

The effects of pathogens, parasites, and familiarity on alarm cell investment in fathead minnows, *Pimephales promelas*

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Abstract

Fishes in the Superorder Ostariophysi have specialized epidermal club cells that contain an alarm substance. Damage to these cells causes the release of the alarm substance which can serve as a useful indicator of predation risk for nearby conspecifics. The majority of research involving alarm substances has investigated the roles that cues play in anti-predator contexts including learned predator avoidance. In this study I tested the effects of non-predatory stressors including pathogens, pathogen conditioned water, social dynamics, and skin-burrowing parasites on epidermal variables including alarm cell investment. In experiment 1, fathead minnows, *Pimephales promelas*, were exposed to different levels of the pathogenic water-mold, *Saprologenia ferax* to determine the effect it would have on the epidermis. Minnows exposed to *Saprologenia* had significantly more alarm cells than those exposed to the control solutions. The treatments had no effect on body condition, alarm cell size, mucous cell density, mucous cell size, or epidermal thickness. In experiment 2, social dynamics were manipulated by pairing minnows with either a familiar partner or an unfamiliar individual and exposing them to *Saprologenia ferax*. The treatments had no effect on body condition, alarm cell density, alarm cell size, mucous cell density, mucous cell size, or epidermal thickness. In experiment 3, minnows were exposed to either *Saprologenia* or *Saprologenia* conditioned water to determine whether the physical presence of zoospores was required to induce a change in epidermal properties. The treatments had no effect on body condition, alarm cell density, alarm cell size, mucous cell density, mucous cell size, or epidermal thickness; suggesting that water conditioned by *Saprologenia* may be sufficient to induce a change in alarm cell investment. In experiment 4, minnows were exposed to three

different infection rates of skin burrowing parasites (trematode cercariae) to determine its effect on the epidermis. Minnows exposed to high levels of trematode cercariae had significantly more alarm cells than those exposed to either low levels or those in the control treatment. The treatments had no effect on body condition, alarm cell size, mucous cell density, mucous cell size, or epidermal thickness. In experiment 5 and 6, cultures of *Saprolegnia ferax* were exposed to skin extracts from various fishes. *S. ferax* cultures exposed to fathead minnow skin extracts, which contain alarm cells showed the least amount of growth, while cultures exposed to swordtail skin extracts, which lack alarm cells, showed an intermediate amount of growth. Cultures exposed to fathead minnow skin extracts showed the least amount of growth compared to cultures exposed to either the synthetic alarm cue or the control. The results of these experiments suggest that disease does appear to influence alarm cell investment and there appears to be some property found in fathead minnow skin that inhibits *S. ferax* growth.

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Table of Contents

Permission to use.....	i
Abstract.....	ii
Acknowledgements.....	iv
Table of Contents.....	v
List of Figures.....	viii
Chapter 1: Introduction.....	1
1.1 Background information.....	1
1.2 Objective.....	2
1.3 Evolutionary hypothesis.....	2
1.4 Factors that affect alarm cell number.....	3
1.4.1 Partner familiarity and alarm cell investment.....	3
1.4.2 Disease and alarm cell abundance.....	5
1.5 Role of mucus glands in immune response.....	6
Chapter 2: Methods.....	8
2.1 Basic experimental protocols.....	8
2.1.1 Fish collection and maintenance.....	8
2.1.2 <i>Saprolegnia</i> cultures.....	8
2.1.3 Zoospore conditioned water preparation.....	10
2.1.4 Digenean trematode collection.....	10
2.1.5 Preparation of minnow and swordtail skin extracts.....	11
2.1.6 Preparation of hypoxanthine 3-N oxide.....	12
2.1.7 Slide preparation.....	12
2.2 Experiment one: The effects of different <i>Saprolegnia ferax</i> concentrations on fathead minnow alarm cell investment.....	15
2.2.1 Experimental protocol.....	15
2.3 Experiment two: The effects of familiarity and <i>Saprolegnia</i> on fathead minnow alarm cell investment.....	16
2.3.1 Experimental Protocol.....	16
2.4 Experiment three: The effects of zoospore conditioned water on fathead minnow alarm cell investment.....	17
2.4.1 Experimental protocol.....	17
2.5 Experiment four: The effects of different levels of trematode cercariae on fathead minnow alarm cell investment.....	18
2.5.1 Experimental protocol.....	18
2.6 Experiment five: The effects skin extracts of fishes on <i>Saprolegnia ferax</i> growth.....	18
2.6.1 Experimental protocol.....	18
2.7 Experiment six: the effect of extracts of fishes and synthetic alarm cues on <i>Saprolegnia ferax</i> growth.....	19
2.7.1 Experimental protocol.....	19

2.8	Statistical analysis.....	20
2.8.1	General analysis.....	20
Chapter 3:	Results.....	21
3.1	Experiment one: The effects of different <i>Saprolegnia ferax</i> concentrations on fathead alarm cell investment.....	21
3.1.1	Body condition.....	21
3.1.2	Alarm cell density and alarm cell size.....	21
3.1.3	Mucous cell density and mucous cell size.....	22
3.1.4	Epidermal thickness.....	22
3.2	Experiment two: The effects of familiarity and <i>Saprolegnia ferax</i> on fathead minnow alarm cell investment.....	26
3.2.1	Body condition.....	26
3.2.2	Alarm cell density and alarm cell size.....	26
3.2.3	Mucous cell density and mucous cell size.....	26
3.2.4	Epidermal thickness.....	27
3.3	Experiment three: The effects of zoospore conditioned water on fathead minnow alarm cell investment.....	31
3.3.1	Body condition.....	31
3.3.2	Alarm cell density and alarm cell size.....	31
3.3.3	Mucous cell density and mucous cell size.....	31
3.3.4	Epidermal thickness.....	32
3.4	Experiment four: The effects of different levels of trematode cercariae on fathead minnow alarm cell investment.....	36
3.4.1	Body condition.....	36
3.4.2	Alarm cell density and alarm cell size.....	36
3.4.3	Mucous cell density and mucous cell size.....	36
3.4.4	Epidermal thickness.....	37
3.5	Experiment five: The effects skin extracts of fishes on <i>Saprolegnia ferax</i> growth.....	41
3.6	Experiment six: The effects skin extracts of fishes and synthetic alarm cues on <i>Saprolegnia ferax</i> growth.....	43
3.7	Comparison of epidermal variables between fathead minnows in experiments one and two.....	45
3.7.1	Body condition.....	45
3.7.2	Alarm cell density and alarm cell size.....	45
3.7.3	Mucous cell density and mucous cell size.....	45
3.7.4	Epidermal thickness.....	46
Chapter 4:	Discussion.....	50
4.1	Experiment one: The effects of different <i>Saprolegnia ferax</i> concentrations on fathead minnow alarm cell investment.....	50

4.2 Experiment two: The effects of familiarity and <i>Saprolegnia ferax</i> on fathead minnow alarm cell investment.....	51
4.3 Experiment three: The effects of zoospore conditioned water on fathead minnow alarm cell investment.....	53
4.4 Experiment four: The effects of different levels of trematode cercariae on fathead minnow alarm cell investment.....	53
4.5 Experiment five: The effects skin extracts of fishes on <i>Saprolegnia ferax</i> growth.....	54
4.6 Experiment six: The effects of skin extracts of fishes and synthetic alarm cues solutions on <i>Saprolegnia ferax</i> growth.....	54
4.7 Significance of thesis work.....	55
4.8 Alarm cells acting in synergy with immune components.....	56
Chapter 5: Conclusions.....	58
Literature cited.....	59
Appendix: Protocol for harvesting zoospores Protocol for making solid and liquid GY media	

List of Figures

Figure 1.1	Location of skin removed from fathead minnows for PAS-H processing.....	14
Figure 1.2	Fathead minnow epidermal cross-section.....	14
Figure 2.1	The effect of <i>Saprolegnia ferax</i> on alarm cell investment. Mean (\pm SE) number of alarm cells of fathead minnows treated with different levels of <i>Saprolegnia</i> . Different letters denote significant differences at $P \leq 0.001$	23
Figure 2.2	The effect of <i>Saprolegnia ferax</i> on alarm cell size. Mean (\pm SE) alarm cell size of fathead minnows treated with different levels of <i>Saprolegnia</i> . Different letters denote significant differences at $P \leq 0.05$	23
Figure 2.3	The effect of <i>Saprolegnia ferax</i> on mucous cell investment. Mean (\pm SE) number of mucous cells of fathead minnows treated with different levels of <i>Saprolegnia</i> . Different letters denote significant differences at $P \leq 0.05$	24
Figure 2.4	The effect of <i>Saprolegnia ferax</i> on mucous cell size. Median (\pm 25th and 75th quartiles) mucous cell size of fathead minnows treated with different levels of <i>Saprolegnia</i> . Different letters denote significant differences at $P \leq 0.05$	24
Figure 2.5	The effect of <i>Saprolegnia ferax</i> on epidermal thickness. Median (\pm 25th and 75th quartiles) epidermal thickness of fathead minnows treated with different levels of <i>Saprolegnia</i> . Different letters denote significant differences at $P \leq 0.05$	25
Figure 3.1	The effect of familiarity and <i>Saprolegnia ferax</i> on alarm cell investment. Median (\pm 25th and 75th quartiles) number of alarm cells of fathead minnows treated with <i>Saprolegnia</i> and different partners. Different letters denote significant differences at $P \leq 0.05$	28
Figure 3.2	The effect of familiarity and <i>Saprolegnia ferax</i> on alarm cell size. Mean (\pm SE) alarm cell size of fathead minnows treated with <i>Saprolegnia</i> and different partners. Different letters denote significant differences at $P \leq 0.05$	28

Figure 3.3	The effect of familiarity and <i>Saprolegnia ferax</i> on mucous cell size. The effect of familiarity and <i>Saprolegnia ferax</i> on mucous cell investment. Median (\pm 25th and 75th quartiles) number of mucous cells of fathead minnows treated with <i>Saprolegnia</i> and different partners. Different letters denote significant differences at $P \leq 0.05$	29
Figure 3.4	The effect of familiarity and <i>Saprolegnia ferax</i> on mucous cell size. Mean (\pm SE) mucous cell size of fathead minnows treated with <i>Saprolegnia</i> and different partners. Different letters denote significant differences at $P \leq 0.05$	29
Figure 3.5	The effect of familiarity and <i>Saprolegnia ferax</i> on epidermal thickness. Mean (\pm SE) epidermal thickness of fathead minnows treated with <i>Saprolegnia</i> and different partners. Different letters denote significant differences at $P \leq 0.05$	30
Figure 4.1	The effect of zoospore conditioned water on alarm cell investment. Mean (\pm SE) number of alarm cells of fathead minnows treated with either <i>Saprolegnia</i> or water conditioned by <i>Saprolegnia</i> . Different letters denote significant differences at $P \leq 0.05$	33
Figure 4.2:	The effect of zoospore conditioned water on alarm cell size. Mean (\pm SE) alarm cell size of fathead minnows treated with either <i>Saprolegnia</i> or water conditioned by <i>Saprolegnia</i> . Different letters denote significant differences at $P \leq 0.05$	33
Figure 4.3	The effect of zoospore conditioned water on mucous cell investment. Median (\pm 25th and 75th quartiles) number of mucous cells of fathead minnows treated either <i>Saprolegnia</i> or water conditioned by <i>Saprolegnia</i> . Different letters denote significant differences at $P \leq 0.05$	34
Figure 4.4	The effect of zoospore conditioned water on mucous cell size. Mean (\pm SE) mucous cell size of fathead minnows treated with either <i>Saprolegnia</i> or water conditioned by <i>Saprolegnia</i> . Different letters denote significant differences at $P \leq 0.05$	34

Figure 4.5	The effect of zoospore conditioned water on epidermal thickness. Median (\pm 25th and 75th quartiles) number of mucous cells of fathead minnows treated either <i>Saprolegnia</i> or water conditioned by <i>Saprolegnia</i> . Different letters denote significant differences at $P \leq 0.05$	35
Figure 5.1	The effect of trematode cercariae on alarm cell investment. The effect of parasite load on observed infection rate. Proportion of fathead minnows infected with cysts followed being treated with different levels of trematode cercariae. Different letters denote significant differences at $P \leq 0.05$	38
Figure 5.2	The effect of trematode cercariae on alarm cell investment. Median (\pm 25th and 75th quartiles) number of alarm cells of fathead minnows treated with different levels of trematode cercariae. Different letters denote significant differences at $P \leq 0.01$	38
Figure 5.3	The effect of trematode cercariae on alarm cell investment. Mean (\pm SE) alarm cell size of fathead minnows treated with different levels of trematode cercariae. Different letters denote significant differences at $P \leq 0.05$	39
Figure 5.4	The effect of trematode cercariae on mucous cell investment. Median (\pm 25th and 75th quartiles) number of mucous cells of fathead minnows treated with different levels of trematode cercariae. Different letters denote significant differences at $P \leq 0.05$	39
Figure 5.5	The effect of trematode cercariae on mucous cell size. Median (\pm 25th and 75th quartiles) mucous cell size of fathead minnows treated with different levels of trematode cercariae. Different letters denote significant differences at $P \leq 0.05$	40
Figure 5.6	The effect of trematode cercariae on epidermal thickness. Median (\pm 25th and 75th quartiles) epidermal thickness of fathead minnows treated with different levels of trematode cercariae. Different letters denote significant differences at $P \leq 0.05$	40

Figure 6.1	The effect of skin extracts on <i>S. ferax</i> growth. Median (\pm 25th and 75th quartiles) growth of <i>Saprolegnia ferax</i> treated with different skin extracts. Different letters denote significant differences at $P \leq 0.001$	42
Figure 7.1	The effect of skin extracts from fishes and synthetic alarm cues on <i>S. ferax</i> growth. Mean (\pm SE) growth of <i>Saprolegnia ferax</i> treated with skin extracts of fishes and synthetic alarm cues. Different letters denote significant differences at $P \leq 0.001$	44
Figure 8.1	Comparison of alarm cell investment between experiments 1 and 2. Mean (\pm SE) alarm cell number of fathead minnows in experiments 1 and 2. Different letters denote significant differences at $P \leq 0.001$	47
Figure 8.2	Comparison of alarm cell size between experiments 1 and 2. Mean (\pm SE) alarm cell size of fathead minnows in experiments 1 and 2. Different letters denote significant differences at $P \leq 0.05$	47
Figure 8.3	Comparison of mucous cell investment between experiments 1 and 2. Median (\pm 25th and 75th quartiles) mucous cell size of fathead minnows in experiments 1 and 2. Different letters denote significant differences at $P \leq 0.001$	48
Figure 8.4	Comparison of mucous cell size between experiments 1 and 2. Mean (\pm SE) mucous cell size of fathead minnows in experiments 1 and 2. Different letters denote significant differences at $P \leq 0.001$	48
Figure 8.5	Comparison of epidermal thickness between experiments 1 and 2. Mean (\pm SE) epidermal thickness of fathead minnows in experiments 1 and 2. Different letters denote significant differences at $P \leq 0.001$	49
Figure 8.6	Comparison of body condition between fish in experiments 1 and 2. Median (\pm 25th and 75th quartiles) body condition of fathead minnows in experiments 1 and 2. Different letters denote significant differences at $P \leq 0.001$	49

Chapter 1: Introduction

1.1 Background Information

Von Frish (1941) made the observation that European minnows (*Phoxinus phoxinus*) exhibited an anti-predator response to a chemical substance released from the skin of conspecifics (see review by Smith 1992). He termed the substance “Schreckstoff” which translates into ‘fear substance’. Since then, researchers have discovered specialized epidermal cells (club cells) in the epidermis of fishes that are known to contain the alarm substance (Smith 1992). These specialized cells appear to be ubiquitous among members of the Superorder Ostariophysi, a group that includes approximately 7,200 species of fishes (Nelson 1994). Many detailed studies over the past 60 years have shown that damage to club cells during capture by a predator releases the alarm substance, which serves as a warning to nearby fishes, enabling them to respond with appropriate anti-predator behaviours (see review by Smith 1992). Consequently, the amount (and concentration) of alarm substance can serve as a useful indicator of predation risk (Wisenden and Smith 1998). The majority of research involving alarm substances has investigated the roles that the cues play in anti-predator contexts including learned predator avoidance (Mathis and Smith 1993, Chivers and Smith 1994).

The evolution of alarm cells has long perplexed researchers because it has been difficult to determine a direct benefit for senders of damage-released alarm cues (Chivers et al. 1996). In order for senders to release their alarm cues they must sustain damage to their epidermis (Wisenden and Smith 1998). The obvious question that arises is: what is the benefit to the sender of the alarm cue? Notwithstanding the possible benefits from kin selection, there is little evidence of a benefit to the sender (see Chivers et al. 1996). In fact, experiments manipulating predation pressure have failed to find a link between alarm cell investment and

predation risk (Hindman 2002). An alternative explanation is that alarm cells evolved for another function altogether. Specifically, Magurran et al. (1996) speculated that alarm cells may have evolved as storage compartments for anti-parasitic or anti-pathogenic agents. In this case, individuals possessing alarm cells may receive a direct benefit; the contents of club cells may lessen the effect of disease, providing fish with enhanced pathogen defence. The alarm-cue role of club cells may have evolved secondarily.

In addition to investigating the effects of predation on alarm cell investment, researchers have also studied the effects of non-predatory stressors on alarm cell abundance. In an experiment that manipulated social dynamics and food availability, Wisenden and Smith (1998) found that both partner familiarity and resource availability influenced alarm cell investment. Minnows from different populations and shoals may represent threats of novel pathogens and parasites; consequently, fish paired with unfamiliar individuals may increase their alarm cell abundance in an attempt to reduce their personal risk of infection. Moreover, the production and maintenance of alarm cells is energetically costly (Wisenden and Smith 1998); therefore alarm cell investment may be a conditional strategy that can only be afforded by individuals that receive adequate resources (Wisenden and Smith 1998).

1.2 Objective

The objective of my thesis research was to determine the effects of non-predatory stressors on alarm cell abundance. In particular, I was interested in determining the effects of social dynamics, and pathogen and parasite exposure on fathead minnow, *Pimephales promelas*, alarm cell abundance.

1.3 Evolutionary Hypothesis

Alarm signaling theory maintains that alarm cells are selected for their signaling role and the number of alarm cells that an individual possess should reflect the predation pressure

that they experience (Hugie 1990). Hugie (1990) suggested that the naturally occurring differences in alarm cell densities among fathead minnow populations might be a result of different predation pressures experienced by minnow populations. However, Hugie (1990) found no correlation between predation pressure and alarm cell abundance. In fact, contrary to Hugie's (1990) predictions, he found that minnows that did not co-occur with piscivorous predators had more alarm cells than those that co-occurred with piscivorous predators. Hugie's findings clearly do not support an alarm-based mechanism for the evolution of alarm cells. To address the discrepancy between alarm signaling theory and his findings, Hugie (1990) claimed that it is difficult to determine the exact level of predation experienced by minnows. He suggested that predator-free fish might in fact experience predation from some unknown source, which would explain their increase in alarm cell densities (Hugie 1990). While it is conceivable that predator-released minnows experience predation from unknown sources, there are no data available to support this hypothesis.

1.4 Factors that affect alarm cell investment

1.4.1 Partner familiarity and alarm cell investment

Wisenden and Smith (1998) argued that there are three mechanisms responsible for facultative alarm cell investment. Specifically, they claim that efficacy of group defence, kin selection, and the benefits of attracting secondary predators lead to facultative alarm cell investment. Using a two-by-two factorial design, Wisenden and Smith (1998) tested the effects of partner familiarity and food availability on alarm cell densities. They found that fathead minnows that were maintained on a high ration diet and paired with familiar partners did not increase their alarm cell densities, while minnows that were maintained on a high ration diet and paired with unfamiliar partners did increase their alarm cell abundance (Wisenden and Smith 1998). Wisenden and Smith (1998) argued that fish that frequently interact with familiar

individuals might forego investing in alarm cells, and instead rely on a highly tuned system of group defense. Fish that are members of a common shoal act more cohesively under predator attack, which may reduce an individual's risk of predation (Chivers et al. 1994).

Wisenden and Smith (1998) also predicted that the personal benefits of attracting secondary predators might contribute to facultative alarm cell investment. They discussed the potential benefits of attracting a secondary predator in terms of kin selection theory (Wisenden and Smith 1998). According to kin selection theory, individuals that frequently interact with related individuals may increase their alarm cell investment in an attempt to increase their inclusive fitness (Wisenden and Smith 1998). While familiarity may not be equal to kinship, familiar individuals may be more likely to be related to each other. Following a predation event, individuals with high densities of alarm cells would release an increased amount of alarm substance into the environment. This may improve their kin's chances of detecting the cue and reacting with the appropriate anti-predator behaviours. However, the experimental findings of Wisenden and Smith (1989) do not support the kin selection theory. Contrary to their predictions, Wisenden and Smith (1998) found that fish paired with familiar partners failed to increase their alarm cell production.

Wisenden and Smith (1998) explained their findings by discussing damage released alarm cues in terms of a selfish escape strategy. Alarm substances can act as predator attractants (Chivers et al. 1996). Damage released cues have been shown to attract secondary predators (Mathis et al. 1995), which may disrupt the predation event, enabling prey to escape (Chivers et al. 1996). The underlying logic behind the argument proposed by Wisenden and Smith (1998) is that while an individual may increase their personal chances of surviving a predation event by increasing their alarm cell abundance, they may simultaneously increase the predation risk of shoalmates. Wisenden and Smith (1998) suggest that fish that frequently

interact with familiar individuals decrease their alarm cell investment as a means to reduce the predation risk of their related shoalmates. This is because newly recruited predators may decide to attack nearby shoalmates, which may be kin. In contrast, fish that frequently interact with unfamiliar fish will not have the same vested interest in the survival of their shoalmates. Consequently, they will increase their alarm cell investment in an attempt to increase the likelihood of attracting a secondary predator and improving their personal chances of escaping attack (Wisenden and Smith 1998). While this hypothesis appears to be supported by the data provided by Wisenden and Smith (1998), the underlying assumptions of the argument should be closely evaluated. Kin selection theory maintains that closely related individuals value their kin's survival in direct proportion to their degree of relatedness. Currently there is no evidence to support the fact that shoals of fish are composed of related individuals. We need a comprehensive understanding of the degree of relatedness among shoal members before addressing kin selection based explanations.

To further study the effects of familiarity on alarm cell abundance, I experimentally manipulated partner familiarity by pairing minnows with either a partner from their 'home' shoal or a distant shoal from the same lake. Minnows were caught using seine nets, which can trap entire shoals of minnows. 'Home' shoal members were defined as individuals that were caught in the same seining attempt. In contrast, unfamiliar fish were defined as fish caught in a separate seining attempt, approximately one kilometer away from the location of the previous shoal. Partner familiarity was manipulated in conjunction with other experimental treatments.

1.4.2 Disease and alarm cell abundance

While predation does not appear to affect alarm cell densities, pathogens do appear to be an important factor in determining alarm cell abundance (Chivers and Jack unpublished data 2003). Chivers and Jack (unpublished data 2003) tested the effects of pathogens on alarm cell

investment by exposing fathead minnows to cultures of *Saprolegnia parasitica* and *S. ferax*. Fish exposed to *Saprolegnia* had significantly more alarm cells than fish in the dilute salt control treatment (Chivers and Jack unpublished data 2003). This is an interesting finding as alarm cells are thought to contain both antibacterial and anti-fungal properties (Smith 1992). Another intriguing finding associated with Chivers and Jack's (2003 unpublished data) pathogen experiment was that despite exposing fish to high levels of the *Saprolegnia*, there was little evidence of infection (hyphal growth). The increased number of alarm cells found in fish challenged with *S. ferax* may have mitigated the deleterious effects of the pathogens.

1.5 Role of mucus glands in immune response

Fish skin appears to have multiple defence systems that help protect the individual from fungal and pathogen infections. By providing a physical barrier to external infection, a fish's integument is its first level of immune defence. Two cell types alarm cells, and mucous cells, appear to play important roles in the innate immune defence in fish (Jones 2001). Excess mucous may reduce infection intensity by making it energetically unfavorable for parasites to penetrate the skin (Buchmann and Bresciani 1998). In addition, the increased sloughing of epithelial cells may further reduce infection rate by physically removing ectoparasites (Jones 2001). In a study investigating the effect of mucous glands on parasite load, Buchmann and Bresciani (1998) found a negative correlation between the investment of mucous cells and number of parasitic monogeneans on the skin surface of rainbow trout, *Oncorhynchus mykiss*. They concluded that mucous glands decreased the infection intensity by the monogenean. Fish mucous glands contain biostatic and biocidal compounds including immunoglobulin complement, C-reactive protein, lectins, lysozyme and haemolysins (Yano 1996). However, it is currently unclear which components play a role in immune defence against skin-burrowing pathogens. In some species, such as the common carp, *Cyprinus carpio*, the mucous gland

compound lysozyme becomes activated in the event of pathogen infection (Cone 1995). In contrast, immunity in rainbow trout is linked with activation of another mucous gland product, complement factor C3 (Harris et al. 1998). Given the importance of mucous glands in pathogen defence, these cells were also counted and measured for each fish in my experiments.

I propose an alternative explanation to predation for the observed differences in alarm cell abundances between minnow populations; differences in ectoparasites and pathogen infection loads may be responsible for maintaining alarm cell differences. Knowing that a high level of pathogen exposure is correlated with high alarm cells densities (Chivers and Jack 2003 unpublished data); it is possible that differences in pathogen infection rates may be responsible for population differences in alarm cell abundances. Here, I manipulated the rate of infection of *S. ferax* in fathead minnows and determine the effect it had on alarm cell abundance. The experimental infection rate of *S. ferax* was manipulated in conjunction with partner familiarity.

Chapter two: Methods

2.1 Basic Experimental Protocol

2.1.1 Fish Collection and Maintenance

Fathead minnows used in experiments one, two, three and five were collected using seine nets from Pike Lake (51° 53'N, 106° 49'W) in May 2004. Pike Lake is a shallow oxbow lake of the South Saskatchewan River located 25 km southwest of Saskatoon, Saskatchewan, Canada. Two separate shoals of fathead minnows were collected approximately 1 km apart from each other. Fish were transported back to the laboratory, divided into their respective 'home' shoals and transferred to 6000 L flow-through ponds containing dechlorinated tap water. Prior to the experiment, fish were maintained on a 14L: 10D photoperiod at approximately 11° C, and were fed flake food ad libitum. Fathead minnows used in experiment four were collected from a pond in State Gamelands 176 in Centre County, Pennsylvania, USA in July 2003. All breeding males and females were removed from the fish traps; consequently minnows used in the experiment were believed to be non-breeding fish. Minnows were held individually in 750 ml plastic cups containing 600 mL of dechlorinated water and maintained under a 16L: 8D photoperiod at 20 °C. Minnows acclimated in the laboratory for two weeks prior to testing and were fed goldfish flake food daily.

2.1.2 *Saprolegnia* cultures

Saprolegnia ferax was the pathogenic agent used in most of the experiments in my thesis. *S. ferax* was used because it can be easily grown and manipulated in the laboratory and it is commonly encountered in areas where fish are stocked at high densities such as hatcheries and fisheries (Richards and Pickering 1978). Using the standard protocol of Laskin and Lechevalier (1978), *S. ferax* was cultured in the laboratory on GY agar plates. These cultures did not sporulate, but they provided culture inoculum (zoospores), which were used as the

pathogen challenge in subsequent experiments. In response to starvation *Saprolegnia* forms asexual secondary zoospores (Neish and Hughes 1980); these secondary zoospores have long hooked hairs that assist them in host recognition (Beakes 1982) and attachment to the host (Pickering and Willoughby 1982). To generate starved colonies, approximately 50 individual 1 mm x 1 mm pieces (solid medium with *S. ferax* hyphae) were cut from the margin of the master culture using sterile technique and transferred to gel slick flasks containing 75 mL of GY liquid solution (see Appendix). Flasks were sealed with aluminum foil and placed on a shaker at 240 rpm for approximately 1 hour. Afterwards, the speed was reduced to 80 rpm and the *Saprolegnia* solutions were allowed to grow overnight. The following day, the liquid was aspirated off and discarded, replaced with several changes of dilute salts DSA and DSB (see appendix). The colonies were stored at 4°C overnight where they released zoospores into the liquid. The zoospores encysted and fell to the bottom of the flask. A comb was used to remove the cysts from the bottom of the flask and the solution was transferred to several 40 mL histology tubes. The solution was then centrifuged (International Equipment Company, Model HN) at $\frac{3}{4}$ speed for 6 minutes. The supernatant was poured off and the pellet was collected. The zoospore abundance was determined using a haemocytometer. Glass distilled dechlorinated water was used to adjust the volumes to the appropriate experimental concentrations. To ensure that the harvested zoospores were viable, 1 mL of zoospore solution was removed using a disposable pipette and applied to a petri dish containing solid GY media. The presence of a *S. ferax* colony on culture plates the following day confirmed the viability of the zoospores. This procedure was repeated every time zoospores were harvested. The control solution in this experiment consisted of glass-distilled water, which was transferred into 80 mL histology jars.

The solutions, including the control, were then divided into the appropriate aliquots (80 mL histology jars) and frozen (-20° C) until needed. To ensure that the zoospores were still viable after freezing, a sample was thawed 48 hours after being initially frozen. To thaw the sample, it was simply removed from the freezer and allowed to reach room temperature. One millilitre of the zoospore solution was removed using a disposable pipette and it was applied to a petridish containing GY agar solid media. The presence of a *S. ferax* colony the following day confirmed that the zoospores could withstand being frozen and thawed. This procedure was also repeated every time zoospores were harvested.

2.1.3 Zoospore Conditioned Water Preparation

To collect the conditioned water from the zoospores used in experiment three, the zoospores were harvested as the previous experiment. However, instead of collecting the pellet and discarding the supernatant after centrifuging as in the previous experiment, the supernatant was transferred to a histology tube. The solution was then recentrifuged at $\frac{3}{4}$ speed for 4 minutes and the supernatant was poured off into a clean histology tube. The supernatant was then examined using a haemocytometer to ensure that there were no zoospores present in the solution. As an extra precaution, 1 mL of solution was removed using a disposable pipette and transferred to a petridish containing GY agar solid media. Had *S. ferax* zoospores been present in the solution, a colony would have established itself on the agar plate. The absence of a *S. ferax* colony several days after the initial inoculation confirmed that there were no zoospores present in the zoospore conditioned water solution.

2.1.4 Digenean Trematode Collection

Snails, *Physa acuta*, were collected from the pond in State Gamelands 176 in Centre County, Pennsylvania, USA in July 2003. Snails were individually housed in 250 ml plastic containers containing dechlorinated water and housed in an incubator at 23 °C under a

12L:12D photoperiod. Digenean cercariae (family Teleorchidae), the free-swimming infectious stage of the parasite are released naturally from the snails. Snail containers were examined daily for the presence of cercariae under a dissecting microscope. To facilitate easier collection of the cercariae, the water level in each cup was reduced to 150 mL prior to being placed using a dissecting scope. Cercariae were removed using a pipette. The numbers of cercariae required for each treatment (0 trematodes, 10 trematodes and 70 trematodes) were counted using a dissection microscope. Following the collection of the cercariae, the snails were then placed in fresh dechlorinated water, fed a pinch of ground up rabbit chow and returned to the incubator.

2.1.5 Preparation of Minnow and Swordtail Skin Extracts

Skin extracts of fishes were prepared by collecting skin from two swordtails (*Xiphophorus helleri*) (mean standard length 38.9 ± 7.2 mm, and two fathead minnows 27.5 ± 5.6 mm.) For each skin extract, donor fish were killed with a blow to the head (in accordance with University of Saskatchewan Animal Care Protocol # 20040016) and skin fillets were removed from both sides of the fish yielding a total of 4.1 cm^2 (in 20 mL) of swordtail skin, and 4.1 cm^2 (in 20 mL) of fathead minnow skin. The skin was immediately placed into a 50 mL glass beaker containing 20 mL of chilled glass distilled water. The solution was homogenized using a Polytron (20 seconds at $\frac{3}{4}$ speed), and then the solution was filtered through a funnel containing glass wool to remove any remaining large particles. To make the [low] skin extract solutions, 1 mL of the stock [high] solution was removed via syringe, and it was added to a clean 100 mL beaker. Forty-nine mL of distilled water was added to the beaker to make the total volume of the solution 50 mL. Glass distilled water was used as a blank control (20 mL in a 50 mL beaker).

In experiment five, I tested the effects of the various skin extracts of fishes on *Saprolegnia ferax* growth. Swordtail skin extract was used as a control in this experiment; swordtails are not ostariophysians, and consequently they lack alarm cells. However, swordtail skin extract still contains several critical ingredients including mucous cells and proteins, which may be used as a food source by *S. ferax*.

2.1.6 Preparation of Hypoxanthine 3-N oxide

In this experiment, I tested the effects of the synthetic ostariophysan alarm substance, hypoxanthine-3-N-oxide (H3NO) on *S. ferax* growth. The concentrations of H3NO used in this study are based on a previous study by Brown et al. (2000). Two concentrations of H3NO were tested; the [high] solution H3NO solution consisted of 0.002 grams of hypoxanthine-3-N-oxide being dissolved in 200 mL of glass-distilled water, while the [low] H3NO solution was made by removing 25 mL of the stock [high] H3NO solution, and transferring it to a clean beaker containing 200 mL of glass-distilled water, making a total solution volume of 225mL. To compare the effects of the synthetic alarm substance and naturally occurring ostariophysan alarm substance on *S. ferax* growth, [high] and [low] of fathead minnow skin extract were also tested. The concentrations and preparation of the fathead minnow skin extracts were the same as those used in the previous experiment. Glass distilled water was used as a blank control (20 mL in a 50 mL beaker). All solutions were kept on ice during the making of the solutions and while inoculating the GY agar Petri dishes.

2.1.7 Slide preparation

Fish were euthanized with an overdose of MS-222 (methane tricaine sulfonate) immediately following the termination of the experiments (#1-4). Fish were then measured, weighed and a body condition index was calculated for each fish (focal fish and partner). The fish were placed in individual histology jars containing 10% formalin before being processed

into slides. Specimen slides were processed by Prairie Diagnostic Services in the same manner as described by Hugie (1990) and Hindman (2002); however the methodology will be briefly reiterated here for purposes of clarification. Cross-sections (3-4 μm thick) were taken from each fish between the pectoral fin and the nape region behind the fish's head (see figure 1.1). The sectioned tissues were stained with Schiff's reagent (periodic acid) and then counterstained with Lillie's haematoxylin (PAS-H). Alarm cells are PAS-H negative and appear white with dark nuclei, while mucous cells are PAS-H positive and stain dark with pink nuclei (see figure 1.2). Alarm cells and mucous cells were identified based on physical characteristics as well as size; alarm cells are typically larger than mucous cells.

Slides were photographed using a Zeiss D-7082 Axioplan Transmitted Fluorescence Universal Microscope equipped with a Zeiss MC100 microscope camera. Epidermal thickness, number of alarm cells, size of alarm cells, number of mucous cells, and size of mucous cells were determined for each fish using ImageJ software (available on the National Institute of Health's web page <http://rsb.info.nih.gov/ij/>). Epidermal thickness was determined by measuring from the basement membrane to the surface of the skin. For each fish, three measurements of epidermal thickness were taken and the average was used in the statistical analysis. All measurement and cell counts were done blind with respect to experimental treatment.

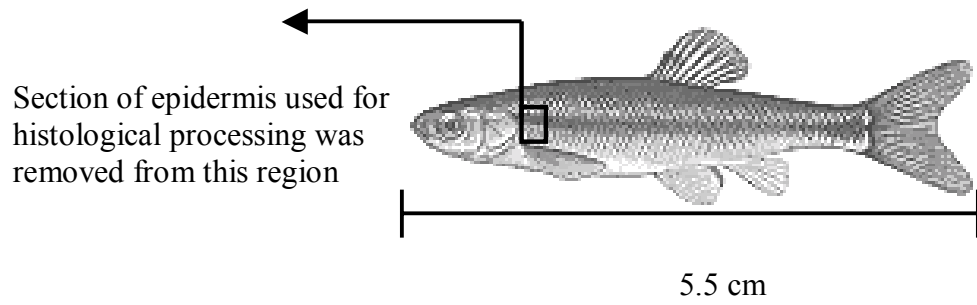


Figure 1.1: Location of skin removed from fathead minnows for PAS-H processing.

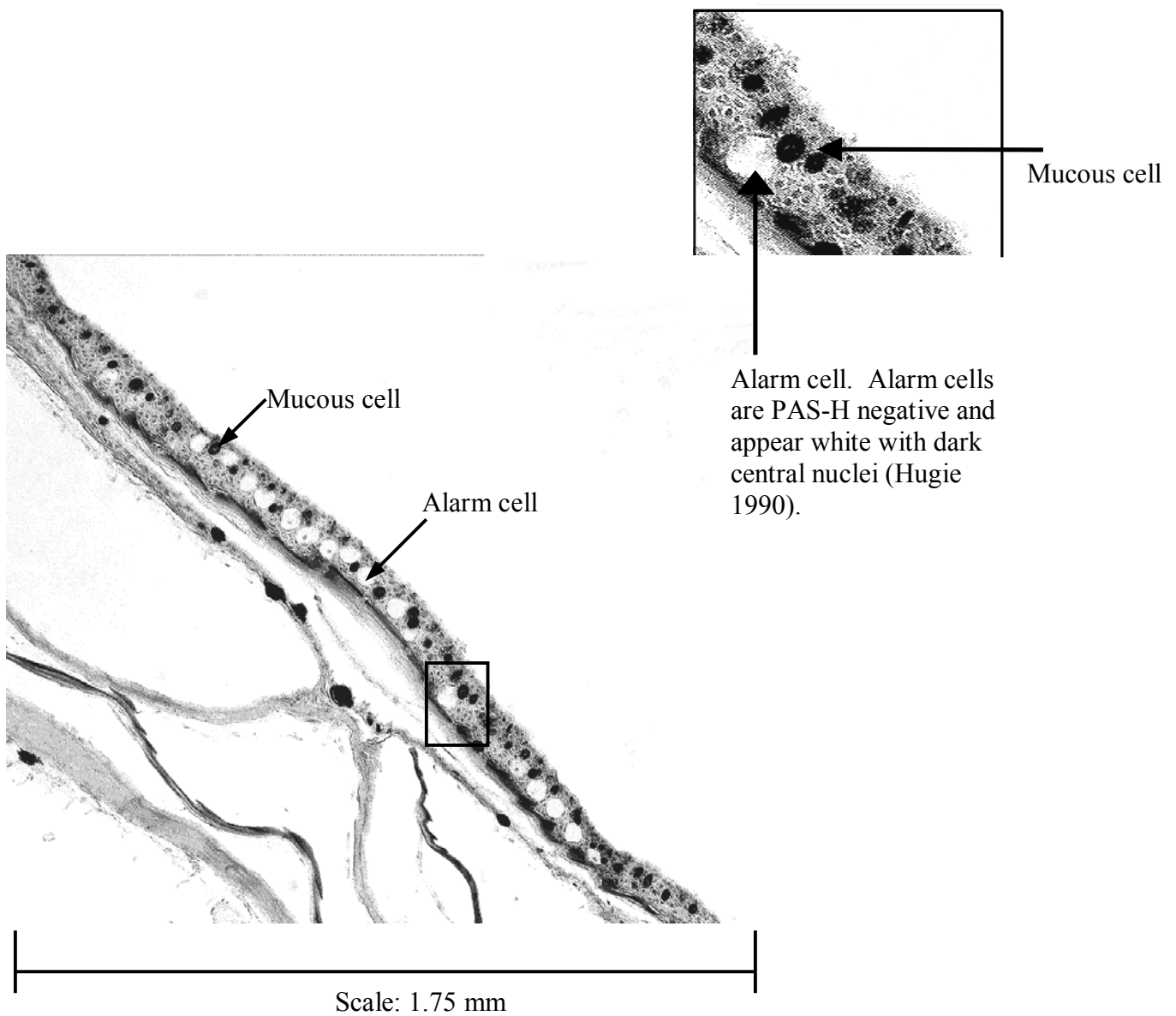


Figure 1.2: Fathead minnow epidermal cross-section. Magnification 100X.

2.2 Experiment one: the effect of *Saprolegnia ferax* concentration of alarm cell investment in fathead minnows

2.2.1 Experimental Protocol

One week before the start of each experiment, fish were randomly assigned to a treatment group and placed in 9.47 L aquaria with their experimental partner, ensuring that the fish acclimated to the experimental tanks prior to the start of the experiment. The tanks were filled with dechlorinated tap water, and the bottom of the tank was covered with approximately a 2.5 cm of gravel. The experimental tanks were aerated with an airstone and had 1 mm x 1 mm mesh screen lids, which were secured with binder clips. The mesh lids served two purposes: they prevented tank contents from being transferred from one tank to another, and they prevented fish from jumping out of their experimental tanks. Fish were maintained in their experimental tanks for 11 days, after which time they were euthanized and processed for further study (for details see section on slide preparation). Mature males were excluded from my study as they lose their alarm cells during breeding season (Hugie 1990). Given that the minnows used in my study were young of the year, enabled me to use only immature fish in my studies. One fish from each experimental tank was randomly selected for histological processing. Fish were not measured prior to the experiments in an attempt to minimize the amount of handling stress on the fish. The excessive handling associated with measuring fish may have damaged the epidermis, which could have facilitated *S. ferax* in penetrating the fish's integument more easily. Consequently, fish were measured and weighed only following the completion of the experiment. Fish were measured using digital Vernier calipers (± 0.01 mm) and weighed using an electronic scale (± 0.01 mg).

Previous studies by Chivers and Jack (2003 unpublished data), found that exposing fish to high doses (4,000-40,000 zoospores in 2L of dechlorinated tap water) of *S. ferax* resulted in

differences in alarm abundance, but did not result in visible fungal infection. While these infection rates induced a dramatic increase in alarm cell abundance, there was no apparent difference in the alarm cell abundance between fish infected with low doses and high doses of the pathogen. The objective of this study was to determine whether *S. ferax* would induce a graded response in alarm cell investment to pathogen infections. The doses used in this experiment were greatly reduced compared to those used by Chivers and Jack (2003 unpublished data). Four different levels of *S. ferax* were tested including 18920 zoospores/day, 1892 zoospores /day, 189 zoospores / day, and 0 zoospores /day. Zoospores were added to experimental tanks containing 9.5L of dechlorinated tap water. Fish were originally intended to be treated with 1mL of their assigned experimental solution daily for 14 days, as this time period has been previously shown to be sufficient to induce differences in alarm cell investment (Chivers and Jack 2002, unpublished data). However, due to unexpected mortality of experimental fish (81 out 220 fish died), the experiment was terminated on the eleventh day. It is important to note that the experimental fish showed no physical signs of disease during the experiment and that there were no differences in mortality among treatments. One fish per tank was randomly selected for analysis. Since social conditions can influence alarm cell investment, only aquaria in which both fish survived were used for PAS-H staining.

2.3 Experiment two: The effect of *Saprolegnia ferax* and familiarity on alarm cell investment

2.3.1 Experimental Protocol

To determine the effects of familiarity and disease on alarm cell investment, fish were randomly assigned to one of two possible experimental treatments (familiar partner +18920 zoospores/ day, or unfamiliar partner + 18920 zoospores/ day). Fish were size matched with

either an experimental partner from their ‘home’ shoal (at time of collection), or a partner from a distant shoal (fish were caught approximately 1 km away from the ‘home’ shoal).

Experimental fish were challenged with cultures of *Saprolegnia ferax*. Cultures were grown in the laboratory and infection rates were based on zoospore numbers. The experimental infection rate was based on previous studies done by Chivers and Jack (2003 unpublished data) and the results of my first experiment. Chivers and Jack (2003 unpublished data) found that fish exposed to high doses (4,000-40,000 zoospores per day) of *S. ferax* resulted in differences in alarm cell abundance, but did not result in visible fungal infection. I exposed fish to 18920 zoospores per day for 11 days. The collection and cultivation of *S. ferax* was the same as described for experiment one. Unlike the previous experiment, there was little mortality of the experimental fish (one fish died out of a total of 186). The testing protocol used in this experiment followed that of experiment one.

2.4 Experiment three: The effects of zoospore conditioned water on alarm cell investment

2.4.1 Experimental Protocol

In a preliminary study I investigated the effects of zoospore conditioned water on alarm cell investment in fathead minnows. Specifically, I was interested in the mechanism that fish use to assess their risk of pathogens and determining whether the physical presence of zoospores was required to elicit alarm cell investment in minnows. To test this, fish were housed in a similar manner as previous experiments and were housed with a partner from their ‘home’ shoal. Experimental fish were exposed daily to either 1 mL of zoospore conditioned water (derived from 18920 zoospores) or 1 mL of a solution containing 18920 zoospores. It is important to note that not only was this experiment conducted in the same experimental facility and the same time as experiment two, but the group of fish that were treated with a familiar

partner +18920 zoospores/ day in experiment two were the same fish used as a comparison in this experiment. As in the previous experiment there was little mortality of the experimental fish (1 out of 152). The testing protocol used in this experiment followed that of experiment one.

2.5 Experiment four: the effect of trematodes cercariae on alarm cell investment

2.5.1 Experimental Protocol

To determine the effect of a skin burrowing trematode on minnow alarm cell investment, minnows were randomly assigned to one of the following experimental treatments: (0 trematodes, 10 trematodes, and 70 trematodes). Minnows were infected every four days for a 16-day period. On the days that minnows were treated with trematodes, their container volume was reduced to 150 ml of water for 24 h; this was done to ensure that the cercariae had the greatest opportunity to enter the minnow host. The controls also had their water levels dropped to 150 ml and were maintained at that level for 24 h to match the experimental treatments. Following the 24 h infectious period, the container volume was restored to 600 ml. On the sixteenth day of the experiment, minnows were euthanized with MS222, measured, weighed, and placed in scintillation vials containing 10% buffered formalin. A section of the minnow skin was removed for histological processing (see Figure 1 and 2 and section on PAS-H staining and slide preparation). Experiment four was a collaboration between Dr. Reehan Mirza (Post doctoral Fellow, Concordia University) and myself. Dr. Mirza collected the fish and conducted the experiment at Pennsylvania State University, while I sectioned the fish and analyzed the results.

2.6 Experiment five: the effects skin extracts of fishes on *Saprolegnia ferax* growth.

2.6.1 Experimental Protocol

In the previous experiments, pathogens or parasites were added to the fish's external aquatic environment to determine the effect it had on alarm cell investment. In experiments five and six I was interested in determining whether there are any properties inherent in fish skin that can influence pathogen growth. To test this, two experiments were conducted. In experiment five, petri dishes containing GY agar were randomly treated with 1 mL of one of the following solutions: blank control (distilled water), swordtail [low] skin extract, swordtail [high] skin extract, fathead minnow [low] skin extract, or fathead minnow [high] skin extract. One milliliter of the experimental solution was applied evenly to the surface of each GY agar Petridish. Treated petri dishes were transferred to a fridge for 24 hours to allow the experimental solutions to diffuse into the GY agar. Petri dishes were then inoculated with a 2 mm x 2 mm² cube of *S. ferax*, which was taken from a master culture. All inoculum cubes were taken from the margin of the same master culture. Following inoculation with *S. ferax*, cultures were measured twice daily (at 9 a.m. and 6 p.m.). Six measurements of *S. ferax* hyphal length were taken for each culture (3 maximum and 3 minimum); the measurements for each culture were averaged and the mean was reported. Cultures were measured using a plastic ruler \pm 1 mm. All measurements were made blind with respect to the experimental treatment.

2.7 Experiment six: the effect of skin extracts of fishes and synthetic alarm cues on *Saprolegnia ferax* growth

2.7.1 Experimental Protocol

As in the previous experiment, GY agar petri dishes were randomly treated with one of the following solutions: hypoxanthine-3-N-oxide (H3NO) [high], H3NO [low], fathead minnow [high], fathead minnow [low], or distilled water. Fathead minnow skin extract concentrations used in this experiment were the same as in the previous experiment. The

application of the solution and the *S. ferax* inoculum cubes, and measuring of the cultures was completed in the same manner as experiment five.

2.8 Statistical Analysis

2.8.1 General analysis

A coefficient of body condition (K) was calculated for each minnow as an indicator of body condition (Busacker et al. 1990). Body condition was calculated using the following equation:

$$K = \frac{\text{weight (mg)}}{\text{Length (cm)}^3}$$

For each aspect of the epidermis, a Kolmogorov-Smirnov test was conducted to determine whether data were normally distributed. Normally distributed data were analyzed using either a one-way ANOVA with Tukey post hoc tests (when there were three or more treatments), or an independent t-test (when there were two treatments). Data analyzed using these statistical tests are presented as means. Non-parametric data were analyzed using either a Kruskal-Wallis test or a Mann-Whitney U test. Data analyzed using these statistical tests are presented as medians. In addition, post-hoc comparisons were conducted between the two treatment groups in experiments one and two in an attempt to explain the negative results of experiment two.

Chapter 3: Results

3.1 Experiment one: The effects of different *Saprolegnia* concentrations on fathead minnow alarm cell investment

As social dynamics may play a role in alarm cell investment, minnows used for statistical analysis were only chosen from aquaria that had two live fish at the end of the experiment. While I originally started the experiment with 55 pairs of fish per treatment, due to unexpected mortality rates, statistical analyses were based on the following sample sizes: 18920 zoospores/ day (n=30), 1892 zoospores/ day (n=37), 189 zoospores/ day (n=35) and 0 zoospores/ day (n=37)

3.1.1 Body condition

A Kolmogorov-Smirnov test determined that the body condition data were normally distributed ($Z=0.94$, $P=0.33$). The results of a one-way ANOVA found no differences in body condition between treatments ($F=1.041$, $P=0.376$).

3.1.2 Alarm cell investment and alarm cell size

A Kolmogorov-Smirnov test established that the data for alarm cell investment were normally distributed ($Z=0.89$, $P=0.283$). The results of the one-way ANOVA showed a significant effect of treatment on alarm cell investment ($F=11.75$, $df=3$, $P<0.001$, see Figure 2.1). However, as there were no differences in alarm cell densities among fish treated with the pathogens, there was no evidence of a graded response. Statistical analyses for alarm cell size were based on following: 18920 zoospores/ day ((mean number of cells =10.34), 1892 zoospores/ day (mean number of cells =14.09), 189 zoospores/ day (mean number of cells =13.47) and 0 zoospores/ day (mean number of cells =5.607). A Kolmogorov-Smirnov test showed that the data for alarm cell size were normally distributed ($Z=0.92$, $P=0.643$). The

results of the one-way ANOVA showed no effect of treatment on alarm cell size ($F=0.460$, $df=3$, $P=0.711$, see Figure 2.2).

3.1.3 Mucous cell investment and mucous cell size

A Kolmogorov-Smirnov test established that the data for mucous cell investment were normally distributed ($Z=0.71$, $P=0.096$). The results of a one-way ANOVA showed no effect of treatment on mucous cell investment ($F=1.045$, $df=3$, $P=0.375$, see figure 2.3). Statistical analyses for mucous cell size were based on following: 18920 zoospores/ day (mean number of cells= 11), 1892 zoospores/ day (mean number of cells =15.5, 189 zoospores/ day (mean number of cells =11.63) and 0 zoospores/ day (mean number of cells= 11). A Kolmogorov-Smirnov test established that the data for mucous cell size were not normally distributed ($Z=2.362$, $P<0.0001$). The results of a Kruskal-Wallis test showed no effect of pathogens on mucous cell size ($X^2=1.665$, $df=3$, $P=0.645$, see figure 2.4).

3.1.4 Epidermal thickness

A Kolmogorov-Smirnov test established that the epidermal thickness data were not normally distributed ($Z=10.64871$, $P<0.001$). The results of a Kruskal-Wallis test showed no effect of pathogens on epidermal thickness ($X^2=1.931$, $df=3$, $P=0.587$, see Figure 2.5).

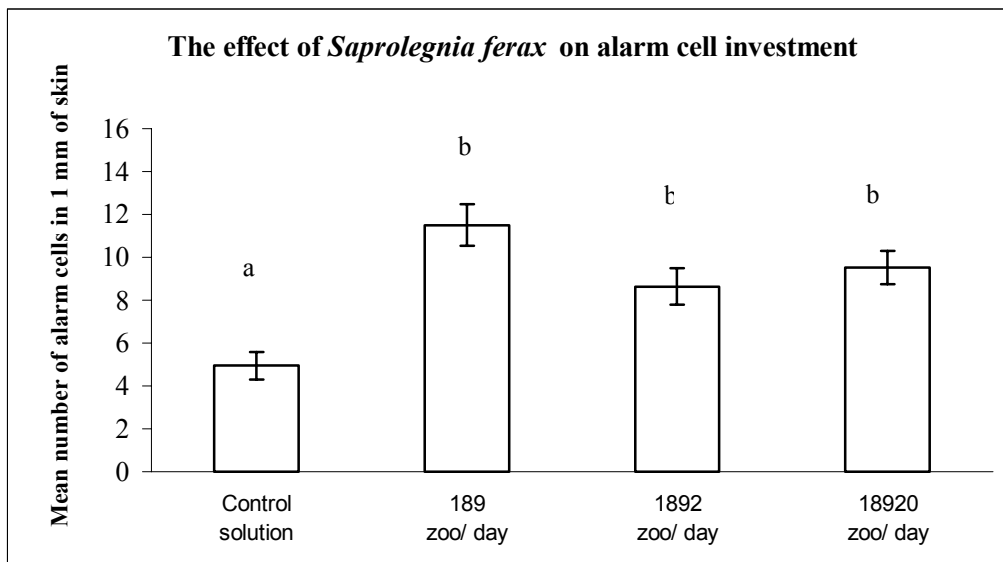


Figure 2.1: Mean (\pm SE) number of alarm cells of fathead minnows treated with different levels of *Saprolegnia*. Zoo/ day= zoospores per day. Statistical analyses were based on the following sample sizes: 18920 zoospores/ day (n=30), 1892 zoospores/ day (n=37), 189 zoospores/ day (n=35) and 0 zoospores/ day (n=37). Different letters denote significant differences at $P \leq 0.001$.

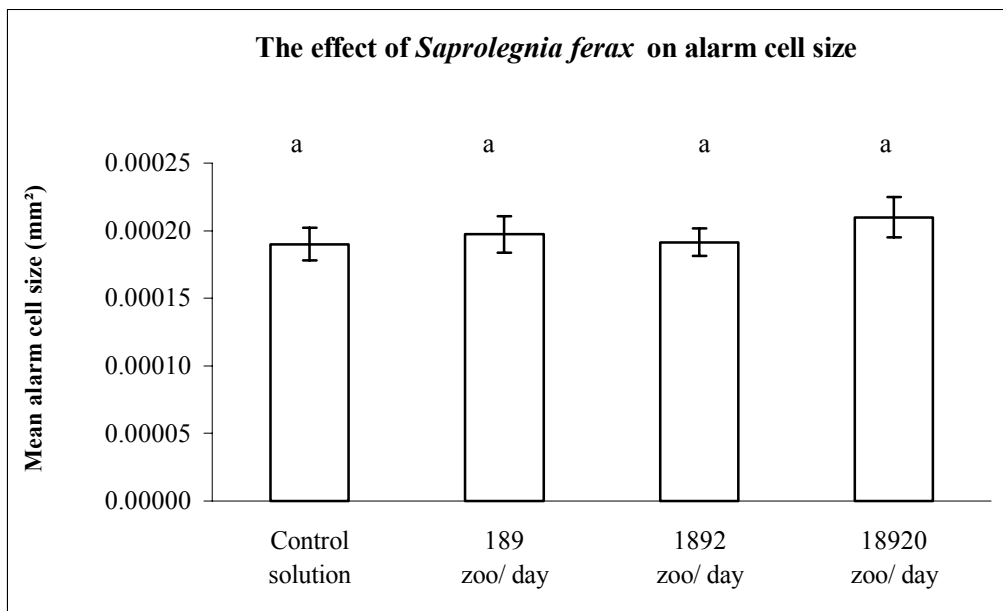


Figure 2.2: Mean (\pm SE) alarm cell size of fathead minnows treated with different levels of *Saprolegnia*. Zoo/ day= zoospores per day. Statistical analyses were based on the following sample sizes: 18920 zoospores/ day (n=30), 1892 zoospores/ day (n=37), 189 zoospores/ day (n=35) and 0 zoospores/ day (n=37). Different letters denote significant differences at $P \leq 0.05$.

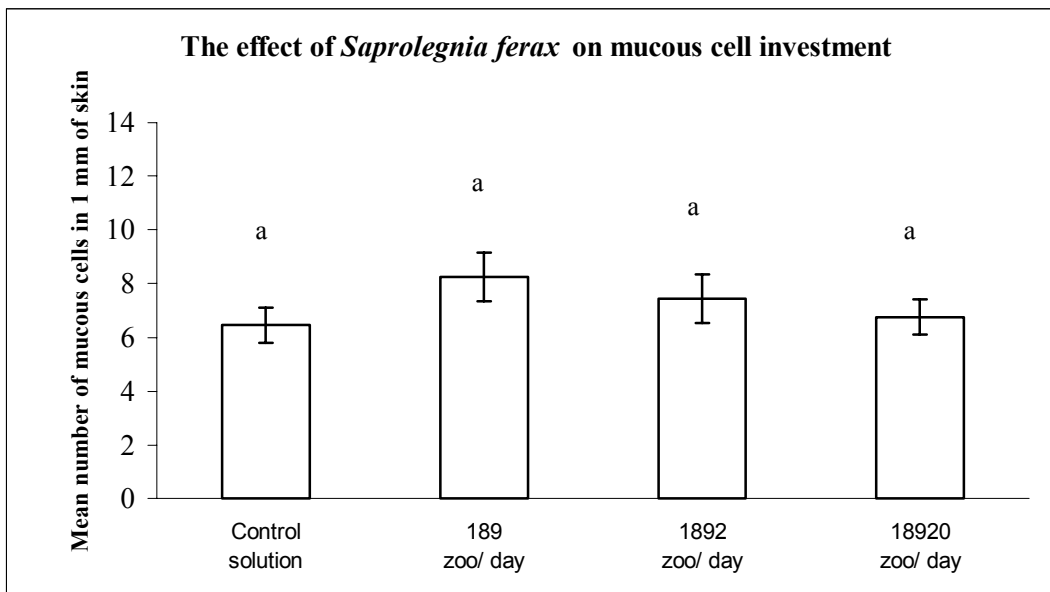


Figure 2.3: Mean (\pm SE) number of mucous cells of fathead minnows treated with different levels of *Saprolegnia*. Zoo/ day= zoospores per day. Statistical analyses were based on the following sample sizes: 18920 zoospores/ day (n=30), 1892 zoospores/ day (n=37), 189 zoospores/ day (n=35) and 0 zoospores/ day (n=37). Different letters denote significant differences at $P \leq 0.05$.

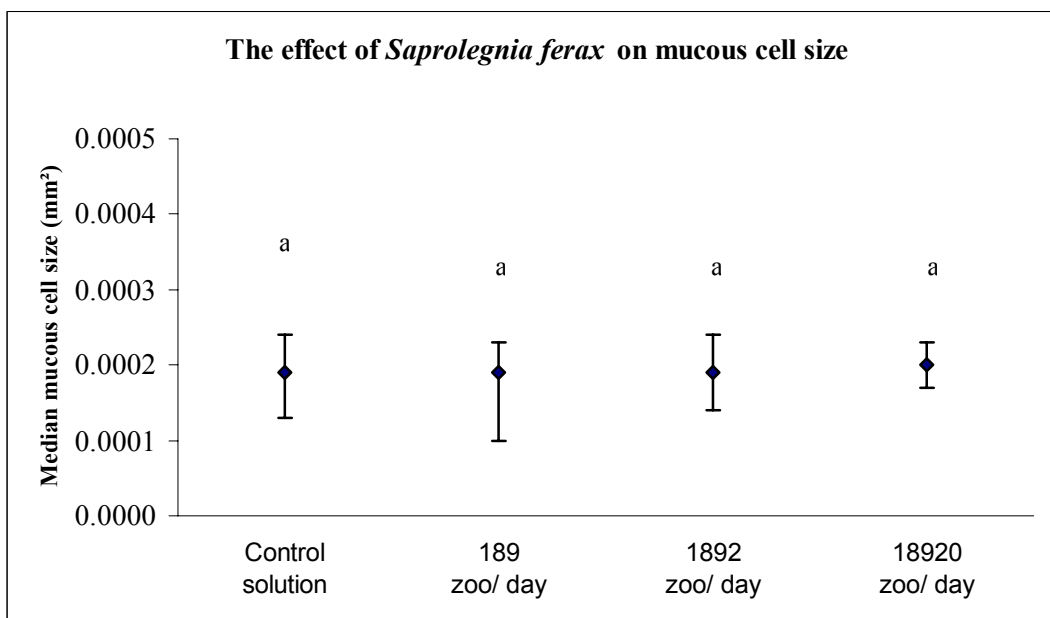


Figure 2.4: Median (\pm 25th and 75th quartiles) mucous cell size of fathead minnows treated with different levels of *Saprolegnia*. Statistical analyses were based on the following sample sizes: 18920 zoospores/ day (n=30), 1892 zoospores/ day (n=37), 189 zoospores/ day (n=35) and 0 zoospores/ day (n=37). Zoo/ day= zoospores per day. Different letters denote significant differences at $P \leq 0.05$.

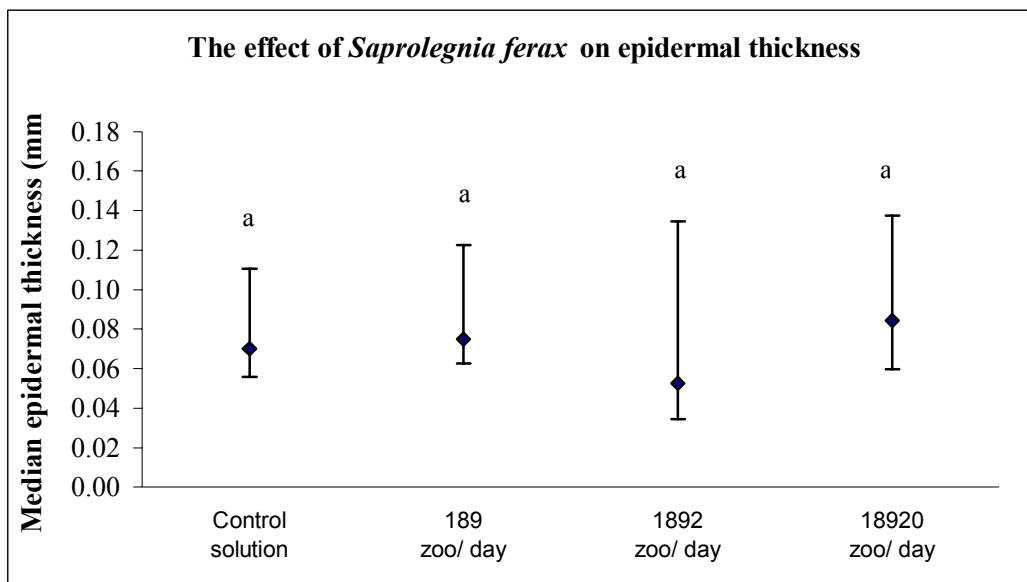


Figure 2.5: Median (\pm 25th and 75th quartiles) epidermal thickness of fathead minnows treated with different levels of *Saprolegnia*. Statistical analyses were based on the following sample sizes: 18920 zoospores/ day (n=30), 1892 zoospores/ day (n=37), 189 zoospores/ day (n=35) and 0 zoospores/ day (n=37). Zoo/ day= zoospores per day. Different letters denote significant differences at $P \leq 0.05$.

3.2 Experiment two: The effects of familiarity and *Saprolegnia* on fathead minnow alarm cell investment

Statistical analyses were based on the following sample sizes: Familiar partner +18920 zoospores/ day (n=38) and Unfamiliar partner + 18920 zoospores/ day (n=55).

3.2.1 Body condition

A Kolmogorov-Smirnov determined that the body condition data were not normally distributed ($Z=4.48$, $P<0.001$). The results of a Mann-Whitney U test found no differences in body condition between treatments ($U=486$, $df=1$, $P=0.34$).

3.2.2 Alarm cell investment and alarm cell size

A Kolmogorov-Smirnov test established that the alarm cell investment data were not normally distributed ($Z=1.673$, $P=0.007$). The results of the Mann-Whitney U test showed no effect of treatment on alarm cell investment ($U=835$, $df=1$ $P=0.247$, See figure 3.1). Statistical analyses for alarm cell size were based on the following sample sizes: Familiar partner +18920 zoospores/ day (mean number of cells=35.24) and Unfamiliar partner + 18920 zoospores/ day (mean number of cells=26.75). A Kolmogorov-Smirnov test showed that the data for alarm cell size were normally distributed ($Z=0.630$, $P=0.822$). The results of the independent sample t-test showed no effect of treatment on alarm cell investment ($t=1.190$, $df=.87$, $P= 0.237$, see figure 3.2).

3.2.3 Mucous cell investment and mucous cell size

A Kolmogorov-Smirnov test established that the data for mucous cell investment were not normally distributed ($Z=1.598$, $P=0.012$). The results of the Mann-Whitney U test showed no effect of treatment on mucous cell investment ($U=866$, $df= 1$, $P=0.479$, see figure 3.3). Statistical analyses for alarm cell size were based on the following sample sizes: Familiar partner +18920 zoospores/ day (mean number of cells =26.94) and Unfamiliar partner + 18920

zoospores/ day (mean number of cells=35.21). A Kolmogorov-Smirnov test established that the data for mucous cell size were normally distributed ($Z= 0.493$, $P=0.622$). The results of the independent sample t-test showed no effect of treatment on mucous cell size ($t=0.088$, $df=86$, $P= 0.930$, see figure 3.4).

3.2.4 Epidermal thickness

A Kolmogorov-Smirnov test established that the data for epidermal thickness were not normally distributed ($Z= 1.816$, $P=0.003$). The results of a Mann-Whitney U test showed no effect of pathogens on epidermal thickness ($U=821$, $df= 1$, $P=0.266$, see figure 3.5).

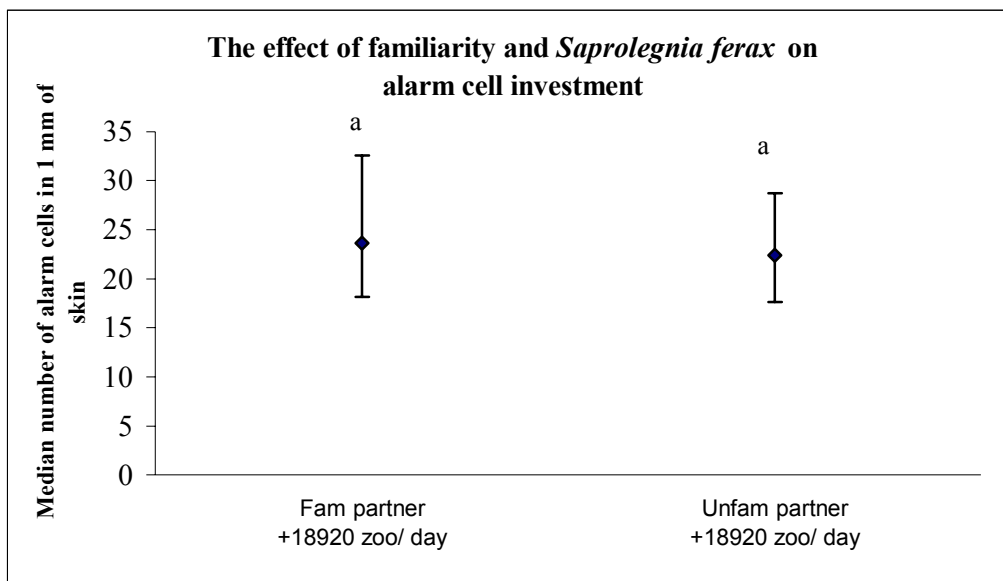


Figure 3.1: Median (\pm 25th and 75th quartiles) number of alarm cells of fathead minnows treated with *Saprolegnia* and different partners. Statistical analyses were based on the following sample sizes: Familiar partner +18920 zoospores/ day (n=38) and Unfamiliar partner + 18920 zoospores/ day (n=55).

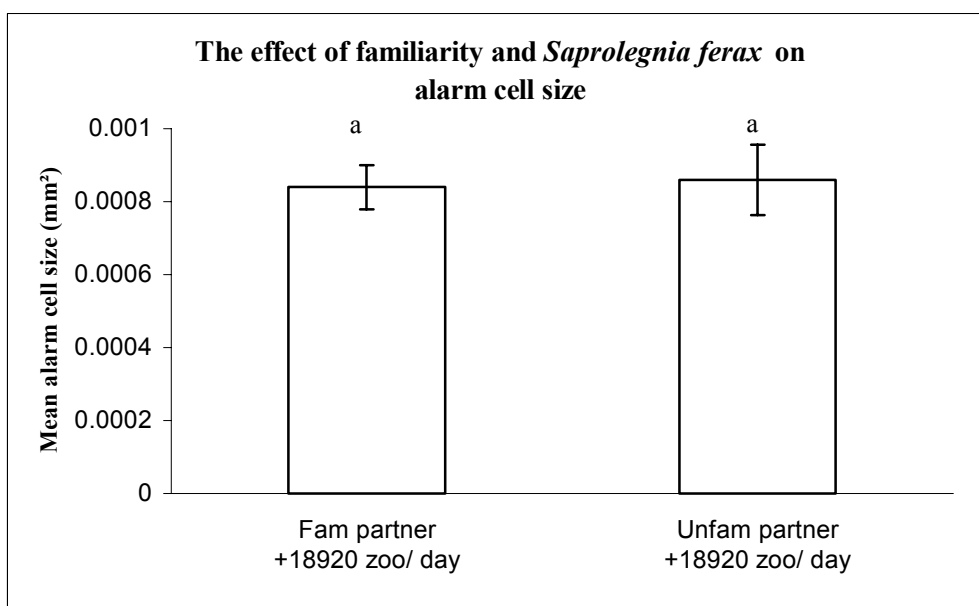


Figure 3.2: Mean (\pm SE) alarm cell size of fathead minnows treated with *Saprolegnia* and different partners. Zoo/ day= zoospores/ day. Statistical analyses were based on the following sample sizes: Familiar partner +18920 zoospores/ day (n=38) and Unfamiliar partner + 18920 zoospores/ day (n=55).

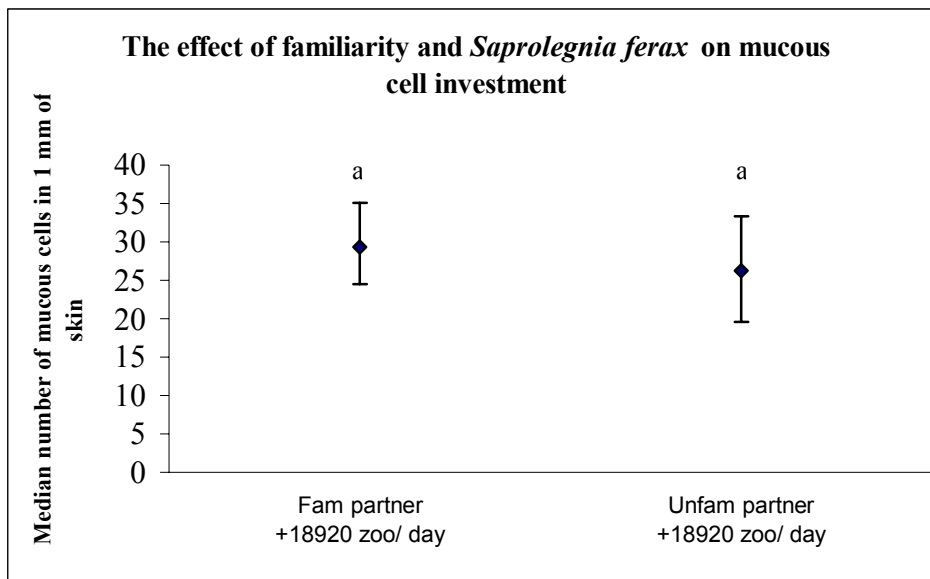


Figure 3.3: Median (\pm 25th and 75th quartiles) number of mucous cells of fathead minnows treated with *Saprolegnia* and different partners. Statistical analyses were based on the following sample sizes: Familiar partner +18920 zoospores/ day (n=38) and Unfamiliar partner + 18920 zoospores/ day (n=55). Zoo/ day= zoospores/ day. Different letters denote significant differences at $P \leq 0.05$.

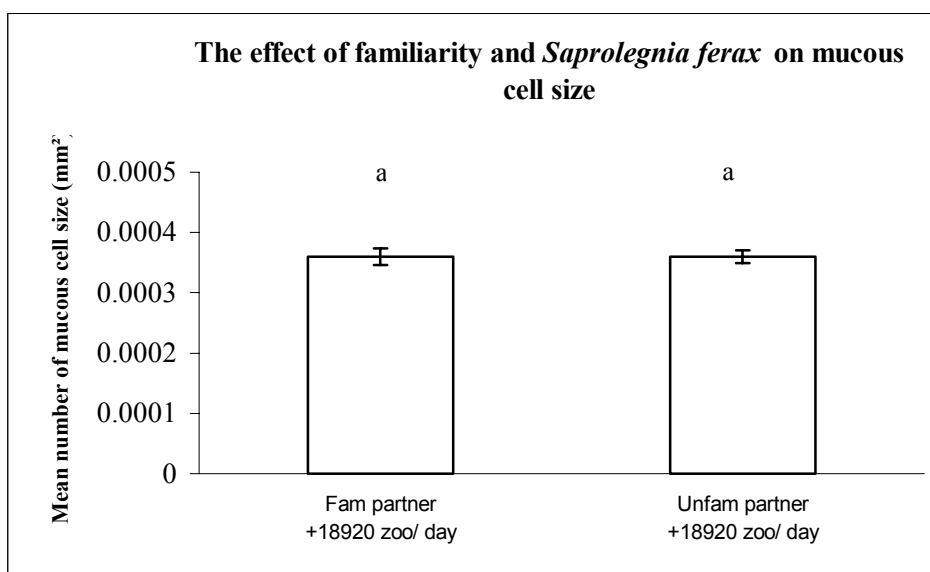


Figure 3.4: Mean (\pm SE) mucous cell size of fathead minnows treated with *Saprolegnia* and different partners. Statistical analyses were based on the following sample sizes: Familiar partner +18920 zoospores/ day (n=38) and Unfamiliar partner + 18920 zoospores/ day (n=55). Zoo/ day= zoospores/ day. Different letters denote significant differences at $P \leq 0.05$.

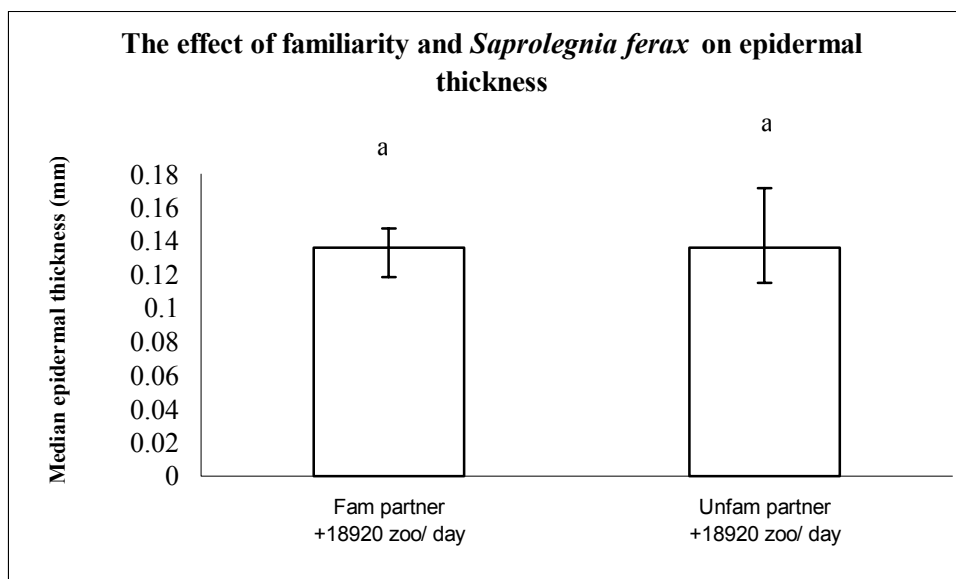


Figure 3.5: Mean (\pm SE) epidermal thickness of fathead minnows treated with *Saprolegnia* and different partners. Statistical analyses were based on the following sample sizes: Familiar partner +18920 zoospores/ day (n=38) and Unfamiliar partner + 18920 zoospores/ day (n=55). Zoo/ day= zoospores/ day. Different letters denote significant differences at $P \leq 0.05$.

3.3 Experiment three: The effects of zoospore conditioned water on fathead minnow alarm cell investment

Statistical analyses were based on the following sample sizes: Familiar partner + zoospore conditioned water (derived from 18920 zoospores)/ day (n=38) and Familiar partner + 18920 zoospores/ day (n=38).

3.3.1 Body condition

A Kolmogorov-Smirnov test determined that the body condition data were not normally distributed ($Z=4.60$, $P<0.0001$). The results of a Mann-Whitney U test found no differences in body condition between treatments ($U=983$, $df=1$, $P=0.11$).

3.3.2 Alarm cell investment and alarm cell size

A Kolmogorov-Smirnov test established that the alarm cell investment data were normally distributed ($Z=1.230$, $P=0.097$). The results of the t-test for independent samples showed no effect of treatment on alarm cell investment ($t=0.078$, $df=67$, $P=0.938$, see figure 4.1). Statistical analyses for alarm cell size were based on the following sample sizes: Familiar partner +18920 zoospores/ day (mean number of cells=35.24) and Familiar partner + water conditioned from 18920 zoospores/ day (mean number of cells=31.29). A Kolmogorov-Smirnov test showed that the data for alarm cell size were normally distributed ($Z=0.626$, $P=0.828$). The results of the independent samples t-test showed no effect of treatment on alarm cell size ($t=1.783$, $df= 67$, $P= 0.08$, see figure 4.2).

3.3.3 Mucous cell investment and mucous cell size

A Kolmogorov-Smirnov test established that the data for mucous cell investment were not normally distributed ($Z=2.045$, $P<0.0001$). The results of the Mann-Whitney U test showed no effect of treatment on mucous cell investment ($U=485$, $df=1$ $P=0.341$, see figure 4.3). Statistical analyses for alarm cell size were based on the following sample sizes: Familiar

partner +18920 zoospores/ day (mean number of cells =26.94) and Unfamiliar partner + 18920 zoospores/ day (mean number of cells=27.54). A Kolmogorov-Smirnov test established that the data for mucous cell size were normally distributed ($Z= 0.758$, $P=0.613$). The results of the independent samples t-test showed no effect of treatment on mucous cell size ($t=1.293$, $df=.87$, $P= 0.20$, see figure 4.4).

3.3.4 Epidermal thickness

A Kolmogorov-Smirnov test established that the data for epidermal thickness were not normally distributed ($Z= 1.816$, $P=0.003$). The results of a Mann-Whitney U test showed no effect of pathogens on epidermal thickness ($U=517$, $df=1$, $P=0.355$, see figure 4.5).

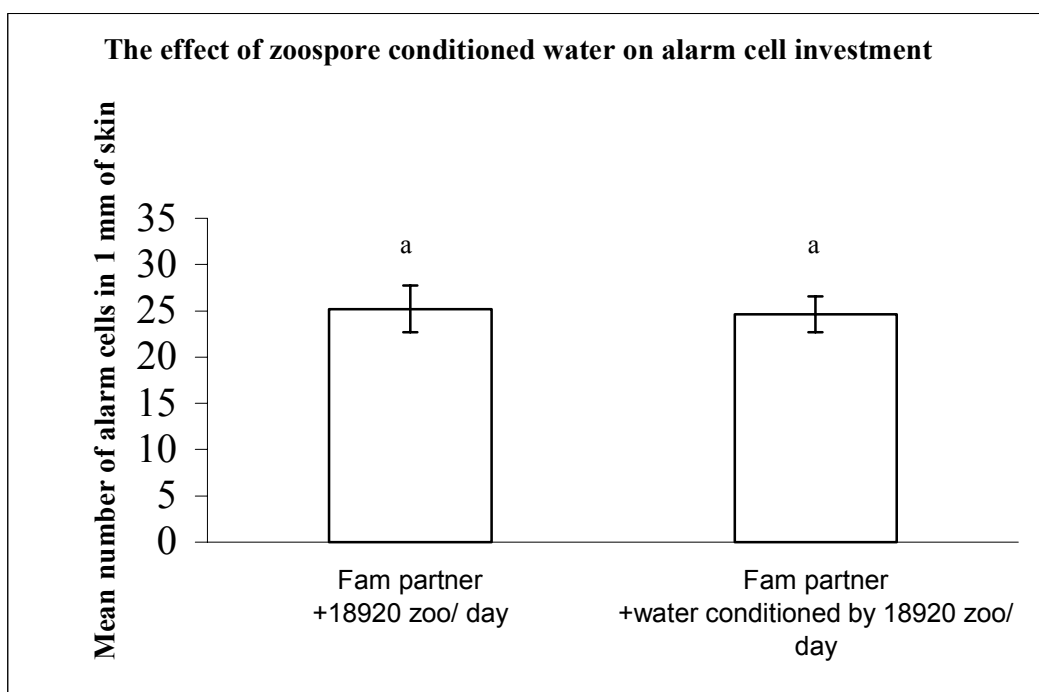


Figure 4.1: Mean (\pm SE) number of alarm cells of fathead minnows treated with either *Saprolegnia* or water conditioned by *Saprolegnia*. Zoo/day= zoospores/ day. Statistical analyses were based on the following sample sizes: Familiar partner + zoospore conditioned water (derived from 18920 zoospores)/ day (n=38) and Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.05$.

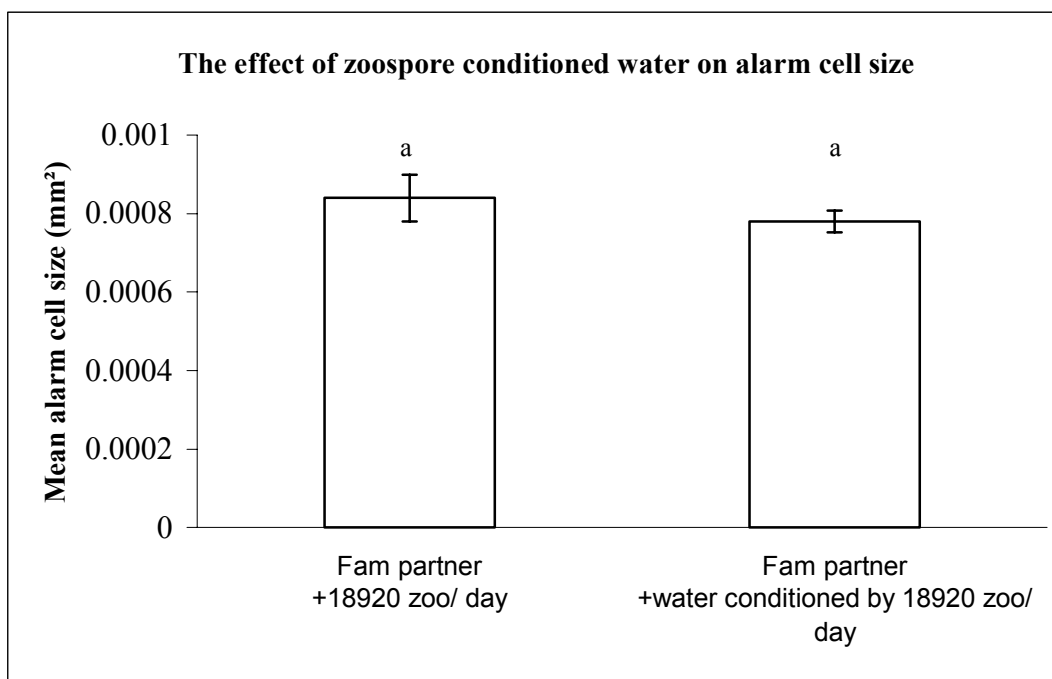


Figure 4.2: Mean (\pm SE) alarm cell size of fathead minnows treated with either *Saprolegnia* or water conditioned by *Saprolegnia*. Zoo/day= zoospores/ day. Statistical analyses were based on the following sample sizes: Familiar partner + zoospore conditioned water (derived from 18920 zoospores)/ day (n=38) and Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.05$.

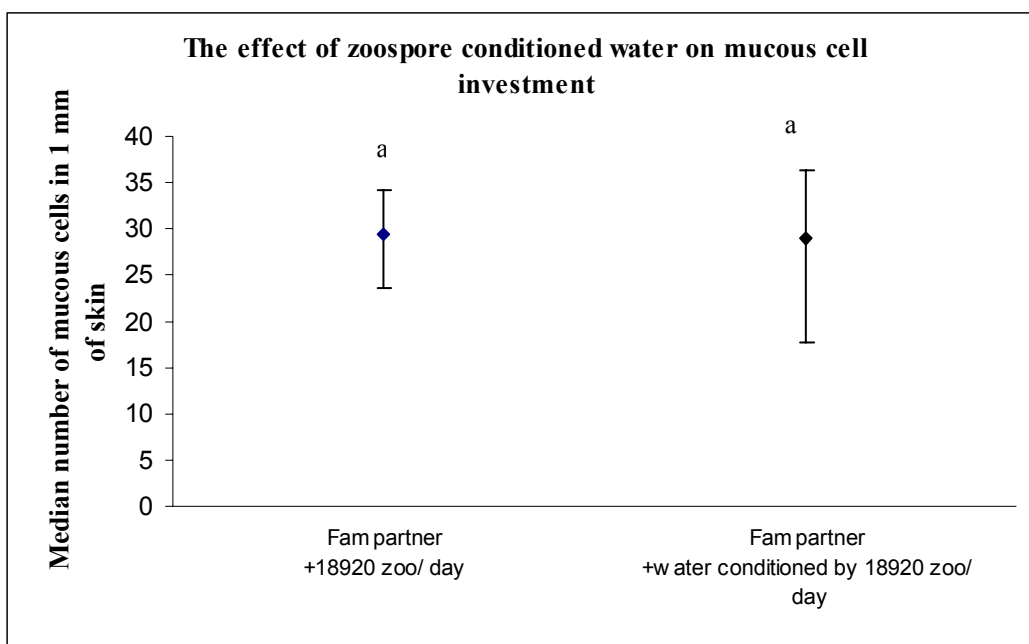


Figure 4.3: Median (\pm 25th and 75th quartiles) number of mucous cells of fathead minnows treated with either *Saprolegnia* or water conditioned by *Saprolegnia*. Zoo/day= zoospores/ day. Statistical analyses were based on the following sample sizes: Familiar partner + zoospore conditioned water (derived from 18920 zoospores)/ day (n=38) and Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.05$.

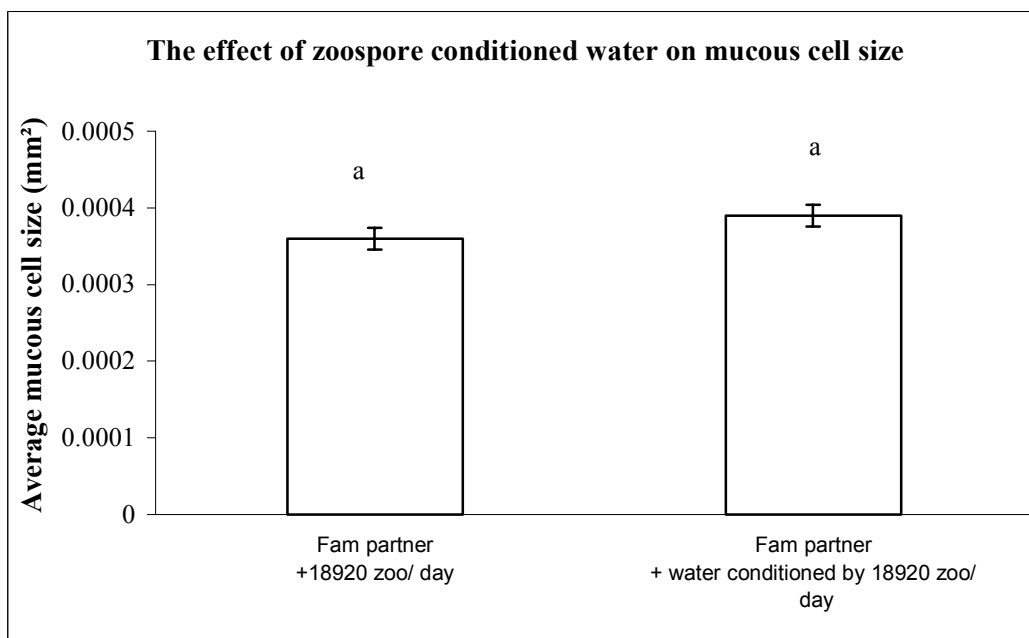


Figure 4.4: Mean (\pm SE) mucous cell size of fathead minnows treated with either *Saprolegnia* or water conditioned by *Saprolegnia*. Zoo/day= zoospores/ day. Statistical analyses were based on the following sample sizes: Familiar partner + zoospore conditioned water (derived from 18920 zoospores)/ day (n=38) and Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.05$.

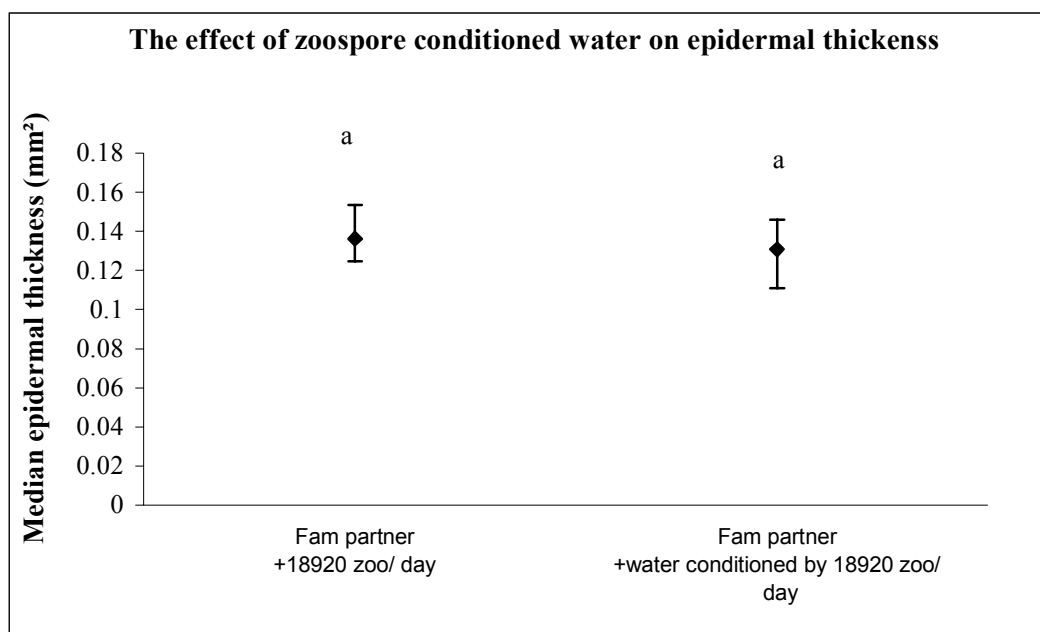


Figure 4.5: Median (\pm 25th and 75th quartiles) number of mucous cells of fathead minnows treated with either *Saprolegnia* or water conditioned by *Saprolegnia*. Zoo/day= zoospores/ day. Statistical analyses were based on the following sample sizes: Familiar partner + zoospore conditioned water (derived from 18920 zoospores)/ day (n=38) and Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.05$.

3.4 Experiment four: The effects of different levels of trematodes cercariae on fathead minnow alarm cell investment.

Statistical analyses were based on the following samples sizes: 0 trematodes (n=39), 10 trematodes (n=42), and 70 trematodes (n=47).

3.4.1 Body condition

A Kolmogorov-Smirnov test determined that the body condition data were not normally distributed ($Z=2.16$, $P<0.0001$). The results of a Kruskal-Wallis test found no differences in body condition between treatments ($X^2=3.71$, $df=2$, $P=0.172$). The result of a chi-squared test showed an effect of parasite load on observed infection rate in fathead minnow skin ($X^2=6.712$, $df=2$, $P=0.035$, see figure 5.1).

3.4.2 Alarm cell investment and alarm cell size

A Kolmogorov-Smirnov test established that the data for alarm cell investment were not normally distributed ($Z=4.89$, $P=0.013$). The results of the Kruskal-Wallis test showed a significant effect of treatment on alarm cell investment ($X^2=12.750$, $df=2$, $P<0.002$, see figure 5.2). Statistical analyses were based on the following samples sizes: 0 trematodes (mean number of cells =15.36), 10 trematodes (mean number of cells =17.23), and 70 trematodes (mean number of cells =21.55). A Kolmogorov-Smirnov test showed that the data for alarm cell size were normally distributed ($Z=1.029$, $P=0.241$). The results of the one-way ANOVA showed no effect of treatment on alarm cell size ($F=2.081$, $df=2$, $P=0.129$, see figure 5.3)

3.4.3 Mucous cell investment and mucous cell size

A Kolmogorov-Smirnov test established that the data for mucous cell investment were not normally distributed ($Z=14.32$, $P<0.001$). The results of a Kruskal-Wallis test showed a trend in the treatment effect on mucous cell investment ($X^2=5.338$, $df=2$, $P=0.069$, see figure 5.4). Statistical analyses were based on the following samples sizes: 0 trematodes (mean

number of cells =13.27), 10 trematodes (mean number of cells =20.81), and 70 trematodes (mean number of cells =21.05). A Kolmogorov-Smirnov test established that the data for mucous cell size were not normally distributed ($Z= 4.17, P= 0.035$). The results of a Kruskal-Wallis test showed no effect of trematodes on mucous cell size ($X^2=4.318, df=2, P=0.115$, see figure 5.5).

3.4.4 Epidermal thickness

A Kolmogorov-Smirnov test established that the epidermal thickness data were not normally distributed ($Z= 2.795, P<0.001$). The results of a Kruskal-Wallis test showed no effect of trematodes on epidermal thickness ($X^2=4.469, df=2, P=0.107$, see figure 5.6).

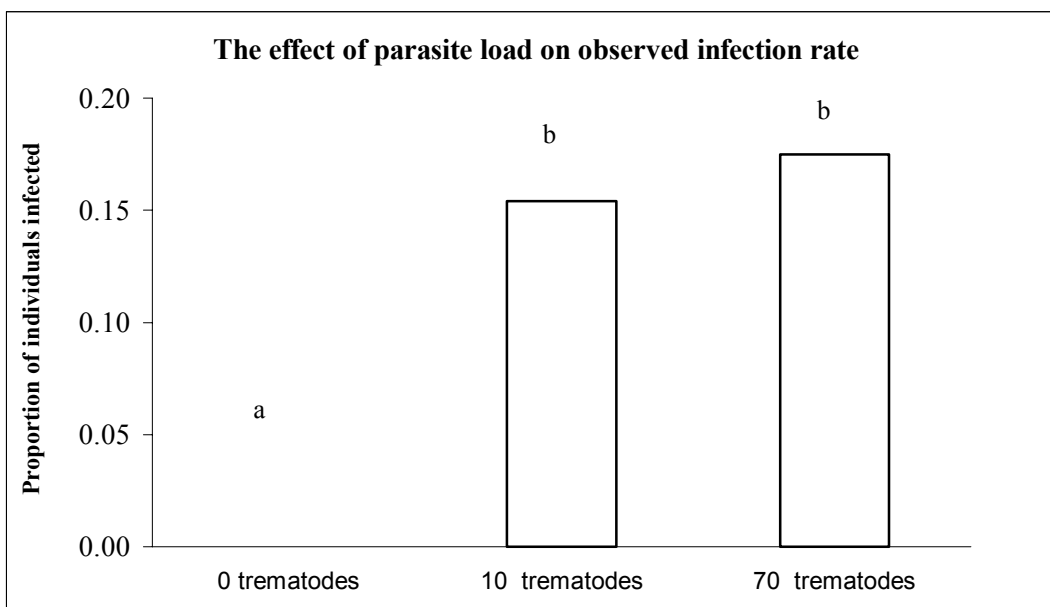


Figure 5.1: Proportion of fathead minnows infected with cysts followed being treated with different levels of trematode cercariae. Statistical analyses were based on the following samples sizes: 0 trematodes (n=39), 10 trematodes (n=42), and 70 trematodes (n=47). Different letters denote significant differences at $P \leq 0.05$.

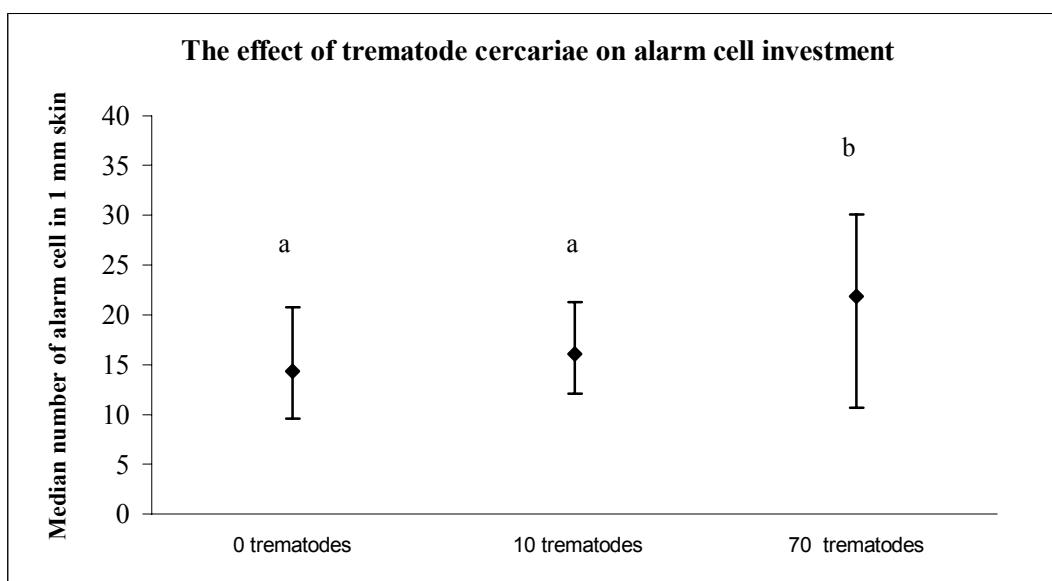


Figure 5.2: Median (\pm 25th and 75th quartiles) number of alarm cells of fathead minnows treated with different levels of trematode cercariae. Statistical analyses were based on the following samples sizes: 0 trematodes (n=39), 10 trematodes (n=42), and 70 trematodes (n=47). Different letters denote significant differences at $P \leq 0.01$.

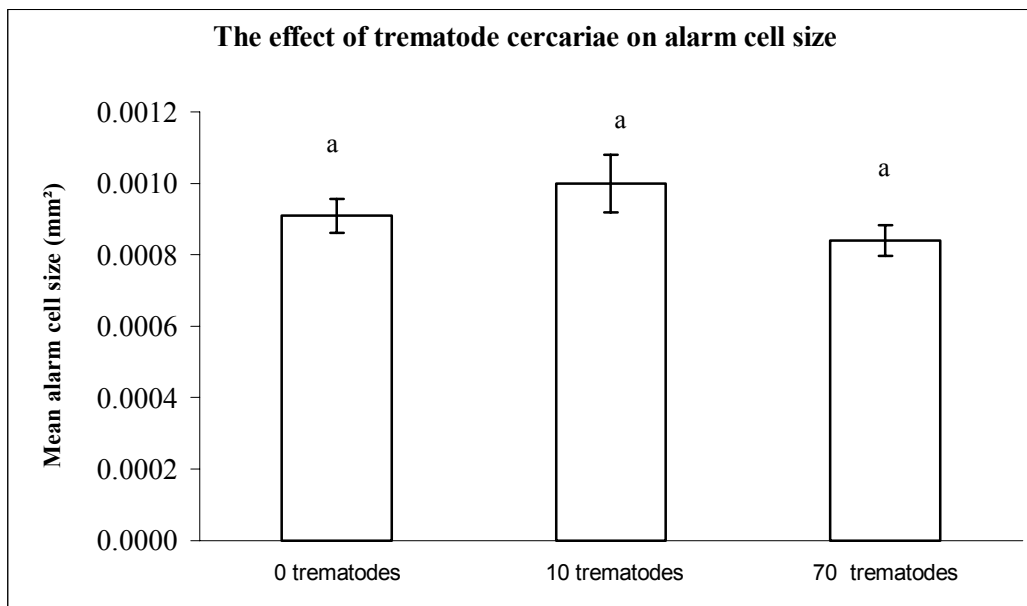


Figure 5.3: Mean (\pm SE) alarm cell size of fathead minnows treated with different levels of trematode cercariae. Statistical analyses were based on the following samples sizes: 0 trematodes (n=39), 10 trematodes (n=42), and 70 trematodes (n=47). Different letters denote significant differences at $P \leq 0.05$.

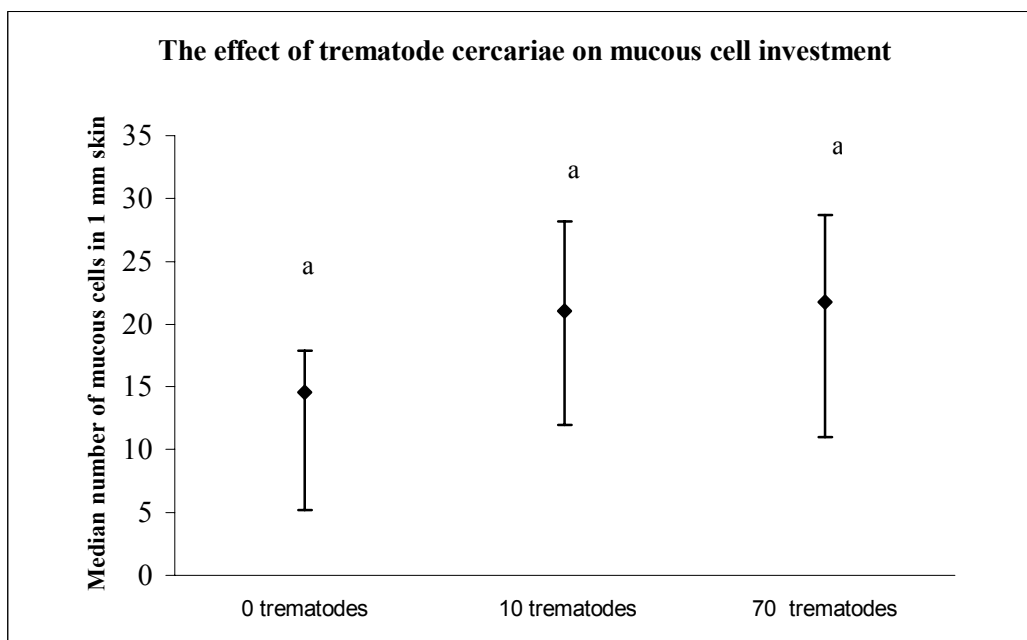


Figure 5.4: Median (\pm 25th and 75th quartiles) number of mucous cells of fathead minnows treated with different levels of trematode cercariae. Statistical analyses were based on the following samples sizes: 0 trematodes (n=39), 10 trematodes (n=42), and 70 trematodes (n=47). Different letters denote significant differences at $P \leq 0.05$.

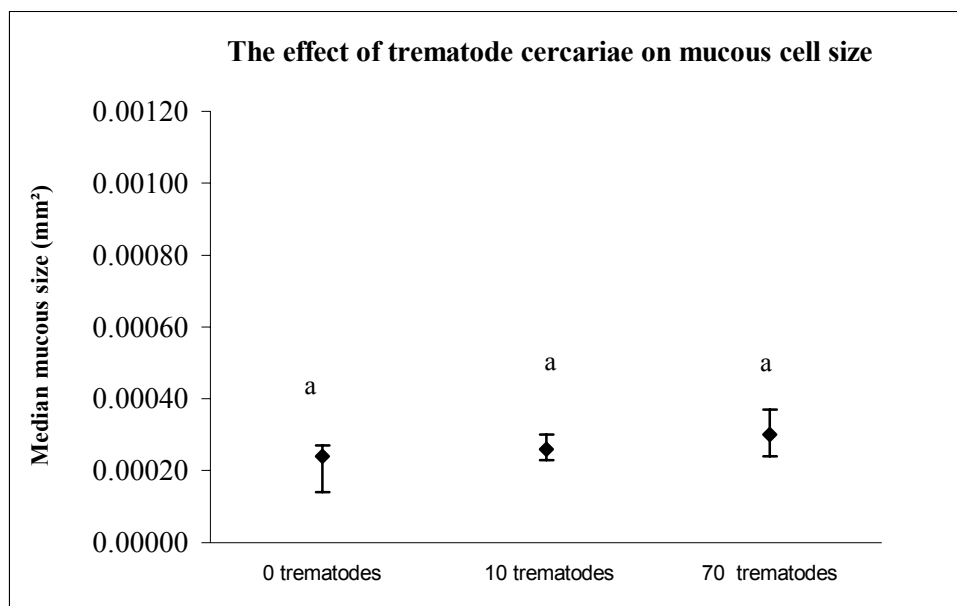


Figure 5.5: Median (\pm 25th and 75th quartiles) mucous cell size of fathead minnows treated with different levels of trematode cercariae. Statistical analyses were based on the following samples sizes: 0 trematodes (n=39), 10 trematodes (n=42), and 70 trematodes (n=47). Different letters denote significant differences at $P \leq 0.05$.

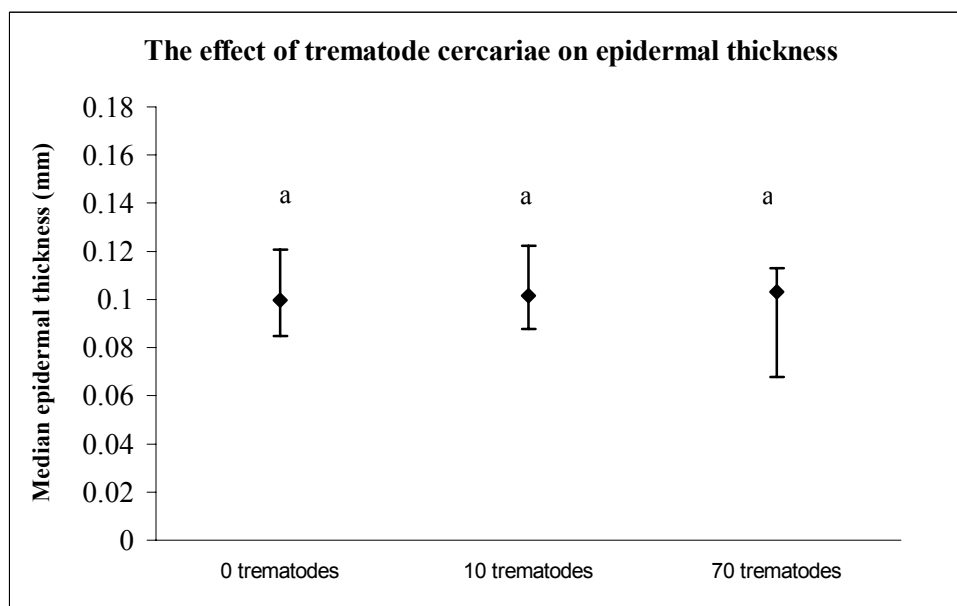


Figure 5.6: Median (\pm 25th and 75th quartiles) epidermal thickness of fathead minnows treated with different levels of trematode cercariae. Statistical analyses were based on the following samples sizes: 0 trematodes (n=39), 10 trematodes (n=42), and 70 trematodes (n=47). Different letters denote significant differences at $P \leq 0.05$.

3.5 Experiment five: The effect of skin extracts of fishes on *Saprolegnia* growth

Experiment five tested the effects of skin extracts on *S. ferax* growth. Statistical analyses were based on the following sample sizes: blank control (distilled water) (n=14), swordtail [low] skin extract (n=13), swordtail [high] skin extract (n=10), fathead minnow [low] skin extract (n=11), and fathead minnow [high] skin extract (n=14). The data reported were obtained 36 hours after inoculating the petri dishes with *S. ferax*. A Kolmogorov-Smirnov test established that the *S. ferax* growth data were not normally distributed ($Z=13.27$, $P<0.0001$). The results of a Kruskal-Wallis test showed a significant effect of skin extracts on *S. ferax* growth ($X^2=16.10$, $df= 3$, $P<0.0001$, see figure 6.1). Cultures exposed to fathead minnow skin extract showed the least amount of growth, while cultures exposed to the high concentration of swordtail skin extract showed the greatest amount of growth. *S. ferax* cultures exposed to either distilled water or a low concentration of swordtail skin extract showed an intermediate amount of growth.

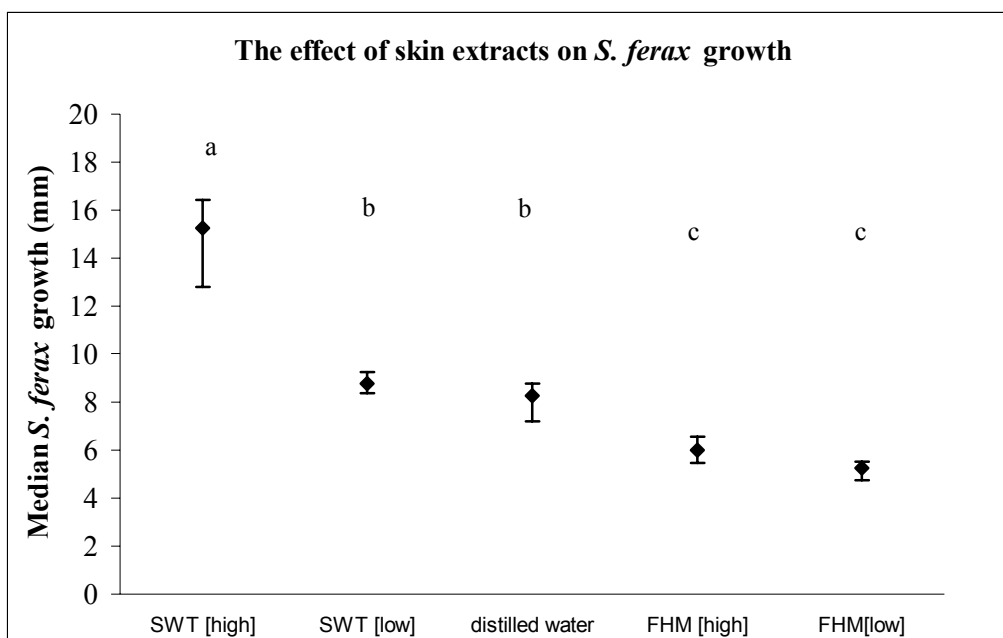


Figure 6.1: Median (\pm 25th and 75th quartiles) growth of *S. ferax* treated with different skin extracts. Statistical analyses were based on the following sample sizes: blank control (distilled water) (n=14), SWT [low] = low concentration of swordtail skin extract (n=13), SWT [high] = high concentration of swordtail skin extract (n=10), FHM [low] = low concentration of fathead minnow skin extract (n=11), and FHM [high] = high concentration of fathead minnow skin extract (n=14). Different letters denote significant differences at $P \leq 0.0001$.

3.6 Experiment six: the effects of skin extracts of fishes and synthetic alarm cues on *Saprolegnia* growth

This experiment tested the effects of skin extracts and synthetic alarm cues on *S. ferax* growth. Statistical analyses were based on the following sample sizes: hypoxanthine-3-N-oxide (H3NO) [high] (n=9), H3NO [low] (n=7), fathead minnow [high] (n=11), fathead minnow [low] (n=11), or distilled water (n=7). The data reported were obtained 24 hours after inoculating the petri dishes with *S. ferax*. A Kolmogorov-Smirnov test established that the *S. ferax* growth data were normally distributed ($Z=1.248$, $P=0.09$). The results of a 1-way ANOVA analysis show a significant effect of treatment on *S. ferax* growth ($F= 91.74$, $df=4$, $P<0.0001$, see figure 7.1).

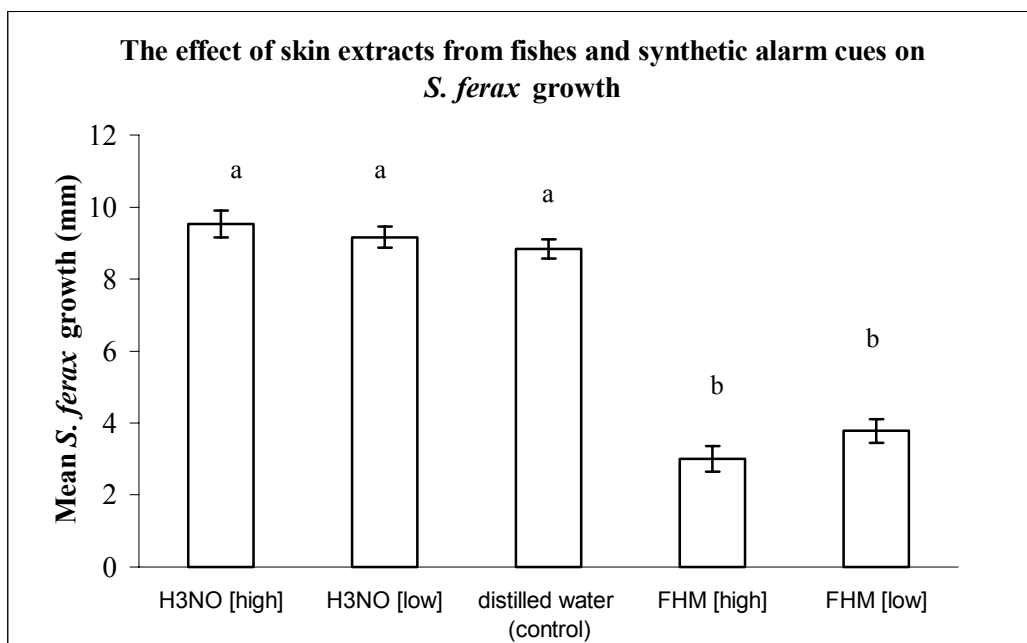


Figure 7.1: Mean (\pm SE) growth of *S. ferax* treated with different solutions. Statistical analyses were based on the following sample sizes: hypoxanthine-3-N-oxide (H3NO) [high] = high concentration of synthetic ostariophysian alarm cue (n=9), H3NO [low] = low concentration of synthetic ostariophysian alarm cue (n=7), FHM[high] = high concentration of fathead minnow skin extract (n=11), FHM[low] = low concentration of fathead minnow skin extract (n=11), or distilled water = blank control (n=7). Different letters denote significant differences at $P \leq 0.001$.

3.7 Comparisons of epidermal variables between experiments one and two

A Kolmogorov-Smirnov test established that the body condition data were not normally distributed ($Z=1.2430$, $P=0.091$). The results of a t-test show a significant difference in body condition between fish in experiments one and two ($t=12.14$, $df=66$, $P<0.001$, see figure 8.6). In addition, statistical tests were performed to test whether the fish from the two experiments differed in both length and mass. The results of a Mann-Whitney U test showed that fish in experiment 1 were significantly shorter in length than fish in experiment two ($t=15.34$, $df=66$, $P<0.001$). Fish from experiment one were also significantly lighter than fish in experiment two ($t=14.19$, $df=66$, $P<0.001$).

3.7.2 Alarm cell investment and alarm cell size

A Kolmogorov-Smirnov test established that the alarm cell investment data were normally distributed ($Z=0.94$, $P=0.56$). The results of a T-test for independent samples showed a significant difference in alarm cell densities between the two groups of fish ($t=5.438$, $df=66$, $P<0.001$, see figure 8.1). A Kolmogorov-Smirnov test showed that the data for alarm cell size were normally distributed ($Z=0.971$, $P=0.34$). The results of a T-test for independent samples showed a trend towards difference in alarm cell size between the two groups of fish ($t=6.61$, $df=66$, $P<0.001$, see figure 8.2).

3.7.3 Mucous cell investment and mucous cell size

A Kolmogorov-Smirnov test established that the data for mucous cell investment were normally distributed ($Z=0.19$, $P=0.812$). The results of a T-test for independent samples showed a significant difference in mucous cell investment between the two groups of fish ($t=10.39$, $df=66$, $P<0.0001$, see figure 8.3). A Kolmogorov-Smirnov test established that the data for mucous cell size were normally distributed ($Z=0.726$, $P=0.668$). The results of a two-

tailed T-test for independent samples showed a significant difference in mucous cell size between fish in the two treatment groups ($t= 5.035$, $df=66$, $P<0.001$, see figure 8.4)

3.7.4 Epidermal thickness

A Kolmogorov-Smirnov test established that the data for epidermal thickness were normally distributed ($Z= 0.556$, $P=0.906$). The results of a T-test for independent samples showed a significant difference in epidermal thickness between fish in the two experiments ($t=6.463$, $df=66$, $P<0.001$, see figure 8.5).

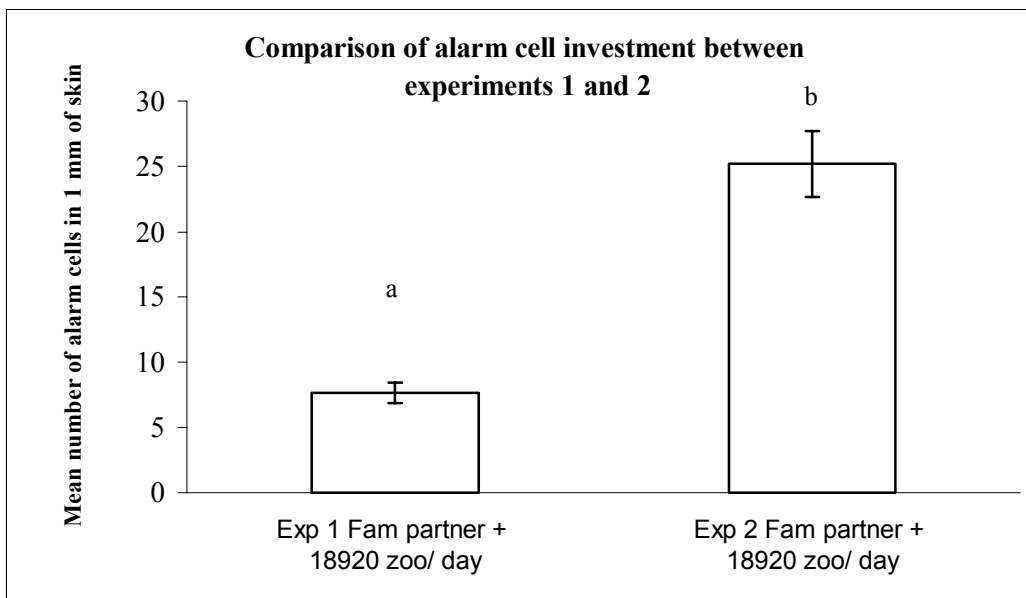


Figure 8.1: Mean (\pm SE) alarm cell number of fathead minnows in experiments 1 and 2. Statistical analyses were based on the following sample sizes: Experiment 1 Familiar partner +18920 zoospores/ day (n=30) and Experiment 2 Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.001$.

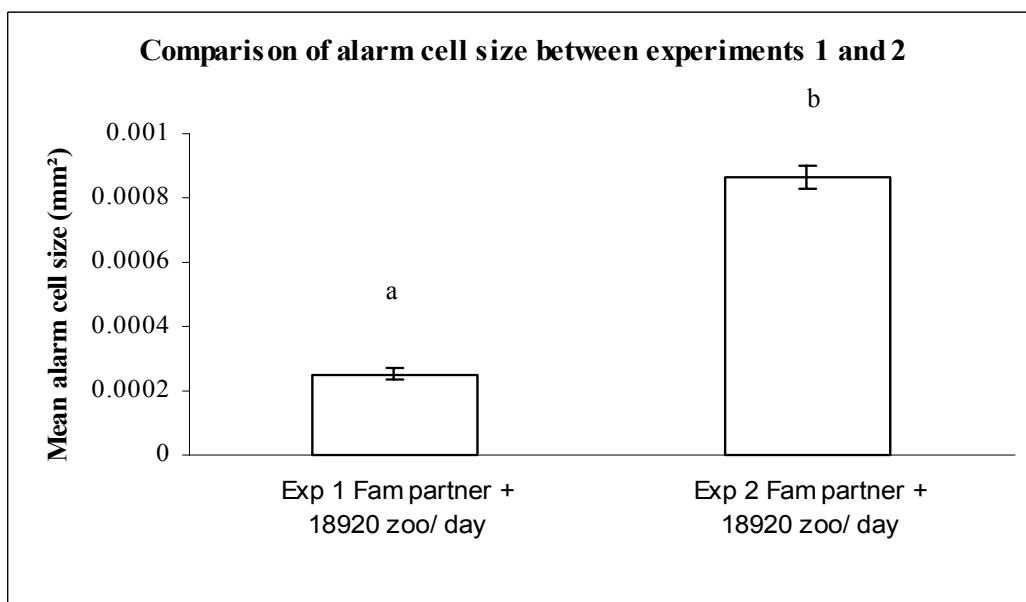


Figure 8.2: Mean (\pm SE) alarm cell size of fathead minnows in experiments 1 and 2. Statistical analyses were based on the following sample sizes: Experiment 1 Familiar partner +18920 zoospores/ day (n=30) and Experiment 2 Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.001$.

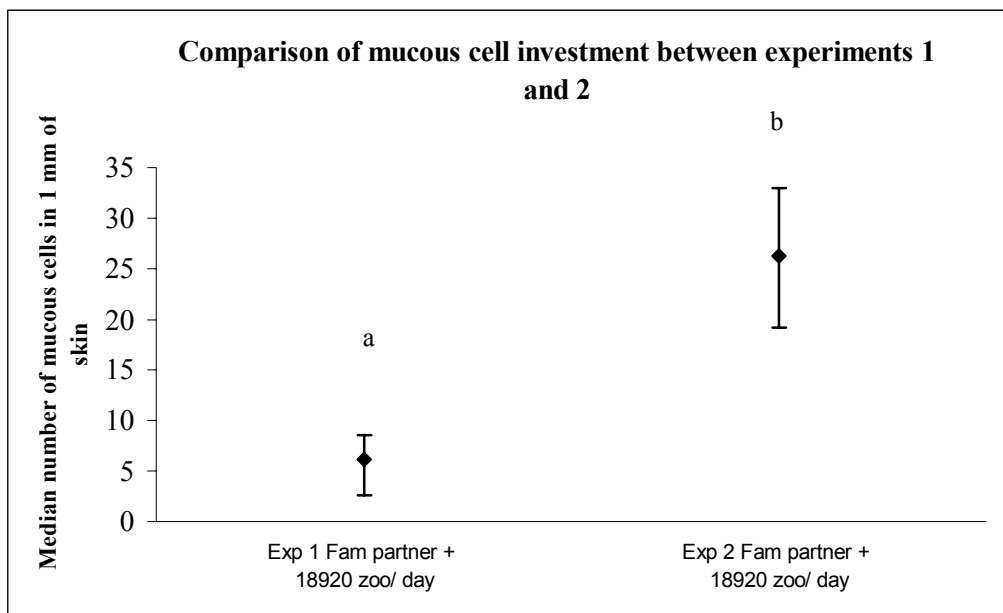


Figure 8.3: Median (\pm 25th and 75th quartiles) mucous cell size of fathead minnows in experiments 1 and 2. Statistical analyses were based on the following sample sizes: Experiment 1 Familiar partner +18920 zoospores/ day (n=30) and Experiment 2 Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.001$.

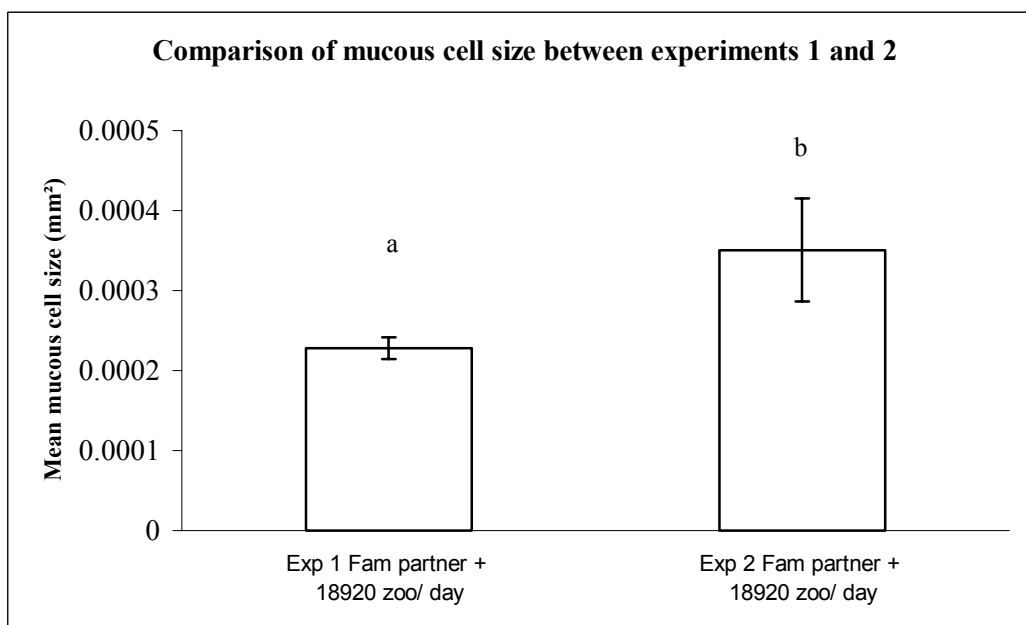


Figure 8.4: Mean (\pm SE) mucous cell size of fathead minnows in experiments 1 and 2. Statistical analyses were based on the following sample sizes: Experiment 1 Familiar partner +18920 zoospores/ day (n=30) and Experiment 2 Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.001$.

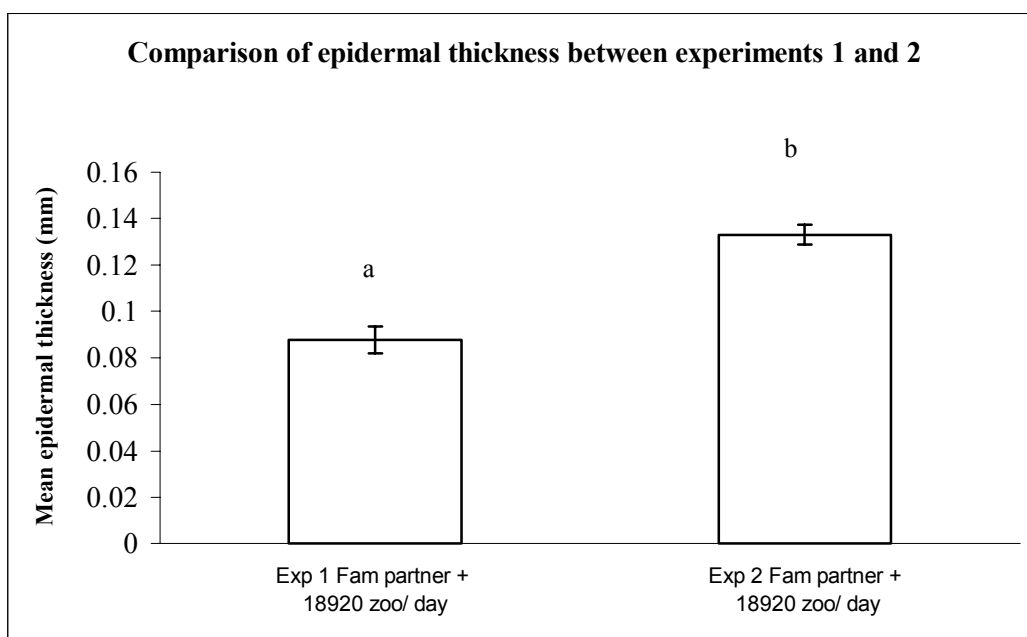


Figure 8.5: Mean (\pm SE) epidermal thickness of fathead minnows in experiments 1 and 2. Statistical analyses were based on the following sample sizes: Experiment 1 Familiar partner +18920 zoospores/ day (n=30) and Experiment 2 Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.001$.

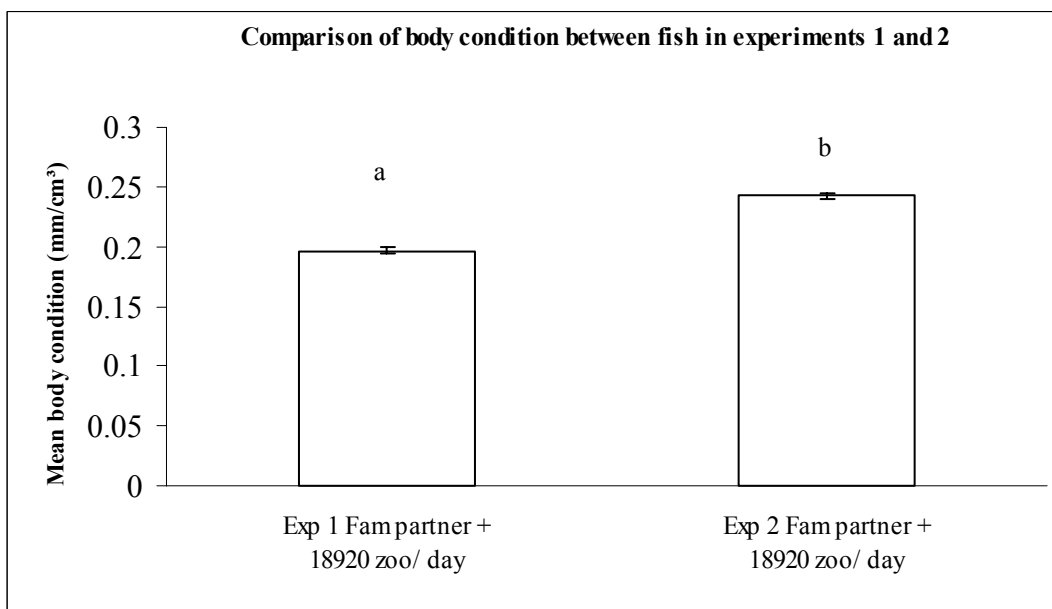


Figure 8.6: Median (\pm 25th and 75th quartiles) body condition of fathead minnows in experiments 1 and 2. Statistical analyses were based on the following sample sizes: Experiment 1 Familiar partner +18920 zoospores/ day (n=30) and Experiment 2 Familiar partner + 18920 zoospores/ day (n=38). Different letters denote significant differences at $P \leq 0.001$.

Chapter 4: Discussion

It was originally hypothesized that alarm cells evolved as specialized structures selected for their alarm-signaling role. Consequently, most experiments that use alarm cues have tested them in anti-predator contexts. However, recent studies by Hindman (2002) failed to find a correlation between predation pressure and alarm cell abundance. The purpose of my thesis was to test the hypothesis that parasites and pathogen influence alarm cell investment in fathead minnows. This study has important implications for alarm-signaling theory as my findings provide some insight into the current role of alarm cells, specifically that they appear to have an anti-parasitic/ anti-pathogenic function, which may inhibit pathogen growth.

4.1 Experiment one: The effects of different *Saprolegnia* concentrations on fathead minnow alarm cell investment

In experiment one I manipulated the concentration of pathogens that minnows were exposed to. I found that while minnows exposed to *Saprolegnia* had significantly more alarm cells than those individuals in the control treatment, there was no evidence of a threat-sensitive response. There are several explanations for this finding including the possibility that minnows may have a threshold level of pathogen/disease tolerance. Exposure beyond this threshold may activate minnows' innate defence mechanisms and once a minnow's defence system has been activated, they may be unable to moderate their response. An alternative explanation is that my study design did not robustly test whether minnows can elicit a threat sensitive response to different pathogen concentrations. Minnows exposed to the lowest *Saprolegnia* concentration (189 zoospores/ day for 11 days = exposure to a total of 2079 zoospores) may have assessed their exposure as a serious threat and responded accordingly. Fathead minnows use chemical cues to assess their risk of predation (for review see Chivers and Smith 1998); information gathered will influence decisions that minnows make, including

alarm cell investment (Lima and Dill 1990). To determine whether minnows can elicit a threat sensitive response to different pathogen concentrations, their perception of risk must be manipulated. I suggest a follow-up study in which both pathogen concentration and the number of days that the minnows are exposed to the pathogens are reduced. By reducing both the pathogen concentration and the duration of exposure to the pathogen, we may be able to manipulate the minnow's perceived threat, and consequently their response to that threat. It is possible that minnows exposed to 50 zoospores/ day for seven days may assess their risk differently than minnows exposed to 50 zoospores/ day for two days. These differences in risk assessment may lead minnows to respond differently.

Four other aspects of epidermal quality were quantified in this experiment; alarm cell size, mucous cell size, mucous cell investment and epidermal thickness. There were no differences in any of these factors between treatments. In addition there were no differences in body condition between treatment groups. From these results it is reasonable to conclude that exposure to pathogens appears to influence alarm cell investment in minnows, however further studies are needed to determine whether minnows can show threat-sensitive responses to pathogens.

4.2 Experiment two: The effects of familiarity and *Saprolegnia* on fathead minnow alarm cell investment

In experiment two, I manipulated the partner familiarity while exposing fish to the same concentration of *Saprolegnia* (18920 zoospores/ day). I found that partner familiarity had no effect on any of the epidermal variables considered in this study. This was an unexpected finding as Wisenden and Smith (1998) have previously demonstrated that partner familiarity influences alarm cell investment in fathead minnows from Pike Lake. In an attempt to understand these negative results, I conducted multiple post hoc comparisons between fish

experiencing the same treatments in experiments one and two. All the fish for experiments one, two, and three were acquired during the same collection attempt, thus they not only originated from the same population, but they were also from the same shoals. Fish were maintained under the same conditions in the laboratory including: being paired with a familiar partner and being exposed to the same pathogen concentration for the same duration of time. However, fish in experiment two had a longer acclimation period in the lab (5 months) compared to experiment one fish that were in the lab for only one month before being tested. In comparing the body conditions of the two groups of fish, I found minnows in experiment two had significantly higher body conditions than fish in experiment one. In addition, fish from experiment two also had higher alarm cell densities, higher mucous cell densities, larger mucous cells, thicker epidermis and larger alarm cells. Fish were fed *ad libitum* food rations while in the laboratory; consequently, minnows in experiment two received *ad lib* food for five months, which may account for their relatively high body condition. Typical investment in the epidermis would involve a trade-off in which individuals must ‘decide’ to allocate resources from one area such as growth to the repair and proliferation of the epidermis. It is possible that both groups of fish in experiment two had sufficient nutritional resources to enable them to maximally invest in their epidermal maintenance and quality. This could result in masking the effect of familiarity between the treatment groups. It is equally possible that larger fish may have more alarm cells in a given section of skin. Large fish may not need to direct substantial proportions of their energy to growth; consequently, they may ‘decide’ to allocate these resources to the maintenance of their epidermal layer.

Another possible explanation for the negative results of experiment two is temporal variation in alarm cell densities of fathead minnows. Individuals may alter their investment in epidermal components at different times of the year. These changes in epidermal maintenance

and investment may be adjusted to reflect local infection risk, food availability, or social dynamics. This hypothesis warrants further testing as it could have important implications for future experiments on alarm cell investment. In particular researchers should consider being consistent in the time of year they conduct their studies, and be mindful of how long they allow their fish to acclimate in the laboratory.

In addition to incorporating temporal variables and acclimation time in the laboratory, future studies should also consider replicating this experiment with a true control of distilled water. A control would have greatly enhanced the experimental design and would have allowed for stronger experimental conclusions.

4.3 Experiment three: The effects of zoospore conditioned water on fathead minnow alarm cell investment

Experiment three was a preliminary experiment in which I attempted to determine the mechanism that minnows use to assess their risk of pathogens. To test this, I exposed minnows to either conditioned water derived from 18920 zoospores/ day or a solution containing 18920 zoospores/ day. I found no difference in treatment on alarm cell investment, alarm cell size, mucous cell investment, mucous cell size, or epidermal thickness. However because of limitations of the study design I cannot state that minnows perceived the two treatments as equal pathogen threats. In order to determine whether the minnows assessed the risk of the zoospore conditioned water equal to the zoospore solution, a control treatment of distilled water is required. Incorporating a control in this study design would have allowed for stronger experimental conclusions. While I cannot make any meaningful biological conclusions based on this study, the proximate mechanism that fish use to access their pathogen threat remains an interesting question that warrants further investigation.

4.4 Experiment four: The effects of different levels of trematode cercariae on fathead minnow alarm cell investment

In experiment four, the number of trematodes that minnows were exposed to was manipulated. I found that exposure to trematodes had a significant effect on observed infection rate, and alarm cell investment. There was also an observed trend on the effect of trematodes on mucous cell investment. Trematode infection level did not influence alarm cell size, mucous cell size or epidermal thickness. The results of this experiment are consistent with those of experiment 1, indicating that alarm cell investment appears to be an important factor determining pathogen/ parasite defence in fathead minnows. The results of this experiment are also consistent with experiment 1 in that minnows did not show a threat-sensitive response to the parasites, rather minnows showed a threshold-type response. While this is an important study because it demonstrates that a skin-burrowing parasite can elicit changes in alarm cell investment in fathead minnows, the minnows in this study were only exposed to one trematode species. Follow-up studies testing the effects of other trematode species on alarm cell investment would verify my findings and lead to more powerful conclusions.

4.5 Experiment five: The effects skin extracts of fishes on *Saprolegnia* growth

In this experiment I exposed *S. ferax* cultures to various solutions including skin extracts of fishes. I found that *S. ferax* cultures exposed to [high] swordtail skin extract showed the greatest amount of growth, cultures exposed to either [low] swordtail skin extract, or distilled water showed an intermediate amount of growth, while cultures exposed to [high] or [low] fathead minnow skin showed the least amount of growth.

4.6 Experiment five: The effects of skin extracts of fishes and synthetic alarm cues on *Saprolegnia* growth

In this experiment, I exposed *S. ferax* cultures to both synthetic (H3NO) and natural alarm substances. I found that cultures exposed to the synthetic alarm cues grew equally well as the distilled water controls. In contrast, cultures exposed to fathead minnow skin extracts showed inhibited growth. These findings suggest that H3NO may not be the active component in fathead minnow skin as the synthetic solution had no effect of *S. ferax* growth.

The effects of fathead minnow skin extracts on pathogen growth were effectively tested twice, with consistent findings. While this means that my hypothesis was robustly tested, I emphasize caution when interpreting the results of this study. While I am confident that some component of fathead minnow skin inhibited *S. ferax* growth, I cannot conclude with any certainty that the factor influencing pathogen growth was the ostariophysan alarm substance. It is equally possible that the bacterial fauna on the minnow's epidermis out-competed *S. ferax* in the petridish and monopolized the food resources, inhibiting its growth. Moreover, the effects of skin extracts were tested against one pathogen species; further studies on other pathogens and bacterium are needed to verify my findings. In addition, isolating the alarm substance from alarm cells would also prove useful, as we would then be able to directly apply the substance to the pathogen cultures.

4.77 Significance of this study

One of the fundamental problems associated with alarm cell evolution is disentangling the multiple functions of alarm cells and assigning priority to one function (Smith 1997). The difficulty in determining the ancestral function of alarm cells has fueled a heated debate about the nature of alarm cell contents. Smith (1997) argued that there are three possible ancestral functions of alarm cells; he claims that alarm cells may have evolved as predator repellents, predator-attractants and/ or anti-pathogenic/anti-parasitic secretory cells. Similarly, Henderson et al. (1997) suggested that the only plausible explanation for a signaling system that relies on

damage to the sender is kin selection. However, there is currently no evidence that shoals are composed of related individuals. Moreover, if alarm cells evolved for their signaling role there should be a correlation between predation risk and alarm cell abundance. However, experiments that manipulated predation risk failed to find a correlation between alarm cell abundance and predation risk (Hindman 2002). While my study did not pinpoint the original selection agent responsible for the evolution of alarm cells, it has provided some insight into the current roles of alarm cells. Specifically my findings suggest that alarm cells appear to have anti-parasitic and anti-pathogenic properties, which may inhibit pathogen growth.

Preliminary studies by Chivers and Jack (2003 unpublished data) have shown that pathogens appear to influence alarm cells abundance. Infections with pathogens and parasites have been shown to affect several important components of animals' physiology (Jones 1998) and behaviour (Barber et al. 1998). Skin disease and infections in terrestrial vertebrates are commonly followed by recruitment of cells with anti-pathogenic properties such as mast cells (Jones 2001). Studies investigating the link between skin disease and mast cell number in humans have found that individuals with dermatitis such as eczema had higher number of mast cells than individuals without dermatitis (Freinkel and Woodley 2002). Mast cells are important components of innate immune responses; their main role is to signal to the immune system that the body is under attack (Malavita et al. 1999). Mast cells are found at the host-environment interface such as under the skin, in the gut, and lungs; their position enables mast cells to interact with dermal cells and allows them to rapidly respond to circulating foreign proteins (Malavita et al. 1999). In addition to alerting the immune system of a foreign agent, mast cells have also been shown to mitigate the effects of certain types of dermatitis (Jones 2001). Alarm cells may function in an analogous manner as mast cells in terrestrial

vertebrates. Alarm cells may work synergistically with the immune system by alerting it to foreign substances and helping to control the potential infection by using specific anti-fungal and antibacterial compounds. Like mast cells, alarm cells are found in the epithelium at the host-environment interface where they would be most effective at detecting and barring entry of pathogens and parasites. Another piece of evidence supporting alarm cells' defence roles comes from studies investigating the effect of testosterone on male fathead minnow alarm cells. Male fathead minnows lose their club cells when they enter breeding condition; this loss of alarm cells has been attributed to increased levels of testosterone in breeding males (Smith 1973). This finding is consistent with my hypothesis that alarm cells contribute to innate defence. Testosterone has also been shown to suppress the immune system in other species (Gonzalez et al. 2001), which may make individuals more susceptible to disease and infection (Folstad and Karter 1992).

In addition to having important implications for alarm signaling theory, this study may also have consequences for the aquaculture industry. Fathead minnows are an economically and ecologically important species; minnows are commonly used by anglers as baitfish and they are also important food sources for large fish predators. Studying fishes' innate defence mechanisms may be useful for fisheries management purposes (Jones 2001). Parasites inhabiting the skin and gills of fish are a common cause of mortality among hatchery-reared fish (Jones 2001). This is because hatchery conditions including stocking fish at higher than natural densities, are conducive to rapid expansion of parasite populations (Jones 2001). In addition, understanding a fish's natural immune defences is not only important because it may help reduce fish mortality in hatcheries, but understanding a fish's natural immunity may be important in conserving naturally occurring fish communities.

Chapter 5: Conclusions:

Six experiments were used to test the hypothesis that pathogens and parasites influence alarm cell investment in fathead minnows. From these experiments, three pieces of evidence support the role of alarm cells in innate epidermal defence: minnows increase their alarm cell investment in response to both pathogens and parasites, and some component found in fathead minnow skin appears to inhibit *S. ferax* growth. I consider the sum of these three pieces of evidence sufficient to conclude that alarm cells appear to play a role in epidermal defence against pathogens and parasites.

While my research only examined alarm cell densities, and alarm cell size, there are several other alarm cell components that should be considered in future studies including determining whether there are treatment differences in the number of alarm cells undergoing mitotic division, changes in cell death rate, or alternatively in cell differentiation. It is possible that in addition to there being differences in alarm cell densities, cells may also differ in their rate of mitotic division. My slides were photographed at 10 X, which precluded me from determining the mitotic state of the alarm cells in my study. In addition, it would be useful to investigate structural features of fathead minnow alarm cells and determine whether there is an increase in an increase in the production of alarm cell substance irregardless of alarm cell number. A similar study of channel catfish found alarm cells engulfing other cells, including microvilli, from surrounding epidermal cells, and intranuclear and intracytoplasmic virus particles (Chapman and Johnson, 1997). Lastly, it would be useful for future studies investigating the effects of fathead minnow skin extracts on bacterium, parasites and pathogens to isolate and determine the chemical composition of the alarm cell substance.

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Appendix

***Saprolegnia* zoospores cultivation:**

1. Cut 1mm x 1 mm cubes from *Saprolegnia* master culture.
2. Add cubes into (liquid) GY media (use GS flasks)
3. Put flasks on shaker @ 240 rpm for approx. 1 hour. Afterwards, reduce speed to 80 rpm and let *Saprolegnia* grow overnight.
4. Aspirate media surrounding the cultures. (The objective is to remove the nutrients from the media so that the zoospores would reproduce).
5. Refill the flask with dilute salt solution. (1 L deionized water, 1.0 mL DSB solution, 0.5 mL DSA solution). Should perform approx. four aspirations in the first hour, followed by 1 aspiration per hour for 3 hours. Always refill flask with dilute salt solution following aspiration.
6. Following the last aspiration cover the flasks and leave in 4° C fridge overnight.
7. Remove colonies with forceps, scrape bottom of flask with comb.
8. Pour solution into 50 mL centrifuge tubes
9. Centrifuge at $\frac{3}{4}$ speed for 6 min.
10. Check for pellet, if no pellet, centrifuge again at full speed for 10 minutes.
11. Aspirate off supernatant.
12. Take sample and check for cysts on slide
13. Quantify the number of cysts using haemocytometer.
14. Dilute stock solution with glass distilled dechlorinated water to achieve appropriate concentrations.
15. Transfer into histology containers and freeze in daily aliquots.

Protocol for making GY media

ATCC medium: 1994 GY agar
 Glucose.....10.0 g
 Yeast extract.....2.5 g
 Agar.....15.0 g (optional, for solid media)
 Distilled water.....1.0 L

The mixture was autoclaved at 121° C for 15 minutes, using the standard temperature/pressure setting. Following autoclaving the bottles were swirled to ensure that they were completely mixed. When the contents had cooled to approximately 45°C, the solution was poured the agar into sterile plastic petri dishes (approximately to about 4 mm per plate). The plates were allowed to cool and once the agar had solidified, they were inverted and stored until needed.

Dilute salts

To make the dilute salt solutions used in starving *Saprolegnia* colonies, 0.5 mL DSA, and 1 mL DSB were added to 1000 mL distilled dechlorinated water.

DSA Dilute Salt Solution A

KH₂PO₄ 136.09

K_2HPO_4 174.18 g/L
 $(\text{NH}_4)_2\text{HPO}_4$ 132.07 g/L

DSB Dilute Salt Solution B

$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 73.50 g/L
 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 101.66 g/L