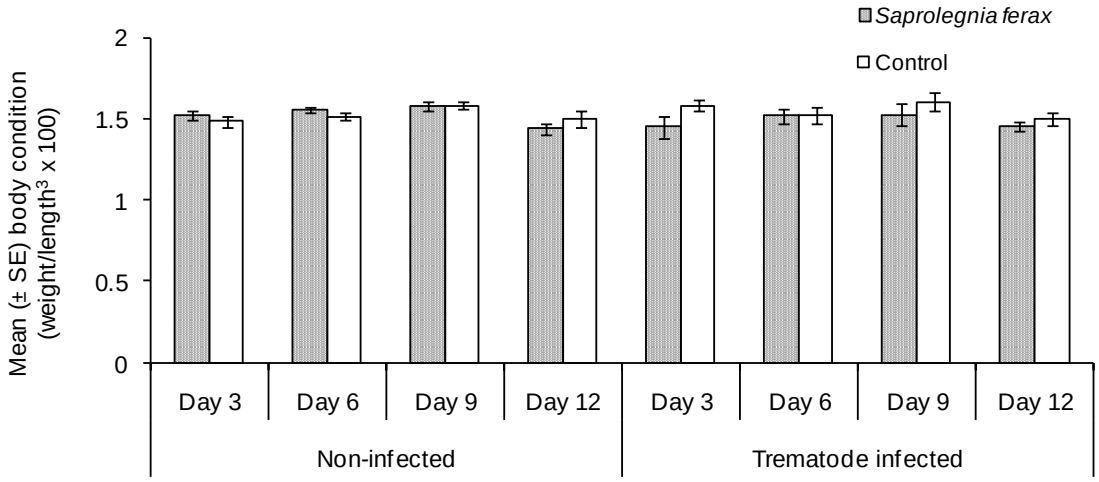

Figure 3.9. Mean (± SE) body condition index of fathead minnows exposed to *Saprolegnia ferax* (200 cysts/L) or a control (dilute salt solution) for a duration of three, six, nine or twelve days.

3.3.2 Mean epidermal thickness

Epidermal thickness ranged from 21 µm to 92 µm. There was no effect of treatment, day or health on the epidermal thickness of the fish (ANOVA: treatment $F_{[1]}$ = 0.254, P = 0.615; ANOVA: day $F_{[3]}$ = 0.696, P = 0.555; ANOVA: health $F_{[1]}$ = 0.418, P = 0.518) (Figure 3.10, Table 3.3).

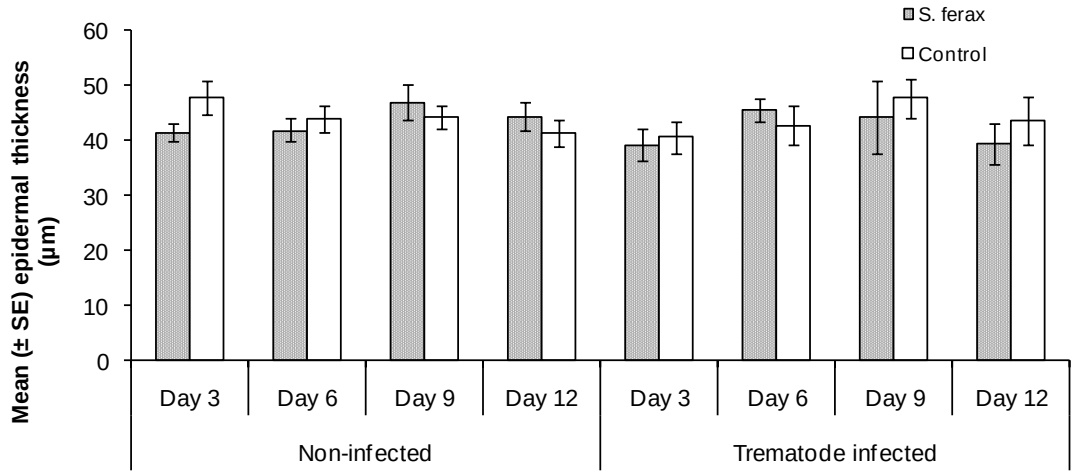

Figure 3.10. Mean (± SE) epidermal thickness of fathead minnows exposed to *Saprolegnia ferax* (200 cysts/L) or a control (dilute salt solution) for a duration of three, six, nine or twelve days.

3.3.3 Mean number of club cells

An ANCOVA was performed with skin area as a covariable examining the treatment, exposure duration and health of the fish as factors that could affect the number of club cells. The number of club cells ranged from 2 to 32. It was determined that whether the fish was trematode infected or not did not significantly affect the number of club cells (ANCOVA: health $F_{[1]} = 0.325$, $P = 0.569$). Further, the day on which the fish was sacrificed, and thus the duration of the exposure to *S. ferax* zoospores/cysts did not affect the number of club cells (ANCOVA: day $F_{[3]} = 0.635$, $P = 0.593$). Treatment did have an effect on the number of club cells (ANCOVA: treatment $F_{[1]} = 4.606$, $P = 0.033$) with the fish treated with the cyst suspension having more club cells than those treated with the control solution. Results are graphically represented as mean number of club cells (Figure 3.11, Table 3.4), as well as the corresponding epidermal area (mm^2) for the *S. ferax* treatment (Figure 3.12) and the control (Figure 3.13).

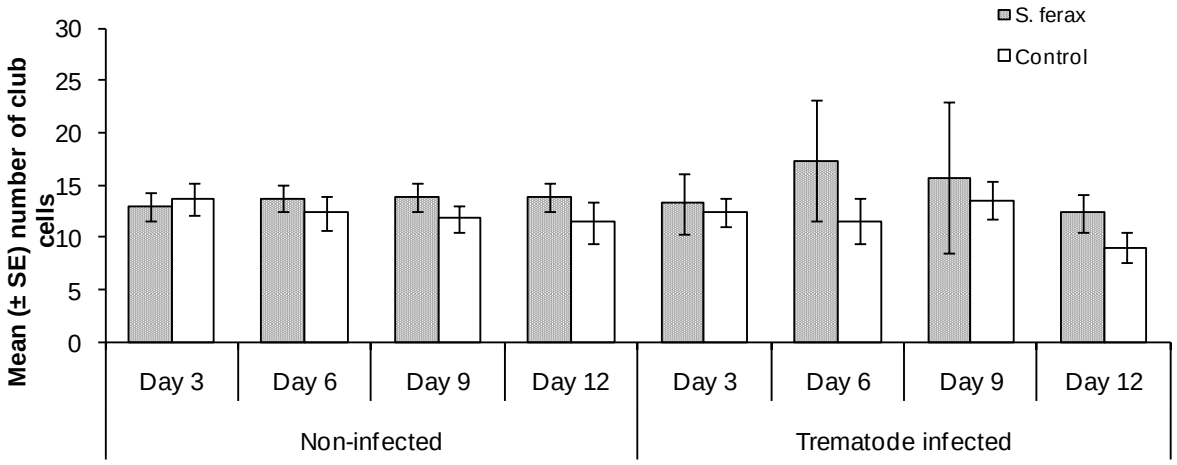

Figure 3.11. Mean (± SE) number of epidermal club cells in fathead minnows exposed to *Saprolegnia ferax* (200 cysts/L) or a control (dilute salt solution) for a duration of three, six, nine or twelve days.

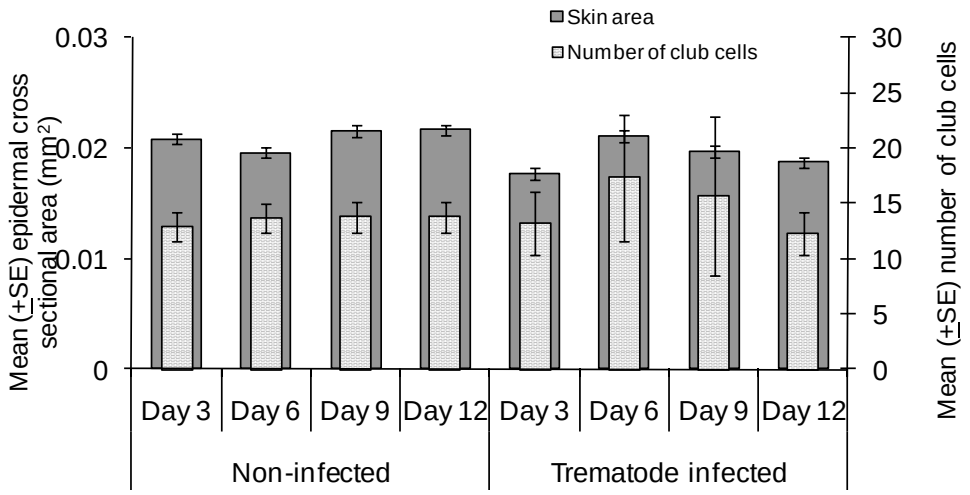

Figure 3.12. Mean (± SE) number of epidermal club cells and corresponding epidermal cross sectional area (mm^2) in fathead minnows exposed to *Saprolegnia ferax* (200 cysts/L) for a duration of three, six, nine or twelve days.

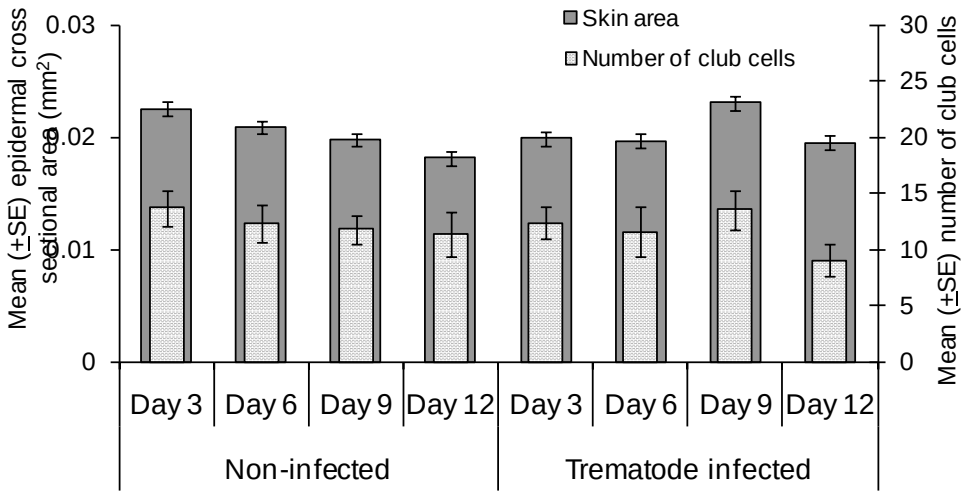

Figure 3.13. Mean (± SE) number of epidermal club cells and corresponding epidermal cross sectional area (mm^2) in fathead minnows exposed to a control (dilute salt solution) for a duration of three, six, nine or twelve days.

3.3.4 Mean area of individual club cells

The area of individual club cells ranged from 88 µm^2 to 334 µm^2. The length of exposure to the *S. ferax* cysts/zoospores did not have an effect on the mean area of individual club cells (ANOVA: day $F_{[3]} = 0.362$, $P = 0.780$). Treatment also did not have an effect on the mean area of individual club cells (ANOVA: treatment $F_{[1]} = 0.207$, $P = 0.649$). However, the "health" of the fish was determined to have an effect (ANOVA health $F_{[1]} = 3.921$, $P = 0.049$) as those fish that were trematode infected had smaller club cells (mean = 182 µm^2) compared to those fish that were not trematode infected and did not exhibit signs of blackspot disease (mean = 201 µm^2) (Figure 3.14, Table 3.3).

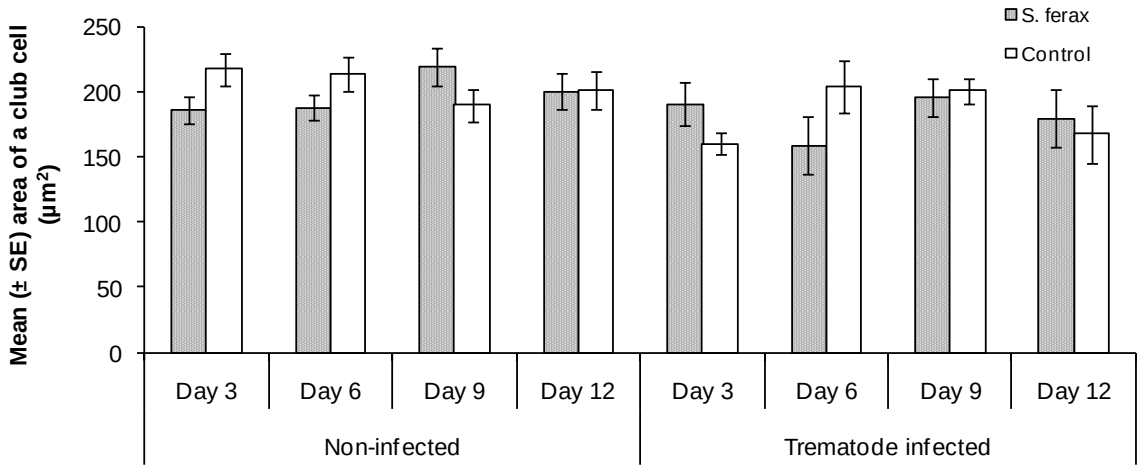

Figure 3.14. Mean (± SE) area of an epidermal club cell in fathead minnows exposed to *Saprolegnia ferax* (200 cysts/L) or a control (dilute salt solution) for a duration of three, six, nine or twelve days.

3.3.5 Mean percentage of epidermal cross section area covered by club cells

The percentage of epidermal cross section area covered by club cells was calculated by summing the area of all club cells in an image and dividing by the total skin area in the image (range: 0.5% to 21%). The percentage of epidermal cross section area covered by club cells was then averaged over the three images for each fish sample. An ANOVA indicated that there was no effect of treatment (ANOVA: treatment $F_{[1]}$ = 2.231, P = 0.137), or health (ANOVA: health $F_{[1]}$ = 0.308, P = 0.580) or day (ANOVA: day $F_{[3]}$ = 0.767, P = 0.514) on the percentage of epidermal cross section area covered by club cells, nor was there an interaction (ANOVA: treatment*health*day $F_{[3]}$ = 0.296, P = 0.828) (Figure 3.15, Table 3.5).

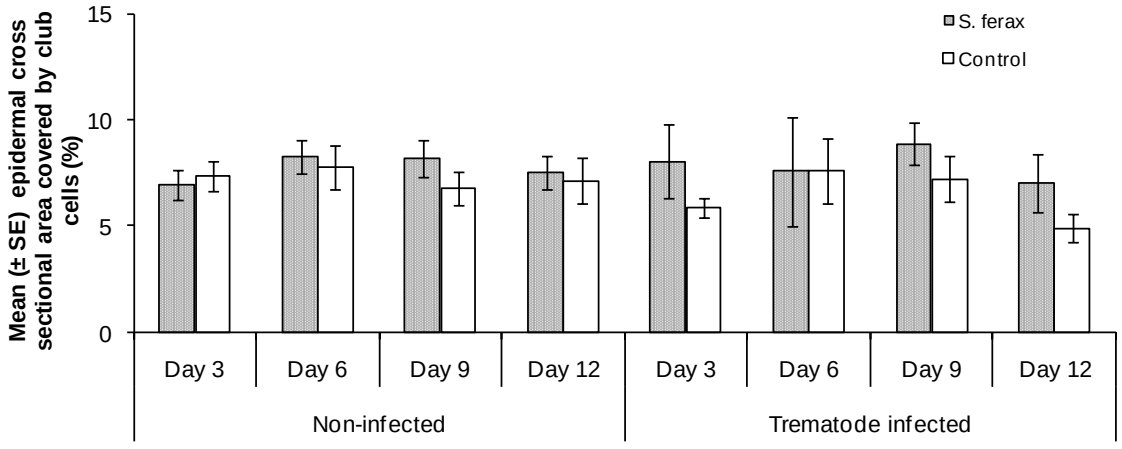

Figure 3.15. Mean (± SE) percentage of epidermal cross section area covered by club cells in fathead minnows exposed to *Saprolegnia ferax* (200 cysts/L) or a control (dilute salt solution) for a duration of three, six, nine or twelve days.

4. DISCUSSION

4.1 FISH SKIN AS A DEFENCE SYSTEM

Fish skin is a metabolically active tissue that responds quickly to a variety of stressors (Whitear 1986; Iger *et al*. 1994a-f), and is the first line of defence against physical damage, as well as disease and parasite infiltration (Iger and Wendelaar Bonga 1994). The skin acts as a physical barrier to external stressors, while specific components such as mucous cells and club cells appear to play important roles in the immune system. Continuous replenishment of the mucus layer on the skin's surface can aid in sloughing off pathogens, potentially making it energetically unfavourable for parasites to penetrate the skin (Buchmann and Bresciani 1998). Mucus also contains several anti-pathogenic compounds including immunoglobulin, complement, C-reactive protein, lectins, lysozyme and haemolysins (Ingram 1980; Ellis 1981; Fletcher 1982; Shephard 1994) which alone or in combination might inhibit pathogen germination and growth. Increased turnover rate and sloughing of epithelial cells can also physically remove ectoparasites, further reducing infection rates (Jones 2001).

Club cells have recently been implicated in a protective role as their production is correlated with skin-penetrating pathogens and parasites (Chivers *et al*. 2007). Due to their location in the surface layers of the skin, club cells are ideally located to release anti-pathogenic substances. Chivers *et al*. (2007) proposed that epidermal injury from pathogens and parasites is a driving force that has maintained these club cells in fish skin. Given the fact that disease is a major source of mortality for fishes, fish that can minimize the effects of parasites and pathogens (e.g. through biochemical, physiological or behavioural means) ultimately will have a reproductive advantage (Moyle and Cech 2004).

The series of experiments in this thesis were undertaken to investigate the responses of the fish skin to manipulated pathogen risk.

4.2 EXPERIMENT 1: THE EFFECTS OF VARYING PATHOGEN RISK ON FATHEAD MINNOW CLUB CELL INVESTMENT

Previous literature (Chivers *et al*. 2007) indicated that fish infected with *Saprolegnia ferax* (20, 200, or 2000 cysts/L) had a higher density of club cells compared to fish treated with a control of DSS; however, there was no significant difference between the various concentration treatments, indicating a threshold-type response. This type of response may be due to the fact that the actual zoospore/cyst concentrations at the end of their experiment may have been considerably higher than reported. They added 1 mL of thawed zoospore/cyst suspension to the tanks every day for eleven days. Although it is not known how long the zoospore/cyst cycle lasts for each individual, motile zoospores are still seen after several days (personal observation). In the current study, the life history of *Saprolegnia* spp. was taken into account, and 1 mL of fresh zoospore/cyst solution was added every three days to ensure a more consistent concentration of zoospores. The objective of the first experiment was two-fold: 1) to test the hypothesis that at lower levels a graded concentration of pathogen would result in a graded pattern of club cell density and/or turnover rate of club cells, and 2) to test the hypothesis that fathead minnows treated with *S. parasitica,* a reportedly more aggressive pathogen, would demonstrate increased club cell density and/or higher turnover rate of club cells compared to those treated with *S. ferax*. In Experiment 1, fathead minnows were treated every third day with varying concentrations (2, 20, or 200 cysts/L) of two pathogens varying in pathogenicity (*Saprolegnia ferax* and *S. parasitica*). Those fish exposed to the higher concentration (200 cysts/L) of either pathogen were predicted to have an increased club cell density compared to those treated with the lower concentration (2 cysts/L); however, there was no statistical difference (P = 0.216). Despite the large sample sizes (n = 31-42/treatment), the high variation in club cell density washed out any statistical significance, indicating that there may be other factors yet to be examined which are impacting club cell density.

Although there was no difference in body condition index between treatments, fish exposed to the highest concentration of pathogens (200 cysts/L) had smaller club cells than those exposed to the low concentration (2 cysts/L). These results are the first to demonstrate a treatment effect on the size of club cells. This is an interesting result that

could indicate an increased turnover rate of cells in the epidermis, and that at the end of the experiment (Day 12), those club cells had not yet reached their full size at maturity. Iger and Abraham (1990) indicated that in carp that were experimentally wounded, the club cells were the last to differentiate during the re-epithelialization period.

Due to the morphological differences between *S. ferax* and *S. parasitica*, it has been implied that the pathogenicity of *S. parasitica* is greater than *S. ferax (*Hatai and Hoshiai 1994; Fregeneda Grandes *et al.* 2000*).* The conclusions from the endpoints measured in this study suggest that at these low concentrations, there is no difference in the pathogenicity of the two species. It is possible that the high turbulence associated with the aeration in the aquaria kept the cysts suspended and reduced any attachment advantages due to morphological differences in the cysts.

In summary, fish treated with 200 cysts/L of a pathogen have smaller club cells and display a trend towards having increased density of club cells compared to those treated with 2 cysts/L. There was no difference in response to *S. parasitica* versus *S. ferax*.

4.3 EXPERIMENT 2: THE EFFECTS OF *S. PARASITICA* CONDITIONED WATER ON FATHEAD MINNOW CLUB CELL INVESTMENT

The objective of Experiment 2 was to determine whether the physical presence of the pathogen was required for an alteration in epidermal parameters, or whether *Saprolegnia parasitica* conditioned water was all that was necessary to evoke a change in the epidermis. There were no significant treatment difference in any of the epidermal parameters measured, including the expected difference between the *S. parasitica* (200 cysts/L) and the control (DSS). The lack of statistical differences between treatments was puzzling as the same concentration of pathogen had resulted in differences in club cell size in Experiment 1. Chivers *et al*. (2007) also reported a significant increase in club cell density in fathead minnows exposed to 200 *S. ferax* cysts/L, and an even lower reported concentration of 20 cysts/L. This discrepancy might be explained by the reported final pathogen concentrations in Chivers *et al*. (2007) having been greatly underestimated since the cysts/zoospore suspensions were added daily, and it is unknown

how many cyst/zoospore cycles that *Saprolegnia* spp. can undergo before exhausting its energy. It is also possible that other factors affecting club cell investment such as population differences, environmental factors, or other unknown infections played a role in the discrepancy.

4.4 EXPERIMENT 3: THE EFFECT OF EXPOSURE DURATION ON FATHEAD MINNOW CLUB CELL INVESTMENT

Studies testing the effects of pathogens on club cell investment have treated the fish with pathogens for 11-16 days prior to quantifying cell investment. The intent of Experiment 3 was to determine at what point in time a significant difference in club cell density or size could be detected following a pathogen challenge. Fish were exposed to a pathogen treatment (200 cysts/L of *S. ferax*) or a control (DSS) for three, six, nine or twelve days, after which epidermal parameters were analyzed. Although the density of club cells was not significantly different over time in those fish treated with the pathogen, graphically it appears that there was an increase on Day 6. A similar pattern of increase on Day 6 or 9 was also observed for club cell size and percentage of skin covered by club cells, although, again there was no overall significant difference. When day was removed as a factor, and those fish exposed to the pathogen treatment were pooled, they had significantly higher club cell densities than fish exposed to the control. During analysis, however, it was noted that a portion of the test fish exhibited signs of blackspot disease.

Blackspot disease is caused by a trematode parasite that has a two-host life cycle, with fish being the second intermediate host (Steedman 1991). Adult worms live in the digestive tract of fish-eating birds. The parasite eggs are released in the bird feces and then eaten by snails. The parasite matures into a free-swimming form that can then bury into the skin and muscle of fish. The metacercariae (infective life stage) provoke the production of a capsule of host tissue around the parasite, which is followed by the migration of melanocytes into the cyst wall resulting in the visible black spots that give the disease its name (Lane and Morris 2000; Tobler and Schlupp 2008). The parasite will remain encapsulated until the fish is eaten by a bird and the life cycle repeats itself (Steedman 1991; Ferrara and Cook 1998).

Those experimental fish that were infected with trematodes and treated with the pathogen treatment or the control treatment were pooled and analyzed separately from the uninfected fish treated with the pathogen and control treatments. Those fish that were trematode infected (exhibited signs of blackspot disease) had smaller club cells than those that were not infected with trematodes. Similar to the results in Experiment 1, perhaps being trematode-infected has resulted in a higher turnover rate of club cells, and the smaller size of the cells may indicate that the club cells had not reached their maximum size at the time of fish sacrifice.

4.5 COMPARISON OF STUDIES

If club cells play a role in the immune system of fish, and have an anti-parasitic/ anti-pathogenic function, exposure to parasite or pathogen challenges should result in increased club cell density. This assumption is supported by Michalak (2006) who demonstrated that fathead minnows exposed to the skin-burrowing trematode *Telorchis* sp. (10 or 70 cercariae) had a higher club cell density than those exposed to the water control.

Results of an experiment by James et al. (2009), however, contrast those reported by Michalak (2006) and Chivers (2007). Exposure of fathead minnows to cercariae of another parasitic trematode, *Ornithodiplostomum* sp., over 6 d did not result in changes in club cell investment. James et al. (2009) suggested that minor differences in the time course of the experiment, in host age (Carreau-Green *et al.* 2008), and in host condition (Wisenden and Smith 1997) could have contributed to the lack of a skin response in their study. James et al. (2009) also suggest that strong and consistent club cell responses may only exist for those parasites or pathogens that remain for extended periods in the epidermis of fish, such as those metacercariae that cause 'black-spot' in the epidermis of their intermediate hosts or some pathogenic water molds (e.g. *Saprolegnia* spp.).

Michalak (2006) demonstrated that fathead minnows exposed to *Saprolegnia* spp. (treatments of approximately 200, 2000 and 20000 cysts/L) had more club cells than those exposed to the control. In a subsequent experiment, Michalak (2006) demonstrated that laboratory cultures of *Saprolegnia ferax* exposed to fathead minnow skin extracts

(containing club cells) responded with reduced growth compared to swordtail skin extract (no club cells) or distilled water, indicating a pathogen-inhibiting component within minnow skin.

In Experiment 1 of this thesis, however, there was no effect of either *S. ferax* or *S. parasitica* pathogen challenges at any of the concentrations (2, 20, or 200 cysts/L) on club cell density. Although these concentrations may seem comparable to those concentrations (20, 200 or 2000 cysts/L of *S. ferax*) presented in Chivers *et al*. (2007) that resulted in significant increases in club cell density, the treatment protocol differed enough to potentially affect the overall concentration. Chivers *et al*. (2007) added zoospores/cysts daily, resulting in the concentration of zoospores/cysts by the end of the exposure period that could have been much higher than reported.

Although a change in club cell density was not observed in Experiment 1, an effect of concentration was noted on the size of the club cells. Those fish treated with the 200 cysts/L concentration, either *S. ferax* or S. *parasitica*, had smaller club cells than those treated with the 2 cysts/L concentration. This is an interesting finding in that there have been no other reports of pathogens affecting the size of club cells. Perhaps those that are under greater infective risk have a higher turnover rate of epidermal cells, and at the time of sacrifice the fish had had a renewal of cells which had not yet had time to obtain full size.

Exposure to the cyst suspension (200 *S. ferax* cysts/L) in Experiment 3 yielded significant results with respect to both club cell density and size. Fish exposed to the *S. ferax* challenge had more club cells than those exposed to the control treatment. However, it was only those fish that exhibited signs of trematode infection that had significantly smaller club cells than those that were uninfected.

After comparing the results of the experiments in this thesis with previous analogous experiments, it is obvious that a consistent epidermal response to a pathogenic/parasitic challenge is not evident. Perhaps it is due to the variation within baseline epidermal characteristics, difference in populations (genetic predispositions) or unknown infective agents confounding the results.

In order to better compare recent studies, the epidermal characteristics of minnows exposed to the control treatment (e.g. distilled water or dilute salt solution) within these experiments have been summarized (Table 4.1). In all cases data are presented for fathead minnow epidermal samples taken posterior to the opercular flap of the fish, with the exception of Hindman (2002), who took samples at the site of the dorsal median, right and left skeletogenous septa (Figures 4.1 and 4.2). For the purposes of comparison, the experimental results from this thesis were converted to number of club cells per area of epidermis (mm^2). Although many of the fish originate from the same water body, and their mean body conditions are relatively consistent, there is a considerable range in epidermal parameters (epidermal thickness: 34 -57 µm; area of a club cell: 71-317 μm^2; number of club cells/mm^2 of epidermis: 300-1042) (Table 4.1). There are many factors which could explain the variation in epidermal characteristics of fish, and these are highlighted in the next section. These factors should be considered in future studies in an attempt to reduce variation within treatments and make similar studies more comparable.

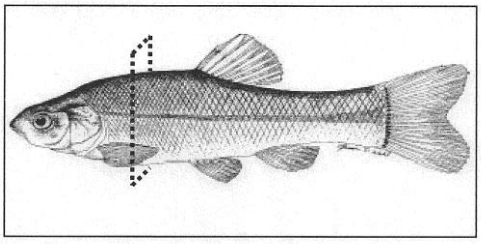

Figure 4.1. Location of histological sectioning of fathead minnows in Hindman (2002).

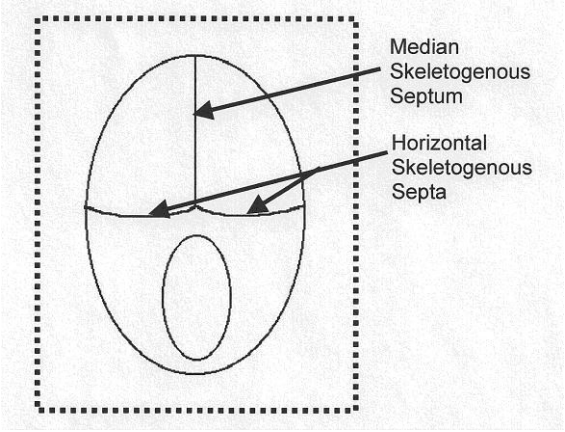

Figure 4.2. Epidermal parameters were measured over the dorsal median, right and left skeletogenous septa in fathead minnows exposed to distilled water Hindman (2002).

Table 4.1. Comparison of epidermal characteristics of fathead minnows exposed to control treatment (e.g. dilute salt solution, distilled water).

	Pollock (this thesis)				*Kusch (unpublished)*		*Halbgewachs 2008*	
Experiment (Control Treatment)	***Exp. 1 (DSS)***	***Exp. 2 (DSS)***	***Exp. 3 Uninfected (DSS)***	***Exp. 3 Trematode infected (DSS)***	***Exp. 1 (DW)***	***Exp. 2 (DW)***	***Exp. 1 (non-injected)***	***Exp. 3 (corn oil injected)***
Club cells/mm	32 ± 1	20 ± 1	26 ± 4	19 ± 3	30 ± 1	9 ± 1	12 ± 2	20 ± n/a
Club cells/mm^2	1042 ± 51	444 ± 25	602 ± 75	502 ± 69	n/a	n/a	688 ± 52†	663 ± 96†
Area of club cell (μm^2)	175 ± 24	177 ± 6	200 ± 14	167 ± 32	83 ± 3	89 ± 3	71 ± 3†	115 ± 4
Epidermal thickness (µm)	34 ± 1	44 ± 1	41 ± 2	44 ± 4	73 ± 16	102 ± 4	40 ± 1†	35 ± 1
Sample size	47	42	15	11	34	50	10	18
Origin	Pike Lake, SK	Pike Lake, SK	Pike Lake, SK	Pike Lake, SK	Feedlot Pond, SK	Feedlot Pond, SK	Feedlot Pond, SK	Feedlot Pond, SK
Date collected	May 2006	Oct. 2006	July 2005	July 2005	Nov. 2004	Nov. 2004	Dec. 2007	May 2007
Date of experiment	June 2006	Oct. 2006	July 2005	July 2005	Dec. 2004	Jan. 2005	Feb. 2008	Jul. 2008
Mean body condition (g/cm^3)	1.7 ± 0.05	1.7 ± 0.03	1.5 ± 0.05	1.5 ± 0.04	2.0 ± 0.02	2.0 ± 0.03	n/a	1.7 ± 0.08
Mean standard length (cm)	3.5 ± 0.5	5.4 ± 0.1	4.6 ± 0.1	4.5 ± 0.1	4.1 ± 0.5	4.4 ± 0.4	n/a	5.2 ± 0.5

Note: In all cases data are presented for epidermal samples taken posterior to the opercular flap of the fish, with the exception of Hindman (2002) (Figures 4.1 and 4.2). The † indicates re-analysis of images by R. Pollock, therefore values differ from those reported in literature. N/a indicates that data is unavailable.

Table 4.1 continued. Comparison of epidermal characteristics of fathead minnows exposed to control treatment (e.g. dilute salt solution (DSS), distilled water (DW)).

	Michalak 2006		*Hindman 2002*		
Experiment (Control Treatment)	***Exp. 1 (DW)***	***Exp. 4 (0 trematodes)***	***Exp. 1 (DW)***	***Exp. 2 (DW)***	***Exp. 3 (DW)***
Club cells/mm	6 ± 1	15 ± 1	7(horizontal septa) 14 (median septa)	39 (median septa)	39 (median septa)
Club cells/mm^2	300 ± 81†	693 ± 115 †	n/a	n/a	n/a
Area of club cell (µm^2)	270 ± 15†	317 ± 17†	n/a	n/a	n/a
Epidermal thickness (µm)	48 ± 5†	48 ± 3†	38 ± n/a	57± n/a	50 ± n/a
Sample size	37	39	11	41	43
Origin	Pike Lake, SK	Centre County, PA, USA	Feedlot Pond, SK	Feedlot Pond, SK	Feedlot Pond, SK
Date collected	May 2004	July 2003	July 2001	Sept.-Oct. 2001	Dec. 2001
Date of experiment	June 2004	n/a	July 2001	n/a	n/a
Mean body condition index	1.3 ± n/a	n/a	0.68 ± n/a	n/a	n/a
Mean standard length (cm)	2.9 ± 0.4	n/a	n/a	n/a	n/a

Note: In all cases data are presented for epidermal samples taken posterior to the opercular flap of the fish, with the exception of Hindman (2002) (Figures 4.1 and 4.2). The † indicates re-analysis of images by R. Pollock, therefore values differ from those reported in literature. N/a indicates that data is unavailable.

4.6 FACTORS AFFECTING EPIDERMAL PARAMETERS IN FISH

4.6.1 Resource availability

Wisenden and Smith (1997, 1998) determined that fish raised under conditions where food was abundant had more club cells, more mucous cells and thicker epidermis than fish raised under conditions where food was limited. These results indicate that the production and maintenance of club cells is energetically costly and that food availability is an important factor when designing an experiment. Michalak (2006) discussed length of acclimation time, and hence length of high quality food intake, in the lab as a potential confounding factor in her experiments. For all of her experiments, fish were captured from a single shoal and, therefore, fish in her first experiment were acclimated in the lab for one month while fish in her second experiment had been held in the lab for five months. Although data from her second experiment is not represented in Table 4.1 due to differing social conditions of experimental fish and lack of a control water treatment, Michalak attributes higher body condition, higher club cell and mucous cell density, larger club cells and mucous cells, and thicker epidermis in fish from her second experiment to this lengthier duration in the lab with *ad libitum* food rations. For this reason, fish in the experiments in this thesis were held in the laboratory and fed *ad libitum* for a maximum of one week prior to starting each experiment.

4.6.2 Shoalmate familiarity

Wisenden and Smith (1998) also found an effect of shoalmate familiarity on investment of club cells. When fathead minnows were placed into a tank with non−familiar shoalmates (fish not belonging to their own shoal) club cell production increased, and conversely, fish placed into a tank with familiar shoalmates had decreased club cell production. Wisenden and Smith (1998) suggested that when in the company of familiar shoalmates, an individual fish can rely more upon the efficacy of a group anti-predator behavioural response, and need not invest as much energy into the maintenance and production of club cells. Non-familiar shoalmates do not respond as effectively as a group to predation risk (Chivers *et al*. 1994). Wisenden and Smith (1998) suggested that non-familiar shoalmates stimulated investment in club cells because an increased importance is placed upon the ability to attract secondary predators. However, this line of reasoning is again anchored in the context of predation. Another explanation that is consistent with their data may be that club cell investment increased in the presence of non-familiar

shoalmates in order to protect the fish from potential exposure to pathogens present on the unfamiliar fish. In order to eliminate the confounding factor of social context, fish in experiments within this thesis were tested alone.

4.6.3 Seasonality and breeding condition

Since fish were wild-caught for these experiments at different times of the year, consideration must be given to the effects of seasonality on club cells. Changes in temperature, UVB radiation and water chemistry (see section 4.6.4) can affect epidermal parameters, and therefore consideration must be given to the season of fish capture. Local changes in pathogen concentration may also change with season and corresponding changes in water quality (Kitancharoen et al. 1996), and therefore fish caught at a certain time of year may have a higher baseline club cell density, and may already exhibit maximum investment of club cells, not allowing for noticeable increases in club cell density when challenged further.

Age of fish must also be a consideration because older fish may not need to input as much energy into growth as younger fish, and therefore may allocate more resources to the maintenance of the epidermis.

Breeding condition of the test subjects is also of importance when designing an experiment. During the breeding season male fathead minnows go through numerous changes in the histology of their gonads and skin (Smith 1974) including development of a dorsal mucous pad and the temporary loss of club cells from the epidermis (Smith 1973). Smith (1973, 1974) speculated that the vigorous abrasive spawning behaviours of the males would release the contents of the club cells or "alarm substance", scaring away potential mates and attracting predators (Mathis *et al*. 1995). Smith (1973, 1974) also noted that loss of club cells can be induced by androgen treatment (intraperitoneal injection of 17α−methyltestosterone) in both males and females. This increase in androgen is consistent with the increased levels of androgen in male minnows during breeding condition, although females do not reach these high levels of androgen in nature.

Field experiments by Pollock *et al*. (2005) support Smith's findings. 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 (Pollock *et al*. 2005). The lack of a behavioural response (e.g. avoidance) to breeding male minnows in these field experiments supports Smith's observations of the loss of club cells in males due to increased androgens during breeding condition.

The mechanism by which club cell investment was inhibited by testosterone was not studied by Smith (1974) or Pollock *et al*. (2005). As testosterone is a well-known immunosuppressant (Slater *et al*. 1995a,b), the decrease in club cell densities might instead be explained by a generalized stress response due to breeding activities which lowered their natural immune level.

Given the confounding nature of breeding condition, fish were visually inspected prior to each experiment in this thesis. If signs of breeding state (tubercles, colour banding and/or presence of dorsal mucous pad on males, or presence of ovipositor on females) were observed, those fish were not included in the experiments. Nevertheless, the time of fish collection as well as the time experiments were conducted was variable throughout the year and there have been no long term studies on how this may affect club cells of both males and females.

4.6.4 Stress

Inadequate diet, social interactions and breeding state can lead to stress, and although the aforementioned factors affecting club cell investment were somewhat controlled for in the experiments, perhaps other stress-inducing factors were not accounted for within and between experiments.

The terms "stress" and "stressors" are difficult to define and, therefore, have been controversial among biologists for many years. The definition used here will follow Wendelaar Bonga's (1997) description of stress as "a condition in which the dynamic equilibrium of animal organisms, called homeostasis, is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors". Stressors can elicit behavioural and/or physiological responses that attempt to allow the animal to cope with the stressor. The animal may reallocate energy and adjust its biological activities involving all levels of its organization including cells, organs, and whole body, enabling it to overcome the threat.

Primary stress responses include activation of brain centers, resulting in release of hormones such as catecholamines and corticosteroids (Wendellaar Bonga 1997). Secondary stress responses are the immediate actions and effects of these hormones at the blood and tissue level, including increases in cardiac output and oxygen uptake, mobilization of energy substrates and disturbance of hydromineral balance. Tertiary responses involve the entire organism, and can include inhibition of growth, reproduction, immune response and reduction in capacity to tolerate subsequent stressors (Wendelaar Bonga 1997).

If club cells are linked to the fish immune system, stress could inhibit the production of club cells. Cortisol, a steroid hormone, plays an important role in energy immobilization, respiration and osmoregulation when fish are in a stressful situation (Pickering 1994), but it is also known as a potent immunosuppressant. An experiment by Halbgewachs (2008) examined cortisol-treated minnows after 12 days, which showed lower densities of club cells than those treated with the control. Club cell area and epidermal thickness of cortisol treated fish were not affected. Evidence that cortisol induced immunosuppression influences epidermal club cell investment provides support for the hypothesis that epidermal club cells may function as part of the fish immune system.

Environmental stresses such as temperature shock, ultra-violet B radiation, and poor water quality are all capable of stimulating the hypothalamic-pituitary-interrenal axis, resulting in the elevation of circulating plasma cortisol levels (Pickering 1994), which can in turn affect epidermal parameters of the fish.

4.6.4.1 Temperature

Temperature shock is a physical stressor that fish may encounter as the result of natural temperature fluctuations (e.g. severe weather fronts) or when fish are captured in the field and transported to a laboratory setting. Although every effort is made to slowly acclimate the fish to within 1 ºC of the holding tank water prior to release into the tank, considerable temperature changes may occur when capturing fish, particularly in the winter months and at peak summer temperatures.

In a study examining the effects of drastic temperature change on skin, a single three hour temperature increase of 7° C induced stress-related physiological changes in rainbow trout

(*Oncorhynchus mykiss*) skin for a duration of 14 days (Iger *et al*. 1994d). Specifically, epidermal thickness decreased compared to control fish within three hours, but then was restored to its initial thickness after 24 hours, followed by an increase beyond control levels from day four onwards (Iger *et al*.1994d). This increase in thickness was associated with an increase in the number of mitotic cells, enlargement of the intercellular spaces and penetration of leucocytes (including lymphocytes and macrophages), responses that are often seen in stressed fish. In a similar study by Nolan *et al*. (2000), the prolonged effect of temperature stress on trout (*Salmo trutta*) skin was verified.

An acute drop in water temperature can also affect epidermal parameters. Channel catfish (*Ictalurus punctatus*) subjected to a water temperature drop from 22° C to 10° C in a 24 hour period had a significant decrease in the number of mucous cells three days after the drop in temperature (Quiniou *et al*.1998). However, recovery of mucous cells to pre-exposure levels occurred after six days at 10° C (Quiniou *et al*. 1998). In an effort to determine the effect of the decrease of mucous cells on disease susceptibility, the authors subjected the catfish to the temperature drop in conjunction with a water mould challenge (*Saprolegnia* sp.). Mucous cells in this case did not return to normal levels, but continued to decline from day three until the fish died from saprolegniasis, a common water mould infection (Quiniou *et al*. 1998).

4.6.4.2 Ultraviolet-B radiation

Solar ultraviolet-B radiation (UVB; 280-320 nm) is a stressful environmental factor that is often overlooked as it not a direct measurement of water quality or chemistry; however, researchers have shown that several fish species are susceptible to UVB-induced skin damage. Reported cases of UVB damaged fish skin involved salmonids (Bullock and Roberts 1979, 1981, 1992; Little and Fabacher 1994), marine flatfish plaice (*Pleuronectes platessa*) (Roberts and Bullock 1981), turbot (*Scophthalmus maximus*) (Bullock 1988), and perhaps even common carp (Bullock *et al*. 1983) and paddlefish (*Polyodon spathula*) (Ramos and Fries 1994).

Blazer *et al*. (1997) compared the skin of UVB-sensitive Lahonton cutthroat trout (*Oncorhynchus clarki henshawi*) and UVB- tolerant razorback suckers (*Xyrauchen texanus*) before and after short term simulated UVB exposure. The cutthroat trout showed visible signs of sunburn within 48 hours including darkening of the skin on the dorsal surface of the fish (due to

a thickening of the melanocyte layer), sloughing of mucous cells, necrosis of the superficial layers of epithelium, and edema in the epidermis and dermis. In a few cases, trout had visible lesions, and signs of secondary fungal infections were evident. Razorback suckers did not show any visible signs of sunburn after 72 hours, and although histological analyses revealed that cell necrosis had occurred, the severe necrosis and sloughing that occurred in the cutthroat trout was not observed. Epidermal thickness did increase in the razorback suckers, mostly due to the increase in size and number of low electron density cells that resembled club cells. Some of these cells were observed at the surface of the skin cross section, and a few appeared to have ruptured, releasing the cell contents. Although occasional melanocytes were present in the area anterior to the dorsal fin, they were not in a distinct layer as seen in the trout. Blazer *et al*. (1997) suggested that club cells may protect against UVB damage simply through the proliferative response or through the production of a substance that appears to protect the suckers against UVB radiation.

Chivers *et al*. (2007) proposed that if club cells are protective against UV damage, then club cell density should be greater on the dorsal surface of the fish compared to the flank or ventral surface of the fish. Indeed, they demonstrated that significantly more club cells were present on the dorsal surface (nape) of wild-collected Johnny darters (*Etheostoma nigrum*), with fewer club cells on the flank, and fewer yet on the ventral surface. A similar pattern of percid club cell distribution was observed in wild caught yellow perch (*Perca flavescens*) with the club cell density ranked as nape>flank>bottom (Chivers *et al*. 2007).

The UVB conditions that fish experience in the field prior to capture (e.g shallow water during the summer months versus under the ice during winter months) may affect the baseline epidermal parameters of experimental fish and may be partly responsible for the variation observed in epidermal parameters in experiments presented in Table 4.1.

4.6.4.3 Epidermal injury, handling and transport

General epidermal injury may also affect epidermal parameters, although experimental results are inconclusive. Physical disturbances, such as handling and transport, can evoke a variety of stress responses including changes in cortisol level (Barton and Iwama 1991) and increased epidermal mucus production (Pickering and Macey 1977). The results of a study by

Pickering and Macey (1977) showed a single incidence of handling is enough to promote an increase in the concentration of mucous cells in the epidermis of char (*Salvelinus alpinus*). The biological significance of this, however, is uncertain because of the delay (up to one week) between the handling stimulus and the peak mucus production. Pickering and Macey (1977) suggest that this delay may represent the time between differentiation of new mucous cells in the basal layer and their appearance at the surface of the epithelium. Repeated handling (every other day for one month) resulted in a maximal number of mucous cells after one week, and no significant effect on the size of the mucous cells or on epidermal thickness. It is not clear from this study whether the response of the mucous cells is a direct effect of the handling, or an overall stress response.

An experiment by Chivers *et al*. (2007) attempted to simulate the damage done by parasite penetration by comparing yellow perch (*Perca flavescens*) that had received needle pokes to control perch that had received no pokes but were handled in an equal fashion. They found no significant differences between treatments in the number of mucous cells, club cells or epidermal thickness. Although Chivers *et al*. (2007) were not able to demonstrate a link between general epidermal injury and changes in epidermal parameters, they suggested that physical injury may only be part of the stimulus required to stimulate club cell production. They proposed that additional chemical cues from the parasite or pathogen may be required to stimulate the club cells. Further studies are required to determine the extent and timing of epidermal responses to epidermal injury.

4.6.4.4 Other stressors

A series of experiments (reviewed in Iger *et al*. 1994a) examined the ultrastructure of club cells of carp after exposure to several stressors including acid water, heavy metals, manure water, brackish water and wounding. Iger *et al*. (1994a) demonstrated that the response of club cells to these stressors was remarkably similar. Club cells responded to stressors with heightened activity including increased size and extensions of endoplasmic reticulum and Golgi areas both of which are consistent with secretory activity. Club cells were more often located nearer the surface of the epidermis after exposure to copper, cadmium, lead, manure, brackish water or low

pH (Iger *et al*. 1994a to f). The environment from which the experimental fish originate, therefore, may also affect club cell parameters. Feedlot Pond, SK for example, is a small pond (~ 5ha) immediately adjacent to a cattle feedlot, and surrounded on two sides by cultivated agricultural land. The fish in this pond may have been exposed to very different water chemistry than those at Pike Lake, SK which is an oxbow lake of the South Saskatchewan River and is located in a provincial park.

Iger *et al*. (1994a) also reported that mitotic and apoptotic activity in stressor−exposed fish was mainly restricted to cells surrounding mature club cells, whereas in control fish mitotic and apoptotic activity was scarce and limited to the upper layer of the epithelium. Another important finding from these studies was that club cells of fish exposed to stressors may be involved in the phagocytosis and lysis (breakdown of cellular membrane) of leucocytes. Iger *et al*. (1994a) reported that up to three leucocytes were seen within a club cell. The presence of two cell membranes around some of the leucocytes within the club cell may indicate phagocytosis of the leucocyte; however, it remains unclear as to whether the leucocyte penetrated the club cell or was taken in by active phagocytosis (Iger *et al*. 1994a). Leucocytes were not seen within club cells in control fish. Iger *et al*. (1994a) also suggested that club cells may be able to influence the turnover rate of the filament cells. This is further evidence that suggests that club cells are an important part of the stress/immune response of fishes, and there is much to be learned about their evolution, function and composition.

4.7 FISH IMMUNE SYSTEM

Saprolegnia spp. is an opportunistic pathogen, and any number of environmental stresses, including the aforementioned sources of stress, can cause an activation of the hypothalamic-pituitary-interrenal (HPI) axis, potentially suppressing the immune defence system and increasing the susceptibility to the infection. Once a stressor is perceived, a cascade of events follows that leads to the elevation of the steroid hormone, cortisol, in circulation, which in turn affects lymphocytes and antibody production.

During an inflammatory response, there is an increased blood supply to the infected area, followed by an increased capillary permeability and then a migration of leucocytes of the capillaries into surrounding tissue (Secombes 1996). When a pathogen enters the tissues of the

host fish, it encounters mucous cells, club cells and a network of phagocytic cells with potent microbicidal activity which limits its spread to other tissue (Secombes 1996). Generally, the acute cellular response of fish exposed to pathogenic agents includes an increase in neutrophils (white blood cells that respond to infection) and monocytes (leucocytes that can differentiate into macrophages) in the blood and at the site of infection (Roberts 1989; Suzuki and Iida 1992; Secombes 1996). Neutrophils are phagocytic, but it is the macrophages that have the largest phagocytic capacity and are able to ingest more particles per cell (Suzuki 1984; Secombes 1996). The majority of phagocytes are seen within the first three to four days post-infection, after which their numbers decline (Secombes 1996). After cellular infiltration and phagocytosis comes tissue repair. If there is a breach in epidermal integrity, there is a risk of secondary infection in addition to an immediate osmotic imbalance (Secombes 1996). Epidermal healing is very quick, and a 2-3 cell thick epidermis can cover the wound within several hours, as a result of migration of pre-existing filament cells from adjacent undamaged skin (Secombes 1996).

If inflammatory stimuli are not eliminated during an acute inflammatory response, a chronic inflammatory response may follow consisting of granulomas (a ball like collection of immune cells) and lymphocytes. Prolonged exposure to stress can be detrimental to the health of fishes, but even brief periods of stress can depress certain aspects of cellular and humoral immune systems, and thus lower the ability to resist pathogens. The ability of fishes to defend themselves against pathogens and respond to stress changes seasonally and through their life stages. Therefore, more work needs to be done to elucidate the relationship between the physical condition of the fish, the conditions under which they have been reared and their ability to resist stress and disease.

4.8 OTHER APPROACHES TO STUDYING CLUB CELLS

Previous studies tended to focus on isolated components of the fish skin defence system, but in order to further understand the effects of complex stressors, alone as well as in combination, a consistent but extensive approach should be used. Scanning electron microscopy and transmission electron microscopy have been used to examine ultra-thin sections of fish skin, focusing on the ultrastructure of the epidermis, cellular necrosis and apoptosis (Iger *et al*. 1994

a-f; Nolan *et al*. 1999), resulting in mainly qualitative observations. These types of procedures should be used in conjunction with those that allow quantitative analysis of skin components. Terminal uridine deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) is a common method for detecting DNA fragmentation that results from apoptosis. The assay relies on the presence of nicks in the DNA of apoptotic cells which are then labelled with a marker. Although in the past this method may have labelled cells undergoing necrosis or cells that had suffered severe DNA damage, advances have improved the method and should only label cells in the last phase of apoptosis (Negoescu *et al*. 1996; 1998). The TUNEL method has several advantages as it is less labour intensive than electron microscopy and larger areas can be examined, although in less detail.

Similarly, monoclonal antibodies can been used to identify and quantify proliferating cells by using proliferating cell nuclear antigen (PCNA) (Van der Salm 2000). The antibody complex can be visualized by staining with a fluorescent marker. Total numbers of PCNA-positive cells can then be counted and expressed per area of epidermis. Monoclonal antibodies can be created to target any proteinaceous component, and can then be used to elucidate the components of a potentially complex mixture (e.g. club cell contents). Using monoclonal antibodies in conjunction with the TUNEL method to target specific skin components (e.g. club cells, apoptotic cells) could allow for quantitative comparison between treatments and between experiments.

Measuring cortisol levels in combination with the above-mentioned quantitative and qualitative methods will allow for a greater understanding of the effects of the stressors physiologically as well as behaviourally. Samples should also continue to be taken over a time series so that a time line can be established for the various effects and the length of recovery. Effects can be noticed as soon as one hour after the stressor has been introduced and can last as long as 30 days (Iger *et al*. 1994 a-f). And as some authors have done (e.g. Carballo and Muñoz 1991; Carballo *et al*. 1995; Quiniou *et al*. 1998; Evans *et al*. 2006), an effort should be made to determine what stressor-related epidermal changes result in an ultimate disease susceptibility and/or mortality. If there is an increase in disease susceptibility, effort should be made to determine whether it could be explained by a stress response (e.g. increase in cortisol levels), or

related to direct effects on a specific defence mechanisms (e.g. increase in club cells), or a combination of mechanisms.

4.9 RELEVANCE OF RESEARCH

Continued club cell research is important in order to understand the evolutionary history of these cells, as well as the dynamics between aquatic pathogen and host. Club cell research may also prove useful in fisheries applications. Oomycetes (e.g. *Saprolegnia* spp.) are second only to bacterial disease in their devastating impact on commercial aquatic facilities (Meyer 1991). With total aquaculture fish production increasing worldwide (Delgado *et al*. 2003), reduction in fish disease will be critically important to the future success of the aquaculture industry. If future studies continue to define the anti-pathogenic properties of club cell contents, this could lead to new aquatic anti-pathogenic control strategies. The use of immunostimulants, most commonly as dietary supplements, in an effort to improve the innate immune response of fish and increase their pathogen resistance during periods of high stress is an area of active research (Bricknell and Dalmo 2005).

Until 2002, malachite green was the most common chemical used to control *Saprolegnia* spp. in aquaculture. It has since been banned due to carcinogenic and toxicological effects at high concentration or following prolonged exposure (Srivastava *et al*. 2004). Other *Saprolegnia* control methods (e.g. sodium chloride) are less effective or can be dangerous and have negative environmental impacts (e.g. ozone, formaldehyde; Cogliano *et al*. 2005). Sodium chloride has the advantage of counteracting osmotic stress in fish with skin damage, but does not totally arrest hyphal growth (van West 2006). Given the current lack of effective *Saprolegnia* control methods, and the evidence collected thus far demonstrating the effects of ostariophysan club cell contents on oomycetes (i.e. Chivers *et al*. 2007; Michalak 2006), this avenue of research will prove beneficial for commercial aquaculture.

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APPENDICES

Appendix A – Dilute Salt Solution Recipe

To make the dilute salt solutions used to induce zoosporogenesis in *Saprolegnia* colonies, 0.5 mL DSA, and 1 mL DSB were added to 1000 mL ultrapure water (Dill and Fuller 1971).

Dilute Salt Solution A (DSA)

KH_2PO_4 136.09 g/L

K_2HPO_4 174.18 g/L

$(NH_4)_2PO_4$ 132.07 g/L

Dilute Salt Solution B (DSB)

$CaCl_2.2H_2O$ 73.50 g/L

$MgCl_2.6H_2O$ 101.66 g/L

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

Prepare solutions and stains prior to stain procedure (see Appendix C). 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 tinfoiled 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 fumehood for several hours.

Appendix C – 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 (21º C in tinfoiled 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 (tinfoiled bottle) at 1-4° C. 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
Hydrochloric acid	18 mL

Saturated Lithium Carbonate

Lithium Carbonate	1.2 g
Distilled water	600 mL

1N Hydrochloric Acid (dilute)

Distilled water	920 mL
Concentrated HCl	80 mL