Field Responses of Prey Fishes to Chemical Alarm Cues

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Abstract

A diversity of fishes release chemical cues upon being attacked by a predator. These cues, commonly termed alarm cues, act as sources of public information warning conspecifics of predation risk. Species which are members of the same prey guild often respond to one another's alarm cues. The purpose of this thesis was to discriminate avoidance responses of fishes to conspecific alarm cues and cues of other prey guild members from responses to unknown damaged fish odours and novel odours. I used a series of trap experiments and underwater video observations to measure avoidance responses of freshwater littoral fish species to chemical alarm cues.

In a series of 6 trap experiments I captured fathead minnows (*Pimephales promelas*) and brook stickleback (*Culaea inconstans*) in traps containing injured fish cues and novel non-fish odours. In addition to documenting the number of fish present, I also recorded length, weight, body condition, and gonadosomatic index. Despite the large sampling effort it was determined that the study had limited power to detect a 20 % difference in the means between treatments.

Avoidance was tested to both injured fish cues and novel non-fish odours in a camera experiment using fathead minnows, finescale dace (*Chrosomus neogaeus*), and brook stickleback. The cyprinids (minnows and dace) showed significant avoidance of minnow cues over swordtail cues, morpholine, and the control of distilled water and tended to avoid fathead cues over cues of known prey guild members (stickleback). Cyprinids also significantly avoided cues of stickleback over unknown heterospecific

cues (swordtail) and tended to avoid stickleback cues over morpholine and the distilled water control. Stickleback significantly avoided fathead minnow extract over the distilled water and tended to avoid stickleback and swordtail over distilled water. I conclude that fishes in their natural environment can show dramatic changes in behaviour upon exposure to alarm cues from conspecifics and prey guild members. These responses were not the result of avoidance of cues of any injured fish or any novel odour.

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Chapter 1: Introduction

1.1 Background Information

Predation is an important agent in the evolution of morphological, physiological, life history and behavioural characteristics of animals (Lima & Dill 1990, Kats & Dill 1998). Morphological adaptations include cryptic colouration, protective armour, and altered body shapes. Physiological adaptations include production of toxins and other chemical defences. Examples of life history adaptations include alterations in time of hatching, metamorphosis, and reproduction; while behavioural adaptations include escape/avoidance, use of certain habitats for feeding and reproduction, and changes in established behaviour patterns (Lima & Dill 1990, Lima 1998).

Since anti-predator responses are costly (Kats & Dill 1998), these responses should only occur when the organism perceives a threat of predation. In terrestrial environments these threats are often realized through alarm signals, specifically audible alarm or distress calls and the visually oriented alarm displays (Aubin 1991, Bshary & Noe 1997). Chemical cues are an efficient means to transfer information in aquatic environments (Chivers & Smith 1998). Within chemical cues there are two distinct signalling systems. There are cues released by prey that are disturbed, but not injured. These cues are termed disturbance signals. Even though disturbance cues do not involve mechanical damage, there is no requirement that they are intentionally released. Damage-released alarm cues, conversely, are cues given when the sender has been

captured by a predator. Therefore, a damage-released alarm cue may indicate a higher risk of predation than the disturbance cue (Chivers & Smith 1998).

Von Frisch (1938) was the first to describe the fright reactions of European minnows (*Phoxinus phoxinus*) to chemicals released from damaged minnow skin. Von Frisch (1938, 1941) coined the term Schreckstoff, which translated means 'fear substance', to describe the chemical responsible for eliciting the response. Since this initial observation, many studies have analysed alarm systems of various fishes (Smith 1992). To summarize the Schreckstoff alarm system, epidermal club cells contain an alarm cue, the functional group of which is thought to be hypoxanthine-3-(N)-oxide (Smith 1992, Brown *et al.* 2000). Once club cells have ruptured, as would occur during a predator attack, the alarm substance is released into the surrounding environment. The cue is detected by smell and receivers perform a "fright reaction" that may be species specific (Smith 1992, Chivers & Smith 1998). The Schreckstoff alarm system appears to be universal among members of the Superorder Ostariophysi, which contains approximately 7200 species. Analogous alarm systems may also occur in other groups of fishes, including stickleback, gobies, cyprinidontids, poeciliids, percids, cichlids, and salmonids (Smith 1992, Chivers & Smith 1998).

Most laboratory studies that examined responses of fishes to chemical alarm cues have shown the anti-predator responses arise strictly from conspecific cues, and are not generalized responses to potential cues of any injured fish or any novel odour (Chivers & Smith 1998). However, cross-species responses are also known to occur between groups (Mathis & Smith 1993, Wisenden *et al.* 1994, Chivers & Smith 1994, Wisenden

et al. 1995). Cross-species responses are explained in one of two ways. First, the individuals may be closely related phylogenetically, thus possessing a chemically similar alarm cue structure. In ostariophysan fishes, the anti-predator response to heterospecific alarm substance is thought to decrease as the fishes become more distantly related (Schütz 1956).

The second way in which species may utilize heterospecific alarm cues is if two species are members of the same prey guild (i.e. syntopic and share common predators) and have learned to recognize each others cues (Brown 2003). However, reaction intensity is often lower in response to heterospecific alarm cues than to those of conspecifics (Mirza & Chivers, 2001 b). This may indicate relative predation risk and help species in risk assessment (Chivers *et al.* 1995, Pollock *et al.* 2003). Information gained from heterospecifics may not be as reliable as information gathered from conspecifics causing a decrease in the intensity of a response.

Most studies that have shown responses to conspecific and heterospecific cues have been carried out in relatively simple environments in the laboratory. The animals are often fed to satiation and tested in water that is clear of pollution or other chemical cues (Chivers & Smith, 1998). Species are also tested in small monospecific groups and only after being held in the absence of predation events for considerable time periods (Chivers & Smith, 1998). It is for this reason that field studies are imperative to our understanding of risk assessment and anti-predator behaviour in a natural setting.

1.2 Objective

The ability to detect and respond to both damaged conspecifics and heterospecific members of a prey guild in the wild would greatly increase the animals' ability to survive (Mirza & Chivers 2001 a). In natural environments fishes must be able to detect these cues over a complex mosaic of odours. The objective of my thesis was to discriminate avoidance responses of fathead minnows (*Pimephales promelas*) and brook stickleback (*Culaea inconstans*) to conspecific alarm cues and cues of other prey guild members from the odour of an unknown damaged fish (swordtail, *Xiphophorus helleri*) and novel non-fish odours. Avoidance for the purposes of this thesis is defined as treatment differences in the number of fish caught in traps labelled with different cues (Exp.1), or a change in the number of fish observed in pre-stimulus period as compared to post-stimulus period (Exp. 2). In my study the addition of morpholine, a known odourant, was used to determine reactions to novel non-biological odours compared to responses to fish skin extract.

Chapter 2

Experiment Series One: Responses of prey fishes to alarm cues from conspecifics, known and unknown heterospecifics and novel odours as assessed using trap experiments.

2.1 Introduction

In 1992, Mathis and Smith devised a trap experiment technique in which the responses of freshwater littoral fishes to chemical alarm cues could be quantified. They attached cellulose sponges, containing fish alarm cue or a control of distilled water, to the inside of the traps and placed the traps in the water for about 4.5 hrs; after this time the traps were pulled and the number of fishes was quantified. They found that the fathead minnows exhibited a very significant avoidance of traps labelled with skin extract. There were 849 fish caught in 16 control traps, while only 27 fish or 4%, were caught in 16 experimental traps. Chivers and Smith (1994) conducted a similar experiment in which avoidance by stickleback to stickleback extract was measured against a control of distilled water. There were 487 stickleback caught in 13 control traps while 335 fish were caught in 13 experimental traps. While the results were significant (p=0.032), the avoidance was much less pronounced in stickleback than as demonstrated in minnows.

In order to test whether syntopic fishes exploit the alarm system of prey guild members, Mathis and Smith (1993) tested whether fathead minnow extract would induce avoidance in the brook stickleback when compared against distilled water. Indeed they

found that stickleback exploit fathead alarm cues and this reduced their own risk of predation (Mathis & Smith 1993, Wisenden *et al.* 1994). In a similar trap experiment, Wisenden *et al.* (1994) demonstrated that stickleback avoided areas marked with fathead minnow extract over distilled water and continued to avoid the area for 2-4 hours before returning to these apparently risky habitats. In a follow-up experiment, Wisenden *et al.* (1995) determined that fishes that were not present at a particular location during the initial release of alarm cue were the first to migrate in the risky area while fish present at the time of the cue release only returned 7 to 8 hours later. These early trap experiments seem to provide field support for laboratory findings concerning cross-species responses between fathead minnows and brook stickleback.

Trap experiments have also been used to determine the chemical structure of the Ostariophysan alarm cue. Brown *et al.* (2000) used traps labelled with fathead minnow extract, hypoxanthine-3-N-oxide, or pyridine-N-oxide to determine which functional group was responsible for the behavioural response. It was demonstrated that the three experimental treatments caught significantly fewer fish than the control of distilled water suggesting that the nitrogen oxide functional group may elicit behavioural responses.

The trap experiments reviewed thus far have demonstrated that brook stickleback avoid fathead minnow or stickleback skin extract over distilled water (Wisenden *et al.* 1994, Chivers & Smith 1994, Wisenden *et al.* 1995, Mathis & Smith, 1992, 1993). It was also demonstrated that fathead minnows avoid stickleback and minnow cues over distilled water (Wisenden *et al.* 1995, Mathis & Smith 1992, Brown *et al.* 2000). These studies support laboratory findings; however trap experiments published within the last

decade used distilled water as the only control. It is difficult to draw any conclusions as to the specificity of the response in these species as they compared avoidance of an odour to an odourless control.

Recently, several studies have included an unknown heterospecific skin extract (usually swordtail) as an additional control to ensure that avoidance by fishes was due to specific cues, and not a generalized response to any fish extract (Tremaine *et al.* 2005). Populations of fathead minnows and stickleback are known to respond to alarm cues in the laboratory (Pollock *et al.* 2003, Chivers & Smith 1995). However, in recent field experiments there have been contradictory results (Tremaine *et al.* 2005, Pollock *et al.* unpublished). Minnows more often than not showed no preferential avoidance between their own cues and the cues of swordtails, an unknown heterospecific. In one experiment, contrary to prediction, minnows avoided swordtail cues over minnow cues, and avoided swordtail cues over stickleback cues. Similarly, sticklebacks more often than not showed no differential avoidance of cues from conspecifics and unknown heterospecifics (swordtails). Again, one experiment was contradictory to predictions with stickleback avoiding swordtail cues over conspecific cues.

These contradictory results (Tremaine *et al.* 2005, Pollock *et al.* unpublished) may be explained in several ways. Given the complex nature of the test environment (i.e. competing chemical cues from mates and competitors) the experimental stimulus must be of high enough concentration to overcome the complex chemical background. In the natural environment, the degree of response may depend on various factors including reproductive state, and age of test fish, presence of other fish, and physical and

biological properties of the environment (Tremaine *et al.* 2005). In fact, Wisenden *et al.* (2003) showed that the presence of a shoal increased the number of fish caught in traps labelled with alarm cues.

The purpose of this experiment was to discriminate avoidance responses of fishes to conspecific alarm cues and cues of prey guild members from responses to unknown damaged fish odours and novel odours.

2.2 Methods

This series of experiments which tested responses of fathead minnows and brook stickleback was carried out during the summer of 2003. Field sites included Feedlot pond (0.5 ha pond located on the University of Saskatchewan campus), and Lakeview pond (1.5 ha pond located in Saskatoon, Saskatchewan). Three experiments were carried out at each pond over the field season. Dates at Feedlot pond experiment were May 28-June 1 (Feedlot 1), July 7-July 11 (Feedlot 2), and September 22- September 26 (Feedlot 3) while dates at Lakeview pond include June 9- June 13 (Lakeview 1), September 2- September 6 (Lakeview 2), and October 6- October 10 (Lakeview 3) respectively. Each experiment consisted of five treatments: fathead minnow skin extract, brook stickleback skin extract, swordtail skin extract, morpholine and distilled water.

Morpholine, classified as a lower aliphatic secondary amine, was chosen for the novel stimulus because zebrafish (*Danio rerio*) have been conditioned using this

chemical in the laboratory (Suboski *et al*, 1990) and coho salmon (*Oncorhynchus kisutch*) likewise showed responses in the field (Hasler & Scholz, 1978). The use of morpholine was questioned by Hara and Brown (1979), who suggested that morpholine, is a non-specific irritant of the olfactory epithelium and that behavioural responses to morpholine involve non-olfactory chemoreceptor system such as taste or general chemical sense. However, in a study by Hirsch (1977) fish with plugged nostrils could not be conditioned to morpholine, suggesting strictly olfaction.

Stimulus Preparation:

Skin extract for each of the damaged fish treatments was created in similar fashion. Stimulus fish used in this experimental series were collected from the respective ponds, ensuring that Lakeview stimulus was used in Lakeview experiments and Feedlot stimulus was used in Feedlot experiments. This was done to control for possible population differences in alarm cue investment. Donor fish were killed with a blow to the head (in accordance with Animal Care Protocol guidelines) and fillets were removed from both sides of the fish ensuring all muscle was removed from the skin. The fillets of skin were then placed in enough distilled water to produce a concentration of $1cm^2$ of skin per 10 ml of distilled water. The solution was then homogenized with a Polytron© homogenizer and the homogenate was filtered through glass wool. Table 1 provides a summary of the standard length (the distance from the nostril tip to the posterior end of the caudal penduncle) and number of fishes used to produce the skin

Table 1: Number and standard lengths ± the standard deviation for donor fish used for experiment series 1.

Species	Experiment	Number of fish	Standard length
fathead minnow	Feedlot 1	5	4.72 ± 0.42
	Feedlot 2	4	5.07 ± 0.22
	Feedlot 3	3	4.99 ± 0.37
	Lakeview 1	4	5.10 ± 0.62
	Lakeview 2	5	4.50 ± 0.41
	Lakeview 3	3	5.43 ± 0.06
stickleback	Feedlot 1	7	4.50 ± 0.15
	Feedlot 2	6	4.32 ± 0.25
	Feedlot 3	3	5.27 ± 0.37
	Lakeview 1	7	4.47 ± 0.65
	Lakeview 2	5	5.20 ± 0.58
	Lakeview 3	4	5.43 ± 0.56
swordtail	Feedlot 1	6	3.62 ± 0.37
	Feedlot 2	3	4.56 ± 0.06
	Feedlot 3	3	2.93 ± 0.35
	Lakeview 1	5	4.02 ± 0.28
	Lakeview 2	3	4.10 ± 0.20
	Lakeview 3	6	3.35 ± 0.26

extracts. The morpholine solution was prepared by adding 0.07 ml of pure morpholine (99%) to 1 litre of glass-distilled water. This concentration was derived from a previous field experiment (Hasler & Scholz, 1978) and a laboratory study (Suboski *et al*, 1990). The distilled water treatment consisted of glass-distilled water. Solutions for each of the treatments were then frozen in 60 ml syringes until needed.

Experimental Protocol:

Each experiment required six days. I caught fish with Gee's Improved Minnow traps, which are roughly cylindrical wire enclosures (43 cm length x 22 cm diameter) with a funnel located at each end leading to a small opening into the trap. Each of the five treatments was assigned randomly around the pond, with the condition that no more than two traps of the same treatment could be in a row. Traps were placed ~10m from the next trap and about 3 m from the shore. Every other day the traps were shifted 5m to the left along the shore to ensure that the same place was not tested twice within 48 hours. Traps were equipped with two sponges (2 cm^3) about 2 cm from each opening using a safety pin. Sponges were injected with thawed stimulus and placed into the pond at two minute and thirty second intervals. Traps were collected after two hours and thirty minutes in the same order in which they were set. Any fishes caught in a trap were euthanized using Ethyl-m-aminobenzoate methanesulfonate salt and stored in appropriately labelled bags of 95% ethyl alcohol.

In the laboratory all fish were counted. If the trap contained more than ten fish of each species, the weight, standard length, and gonad weight of ten randomly chosen

fish from of each species from each trap was measured. If the trap contained fewer than ten fish of a species, all fish present were measured for these variables. The level of replication with respect to this study is the trap. Sample sizes are shown in tables 2-7 for all experiments. From this data it is possible to attain information about the different factors influencing avoidance behaviours.

First, avoidance was determined between the treatments using the number of fish caught in each treatment. Second, the length measurements allow conclusions to be made about experience and avoidance, larger fish are typically older and have more life experience. Third, the length and weight was used to determine the body condition (weight/length^3) of the fish of each species from each trap. This information can be used to draw conclusions about the health of the fish and the risks both low and high condition fish will take. Finally, the gonadosomatic index (gonad weight/ body weight as expressed as a percent) was calculated to determine whether reproductive state affects avoidance behaviours, in particular whether reproductive or non-reproductive fish are more or less prone to participate in avoidance behaviours.

2.3 Statistical Analysis

Initial analyses consisted of paired comparisons made between treatments for each experiment independently. Kolomogorov-Smirnov tests were done for each variable. If data met parametric assumptions I used a series of T-tests to compare between the treatments. If data failed to meet parametric assumptions I used a series of

Mann-Whitney tests to compare between treatment pairs. The family-wise error rate was assessed and controlled using the modified Bonferroni test following Keppel (1982). The modified Bonferroni test specifies that corrections to the family-wise error rate be introduced only when the number of comparisons exceeds $k - 1$, where k is the number of treatments (Keppel 1982). In this experiment, there were a total of five treatments. Since the analysis was restricted to 9 pre-planned (see tables 2-7) comparisons that were based on specific a priori comparisons, the rejection probability (P) was set at, alpha of 0.05 (k-1)/comparisons = 0.022 for each comparison (Keppel, 1982).

Power analysis for each comparison was done using Power and Precision ™ computer program from Biostat, Inc. Power was determined for each comparison using an effect size of 20%, representing an ability to detect a 20 % difference between the two means, alpha was set to 0.022 and sample size varied for each comparison.

Since all six studies shared identical protocols Combined P-values were calculated where the conditions were met, using the formula $-2\sum \ln P$. This X^2 value was then compared to the X^2 critical value for alpha 0.05 with degrees of freedom defined as two degrees of freedom for every experiment included. The Combined P-value offers the probability that all six similar comparisons are found in the same area of the standard normal curve. This method is employed when the probabilities may be low enough to be suggestive, while not yielding statistically significant results.

2.4 Results

2.4.1 Feedlot pond 1-3

Avoidance

Fathead minnows showed no significant avoidance of any one treatment over another in all three Feedlot pond experiments (Tables 2-4). Stickleback showed no significant results in the Feedlot 1 and 2, however in Feedlot 3 (Table 4), stickleback showed a significant avoidance of the fathead minnow extract treatment compared to the distilled water control (Mann-Whitney, Z= -2.9, p=0.003). Power was calculated for each comparison and yielded limited power to detect a 20% difference (Tables 2-4). These power values suggest that the experiment requires an increased sample size or reduced comparisons in order to gain the power necessary to detect a 20 point difference between any two means.

Length

There were no significant results found for stickleback concerning the length between treatments in all three Feedlot pond experiments (Tables 2-4). Fathead minnows showed no significant results in Feedlot 1 and 2, however there was significant result detected in the length of fish caught between the fathead minnow extract treatment and the distilled water control (T-test, Z= -2.8, p=0.005). Power for all three experiments is reported in Tables 2-4.

Table 2: P-values and (power to detect 20% difference between means) for fathead minnows and stickleback in response to stickleback (SB), fathead minnow (FHM), swordtail (SWT) skin extracts, morpholine (M), and distilled water (DW) for fish in Feedlot 1. (Sample size for avoidance: SB=18, FHM=18, SWT=18, M=18, DW=18. Sample size for FHM length, weight, body condition, gonadosomatic index: SB=11, FHM=13, SWT=12, M=16, DW=12. Sample size for SB length, weight, body condition, gonadosomatic index: SB=17, FHM=17, SWT=17, M=18, DW=18. [a]=nonparametric, [b]=parametric, see text for details).

Species	Treatment Comparisons	Avoidance[a]	Length (mm)[b]	Weight (g)[b]	Body Condition[b]	GSI[a]
FHM	SB - FHM	0.649 (5%)	0.541 (4%)	0.427 (4%)	0.664 (4%)	0.543 (4%)
	SB - SWT	0.449 (5%)	0.541 (4%)	0.635 (4%)	0.717 (4%)	0.157 (4%)
	SB - M	0.680 (5%)	0.372 (4%)	0.094 (4%)	0.109 (4%)	0.374 (4%)
	SB - DW	0.164 (5%)	0.392 (4%)	0.859 (4%)	0.238 (4%)	0.498 (4%)
	FHM - SWT	0.728 (5%)	0.341 (4%)	0.277 (4%)	0.336 (4%)	0.415 (4%)
	FHM - M	0.329 (5%)	0.844 (4%)	0.507 (4%)	0.138 (4%)	0.539 (4%)
	FHM - DW	0.446 (5%)	0.245 (4%)	0.497 (4%)	0.303 (4%)	1.000 (4%)
	SWT - M	0.223 (5%)	0.216 (4%)	0.062 (4%)	0.024 (4%)	0.781 (4%)
	SWT - DW	0.671 (5%)	0.98 (4%)	0.538 (4%)	0.091 (4%)	0.817 (4%)
SB	Treatment Comparisons	Avoidance[a]	Length (mm)[b]	Weight (g)[a]	Body Condition[a]	GSI[a]
	SB - FHM	0.520 (5%)	0.588 (4%)	0.963 (4%)	0.135 (4%)	0.607 (4%)
	SB - SWT	0.039 (5%)	0.457 (4%)	0.614 (4%)	0.875 (4%)	0.407 (4%)
	SB - M	0.868 (5%)	0.506 (5%)	0.815 (5%)	0.439 (5%)	0.708 (5%)
	SB - DW	0.584 (5%)	0.825 (5%)	0.663 (5%)	0.853 (5%)	0.318 (5%)
	FHM - SWT	0.129 (5%)	0.812 (4%)	0.577 (4%)	0.056 (4%)	0.184 (4%)
	FHM - M	0.493 (5%)	0.704 (5%)	0.852 (5%)	0.651 (5%)	0.988 (5%)
	FHM - DW	0.952 (5%)	0.635 (5%)	0.699 (5%)	0.180 (5%)	0.086 (5%)
	SWT - M	0.072 (5%)	0.527 (5%)	0.428 (5%)	0.330 (5%)	0.276 (5%)
	SWT - DW	0.140 (5%)	0.635 (5%)	0.286 (5%)	0.711 (5%)	0.978 (5%)

Table 3: P-values and (power to detect 20% difference between means) for fathead minnows and stickleback in response to stickleback (SB), fathead minnow (FHM), swordtail (SWT) skin extracts, morpholine (M), and distilled water (DW) for Feedlot 2. (Sample size for avoidance: SB=18, FHM=18, SWT=18, M=18, DW=18. Sample size for FHM length, weight, body condition, gonadosomatic index: SB=11, FHM=7, SWT=10, M=11, DW=10. NA= Not Available. [a]=nonparametric, [b]=parametric, see text for details).

Species	Treatment Comparisons	Avoidance[a]	Length (mm)[b]	Weight (g)[b]	Body Condition[b]	GSI[a]
FHM	SB - FHM	0.097 (5%)	0.801 (3%)	0.842 (3%)	0.479 (3%)	0.821 (3%)
	SB - SWT	0.650 (5%)	0.910 (3%)	0.630 (3%)	0.577 (3%)	0.778 (3%)
	SB - M	0.673 (5%)	0.587 (4%)	0.932 (4%)	0.857 (4%)	0.909 (4%)
	SB - DW	0.628 (5%)	0.463 (4%)	0.356 (4%)	0.324 (4%)	0.888 (4%)
	FHM - SWT	0.152 (5%)	0.789 (3%)	0.630 (3%)	0.122 (3%)	0.922 (3%)
	FHM - M	0.097 (5%)	0.819 (3%)	0.944 (3%)	0.692 (3%)	0.427 (3%)
	FHM - DW	0.152 (5%)	0.858 (3%)	0.720 (3%)	0.844 (3%)	0.845 (3%)
	SWT - M	0.913 (5%)	0.593 (4%)	0.706 (4%)	0.522 (4%)	0.683 (4%)
	SWT - DW	0.864 (5%)	0.545 (4%)	0.232 (4%)	0.055 (4%)	0.65 (4%)
SB	Treatment Comparisons	Avoidance	Length (mm)	Weight (g)	Body Condition	GSI
	SB - FHM	NA	NA	NA	NA	NA
	SB - SWT	NA	NA	NA	NA	NA
	SB - M	NA	NA	NA	NA	NA
	SB - DW	NA	NA	NA	NA	NA
	FHM - SWT	NA	NA	NA	NA	NA
	FHM - M	NA	NA	NA	NA	NA
	FHM - DW	NA	NA	NA	NA	NA
	SWT - M	NA	NA	NA	NA	NA
	SWT - DW	NA	NA	NA	NA	NA

Table 4: P-values and (power to detect 20% difference between the means) for fathead minnows and stickleback in response to stickleback (SB), fathead minnow (FHM), swordtail (SWT) skin extracts, morpholine (M), and distilled water (DW) for Feedlot 3. (Sample size for avoidance: SB=17, FHM=18, SWT=16, M=18, DW=18. Sample size for FHM length, weight, body condition, gonadosomatic index: SB=11, FHM=13, SWT=12, M=16, DW=12. Sample size for SB length, weight, body condition, gonadosomatic index: SB=14, FHM=13, SWT=14, M=15, DW=16. [a]=nonparametric, [b]=parametric, see test for details).

Species	Treatment Comparisons	Avoidance[a]	Length (mm)[a]	Weight (g)[b]	Body Condition[b]	GSI
FHM	SB - FHM	0.938 (5%)	0.500 (3%)	0.857 (3%)	0.119 (3%)	0.687 (3%)
	SB - SWT	0.235 (5%)	0.404 (3%)	0.349 (3%)	0.156 (3%)	0.168 (3%)
	SB - M	0.479 (5%)	0.397 (4%)	0.255 (4%)	0.050 (4%)	0.645 (4%)
	SB - DW	0.437 (5%)	0.128 (4%)	0.162 (4%)	0.075 (4%)	0.434 (4%)
	FHM - SWT	0.408 (5%)	0.064 (3%)	0.123 (3%)	0.415 (3%)	0.126 (3%)
	FHM - M	0.643 (5%)	0.075 (3%)	0.108 (3%)	0.861 (3%)	0.941 (3%)
	FHM - DW	0.536 (5%)	**0.005**	0.034 (3%)	0.764 (3%)	0.343 (3%)
	SWT - M	0.556 (4%)	0.815 (4%)	0.912 (4%)	0.518 (4%)	0.047 (4%)
	SWT - DW	0.554 (4%)	0.477 (4%)	0.657 (4%)	0.671 (4%)	0.746 (4%)
SB	Treatment Comparisons	Avoidance[a]	Length (mm)[b]	Weight (g)[b]	Body Condition[b]	GSI[b]
	SB - FHM	0.205 (5%)	0.390 (4%)	0.543 (4%)	0.836 (4%)	0.372 (4%)
	SB - SWT	0.328 (5%)	0.320 (4%)	0.467 (4%)	0.928 (4%)	0.532 (4%)
	SB - M	0.631 (5%)	0.199 (4%)	0.628 (4%)	0.466 (4%)	0.188 (4%)
	SB - DW	0.069 (5%)	0.210 (4%)	0.112 (4%)	0.208 (4%)	0.748 (4%)
	FHM - SWT	0.754 (5%)	0.965 (4%)	0.907 (4%)	0.762 (4%)	0.267 (4%)
	FHM - M	0.091 (5%)	0.962 (4%)	0.876 (4%)	0.574 (4%)	0.680 (4%)
	FHM - DW	**0.003**	0.152 (4%)	0.082 (4%)	0.274 (4%)	0.205 (4%)
	SWT - M	0.231 (4%)	0.916 (4%)	0.777 (4%)	0.417 (4%)	0.178 (4%)
	SWT - DW	0.010 (4%)	0.107 (4%)	0.066 (4%)	0.177 (4%)	0.633 (4%)

Weight

No significant results were demonstrated in all three experiments for either species but Power to detect a 20% difference was minimal (Tables 2-4).

Body Condition

Neither species in all three Feedlot experiments demonstrated significant differences in body condition between the treatments (Tables 2-4). The body condition was calculated using the formula Weight/Length^3. The associated power values are also reported in Tables 2-4.

Gonadosomatic index

The gonadosomatic index calculated by the gonad weight/ body weight x 100; the gonadosomatic was used to determine the reproductive state of the fish. No significant differences were detected between treatments in either species through all three Feedlot experiments; however the power to detect a difference was minimal (Tables 2-4).

2.4.2 Lakeview 1-3

Avoidance

Stickleback did not demonstrate significant avoidance of any one treatment over another in all three Lakeview pond experiments (Tables 5-7). Fathead minnows did not

show significant avoidance in Lakeview 2 and 3. In Lakeview 1 fathead minnows avoided fathead minnow extract over morpholine (Mann-Whitney, Z= -2.7, p=0.006). Power analysis for each comparison yielded limited power to detect a 20% difference (Tables 5-7).

Length

There were no significant results found for fathead minnows concerning the length between treatments in Lakeview 2 and 3 (Tables 5-7). However in Lakeview 1 stickleback were found to be shorter in the stickleback skin extract treatment compared to the distilled water control (Mann-Whitney, Z= -3.2, p=0.002), in the fathead minnow extract treatment when compared to the distilled water control (Mann-Whitney, Z= -2.5, p=0.013) and swordtail skin extract when compared to the distilled water control (Mann-Whitney, Z= -2.8, p=0.006). Power for all three experiments is reported in Tables 5-7.

Weight

Fathead minnows in all three Lakeview experiments failed to demonstrated significant results. Stickleback from Lakeview pond 1 were significantly lighter in the stickleback skin extract treatment compared to the distilled water control (T-test, t= -2.8, p=0.007) and in the fathead minnow skin extract treatment when compared to control of distilled water. (T-test, t= -2.7, p=0.010). Power to detect a 20% difference was minimal (Table 5-7).

Table 5: P-values and (power to detect 20% difference between means) for fathead minnows and stickleback in response to stickleback (SB), fathead minnow (FHM), swordtail (SWT) skin extracts, morpholine (M), and distilled water (DW) for Lakeview 1. (Sample size for avoidance: SB=24, FHM=24, SWT=24, M=24, DW=24. Sample size for FHM length, weight, body condition, gonadosomatic index: SB=22, FHM=18, SWT=22, M=23, DW=22. Sample size for SB length, weight, body condition, gonadosomatic index: SB=23, FHM=24, SWT=24, M=24, DW=24. [a]=nonparametric, [b]=parametric, see text for details).

Species	Treatment Comparisons	Avoidance[a]	Length (mm)[a]	Weight (g)[a]	Body Condition[a]	GSI[a]
FHM	SB - FHM	0.118 (5%)	0.148 (5%)	0.256 (5%)	0.183 (5%)	0.15 (5%)
	SB - SWT	0.396 (5%)	0.741 (5%)	0.853 (5%)	0.023 (5%)	0.76 (5%)
	SB - M	0.157 (5%)	1.000 (5%)	0.837 (5%)	0.364 (5%)	0.238 (5%)
	SB - DW	0.501 (5%)	0.332 (5%)	0.483 (5%)	0.324 (5%)	0.453 (5%)
	FHM - SWT	0.031 (5%)	0.166 (5%)	0.179 (5%)	0.415 (5%)	0.041 (5%)
	FHM - M	**0.006**	0.103 (5%)	0.208 (5%)	0.033 (5%)	0.713 (5%)
	FHM - DW	0.05 (5%)	0.363 (5%)	0.476 (5%)	0.605 (5%)	0.328 (5%)
	SWT - M	0.605 (5%)	0.621 (5%)	0.665 (5%)	**0.001**	0.146 (5%)
	SWT - DW	0.852 (5%)	0.536 (5%)	0.343 (5%)	0.205 (5%)	0.398 (5%)
SB	Treatment Comparisons	Avoidance[b]	Length (mm)[a]	Weight (g)[b]	Body Condition[b]	GSI[b]
	SB - FHM	0.589 (5%)	0.386 (5%)	0.496 (5%)	0.137 (5%)	0.404 (5%)
	SB - SWT	0.924 (5%)	0.303 (5%)	0.079 (5%)	0.173 (5%)	0.587 (5%)
	SB - M	0.435 (5%)	0.058 (5%)	0.063 (5%)	0.606 (5%)	0.365 (5%)
	SB - DW	0.992 (5%)	**0.002**	**0.007**	0.788 (5%)	0.205 (5%)
	FHM - SWT	0.414 (5%)	0.902 (5%)	0.172 (5%)	**0.006**	0.724 (5%)
	FHM - M	0.142 (5%)	0.353 (5%)	0.139 (5%)	0.292 (5%)	0.936 (5%)
	FHM - DW	0.442 (5%)	**0.013**	**0.010**	0.154 (5%)	0.669 (5%)
	SWT - M	0.390 (5%)	0.364 (5%)	0.773 (5%)	0.054 (5%)	0.663 (5%)
	SWT - DW	0.872 (5%)	**0.006**	0.099 (5%)	0.067 (5%)	0.406 (5%)

Table 6: P-values and (power to detect 20% difference between means) for fathead minnows and stickleback in response to stickleback (SB), fathead minnow (FHM), swordtail (SWT) skin extracts, morpholine (M), and distilled water (DW) for Lakeview 2. (Sample size for avoidance: SB=30, FHM=29, SWT=30, M=29, DW=28. Sample size for SB length, weight, body condition, gonadosomatic index: SB=26, FHM=25, SWT=24, M=25, DW=25. NA= Not Available. [a]=nonparametric, [b]=parametric, see text for details).

Species	Treatment Comparisons	Avoidance	Length (mm)	Weight (g)	Body Condition	GSI
FHM	SB - FHM	NA	NA	NA	NA	NA
	SB - SWT	NA	NA	NA	NA	NA
	SB - M	NA	NA	NA	NA	NA
	SB - DW	NA	NA	NA	NA	NA
	FHM - SWT	NA	NA	NA	NA	NA
	FHM - M	NA	NA	NA	NA	NA
	FHM - DW	NA	NA	NA	NA	NA
	SWT - M	NA	NA	NA	NA	NA
	SWT - DW	NA	NA	NA	NA	NA
SB	Treatment Comparisons	Avoidance[a]	Length (mm)[a]	Weight (g)[b]	Body Condition[b]	GSI[b]
	SB - FHM	0.668 (6%)	0.559 (6%)	0.280 (6%)	0.072 (6%)	0.874 (6%)
	SB - SWT	0.165 (6%)	0.683 (6%)	0.560 (6%)	0.030 (6%)	0.686 (6%)
	SB - M	0.733 (6%)	0.638 (6%)	0.850 (6%)	0.069 (6%)	0.515 (6%)
	SB - DW	0.379 (6%)	0.910 (6%)	0.959 (6%)	0.253 (6%)	0.695 (6%)
	FHM - SWT	0.258 (6%)	0.222 (6%)	0.559 (6%)	0.418 (6%)	0.579 (6%)
	FHM - M	0.392 (6%)	0.204 (6%)	0.344 (6%)	0.942 (6%)	0.645 (6%)
	FHM - DW	0.216 (6%)	0.516 (6%)	0.249 (6%)	0.198 (6%)	0.846 (6%)
	SWT - M	0.070 (6%)	0.968 (6%)	0.678 (6%)	0.478 (6%)	0.260 (6%)
	SWT - DW	0.024 (6%)	0.617 (6%)	0.539 (6%)	0.036 (6%)	0.361 (6%)

Table 7: P-values and (power to detect 20% difference between means) for fathead minnows and stickleback in response to stickleback (SB), fathead minnow (FHM), swordtail (SWT) skin extracts, morpholine (M), and distilled water (DW) for Lakeview 3. (Sample size for avoidance: SB=24, FHM=30, SWT=29, M=29, DW=29. Sample size for SB length, weight, body condition, gonadosomatic index: SB=23, FHM=27, SWT=27, M=24, DW=28. NA=Not Available. [a]=nonparametric, [b]=parametric, see text for details).

Species	Treatment Comparisons	Avoidance	Length (mm)	Weight (g)	Body Condition	GSI
FHM	SB - FHM	NA	NA	NA	NA	NA
	SB - SWT	NA	NA	NA	NA	NA
	SB - M	NA	NA	NA	NA	NA
	SB - DW	NA	NA	NA	NA	NA
	FHM - SWT	NA	NA	NA	NA	NA
	FHM - M	NA	NA	NA	NA	NA
	FHM - DW	NA	NA	NA	NA	NA
	SWT - M	NA	NA	NA	NA	NA
	SWT - DW	NA	NA	NA	NA	NA
SB	Treatment Comparisons	Avoidance[a]	Length (mm)[a]	Weight (g)[a]	Body Condition[b]	GSI[b]
	SB - FHM	0.828 (6%)	0.096 (6%)	0.263 (6%)	0.963 (6%)	0.835 (6%)
	SB - SWT	0.851 (6%)	0.28 (6%)	0.477 (6%)	0.655 (6%)	0.565 (6%)
	SB - M	0.236 (6%)	0.932 (6%)	0.949 (6%)	0.737 (6%)	0.717 (6%)
	SB - DW	0.648 (6%)	0.222 (6%)	0.272 (6%)	0.419 (6%)	0.895 (6%)
	FHM - SWT	0.952 (6%)	0.562 (6%)	0.924 (6%)	0.769 (6%)	0.628 (6%)
	FHM - M	0.301 (6%)	0.131 (6%)	0.18 (6%)	0.846 (6%)	0.512 (6%)
	FHM - DW	0.649 (6%)	0.946 (6%)	0.272 (6%)	0.567 (6%)	0.706 (6%)
	SWT - M	0.193 (6%)	0.439 (6%)	0.417 (6%)	0.885 (6%)	0.343 (6%)
	SWT - DW	0.663 (6%)	0.84 (6%)	0.699 (6%)	0.699 (6%)	0.457 (6%)

Body Condition

No significant differences in body condition between the treatments were found in Lakeview 2 and 3 for either species (Tables 6-7). In Lakeview 1, fathead minnows in the swordtail skin extract treatment demonstrated a lower body condition than fatheads in the morpholine treatment (Mann-Whitney, $Z= -3.2$, $p=0.001$). Stickleback in the fathead minnow skin extract treatment demonstrated a lower body condition relative to the swordtail skin extract treatment (T-test, $t= -2.9$, $p=0.006$). The associated power values are also reported in Tables 5-7.

Gonadosomatic index

No significant differences were detected between treatments in either species through all three Lakeview experiments; however the power to detect a difference was minimal (Tables 5-7).

2.4.3 Combined Probabilities

Fathead Minnows

One comparison met all requirements for a combined P-value within the minnow response. Within avoidance in all six experiments fathead minnows were shown to significantly avoid minnow skin extract over morpholine (Table 8, Combined P, $df=8$, $X^2=18.0$, $p<0.025$).

Stickleback

There were five combined P-values among the stickleback which are worth noting (Table 8). A significant result was yielded in avoidance of swordtail skin extract over morpholine (Combined P, df =10, X^2=18.6, $p<0.05$). Shorter stickleback were found in the stickleback skin extract treatment when compared to the distilled water treatment (Combined P, df =10, X^2=19.1, $p<0.05$). A noteworthy trend in avoidance was also detected, stickleback demonstrate a trend in avoiding fathead minnow extract over the control of distilled water (Combined P, df =10, X^2=17.2, $0.05<p<0.10$). A trend within the length combined P-values was displayed between the swordtail skin extract and the control of distilled water (Combined P, df =10, X^2=16.9, $0.05<p<0.10$) with shorter fish being caught in the swordtail skin extract treatment. Only a trend was noted concerning the weight of the fish within the stickleback species. Stickleback in the fathead minnow extract treatment were lighter than the distilled water control (Combined P, df =10, X^2=17.8, $0.05<p<0.10$).

Table 8: Combined P-values for fathead minnows and stickleback from relevant studies. Table shows the comparison, associated test and P-value. FHM Combined P-value X^2 critical value, df = 8, $\alpha = 0.05$ = 15.507. SB Combined P-value X^2 critical value, df = 10, $\alpha = 0.05$= 18.307. For details see text.

Species	Comparison	Test	P-value	X^2 value	Combined P value
FHM	Avoidance				
	FHM-M	1. Mann-Whitney, Z = -1.0	0.329	18.0	p<0.025
		2. Mann-Whitney, Z = -2.7	0.006		
		3. Mann-Whitney, Z = -5.4	0.097		
		4. Mann-Whitney, Z = -1.8	0.643		
SB	Avoidance				
	FHM-DW	1. t-test, t = -0.1	0.952	17.2	0.05<p<0.10
		2. Mann-Whitney, Z = -2.9	0.442		
		3. t-test, t = -0.8	0.003		
		4. Mann-Whitney, Z = -1.2	0.216		
		5. Mann-Whitney, Z = -4.5	0.649		
	SWT-M	1. t-test, t = -1.9	0.072	18.6	p<0.05
		2. Mann-Whitney, Z = -1.2	0.390		
		3. t-test, t = -0.9	0.231		
		4. Mann-Whitney, Z = -1.8	0.070		
		5. Mann-Whitney, Z = -1.3	0.193		
	Length				
	SB-DW	1. Mann-Whitney, Z = -0.2	0.825	19.1	p<0.05
		2. t-test, t = -1.3	0.002		
		3. Mann-Whitney, Z = -3.1	0.210		
		4. Mann-Whitney, Z = -0.1	0.910		
		5. Mann-Whitney, Z = -1.2	0.222		
	SWT-DW	1. Mann-Whitney, Z = -0.5	0.635	16.9	0.05<p<0.10
		2. t-test, t = -1.8	0.006		
		3. Mann-Whitney, Z = -2.8	0.107		
		4. Mann-Whitney, Z = -0.5	0.617		
		5. Mann-Whitney, Z = -0.2	0.840		
	Weight				
	FHM - DW	1. t-test, t = -0.4	0.663	17.8	0.05<p<0.10
		2. t-test, t = -1.8	0.007		
		3. t-test, t = -2.7	0.112		
		4. t-test, t = -1.2	0.959		
		5. Mann-Whitney, Z = -0.1	0.272		

2.5 Discussion

The purpose of this study was to discriminate avoidance responses of fishes to conspecific alarm cues and cues of prey guild members from responses to unknown damaged fish odours and novel odours. Previous contradictory results led to the design of this study (Tremaine *et al.* 2005, Pollock *et al.* unpublished). Limited statistical significance found in the initial statistical tests led to an analysis of the power of each comparison. Using Power and Precision, it was determined that all six studies independently yielded limited power to detect a 20% difference in the means. This is despite the fact that the sample size per treatment in each trap experiment was rather large (Tables 2-7) in comparison to other trap studies. With such reduced power it is difficult to determine whether significant results are merely chance or whether the results are indeed significant. To draw conclusions about the null hypothesis when the power is so reduced would greatly increase the probability of making a type II error.

When the P-values were combined there were some notable significant interactions (Table 8). Fathead minnows were shown to significantly avoid minnow skin extract over morpholine (Combined P, $df = 8$, $X^2 = 18$, $p<0.025$). It would be expected that fathead minnows would avoid their own skin extract over a novel odour (Wisenden *et al.* 1995, Mathis & Smith 1992, Brown *et al.* 2000), however, because there was no difference between fathead minnow extract and distilled water it is difficult to draw any conclusions. Within the four experiments, where minnows were caught in large enough numbers to warrant statistical testing, minnows are either avoiding fathead

minnow skin extract or they are attracted to the novel non-biological odour of morpholine.

The combined P-values for stickleback included a trend in the avoidance of fathead minnow skin extract over the control of distilled water and a significant avoidance of swordtail skin extract over the novel odour of morpholine. I predicted that stickleback would avoid fathead minnow extract over the control of distilled water as fathead minnows are prey guild members and this cross-species recognition has been demonstrated in past studies (Wisenden *et al.* 1994, Chivers & Smith 1994, Wisenden *et al.* 1995, Mathis & Smith, 1992, 1993). The interesting significant response of avoiding swordtail skin extract over morpholine raises some questions. Are stickleback recognising the odour of damaged fish? Are they simply attracted to morpholine? Or is there some innate recognition of swordtail alarm cue structure? Because there was no difference between swordtail skin extract and control of distilled water it is difficult to draw conclusions as stickleback may simply be attracted to the novel odour of morpholine.

Length, with respect to this study, was an indication of experience. The underlying hypothesis was that fish with increased experience i.e. longer, would be more likely to avoid dangerous odours, namely those from conspecific and known heterospecific extracts. Stickleback were significantly shorter in the stickleback skin extract treatment than in the distilled water control (Combined P, df = 10, $X^2 = 19.1$, $p<0.05$). Inexperienced fish being present in the stickleback skin extract trap may suggest that younger fish may have to increase risky behaviour as competition for

resources, such as mates and food, may be high (Lima & Dill 1990, Lima 1998, Kats & Dill 1998). This may also indicate that fish with less experience have had fewer encounters with predatory attacks and this may lead to a decreased response to the cue.

Previous questions raised by the results of this study continue with respect to fish length. There is a trend for shorter stickleback to be found in the swordtail skin extract treatment than in the blank control of distilled water (Combined P, df = 10, $X^2 = 16.9$, $0.01<p<0.05$). This result combined with the avoidance of swordtail skin extract over the morpholine treatment leads to some interesting hypotheses. Since it has been shown that species which are more closely related tend to recognise each others alarm cue, and this may be a result of similar structure in chemical alarm (Schütz 1956), it may be argued that because swordtail and stickleback are both members of the Superorder Acanthopterygii, they are more closely related to one another than to fathead minnows (Superorder Ostariophysii). This relatedness may account for an innate ability to detect the functional group associated with alarm cue structure in that Superorder. This is an avenue which needs to be investigated as it would assist in the understanding of phylogenetic relatedness of species and could offer crucial information as to the chemical functional groups being detected in the alarm substance. Although genetics and morphology are more often used in such cases, there may be opportunity to use behavioural studies to further understand the relatedness and evolution of species.

The results of this study have raised some important ecological questions and several experimental questions as well. The ecological importance of the apparent recognition of swordtail skin extract by stickleback has already been mentioned;

however, without a powerful enough experimental design these interactions are only alluded to. Several experimental questions which should be addressed are the use of swordtail skin extract as a control when analysing the responses of brook stickleback. This may explain ambiguous results from previous studies. Lab studies which used swordtail to draw any conclusions about the response of stickleback should also re-evaluate the use of this type of control.

Future research should analyse the use of other observational techniques. The use of the minnow traps for behavioural observations is questionable as shoaling and shelter use are recognised antipredatory behaviours. The combination of a threatening stimuli and conspecifics actually increased the number of minnows caught in an experiment by Wisenden et al. (2003). The traps themselves may be viewed as preferable habitat when faced with predation cues (Layman & Smith 2001). Recently underwater video has been used to observe the reaction of numerous fish species to determine avoidance behaviour of various chemical stimuli (Magurran *et al.* 1996, Wisenden *et al.* 2004, Wisenden & Barbour 2005). This technology would offer a direct observation of fathead minnow's and brook stickleback's reaction to the chemicals in the scope of this study. Although the comparisons about morphological characteristics are lost in this strategy, the direct observation of fish in a natural environment allows for the validation of previous field studies. The next chapter of this thesis deals with the issues and concerns raised by this study.

Chapter 3

Experiment Two: Responses of prey fishes to alarm cues from conspecifics, and unknown heterospecifics and novel odours as assessed using underwater video

3.1 Introduction

The field experiments outlined in Chapter 2 utilized minnow traps to assess avoidance of fishes to alarm cues. Recently, Layman and Smith (2001) determined that minnow traps are not an effective means of passive sampling. This conclusion is based on fieldwork done at the Virginia Coast Reserve Long-Term Ecological Research Site. They compared number of fish and species caught using both seining and minnow trapping and found that there was a significant difference in percentage and types of fishes caught. Layman and Smith (2001) hypothesised that three factors may bias trap sampling including attraction to traps, frequency of encounter, and fish size and/or morphology. They concluded that fishes show preferential attraction to traps and consequently caution is needed when they are used for sampling. Such a bias may contribute to the contradictory results observed in previous trap experiments. Instead underwater video observations may allow observations with a reduced observer affect.

Magurran *et al.* (1996) used underwater video cameras to observe the behavioural responses of European minnows (*Phoxinus phoxinus*) and found limited evidence of antipredator behaviour. This finding brought about a debate between

researchers as to the importance of alarm cue, and whether or not responses were an artefact of the laboratory setting (Henderson *et al.* 1997, Smith 1997). Henderson *et al.* (1997) concluded more field experiments using underwater video were required.

Wisenden *et al.* (2004) and Wisenden and Barbour (2005) met the challenge and set out to record the response of wild populations of free swimming fish to Ostariophysan skin extract and a control of lake water. They found that chemical cues in ostariophysan skin alerted wild free-ranging populations of littoral fishes and concluded these responses were not an artefact of the laboratory. However, like early trap experiments, Wisenden *et al.* (2004) and Wisenden & Barbour (2005) could not demonstrate that the avoidance of the specific cue is not a generalized response to any damaged fish odour.

In this experiment I set out to use underwater video to discriminate avoidance responses of fishes to conspecific alarm cues and cues of prey guild members from responses to unknown damaged fish odours and novel odours.

3.2 Methods

Field data were collected between June 16 and July 6, 2004 from Oscar Creek, which is a small body of water located 75 km northwest of Saskatoon, Saskatchewan (52°46′N, 107°07′W). The creek ranges from 1-10 m in width and contains populations of brook stickleback, fathead minnows and finescale dace (*Chrosomus neogaeus*). Current speed and water depth varied with basin morphology.

The response of littoral fishes to five treatments, including fathead minnow, stickleback, and green swordtail skin extracts, morpholine and distilled water, was viewed using the Aqua-Vu underwater viewing system. The Aqua-Vu system consists of a small water resistant camera, ~10 cm x 5 cm, attached to a viewing screen via an extension cord. The viewing screen produces a black and white image on a screen measuring 8.5 cm x 6.5 cm.

The Aqua-Vu underwater camera was mounted on a metal tri-pod frame 0.4 m high, facing downward creating a substrate viewing area of 51.5 x 37 cm. An injection hose ran along the length of the camera wiring and attached approximately 10cm from the substrate on upstream leg of the tri-pod. Ten sites were chosen arbitrarily and treatments were assigned at random, with the conditions that the camera was to be submerged, sites were to have minimal plant cover (<15% coverage), and there was at least 10 m between sites. Trials on the same day were done in such a manner that each trial took place upstream from the previous, ensuring that fish were not exposed to any chemical before they were to be tested.

Stimulus Preparation

Fathead minnow extract was produced from six fish ($X \pm SD$ standard length = 5.32 ± 0.29cm), stickleback extract was produced from seven fish (5.05 ± 0.57cm) and swordtail extract was created using six fish (4.51 ± 0.71cm). Skin extract for each of the damaged fish treatments was created in similar fashion. Donor fish were killed with a blow to the head (in accordance with Animal Care Protocol guidelines) and skin fillets

were removed from both sides of the fish. Fillets of skin were placed in enough distilled water to produce a concentration of 1cm^2 of skin per 5 ml of distilled water. This value differs from the previous study because stimuli are injected directly into the water and not applied to two sponges. The active space created is similar between the two experiments. The solution was then homogenized with a Polytron© homogenizer and the homogenate was filtered with glass wool. The morpholine solution was prepared by adding 0.07 ml of pure morpholine (99%) to 500 ml of glass-distilled water. This concentration was derived from a previous field experiment (Hasler & Scholz 1978) and a laboratory study (Suboski *et al.* 1990). The distilled water treatment consisted of glass-distilled water. The solutions for each of the treatments were then frozen in 5 ml aliquots in VWR sterile sampling bags until they were used.

Experimental Protocol

The camera was placed in the creek such that the injection hose was upstream from the viewing area, ensuring the stimulus plume extended through the viewing area. Before the experiment commenced, 60 ml of creek water were withdrawn through the injection hose using a 60 ml plastic syringe and discarded, and then another 60 ml were withdrawn and retained. An experimental trial was not started until a fish of any species was viewed, therefore acclimation times varied. Trials consisted of an eight minute pre-stimulus and eight minute post-stimulus period, separated by a 90 s injection interval. During the pre- and post-stimulus periods, those fishes which were visible in the camera's viewfinder were counted every 15 s. During the injection time 5 ml of thawed

stimulus was injected into the injection hose and 60 ml of creek water was then injected to ensure that the stimulus reached the observation area. We conducted a total of 60 trials, consisting of 12 replicates in each of the 5 treatments.

3.3 Statistical Analysis

The number of each species, both in the pre- and post-stimulus periods was counted and the differences were compared. Because it was difficult to differentiate fathead minnows and finescale dace, these two species were counted together and categorized as cyprinids. Mann-Whitney tests were used to compare among treatment pairs. The family-wise error rate was assessed and controlled using a modified Bonferroni test following Keppel (1982). The modified Bonferroni test specifies that corrections to the family-wise error rate be introduced only when the number of comparisons exceeds $k - 1$, where k is the number of treatments (Keppel 1982). In this experiment, there were five treatments. The analysis was restricted to 9 pre-planned comparisons (see experimental series 1) that were based on specific a priori comparisons, so rejection probability (P) was set at 0.022 for each comparison (Keppel 1982).

3.4 Results

Upon release of fathead minnow or stickleback extract there was a reduction in the number of fishes observed in the area. The percent reduction in number of minnows observed in the fathead minnow treatment, for example, was 43%; the reduction in the percent of stickleback observed was 14%. There was a greater reduction observed in cyprinid fishes after injecting fathead minnow extract treatment than in the swordtail extract treatment ($Z = -3.725$, $N = 12$, $p < 0.001$, Figure 1), morpholine treatment ($Z = -3.557$, $N = 12$, $p < 0.001$), or distilled water treatment ($Z = -3.583$, $N = 12$, $p < 0.001$). When comparing change in number of cyprinids between the fathead minnow treatment and stickleback treatment there was a trend ($Z = -1.971$, $N = 12$, $p = 0.049$) with a greater reduction observed in the minnow treatment. Cyprinids also displayed significant avoidance of the stickleback treatment compared to the swordtail treatment ($Z = -2.378$, $N = 12$, $p = 0.017$). Despite this, only trends were noted when comparing the change in number in the stickleback treatment compared to morpholine treatment ($Z = -2.085$, $N = 12$, $p = 0.037$), and stickleback compared to distilled water ($Z = -1.918$, $N = 12$, $p = 0.055$).

Within the stickleback response (Figure 2), change in number of fish present was significantly different between the fathead minnow treatment and distilled water ($Z = -2.292$, $N = 12$, $p = 0.022$). There was also a trend ($Z = -2.003$, $N = 12$, $p = 0.045$) between the stickleback and distilled water treatments. A notable trend also occurred between the swordtail and distilled water treatments ($Z = -1.711$, $N = 12$, $p = 0.087$).

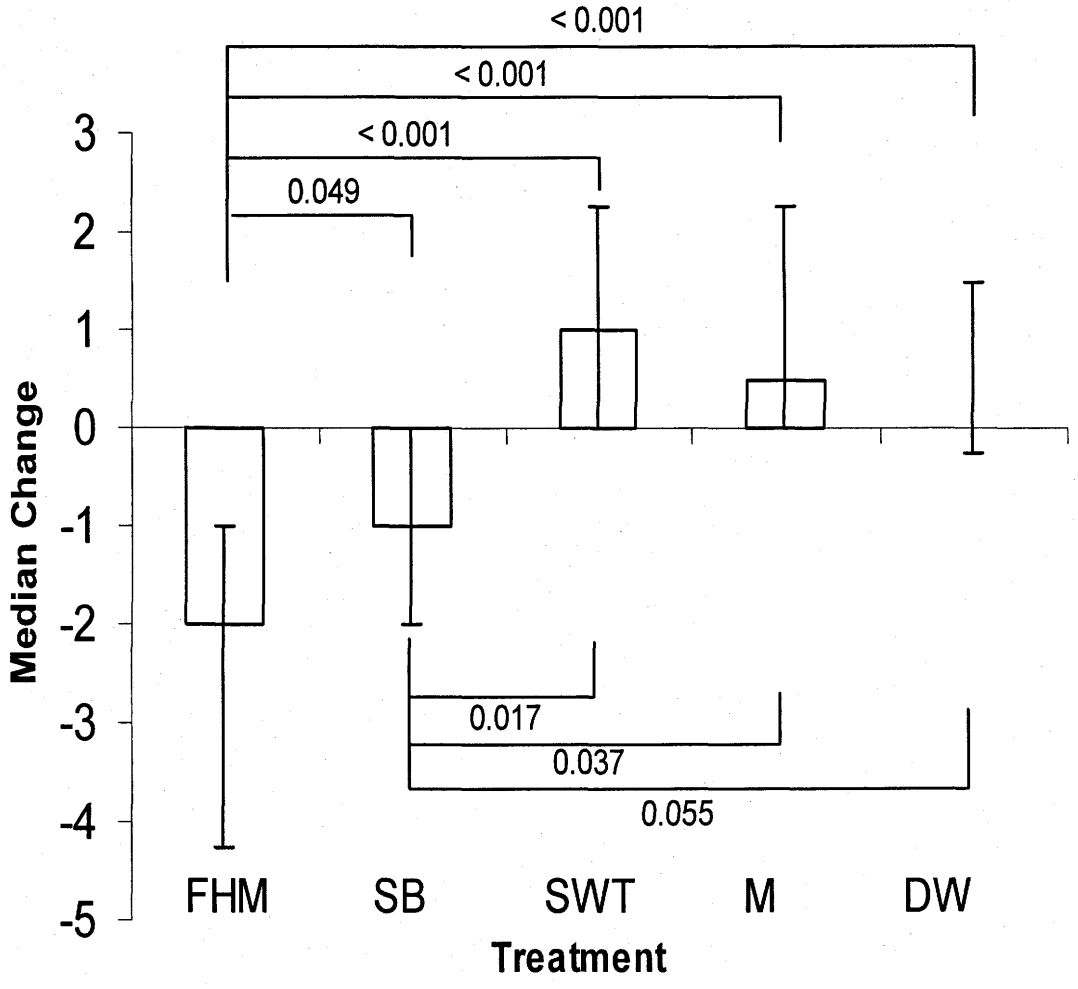

Figure 1: Median Graph (1st and 3rd quartile) of the change in number of cyprinid species from pre to post stimulus among five treatments. FHM = fathead minnow extract, SB = stickleback extract, SWT = swordtail extract, M = morpholine, and DW = distilled water.

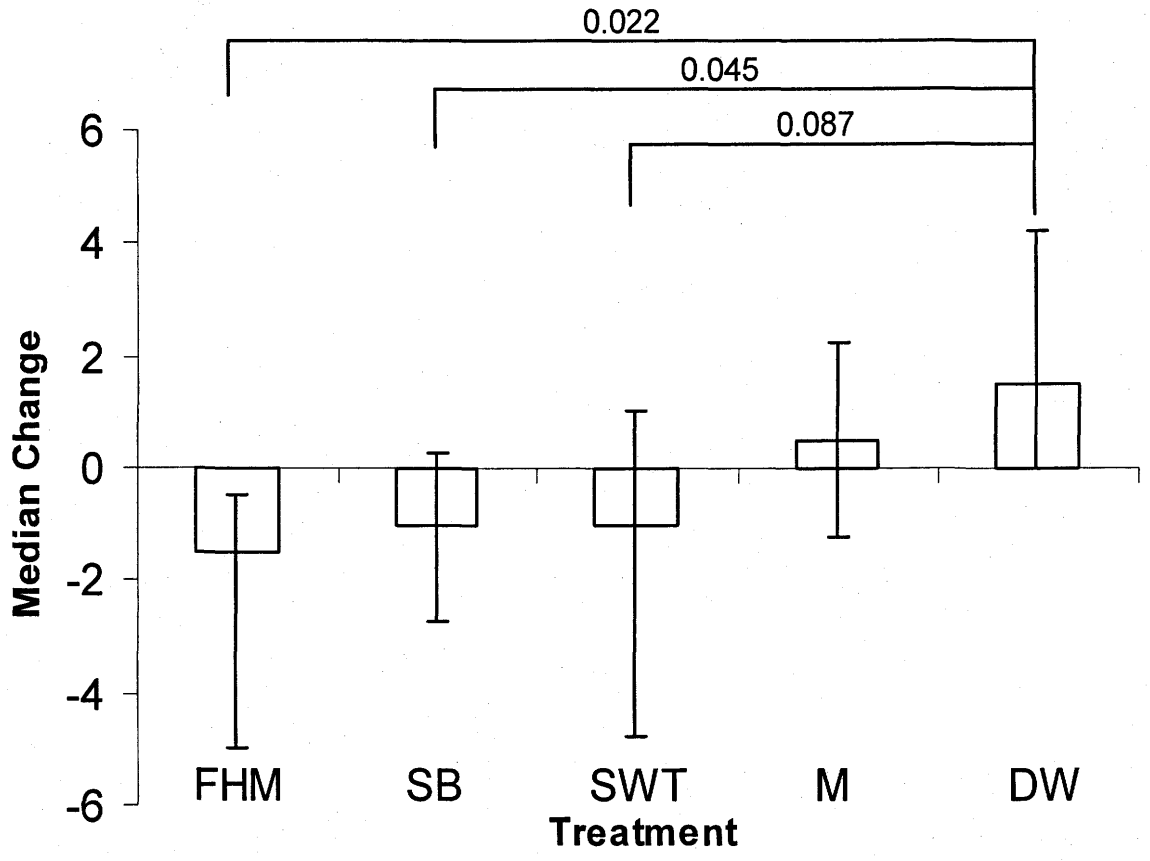

Figure 2: Median Graph (+ 1st and 3rd quartile) of the change in number of stickleback from pre to post stimulus among five treatments. FHM = fathead minnow extract, SB = stickleback extract, SWT = swordtail extract, M = morpholine, and DW = distilled water.

3.5 Discussion

My experiment provided strong evidence that fishes in a natural environment perform avoidance behaviour when faced with both conspecific and heterospecific skin extracts over the controls. This study is the first camera experiment in which the responses of fishes to conspecific alarm cues and cues of prey guild members are compared to unknown damaged fish odours and novel odours. The response of cyprinids clearly demonstrated that minnow skin extract elicited a greater avoidance than did all control treatments (distilled water, morpholine, and swordtail). There was also a trend for cyprinids to avoid minnow extract over stickleback extract, which are members of the same prey guild. Either minnow extract indicated a higher risk to these species than did stickleback extract or minnow alarm cues were easier to detect or persisted longer in the environment.

This experiment showed that stickleback avoided fathead minnow extract more than distilled water. However, only trends were noted between the response to stickleback extract and swordtail extract when compared to distilled water. There may be several explanations for the apparent difference between the cyprinid response and the stickleback response. Stickleback were observed to be defending nest sites indicating that stickleback were in breeding season. This may account for the lack of avoidance between the skin extracts. When reproductive success is at stake, stickleback may be more likely to ignore indications of danger (Lima & Dill 1990, Kats & Dill

1998). Sticklebacks are also an armoured fish and perhaps with this advantage avoidance of predators is not as crucial to survival.

My findings support Wisenden *et al.*'s (2004) field work with cameras. Although Wisenden *et al.* (2004) used a model predator in their experiment, they found that the effect of the chemical alarm cue alone was the same as the effect of the model predator. Our results and those of Wisenden *et al.* (2004) contradict the experiment of Magurran *et al.* (1996). Wisenden *et al.* (2004) charged that "given the published data from Magurran *et al.* (1996) for any given level of area use before cue release less than half as many fish were in the same area after the release of alarm cue as compared to the number before and after the release of the muscle tissue control." The control of muscle extract used in Magurran *et al.* (1996) is indeed questionable as the metabolic pathways which produce the alarm substance and the identity of the alarm substance itself are not yet fully understood. Magurran *et al.* (1996) also summed videotaped behavioural responses over 30 min observation periods. As stated by Wisenden *et al.* (2004) this observation time may have contained 2 min of response followed by 28 min of normal activity. Extended response times may be costly as they reduce time available for reproduction, territory defense and foraging (Lima & Dill 1990, Lima 1998, Kats & Dill 1998, Magnhagen 1991).

I encourage future researchers to take considerable care to provide details on water body characteristics as this information may provide insights into why responses are seen in some but not all studies. I hypothesize that with a decrease in volume of water as occurs at streams like Oscar creek in mid-late summer, avoidance behaviour

may not be possible and the environment may be confined creating an environment similar to lab studies (Magurran *et al.* 1996). Perhaps other antipredator responses such as shoaling or shelter use would increase under such circumstances. This can be seen in laboratory experiments, where fish are usually confined to a relatively small body of water (Chivers & Smith 1998, Smith 1992). Although there is a response, the type of response seems to depend on the environment (Lima & Dill 1990, Kats & Dill 1998, Hartman & Abrahams 2000).

I chose to use the swordtail treatment in my experiment because its past use as a control in laboratory experiments and because of the contradictory results found in previous field studies (Tremaine *et al.* 2005, Pollock *et al.* unpublished). My study indicates that the ostariophysan fish and stickleback in Oscar creek respond to swordtail extract differently. This may have been because 1) stickleback were defending nests during this study 2) these species may have different antipredator strategies, and 3) the phylogenetic relatedness of the species. Schütz (1956) found that for fishes in the Superorder Ostariophysii, the anti-predator response to heterospecific alarm substance decreases with phylogenetic distance. Because both swordtails and stickleback belong to Superorder Acanthopterygii, they are more closely related to one another than to fathead minnows and finescale dace (Superorder Ostariophysii). The recognition of swordtail extract by stickleback may be innate if stickleback and swordtail share similar alarm cue chemistry (Schütz 1956, Smith 1992). This might explain why the cyprinids treated the swordtail extract as a control and the stickleback showed a trend to avoid the treatment when compared to the control. The addition of morpholine allowed for more

discrimination of antipredator responses. It demonstrated that ostariophysan fish and stickleback did not react in the same manner to novel odours and odours that do not indicate risk. Both groups responded similarly to the novel odour and to the control of distilled water indicating that the difference in response to the swordtail treatment is of biological importance.

I encourage additional field work examining the responses of fishes to alarm cues. There are hundreds of laboratory studies, yet we are just beginning to understand the dynamic responses that occur in nature.

Chapter 4: General Discussion

The results of the studies presented in this thesis provide some experimental evidence that fishes in a natural environment perform avoidance behaviour when faced with either conspecific or heterospecific skin extracts over the controls. The lack of power in the first experimental series called into question the experimental design and protocol. However, the combined P-values were an excellent way to draw conclusions as to the possible interactions and allowed for the design of a more powerful experiment. The initial observations led to the camera experimental design which elicited significant results that may both validate previous field studies and explain some of the unexpected results seen.

The use of traps in the experiments discussed in Chapter 2 demonstrated that an increased number of treatments, which is required to discriminate behaviours, led to a decrease in power with the sample sizes that were possible. This lack of power did not allow for any viable conclusions to be made concerning all variables of interest.

The camera experiment in Chapter 3 showed the response of cyprinids clearly demonstrates that minnow skin extract elicits a greater avoidance than all of the control treatments (distilled water, morpholine, and swordtail). This study validates earlier findings reported by Mathis and Smith (1992) where fathead minnows showed significant avoidance of fathead minnow extract over a control of distilled water. Not only did this thesis replicate those findings, it determined that these results were not a

generalised response to something that has an odour. This study also confirms that cyprinids avoid minnow skin extract over swordtail skin extract, strengthening the hypothesis that these responses are not generalized responses to the odour of any damaged fish. The addition of morpholine in this study was used to clarify the ambiguous results of previous studies (Tremaine *et al.* 2005, Pollock *et al.* unpublished). This information was used to demonstrate that cyprinids are definitely responding to minnow extract over a blank control, an unknown heterospecific, and novel non-biological odours.

There was a trend for cyprinids to avoid minnow skin extract over stickleback skin extract. This reinforces the hypothesis that prey-guild members' alarm cue, although valuable information, may not indicate as high a level of risk as the conspecific extract (Chivers *et al.* 1995, Mirza & Chivers 2001, Pollock *et al.* 2003) or, alternatively, minnow alarm cues are easier to detect and last longer in the environment.

Cyprinids were also shown to avoid stickleback over the unknown heterospecific treatment, with trends towards avoiding stickleback skin extract over both distilled water and morpholine. This strengthens the hypothesis that known heterospecific cues are responded to less intensely than conspecific cues (Chivers *et al.* 1995, Mirza & Chivers 2001 b, Pollock *et al.* 2003). It also shows that cyprinids are utilising the odour of damaged stickleback in their antipredator strategy.

This thesis shows that stickleback avoided fathead minnow extract over distilled water. However, only trends were noted between the response to stickleback extract and swordtail extract when compared to distilled water. This seems to oppose the hypothesis

that stickleback should deem conspecific skin extract more dangerous than that of the known heterospecific, fathead minnow, extract (Chivers *et al.* 1995, Mirza & Chivers 2001 b, Pollock *et al.* 2003). This apparent reversal may be a direct result of the stickleback breeding condition. Species in breeding condition may be more likely to assert risky behaviour to defend nest sites from conspecific competitors (Magnhagen 1991).

Perhaps the most interesting aspect of this thesis is the inconclusive results seen in the stickleback response to the swordtail skin extract treatment. Inexperienced stickleback treated the swordtail skin extract the same as stickleback extract compared to the control of distilled water in the first experimental series. They were also shown to avoid swordtail extract over the novel non-biological odour of morpholine. These findings are validated in the second study where sticklebacks are demonstrated to treat the swordtail extract different than the cyprinids. Cyprinids respond to the swordtail treatment the same as the blank control, indicating that there is no recognition of the odour present. Stickleback conversely show a trend towards treating the swordtail extract like other fish extracts in the study. Numerous explanations have already been discussed throughout the course of this thesis, however, I hypothesize that since these two species are members of the same Superorder and therefore are phylogenetically related, they probably share similar functional groups used in the alarm system as stated by Schütz (1956).

Although the first underwater camera experiment examining the responses of fishes to alarm cues was in 1996 (Magurran *et al.* 1996) relatively few studies have been

conducted using this technology. In the Magurran *et al.* (1996) study they concluded that there was no response demonstrated by European minnows to conspecific extract. Several problems with the study are outlined in section 3.5. Since then, however, Wisenden *et al.* (2004) and Wisenden & Barbour (2005) have clearly demonstrated that chemical cues in ostariophysan skin alert wild free-ranging populations of littoral fishes and concluded that these responses were not an artefact of the laboratory. These findings are replicated in this thesis. However, this thesis demonstrates that the avoidance of the specific cue is not a generalized response. Like early trap experiments, Wisenden *et al.* (2004) and Wisenden & Barbour (2005) have failed to demonstrate this.

Future research should consider many things when conducting behavioural assays on fish species in the wild. The observational technique and variables within the environment are the two most important factors to consider, as highlighted in this thesis. The use of traps, a means of observation in which there is no actual observation, should be closely analysed. Although previous studies have shown significant and important ecological findings (Mathis & Smith 1992, Chivers & Smith 1994, Chivers *et al.* 1995) which were validated within this thesis, these early studies were lacking all of the possible controls. Numerous ambiguous results (Tremaine *et al.* 2005, Pollock *et al.* unpublished), shoals affecting responses (Wisenden *et al.* 2003), and cues from the traps themselves (Layman & Smith 2001) are all problems which can be rectified by using underwater video observation.

Literature Cited:

Aubin, T. 1991. Why do distress calls evoke interspecific responses? An experimental study applied to some species of birds. *Behav. Process*. **23**: 103-111.

Brown, G.E. 2003. Learning about danger: chemical alarm cues and local risk assessment in prey fishes. *Fish and Fisheries*. **4**: 227-234.

Brown, G.E., Adrian, J.C. Jr., Smyth, E., Leet, H. & Brennan, S. 2000. Ostariophysan alarm pheromones: laboratory and field tests of the functional significance of nitrogen oxides *J. Chem. Ecol.* **26**:139-154.

Bshary, R. & Noe, R. 1997. Red colobus and Diana monkeys provide mutual protection against predators. *Anim. Behav.* **54**: 1461-1474.

Chivers, D.P. & Smith, R.J.F. 1994. Intra and interspecific avoidance of areas marked with skin extract from brook sticklebacks (*Culaea inconstans*) in a natural habitat. *J. Chem. Ecol.* **20**:1517-1524.

Chivers, D.P. & Smith, R.J.F. 1998. Chemical alarm signalling in aquatic predator-prey systems: A review and prospectus. *Ecoscience* **5**:338-352.

Chivers, D.P., Wisenden, B.D. & Smith, R.J.F. 1995. The role of experience in the response of fathead minnows (*Pimephales promelas*) to skin extract of Iowa darters (*Etheostoma exile*). *Behaviour* **132**:665-674.

Frisch, K. Von 1938. Zur psychologie des Fishe-Schwarmes. *Naturwissenschaften.* **26**:601-606.

Frisch, K. Von 1941. Über einen Shreckstoff der Fischhaut and seine biologishe Bedeutung. *Z. vergl. Physiol.* **29**:46-145.

Hara, T.J. & Brown, S.B. 1979. Olfactory bulbar electrical responses of rainbow trout (*Salmo gairdneri*) exposed to morpholine during smoltification. *J. Fish. Res. Bd. Can.* **36**:1186-1190.

Hartman, E.J. & Abrahams, M.V. 2000. Sensory compensation and the detection of predators: The interaction between chemical and visual information. Proc. Roy. Soc., London, Series B 264: 451-453.

Hasler, A.D. & Sholz, A.T. 1978. Olfactory imprinting in coho salmon (*Oncorhynchus kisutch*). In: Schmidt-Koenig, K., Keeton, W.T. (eds) Animal migration, navigation, and homing. New York, pp. 356-369.

Henderson, P.A., Irving, P.W., Magurran, A.E. 1997. Fish pheromones and evolutionary enigmas: A reply to Smith. *Proc. Roy. Soc.*, London, Series **B** 264: 451-453.

Hirsch, P.J. 1977. Conditioning of heart rate of coho salmon (*Oncorhynchus kisutch*) to odors. PhD Diss, Univ. Wisc, Madison.

Kats, L.B. & Dill, L.M. 1998. The scent of death: Chemosensory assessment of predation risk by prey animals. *Ecoscience* **5**:361-394.

Keppel, G. 1982. Design & analysis: A researcher's handbook. Prentice-Hall, Englewood Cliffs, New Jersey.

Layman, C.A. & Smith, D.E. 2001. Sampling bias of minnow traps in shallow aquatic habitats on the eastern shore of Virginia. *Wetlands* **21**:145-154.

Lima, S.L. & Dill, L.W. 1990. Behavioural decisions made under risk of predation: a review and prospectus. *Can. J. Zool.* **68**:619-640.

Lima, S.L. 1998. Stress and decision making under the risk of predation: Recent developments from behavioural, reproductive and ecological perspectives. *Adv. Stud. Behav.* **27**:215-290.

Magnhagen, C. 1991. Predation risk as a cost of reproduction. *Trends Ecol. Evol.* 6: 183-186.

Magurran, A.E., Irving, P.W. & Henderson, P.A. 1996. Is there a fish alarm pheromone? A wild study and critique. *Proc. R. Soc. Lond.* **B 263**:1551-1556.

Mathis, A. & Smith, R.J.F. 1992. Avoidance of areas marked with a chemical alarm substance by fathead minnows (*Pimephales promelas*) in a natural habitat. *Can. J. Zool.* **70**:1472-1476.

Mathis, A. & Smith, R.J.F. 1993. Intraspecific and cross-superorder responses to chemical alarm signals by brook stickleback. *Ecology* **74**:2395-2404.

Mirza, R.S. & Chivers, D.P. 2001 a. Chemical alarm signals enhance survival of brook char (*Salvelinus fontinalis*) during encounters with predatory chain pickerel (*Esox niger*). *Ethology* **107**:989-1005.

Mirza, R.S. & Chivers, D.P. 2001 b. Differential responses of male and female red swordtails to chemical alarm cues. J. Fish Biol. 59: 716-728.

Pollock, M.S., Chivers, D.P., Mirza, R.S. & Wisenden, B.D. 2003. Fathead minnows, *Pimephales promelas*, learn to recognize chemical alarm cues of introduced brook stickleback, *Culaea inconstans*. *Env. Biol. Fishes*. **66**:313-319.

Schutz, F. 1956. Vergleichende untersuchungen über die Schreckreaktion bei Fischen Und deren verbreitung. *Z. vergl. Physiol* **38**: 84-135.

Smith, R.J.F. 1992. Alarm signals in fishes. *Rev. Fish. Biol. Fish.* **2**: 33-63.

Smith, R.J.F. 1997. Does one result trump all others? A response to Magurran, Irving, and Henderson. *Proc. Roy. Soc.*, London, Series B 264: 445-450.

Suboski, M.D., Bain, S., Carty, A.E., McQuid, L.M., Seelen, M.I. & Seifert, M. 1990. Alarm reaction in acquisition and social transmission of simulated-predator recognition by zebra danio fish (*Brachydanio rerio*). *J. Comp. Psych.* **104:**101-112.

Tremaine, R.J., Pollock, M.S., Friesen, R.G., Kusch, R.C. & Chivers, D.P. 2005. The response of prey fishes to chemical alarm cues: what recent field experiments reveal about the old testing paradigm. *Chemical Signals in Vertebrates, Volume 10*. pp. 328-333 (Mason, R.T., LeMaster, M. and Müller-Schwarze, D., eds.). Plenum Press: New York.

Wisenden, B.D., Chivers, D.P. & Smith, R.J.F. 1994. Risk-sensitive use by brook stickleback (*Culaea inconstans*) in areas associated with minnow alarm pheromone. *J. Chem. Ecol.* **20:** 2975-2983.

Wisenden, B.D., Chivers, D.P., Brown, G.E. & Smith, R.J.F. 1995. The role of experience in risk assessment: avoidance of areas chemically labelled with fathead minnow alarm pheromone by conspecifics and heterospecifics. *Écoscience.* **2**: 116-124.

Wisenden, B.D., Pollock, M.S., Tremaine, R.J., Webb, J.M., Wismer, M.E. & Chivers, D.P. 2003. Synergistic interactions between chemical alarm cues and the presence of conspecific and heterospecific fish shoals. *Behav. Ecol. Sociobiol.* **54**. 485-490.

Wisenden, B.D., Vollbrecht, K.A. & Brown, J.L. 2004. Is there a fish alarm cue? affirming evidence from a wild study. *Anim. Behav.* **67**, 59-67.

Wisenden B.D. & Barbour K.A. (2005). Antipredator responses to skin extract of redbelly dace by free-ranging populations of redbelly dace and fathead minnows. Environmental Biology of Fishes. 72: 227-233.