

**Chemosensory assessment of predation risk by fishes: ecological and social factors
affecting learning and use of heterospecific alarm cues**

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By

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Abstract

Throughout their lives, prey organisms must balance the tradeoff between fitness-related activities and the risk of predation. To successfully mediate such tradeoffs, prey must have an accurate method to gauge current predation risk. For many aquatic organisms, the use of chemosensory information has been shown to be a ubiquitous and useful tool in mediating predation risk. The chemical cues to which aquatic organisms respond include the odour of known predators and the odour of a damaged conspecific or known or closely related heterospecific. In fishes, the response to damage-released cues from conspecifics or closely related heterospecifics has been shown to be innate, while the response to distantly related unknown heterospecific cues are likely learned. In a series of laboratory and field studies I examined the role of learning in the ability of fathead minnows to respond to damage-released cues of brook stickleback as an indication of predation risk. My results indicate that minnows from a population without stickleback do not recognize stickleback cues as dangerous. However, following the introduction of stickleback, minnows learn to recognize stickleback cues as dangerous. Further study indicated a low ratio of stickleback to minnows in a given population will decrease the likelihood of learning when compared with a similar sized population containing a higher ratio of stickleback to minnows. I also demonstrated that an increase in habitat complexity decreases the ability of minnows to learn to recognize stickleback cues. Studies have further demonstrated that in the face of predation (as indicated by

chemical cues from minnows and stickleback) minnows will decrease their antipredator response when in the presence of a fish shoal, especially a shoal of conspecifics. Finally, an examination of the effects of a minnow's length, body condition and breeding status indicate that morphological parameters can play a significant role in the intensity of response to heterospecific and conspecific damage-released cues. Previous works have demonstrated that behavioural responses to heterospecific damage-released cues increase the probability of the 'receiver' surviving a predation event. This has important implications for predator/prey interactions and consequently factors affecting the learning and use of such cues deserve increasing attention from ecologists.

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Chapter 1: General introduction

1.1 Predation and the assessment of risk

Regardless of trophic level, all organisms must overcome the same challenges within their lives. They must all acquire energy, obtain a suitable place to live, and at the appropriate time, reproduce. For prey organisms, such fitness-related activities are complicated by the risk of predation (Lima & Dill 1990; Kats & Dill 1998). Predation can directly or indirectly have an effect on a prey organism's timing or choice of food (Marcus & Brown 2003), choice of habitat (Candolin & Voigt 2003), availability of mates, or the number or quality of their offspring (Kusch & Chivers 2004). Predation can therefore be a strong selective force on prey organisms (Lima & Dill 1990). It is also known to be a force in constant fluctuation; fluctuations which can last days, years or centuries (Lima & Bednekoff 1999). In order to balance fluctuating predation risk with fitness gains prey organisms must, at all times, be aware of their current predation risk.

While most prey organisms possess multiple defences to decrease the probability of becoming a victim, including aposematic or cryptic colouration, armour or chemical defences, they may also use adaptations in life history or a change in behaviour to manipulate predatory outcomes. Life history changes can include alterations in time of hatching, metamorphosis or reproduction (Chivers et al. 1999). Behavioural adaptations may include choosing a specific habitat in which to forage and reproduce or the development of social systems (Jones & Paszkowski 1997; Lima 1998). All

morphological, and to a lesser extent behavioural antipredator adaptations must develop over time, becoming acquired and improved with successive generations. As predation pressures may fluctuate, at times the cost of possessing or maintaining certain antipredator strategies or morphologies may outweigh the benefits. In such cases, it makes adaptive sense for an animal to perform a change in their life history, morphology or behaviour to balance the cost of predation with the cost of antipredator strategies. Studies have indicated that many animals have the ability to change their morphology (Peterson & Brönmark 1999, Pollock et al. 2005a), life history (Pollock et al. 2005a, Chivers et al. 2001) or behaviour (Chivers & Smith 1998) to maximize fitness.

For a prey animal to perform an adaptive change they must first possess the ability to accurately assess their predation risk. Prey organisms are often equipped with one or more highly adaptive senses which can be used to detect an early indication of predation risk (i.e. Kats & Dill 1998). The sense, or senses, an animal relies on depends, at least in part, on the environment in which they exist. For example, research has shown that aquatic animals have a strong dependence on the use of chemical cues as a means of assessing their current threat of predation. In fact, the most recent review on the use of chemical cues by aquatic organisms has demonstrated that the mediation of predation risk via such cues may be a ubiquitous phenomenon (Chivers & Smith 1998).

1.2 The use of chemical cues by fishes

The aquatic environment has many attributes that have led to the adaptive use of chemical cues by aquatic organisms. First, while studies have demonstrated a dependence on visual cues by aquatic species (i.e. Hartman & Abrahams 2000), visual

cues are often unavailable due to the cluttered nature of aquatic environments, the inability of light to penetrate depths or eutrophic waters, or time of day or year. Secondly, many of the other senses available to aquatic animals, such as electrical abilities, the acoustical lateral line, or auditory cues are too limited in space and time to offer utility when faced with many types of predation, such as an ambush by a predator like the northern pike (*Esox lucius*). Chemical cues, however, travel well in aquatic media, can persist for long periods of time, are available 24 hours per day and throughout the year, and as numerous studies have demonstrated, offer information that other senses cannot provide (i.e. Chivers & Smith 1995; Kusch et al. 2004). Chemical cues are also available to organisms with rudimentary sensory systems which are unable to gather information available to more derived animals (Wisenden & Millard 2001).

The use of chemical cues may also benefit prey organisms by ending the predation sequence at an earlier point than other antipredator strategies. Lima and Dill (1990) describe the predation sequence as including an encounter, attack, capture and ingestion phase. The use of chemical cues should allow prey organisms to halt the predation sequence earlier (the encounter phase) resulting in an increased survival rate and fitness.

1.2.1 The use of disturbance-released cues

The chemical cues aquatic organisms are known to respond to include the odour of a damaged or disturbed conspecific or heterospecific as well as the odour of a predator (Chivers & Smith 1998). As the name suggests, disturbance-released cues are released early in the predation sequence, usually when the prey initially encounters the predator.

Detection of disturbance-released cues may aid receivers in increasing vigilance or may aid the sender in signalling to the predator that it has been discovered, decreasing its chances of a surprise attack (Wisenden 2000a, b). Disturbance-released cues have been documented in crayfish (i.e. *Orconectes virilis*; Hazlett 1990a), hermit crabs (*Calinus laevimanus*; Hazlett 1990b), fishes (i.e. *Salvelinus fontinalis*; Mirza & Chivers 2002) and amphibians (Kiesecker et al. 1996). While these cues are undoubtedly important to the animals known to respond (i.e. Mirza & Chivers 2002), the breadth of knowledge on disturbance-released cues is limited, as are the taxa known to respond.

1.2.2. The use of damage-released cues

In fishes, a response to damage-released cues is noted when a nearby individual is damaged or consumed during a predation event resulting in the release of damage-released cues into the surrounding environment, thereby “warning” other prey of an impending predation threat. Damage-released cues have been found in organisms ranging from simple flatworms (Wisenden & Millard 2001) to more derived organisms including most aquatic and semi-aquatic invertebrates and vertebrates (Chivers & Smith 1998). The use of damage-released cues has been most heavily studied in fishes, in particular the Superorder Ostariophysi, which comprises approximately 70% of freshwater fishes (Chivers & Smith 1998).

Upon detection of damage-released cues, prey fishes may perform a number of differing antipredator responses, including an immediate or longer term change in behaviour (i.e. dashing or hiding; Smith 1979), versus a shift in habitat use; Chivers & Smith 1994b), morphology (Zhao & Chivers 2005) or life history (Chivers et al. 1999).

The ability to respond to conspecific damage-released cues is known to be innate in some species (Smith 1979), as is the response to closely related heterospecific damage-released cues (i.e. Mirza & Chivers 2001a). However, many prey organisms, including fishes, may also respond to the damage-released cues of distantly related heterospecifics (i.e. Mathis & Smith 1993).

1.3. Responses to heterospecific damage-released cues

Responses to heterospecific alarm cues are common not only in fishes but also in primates (Seyfarth & Cheney 1986), amphibians (Lutterschmidt et al. 1994, Marvin & Hutchinson 1995, Chivers et al. 1997) and gastropods (Snyder & Snyder 1970). The response by animals to heterospecific cues are usually explained in one of two ways.

First, in closely related species, it is thought that the cue responsible for instigating the response is chemically similar enough to the individual's own species cue as to cause the antipredator behaviour (i.e. Mirza & Chivers 2001a). Second, for distantly related species it has been hypothesized that animals sharing habitat and common predators may benefit from responding to each other's cues just as they would to their own, and therefore learn to respond to the chemical in question. It is hypothesized that in such cases an animal will learn to respond by initially associating the odour with a known indication of danger, such as a conspecific damage-released cue or the odour of a predator (see Chapter 2).

In fishes, indirect evidence has indicated that learning may be the explanation behind distantly related cross-species responses. Chivers et al. (1995) compared the response of fathead minnows (*Pimephales promelas*) that co-occurred with Iowa darters

(*Etheostoma exile*) to minnows from a pond lacking darters. Only minnows from the pond containing darters avoided cues of injured darters.

Further studies examining the importance of responding to heterospecific damage-released cues have documented that responding individuals do gain survival benefits equal to those gained by responding to conspecific damage-released cues (Chivers et al. 2002). The results of such studies, in addition to the fact that fishes in natural conditions rarely, if ever, occur in monospecific conditions (Scott & Crossman 1973), underscore the importance of gaining a complete understanding of the complexity involved in the learning and use of heterospecific alarm cues.

1.4 Terminology

In the discipline of chemical ecology the terminology that researchers use has been a major source of controversy (Magurran et al. 1996, Smith 1997). Most early papers describing the responses of fishes to chemical cues released as a result of damage to a conspecific used the term pheromones to describe the chemicals involved. Many equated the term pheromone with signal and for a while researchers often referred to the chemicals as chemical alarm signals. In doing so, the theoretical framework for some researchers became signalling theory and design. Nevertheless, there is little evidence to suggest that the chemicals in question are true signals. Following the arguments of Wisenden and Chivers (2005), “These chemicals are not signals in the strict sense because their production and release are not shaped by natural selection by benefits to the sender that accrues from receiver responses”. For much of the past decade many researchers have referred to the chemicals released as a result of damage to conspecifics

as alarm cues instead of alarm signals. This was an attempt to move away from the mentality of alarm signalling theory. While this is a considerable improvement, we still have the implication of predation as the driving force behind the evolution of chemical cues. Recent findings (Chivers et al. unpub. data) suggest that the chemicals in question may have evolved as antiparasitic or antipathogenic agents and that the antipredator functions evolved secondarily. I often use the terms alarm cue and damage-released cue throughout my thesis. Using the term alarm cue provides continuity in the field while avoiding signalling theory; the most appropriate, although somewhat cumbersome, terminology may be damage-released cues.

1.5. Research objectives

The objectives of my research are to understand the ecological and social factors influencing the learning and use of heterospecific damage-released cues in fishes. Specifically, I first designed and conducted experiments to examine the hypothesis that learning is required for distantly related fishes to exhibit cross-species responses. I then examined factors that could affect the rate or ability of fishes to learn to use a novel odour as an indication of predation. Specifically, I examined the effects that relative density would have on learning. For example, if two prey fishes co-occur over a wide range they will almost certainly exist with varying ratios in different areas. I examined the effect that varying ratios of two prey species would have on the rate or ability of a species to learn to recognize a novel heterospecific odour. Similarly, I also examined the effect of habitat complexity on the learning curve. For example, if two species co-occur in various habitats throughout their range what effect would this have on their learning

abilities? Prey species may use available habitats in different ways, accelerating learning in one habitat type while inhibiting it in another.

I also examined several factors that may influence the probability of fishes to respond to learned cues. If learning must occur before a response to distantly related heterospecific cues can occur, then older fish should be more likely to respond to such cues than younger fish. Along with testing this hypothesis I examined the effect that body condition and breeding condition have on a fish's ability or willingness to respond to not only learned cues but also conspecific cues.

Finally, I examined the effects of social context on a fish's response to both learned and innate cues. In particular, I examined how a fish will mediate an indication of predation risk (i.e. receiving a damage-released cue) with the visual stimuli of a shoal of fish that have not received any indication of danger and are therefore unafraid.

1.6 Research approach

My studies concentrated on the abilities of fathead minnows to learn and use brook stickleback (*Culaea inconstans*) damage-released cues. These fishes are ideal for such studies as they are small, distantly related prey fishes (minnows: superorder Ostariophysi; stickleback: superorder Acanthopterygii), that co-occur throughout most of their native range (Scott & Crossman 1973) and more importantly they share the same suite of predators (Mathis & Smith 1993; Mathis et al. 1996). Therefore, a fitness benefit should exist for either species upon learning to use the damage-released cues of the other as an indication of predation risk. My experiments followed a flow from the initial hypothesis that learning is responsible for some heterospecific responses (Chapter 2) to

the investigation of factors affecting the learning and use of heterospecific alarm cues (Chapters 3-6, Figure 1.1).

I conducted my studies using two minnow populations. Minnows that do not occur with stickleback and therefore do not respond to stickleback damage-released cue (used for Chapters 2, 3 and 4) and minnows that co-occur with stickleback and do respond to stickleback damage-released cues (Chapters 5 and 6). The stickleback-naïve minnows allowed me to test a natural population's ability to learn, which is preferable to rearing naïve fish in the laboratory. On the other hand, a population that has co-occurred with stickleback will allow me to test the response of minnows that have learned to recognize the cues in the wild, a preferable situation to laboratory-taught minnows.

I also compared and contrasted the responses of minnows to conspecific cues with those to stickleback damage-released cues. Therefore, in most of my studies I include minnow damage-released cues as a treatment. This treatment allows me to not only compare the intensity of a learned versus innate response, but also adds an additional layer of novelty to my work, as many of my hypotheses regarding factors affecting minnows' responses to heterospecific cues have also not been tested with conspecific cues.

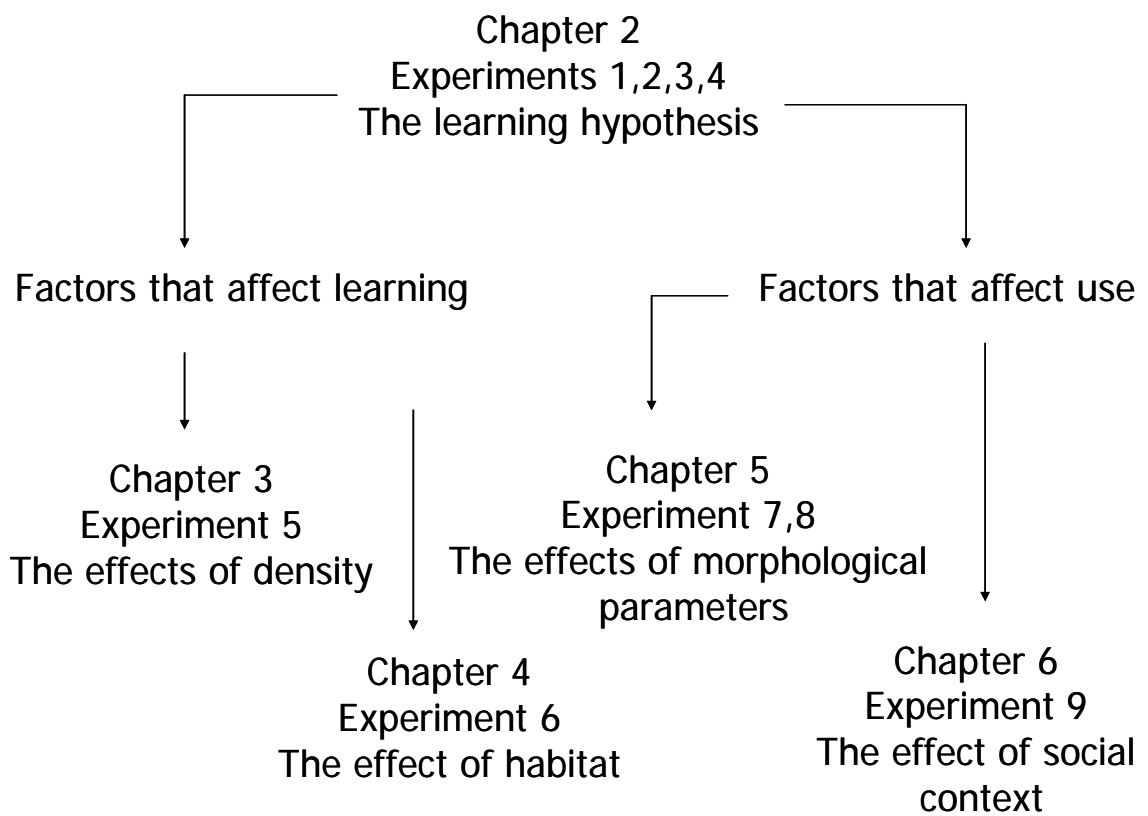


Figure 1.1. Flow chart of experiments conducted in the thesis.

Chapter 2: Fathead minnows learn to recognize the damage-released cues of introduced brook stickleback

2.1. Introduction

The importance of learning in the recognition of heterospecific damage-released cues in fishes has received little attention. Several of the documented examples of cross-species responses appear to suggest that closely related prey animals are responding to evolutionarily conserved homologous alarm cues (Brown et al. 2000, Mirza & Chivers 2001a). For example, Brown et al. (2000) showed that a nitrogen oxide functional group might act as the chief molecular trigger in the alarm systems of fishes in the Superorder Ostariophysi. Likewise, Mirza and Chivers (2001a) suggest an analogous chemical may be conserved within the salmonids. However, some authors have suggested that cross-species responses to damage-released cues may be learned when the cues are not homologous.

Chivers & Smith (1994a) provided indirect evidence that fathead minnows and finescale dace (*Phoxinus neogaeus*) can learn to identify brook stickleback damage-released cues. In their field study, traps marked with brook stickleback skin extract caught fewer and smaller dace and minnows than traps marked with distilled water. Larger fish are typically older and more experienced, suggesting that minnows and dace may have learned to recognize stickleback cues. Chivers et al. (1995) compared the

response of fathead minnows that co-occurred with Iowa darters (*Etheostoma exile*) to minnows from a pond lacking darters. Only minnows from the pond containing darters avoided cues of injured darters, and larger fish avoided darter alarm cues more often than smaller fish.

In the current study I tested whether fathead minnows can recognize the damage-released cues of brook stickleback following their introduction. When minnows and stickleback co-occur, they are exposed to many of the same predators (Mathis & Smith 1993) and should benefit by responding to heterospecific cues. Previous studies (Mathis & Smith 1993, Chivers & Smith 1994a, Brown et al. 1995) provide mixed results regarding whether minnows from populations containing stickleback respond to stickleback alarm cues. No studies have tested whether minnows from locations without stickleback respond to stickleback cues.

Three studies were conducted to explore the role of experience in response to stickleback damage-released cues by minnows. 1) In a laboratory experiment, minnows from a pond that did not contain stickleback were tested to determine if they would respond to stickleback damage-released cues any differently than they would to an unknown distantly related control stimulus (swordtail, *Xiphophorus helleri*). 2) Stickleback were then introduced into the pond, and after five years the minnows were retested for a response to stickleback damage-released cues in both laboratory and field trials. 3) In a final experiment, minnows were raised from eggs collected from a population known to respond to stickleback cues and tested to determine if they responded to stickleback damage-released cues.

I predicted that minnows from a pond without stickleback would not respond to stickleback damage-released cues, but following introduction and subsequent association a response would be noted. I further predicted that minnows reared from eggs collected from the now responding population would fail to respond to the stickleback damage-released cue, ruling out a genetically driven response, leaving learning as the only explanation.

2.2. General methods

2.2.1. Collection and maintenance of animals

Fathead minnows for Experiment 1 were collected from a 1 ha pond, known as Feedlot pond, located on the University of Saskatchewan campus (Saskatoon, SK, Canada). The pond is known to contain other fish species, including finescale dace (*Phoxinus neogoeus*), white suckers (*Catostomus commersoni*), and Iowa darters, but does not contain brook stickleback. Minnows were transported to the laboratory and maintained in a 350 l stream tank at approximately 13°C on a 14:10 h light:dark cycle, where they were fed daily with commercial fish flakes.

Following Experiment 1, 2008 brook stickleback were collected from Bradwell Reservoir in south central Saskatchewan, and released into Feedlot pond. Minnows from Feedlot pond were collected in the winter five years later for use in Experiment 2. Minnows collected for Experiment 2 were housed in the same conditions as in Experiment 1.

In Experiment 3, stickleback-naïve minnows were raised from eggs collected from Feedlot pond in the summer five years following the introduction of the brook

stickleback. My laboratory population of naïve minnows originated from eight different broods but I do not know how many test fish came from each brood. The experienced minnows were collected from Feedlot pond the following winter. Both laboratory minnows and wild caught individuals were housed in the same manner as in Experiments 1 and 2.

2.3. Experiment 1: Response of stickleback-naïve minnows to stickleback damage-released cues

The purpose of this experiment was to determine if minnows allopatric with stickleback will respond with an antipredator response to stickleback damage-released cues.

2.3.1. General set-up and behavioural assays

Most experiments in my thesis followed the same general set-up and behavioural assay. I will describe the details of those here and refer to them in future chapters along with any modifications.

Behavioural observations were completed in 37l glass aquaria filled with dechlorinated tap water. Each tank contained a gravel substrate and a centrally located shelter object consisting of a 10 X 20 cm ceramic tile mounted on three 5.5 cm long cylindrical glass legs. Each tank also contained an airstone near which I attached a two metre long piece of plastic tubing used to introduce the test stimuli into the tanks. The injection line allowed the observer to inject test stimuli at approximately 1.5 m from the test aquaria.

2.3.2. Preparation of stimuli

Ten adult stickleback (SL = mean \pm 1 SD = 6.74 \pm 0.27 cm) were sacrificed by a blow to the head (in accordance with the guidelines set by the Canadian Council on Animal Care) and a skin fillet was removed from both sides of each fish producing approximately 45 cm² of skin in total. The skin was placed into 100 ml of distilled water and homogenized using a polytron homogenizer. The sample was filtered through glass wool to remove any solid particles and diluted with 350 ml of distilled water for a total volume of 450 ml. The extract was divided into sample bags containing 50 ml each and immediately frozen at -20°C . I used the same procedure to produce a control extract from swordtails, (SL = 5.31 \pm 0.33 cm). I used swordtail skin extract as a control stimulus to test for an alarm response specific to stickleback and not a general response to injured fish cues. Swordtails are an ideal control because they are easily obtained, similar in size to stickleback, and are allopatric from, and distantly related to minnows (Chivers & Smith 1998).

2.3.3 Experimental protocol

Immediately prior to the start of each trial, 60 ml of water was drawn through the stimulus tube and discarded to ensure the tube was clean before each trial. Another 60 ml of water was then drawn, retained, and later used to flush the stimulus into the tank during the trial. Each trial consisted of an 8-minute pre-stimulus period followed by injection of the stimulus and an 8-minute post-stimulus period. I recorded the time spent

under shelter in both the pre- and post-stimulus periods. The occurrence of freezing (the cessation of movement where the fish drops to the substrate and remains immobile for a minimum of 30 s) and dashing (very rapid, erratic swimming) were also recorded following injection of the stimulus. I did not test fish that either froze or dashed in the pre-stimulus period. Shelter use, freezing and dashing are known antipredator responses in minnows (review Chivers & Smith 1998).

I observed the response of 15 minnows to each of two different stimuli: (1) skin extract from stickleback and (2) skin extract from swordtails. Tests were conducted after fish had acclimated for 48 hours. Rehnberg et al. (1987) demonstrated that minnows exposed to a frightening stimulus may show a physiological stress response for extended periods (i.e. for hours or days). Consequently, I tested all fish (individually) for a response to the control stimulus (swordtail skin extract) two days before I tested them for a response to the experimental stimuli (stickleback skin extract).

2.3.4. Statistical analysis

I calculated changes in time spent under shelter (post-stimulus minus pre-stimulus) for each treatment and compared the responses using a Wilcoxon signed-rank test (Siegel & Castellan 1988). The occurrence of dashing and freezing were compared using a McNemar change test (Siegel & Castellan 1988). I used one-tailed probability distributions because I predicted that if minnows could recognize stickleback damage-released cues then they would increase shelter use and exhibit dashing and freezing behaviour.

2.3.5. Experiment 1 results

There was no significant change in time spent under shelter in response to stickleback versus swordtail skin extract ($Z = 0.12$, $n = 14$, $p > 0.45$, Figure 2.1A). In addition, there was no significant difference in the frequency of dashing ($X^2 = 0$, $df = 1$, $p = 0.5$) or freezing behaviour ($X^2 = 0$, $df = 1$, $p = 0.5$, Table 2.1) between the two treatments. These results indicate that minnows naïve to stickleback do not recognize stickleback alarm cues as an indicator of predation risk.

2.4. Experiment 2: Acquired recognition of stickleback damage-released cues by fathead minnows: a laboratory test

The purpose of this experiment was to determine whether fathead minnows from Feedlot pond could acquire the ability to recognize stickleback damage-released cues following the introduction of stickleback.

2.4.1. General set-up and behavioural assays

I used a similar experimental procedure as in Experiment 1. However, instead of testing the response of the same minnows to cues of swordtails (control) and stickleback (experimental), each fish was tested only once. Moreover, I added fathead minnow damage-released cues as a third treatment in order to compare the intensity of any learned response to heterospecific cues against the innate response to conspecific cues. My sample size was 20 for each of the three treatments.

2.4.2. Preparation of stimuli

Skin extracts were prepared as in the first experiment. I used the skin from ten swordtails (SL = mean \pm 1 SD = 3.34 \pm 0.54 cm), seven stickleback (SL = 4.90 \pm 0.42 cm) and five minnows (SL = 5.30 \pm 0.51 cm). In all cases I added the appropriate amount of distilled water to the skin extract I collected to create a stimulus with a concentration of 1.0 cm² per 10 ml of distilled water.

Table 2.1: Comparisons of the occurrence of dashing and freezing by stickleback-naïve fathead minnows exposed to swordtail and stickleback skin extract (McNemar change test) (n = 14).

Treatment	Dashes	X² p-values	Freezes	X² p-values
Swordtail	0	X ² = 0	0	X ² = 0
Stickleback	4	p = 0.5	1	p = 0.5

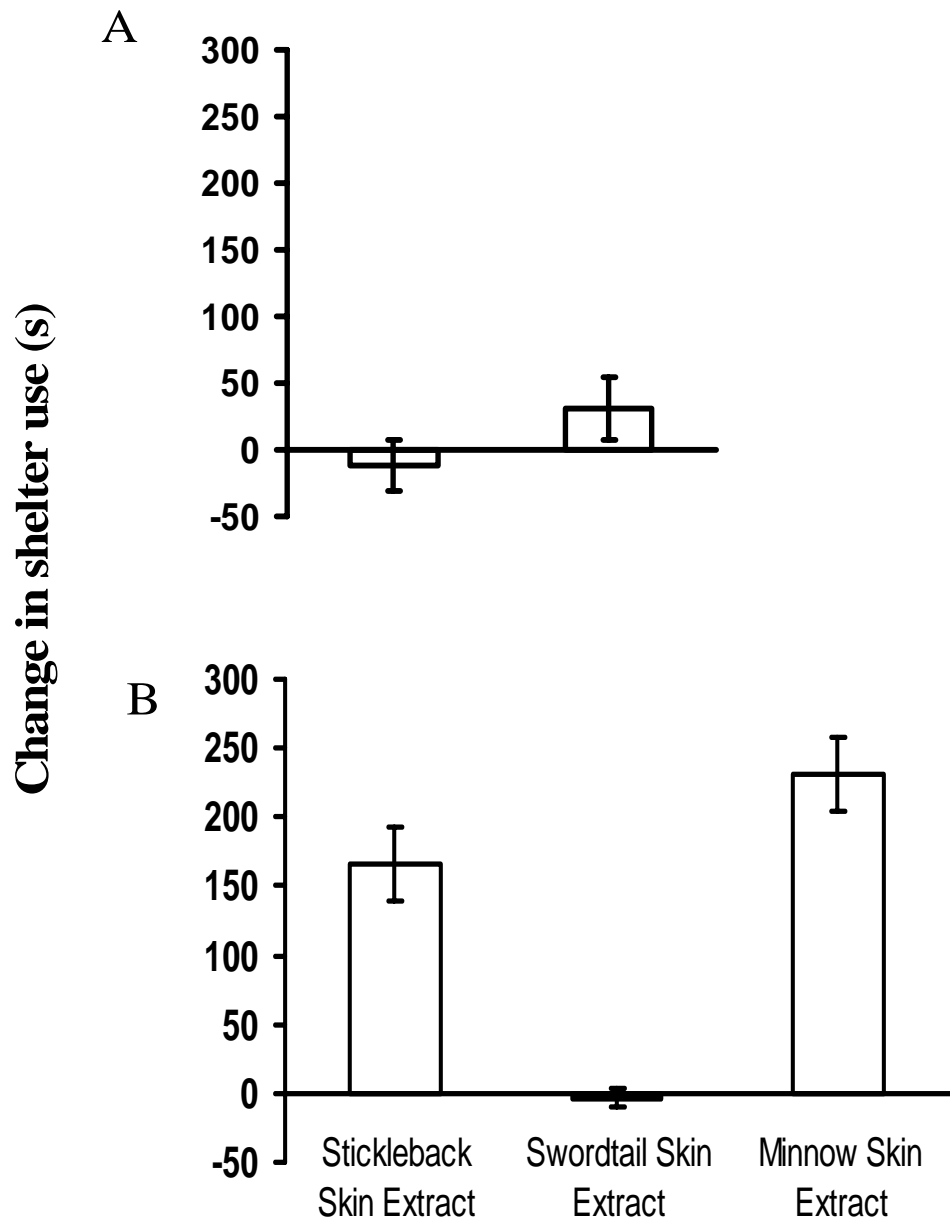


Figure 2.1 Mean (\pm SE) change in shelter use (s) by A) stickleback-naïve (n = 14) and B) stickleback-experienced fathead minnows (n = 20) exposed to stickleback and swordtail damage-released cues.

2.4.3. Statistical analysis

The change (post-stimulus minus pre-stimulus) in time spent under shelter was compared using a Kruskal-Wallis one-way analysis of variance on ranks (Siegel & Castellan 1988). The occurrence of dashing and freezing was analyzed with a Fisher exact probability test.

2.4.4. Results

There was an overall significant difference in time spent under shelter among the three treatments (KW = -28.90, df = 2, $p < 0.001$, $n = 20$, Figure 2.1B). Post-hoc multiple comparisons among treatments showed that there was no difference in shelter use between the stickleback and minnow treatments but both differed from the swordtail treatment. Fathead minnows exposed to minnow extract dashed more frequently (and tended to freeze more frequently) than those exposed to swordtail cues (Table 2.2). Similarly, minnows that were exposed to stickleback extract showed a tendency to dash more frequently than those exposed to swordtail extract (Table 2.2).

2.5. Experiment 3: Acquired recognition of stickleback damage-released cues by fathead minnows: a field test

The purpose of this experiment was to determine if fathead minnows from Feedlot pond would avoid traps labelled with stickleback damage-released cues.

2.5.1. General set-up and behavioural assay

Wires containing stimulus sponges were connected to the inside of 34 minnow traps (17 traps had sponges labelled with stickleback cues and 17 traps had sponges

labelled with swordtail cues). The minnow traps were roughly cylindrical wire enclosures (43 cm long X 22 cm wide) with an inverted funnel located at each end. The two sponges in each trap were positioned such that they were approximately four cm from each trap entrance. Pairs of traps (consisting of one control and one experimental trap) were placed into the water along the edge of the pond approximately ten metres apart. Pairs of traps were placed into the water at five-minute intervals until all 17 pairs were set. The order of placement of the control and experimental traps along the pond edge was determined randomly for each pair.

Trap pairs were removed from the water at five-minute intervals, starting two hours after the first pair was set. All fish found in the traps were removed, identified to species, and counted.

2.5.2. Preparation of stimuli

I used the same general methodology as in Experiments 1 and 2 to produce the swordtail and stickleback skin extracts. The skin from six adult swordtails (mean \pm 1 SD = 3.42 \pm 0.32 cm) and seven adult stickleback (4.2 \pm 0.29 cm) was added to the appropriate amount of distilled water to create a stimulus with a concentration of 1.0 cm² per 10 ml of water. I attached two sponges (2 X 2 X 2 cm) onto each of 34 stainless steel wires. I added 5 ml of stickleback skin extract (experimental) to each of the sponges on the 17 traps and 5 ml of swordtail skin extract (control) to each of the sponges on the remaining 17 traps.

Table 2.2: Comparisons of the occurrence of dashing and freezing by stickleback-experienced fathead minnows exposed to swordtail, stickleback, or fathead minnow skin extract (Fisher exact test) (n = 20).

Treatment	Dashing	p-value	Freezing	p-value
Swordtail	0		0	
vs.				
Stickleback	4	0.053	1	0.500
Swordtail	0		0	
vs.		0.010		0.053
Fathead	6		4	
Stickleback	4		1	
vs.		0.530		0.200
Fathead	6		4	

2.5.3. Statistical analysis

A Wilcoxon signed-rank test was used to compare the number of minnows captured in traps marked with swordtail extract compared with the number of minnows captured in traps marked with stickleback extract. A one-tailed test was used because I predicted that fathead minnows would be captured significantly less often in experimental traps than control traps.

2.5.4. Results

A total of 119 fathead minnows and 161 stickleback were captured in this experiment. Significantly fewer fathead minnows were captured in traps marked with

stickleback skin extract than in traps marked with swordtail skin extract ($Z = 1.82$, $n = 13$, $p = 0.034$, Figure 2.2). Stickleback showed a non-significant trend toward avoiding stickleback traps, ($Z = 1.38$, $n = 11$, $p = 0.083$). However, due to the fact that six trap-pairs failed to capture stickleback the sample size for stickleback avoidance was reduced to 11.

2.6. Experiment 4: The role of experience in acquired recognition of stickleback damage-released cues by fathead minnows

The purpose of this experiment was to determine if the acquired recognition of stickleback damage-released cues is a learned or, alternatively, a genetically-based behaviour promoted via selection.

2.6.1. General set-up and behavioural assay

The experimental protocol for this experiment was nearly identical to that used in Experiment 1, except both stickleback-naïve and stickleback-experienced minnows were tested. Due to the limited availability of fish at the time of testing, 12 wild-caught experienced minnows and 11 laboratory-reared naïve minnows were tested. Experienced and stickleback-naïve test fish were of similar size when tested (SL: naïve minnows = mean \pm 1 SD = 4.1 ± 0.5 cm, experienced minnows = 4.4 ± 1.0 cm).

2.6.2. Stimulus preparation

I used the same general methodology as in previous experiments to produce the swordtail and stickleback skin extracts. The skin from eight adult swordtails (mean \pm 1 SD = 3.9 ± 0.5 cm) and six adult stickleback (4.8 ± 0.4 cm) was added to the appropriate

amount of distilled water to create a stimulus with a concentration of 1.0 cm² per 10 ml of water.

2.6.3. Statistical analysis

The change (post-stimulus minus pre-stimulus) in time spent under shelter was compared using a Mann-Whitney U test (Siegel & Castellan 1988). The occurrence of dashing and freezing was analyzed with a McNemar change test (Siegel & Castellan 1988).

2.6.4. Results

There was no significant difference between treatments for change in time spent under shelter ($Z = -0.561$, $n = 11$, $p > 0.25$, Figure 2.3) for laboratory-reared minnows. In contrast, wild-caught minnows showed a significantly stronger increase in shelter use following exposure to the stickleback extract in comparison to the swordtail skin extract ($Z = -2.490$, $n = 12$, $p = 0.007$, Figure 2.3). Dashing and freezing were exhibited less frequently in this experiment than in the previous experiment with experienced fish. There were no significant treatment differences in the frequency of dashing or freezing behaviour between the two treatments for either wild-caught or laboratory-reared minnows (McNemar change test, $p > 0.10$ for all comparisons, Table 2.3).

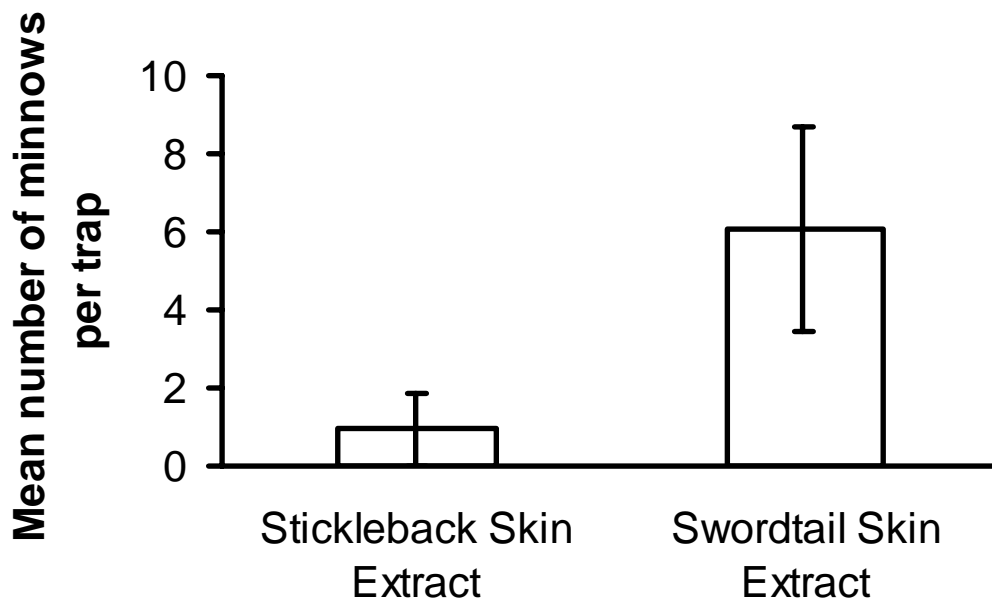


Figure 2.2. Mean (\pm SE) number of fathead minnows captured in traps marked with skin extract from stickleback ($n = 11$) or swordtails ($n = 13$).

2.7. Discussion

The results of Experiment 1 indicate that fathead minnows from a population without stickleback do not respond to stickleback damage-released cues with antipredator behaviour. However, laboratory and field experiments show that minnows from the test population (Feedlot pond) acquired recognition of stickleback cues following the introduction of stickleback. Testing the response of minnows collected as eggs allowed

me to conclude that acquired recognition was the result of learning and was not an evolved response. These data are among the first to provide clear evidence that cross-species responses to damage-released cues of fishes must be learned in at least some instances. Minnows should receive a survival benefit by responding to alarm cues from brook stickleback (Mathis & Smith 1993). Although mixed associations of fishes are common (Nursall & Pinsent 1969), few studies have examined the costs or benefits of these associations (Pitcher 1986). My study illustrates a possible benefit for mixed species aggregations of fishes.

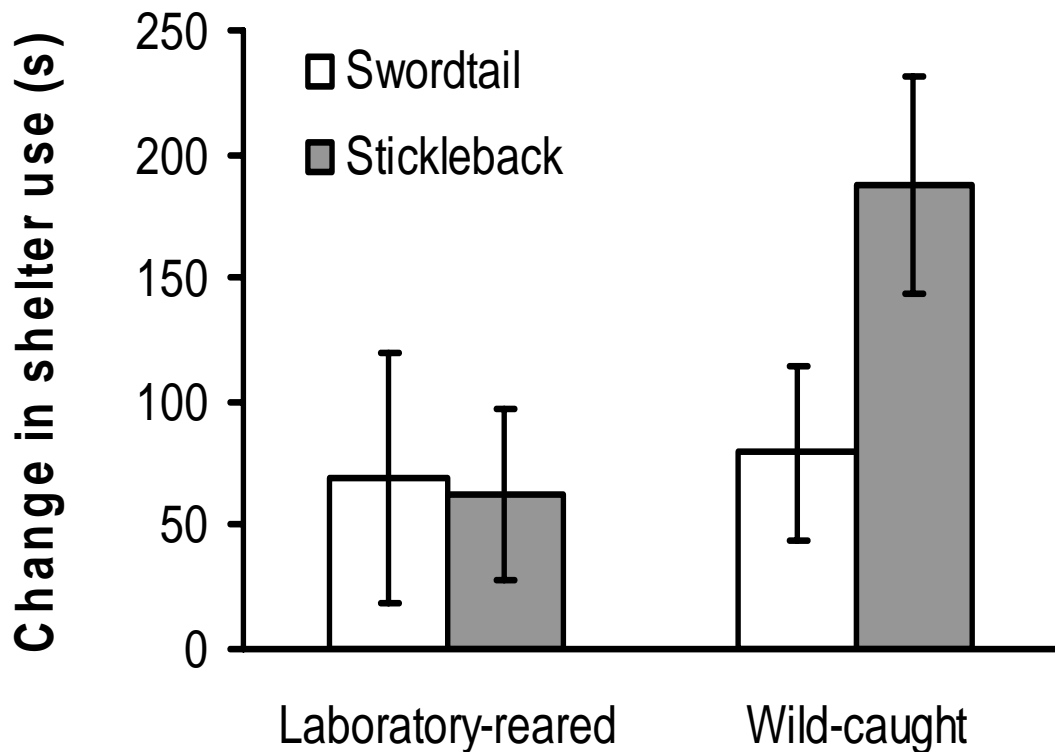


Figure 2.3 Mean (\pm SE) change in shelter use by laboratory-reared ($n = 11$) (naïve to stickleback) or wild-caught ($n = 12$) (experienced with stickleback) minnows following exposure to damage-released cues from swordtails and stickleback.

Table 2.3. Comparisons of the occurrence of dashing and freezing by wild-caught and laboratory-reared fathead minnows exposed to swordtail and stickleback skin extract (McNemar change test).

	Treatment	Dashes	X² p-values	Freezes	X² p-values
Wild-caught	Swordtail	0	-	1	X ² =0
	Stickleback	0	-	0	p > 0.9
Laboratory-reared	Swordtail	0	X ² =2.25	0	X ² =0
	Stickleback	4	p = >0.1	1	p > 0.9

Three previous studies have tested whether fathead minnows respond to damage-released cues of brook stickleback. In a laboratory study, Mathis and Smith (1993) observed no significant difference in response by minnows experienced with stickleback to stickleback damage-released cues. In a subsequent field experiment, Chivers and Smith (1994a) caught fewer minnows in traps marked with stickleback cues than traps marked with a control extract. While providing tentative evidence that minnows avoid stickleback cues, these results could have occurred if minnows were attracted to stickleback captured in control traps. A third study by Brown et al. (1995) showed that minnows experienced with stickleback recognize stickleback cues in the diet of a pike and avoided areas containing such cues.

The results of my current study provide insights into previously conflicting results. If minnows must learn to recognize stickleback cues, then the recognition by individuals in any population must be a consequence of the potential opportunities to learn. The degree of overlap in microhabitat use between these species and the degree to which they share predators are likely important variables to consider when examining whether there are sufficient opportunities for animals to learn to recognize each other's damage-released cues. Likewise, the relative density of the two prey species may be an important variable to consider because the greater the number of heterospecifics the greater the opportunity for acquired recognition. Species abundance, microhabitat overlap, the dietary overlap of predators, and the relative density of prey animals vary across short and long-term spatial and temporal scales. Chapters 2 and 3 will examine such variables, however, future studies should also attempt to address the mechanisms by which prey learn to recognize and use such cues as little is known on this topic (but see Mirza & Chivers 2001a).

Chapter 3: The effect of relative density on the ability of fathead minnows to learn to recognize damage-released cues of brook stickleback

3.1. Introduction

Several studies have examined factors affecting the response of various fishes to known conspecific damage-released cues; some examples include gender differences (Matity et al. 1994, Mirza et al. 2001), hunger level (Brown & Smith 1996, Chivers et al. 2000), water conditions (Lemly & Smith 1985), reproductive status (Smith 1976), and visual signals (Hartman & Abrahams 2000). Little attention has been paid to factors affecting the responses of fishes to heterospecific cues. We know that cross-species responses to heterospecific alarm cues among closely related species are often innate (Mirza & Chivers 2001a), while the recognition of cues from distantly related heterospecifics must be learned (see Chapter One, Chivers et al. 1995). Very little is known about how ecological variables may influence learned responses.

The fathead minnow / brook stickleback system has become established as an ideal test system for understanding the role of learning heterospecific alarm cues (Pollock et al. 2005b). The two species commonly co-occur throughout much of their range and often share the same suite of predators (Mathis & Smith 1993). In Chapter 2, it was demonstrated that minnows from ponds without stickleback do not recognize stickleback cues as dangerous. However, following the introduction of stickleback, minnows learned

to exhibit antipredator responses to the cues. Mirza and Chivers (2001b) and Chivers et al. (2002) investigated the mechanisms by which minnows learn to recognize stickleback alarm cues. Both studies show that minnows learn to identify stickleback cues when they are associated with conspecific damage-released cues in the diet of a predator. These learned responses provide the minnows with a survival advantage. Minnows 'warned' by stickleback cues survive longer in the presence of an unknown predator than minnows that are not 'warned' (Chivers et al. 2002).

The current study examined the effects that varying densities of stickleback had on the ability of minnows to learn to recognize stickleback alarm cues. The premise of the current study was based on two well-documented facts. First, the use of heterospecific alarm cues as indicators of predation risk is a wide spread phenomenon found in both terrestrial (Kats & Dill 1998) and aquatic systems (Chivers & Smith 1998). Second, due to many variables, animals are distributed in a heterogeneous manner throughout their range (Elton 1927). Consequently, the ratio of individuals of one species to the other species should be variable through time and space. The variance in these ratios may be an important factor in an animal's ability to learn to use heterospecific damage-released cues. Minnows can learn to associate the chemicals from a damaged stickleback with the odour of a known predator or damage-released cues from a conspecific (Mirza & Chivers 2001b, Chivers et al. 2002).

In the following study, minnows naïve to stickleback will be placed into artificial ponds with differing numbers of stickleback and a predator. I predict that minnows associating with low numbers of stickleback will be afforded fewer chances to learn. In contrast, minnows associated with relatively high numbers of stickleback will

be afforded several opportunities to associate the heterospecific cue with danger and will subsequently learn to recognize the stickleback damage-released cues as an indication of danger.

3.2. General methods

3.2.1. Collection and maintenance of animals

All minnows used in the experiment were collected from Briarwood pond, located in Saskatoon, Saskatchewan. Extensive trapping over the summer of 2000, 2001 and 2002 revealed that the pond is monospecific, containing only fathead minnows.

Minnows were collected on the same day the conditioning was to begin and transported immediately to the outdoor conditioning pools. All stickleback were collected from Lakeview pond, also located in Saskatoon, Saskatchewan. Stickleback were collected on the same day the conditioning was to begin and immediately transported to the same outdoor conditioning pools.

Following 14 days of conditioning, the pools were drained and all surviving minnows were collected, measured, and taken into the laboratory. Minnows were kept in 55 l tanks with a 14:10 h light:dark cycle photoperiod. Minnows were transferred to the observation tanks 48 hours prior to the start of the trials to allow for acclimation. The photoperiod in the testing tanks was also 14L:10D.

3.2.2. General set-up and conditioning regime

The experiment was conducted in two parts; the conditioning, which took place in large outdoor pools, and the subsequent observations, conducted in 37 l tanks in the laboratory.

The circular conditioning pools were 18,000 l in size (~ 1 m deep X 4.6 m diameter) and had a flow-through rate of 5 l/min. All conditioning pools contained a single pike (SL = mean \pm 1 SD = 19.0 cm \pm 14.1). There were no lids on the pools so the fishes were also exposed to terrestrial predators (Butt et al. 2005). In particular I observed magpies (*Pica pica*) catching fishes from the pools. The pools were supplied with 40 l of aquatic plants for cover, and a 7 x 7 cm airstone. Minnows and stickleback were stocked in the pools in three ratios (400 minnows: 100 stickleback, 475 minnows: 25 stickleback, and 500 minnows: 0 stickleback) and in all cases left for 14 days. Fishes were supplemented daily with commercial flake food and frozen brine shrimp (*Artemia sp.*).

3.2.3 General set-up and behavioural assays

We used a similar experimental procedure as in Experiment 2 of Chapter 1. Treatments included skin extracts of fathead minnow, brook stickleback and swordtail and each fish was tested only once. My sample size was 25 for each of the three treatments, though my sampling unit was the pool itself which was replicated three times.

3.3. Preparation of stimulus

Skin extracts were prepared as in Chapter 1 using the skin from 52 swordtails (SL = mean \pm 1 SD = 4.2 \pm 0.3 cm), 54 stickleback (SL = 4.5 \pm 0.4 cm) and 49 minnows (SL = 4.5 \pm 0.6). In all cases I added the appropriate amount of distilled water to the skin extract I collected to create a stimulus with a concentration of 1.0 cm² per 10 ml of distilled water.

3.4. Statistical analysis

A sample of 10% of the minnows and 50% of the stickleback were measured prior to conditioning; more stickleback were measured because there were fewer of them. I used a one-way ANOVA to test for survival effects, and a one-way ANOVA (for minnows) and t-test (for stickleback) to be sure all pools started with fishes of equal length.

The data pertaining to the difference in shelter use (post- minus pre-stimulus) was analyzed using a 2-way ANOVA with the pools as replicates (Zar 1999). The dashing and freezing data, which compared the average number of occurrences between treatments is presented in table form (Table 3.1) but was not analyzed due to rarity of occurrence.

3.5. Results

3.5.1. Size effects and survival rate

The mean standard length (\pm SD) of both fathead minnows (4.78 \pm 0.21 cm) and brook stickleback (4.62 \pm 0.16 mm) were equal across all pool types at the outset of the

experiment (minnows: one-way ANOVA, $df = 2$, $F = 1.549$, $p = 0.287$; stickleback: t-test, $df = 4$, $t = -1.01$, $p = 0.41$). The survival rate of minnows from most pools was relatively low (Table 3.2). This probably indicates a large amount of predation by birds as a pike of the size used would be unable to consume that number of prey in the given time. There was no difference in survival rate among pools (one-way ANOVA, $df = 2$, $F = 1.868$, $p = 0.234$). Likewise, the survival rate of stickleback from pools containing either 25 or 100 individuals did not differ significantly (t-test, $df = 4$, $t = 1.369$, $p = 0.243$). Following the conditioning period there was no difference among pools in the length of minnows (one-way ANOVA, $df = 2$, $F = 0.988$, $p = 0.426$).

3.5.2. Behavioural assay for learned recognition

Prior to conducting the ANOVA, the data were analyzed and found to be normal and variances were found to be equal. The results of the two-way ANOVA revealed a significant interaction between the cue given during the assay and the ratio of stickleback to minnows in the conditioning pools (See Figure 3.1) (two-way ANOVA, $df = 4$, $F = 3.181$, $p = 0.038$). Figure 3.1 shows that minnows from pools containing high numbers of stickleback responded to stickleback damage-released cues with an antipredator response (increase in shelter use). In contrast, minnows from the pool containing zero, or low numbers of stickleback, did not respond to the stickleback alarm cues differently than they did to the control.

More dashing and freezing occurred when minnows were exposed to minnow extract than to the control extract, regardless of which pool they came from (see Table 3.1). In contrast, I observed a difference in occurrence of dashing and freezing between

stickleback extract and control swordtail extract only when minnows came from a pool with high numbers of stickleback. The minnows from pools with high numbers of stickleback appeared to show no difference in either dashing or freezing behaviour when exposed to either stickleback or minnow cues. Similarly, minnows showed no obvious difference between minnow and stickleback cues if they originated from pools with low numbers of stickleback.

Table 3.1 Percentage of occurrences (± 1 SD) of dashing and freezing by minnows exposed to fathead minnow (FHM), stickleback (SB) and swordtail (SWT) cues following the conditioning phase (n = 3).

Ratio (FHM:SB)	Cue	Percentage of dashes	Percentage of freezes
400:100	FHM	12 \pm 4.0%	9 \pm 0.58 %
	SB	8 \pm 2.0 %	12 \pm 2.0 %
	SWT	0%	1 \pm 0.58 %
475:25	FHM	13 \pm 4.36 %	19 \pm 2.52
	SB	4 \pm 1.0%	4 \pm 1.0%
	SWT	1 \pm 0.58 %	1 \pm 0.58 %
500:0	FHM	17 \pm 0.58 %	17 \pm 0.58 %
	SB	4 \pm 0.0 %	4 \pm 0.0 %
	SWT	0%	0%

Table 3.2 Mean survival rate (percent survived \pm 1 SD) of minnows (FHM) and stickleback (SB) from the three conditioning regimes (n = 3).

	Conditioning regime		
	400:100	475:25	500:0
SB Survival	27 \pm 13%	52 \pm 32%	-
FHM Survival	48 \pm 19%	25 \pm 19%	28 \pm 21%

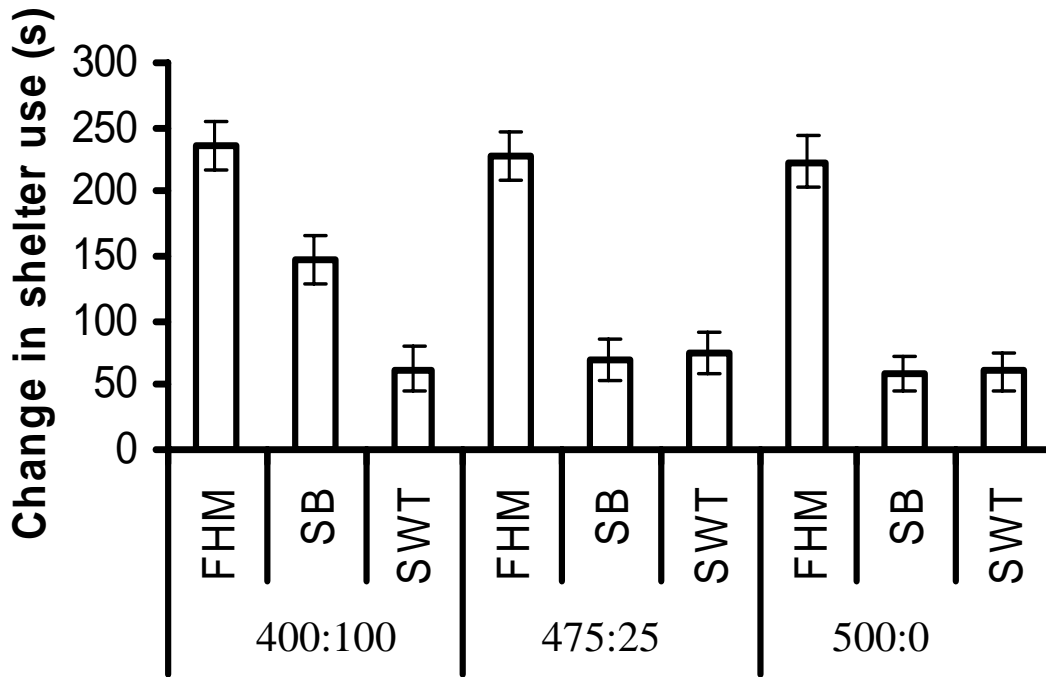


Figure 3.1. Mean (\pm SE) change in time spent under shelter by minnows exposed to fathead minnow (FHM), stickleback (SB) and swordtail (SWT) cues following the conditioning phase (n = 3).

3.6. Discussion

The current study has shown that the relative density of two co-habiting prey species may be very important in mediating the learning of heterospecific cues. The data has also shown that the learning of heterospecific damage-released cues can occur in as short a time as two weeks. With regard to density, I know of no other studies which examine the effects of relative density on the learning, or use, of heterospecific alarm cues. However, some researchers have speculated on the importance of relative density in the use of conspecific damage-released cues. For example, Chivers et al. (1997) speculated that the conspicuous absence of alarm responses in Dunn's salamanders (*Plethodon dunni*) but not other plethodontid salamanders may be a consequence of low density. If the salamanders lived in an area where they would not frequently come into contact with conspecifics, there may be little selection to recognize these cues.

The results of my current study, as well as those of Chapter 2, may provide insights into previously conflicting results in a minnow's response to stickleback damage-released cues (Mathis & Smith 1993, Brown et al.1995). If minnows must learn to recognize stickleback cues, then recognition must be a consequence of the potential opportunities to learn, which may be affected by the ratio of stickleback to minnows in a given population. Since the ratio of any two species will vary on both spatial and temporal scales, future studies must consider these variables when explaining the presence or absence of cross-species responses.

Chapter 4. The effects of habitat complexity on the ability of fathead minnows to learn to recognize stickleback damage-released cues

4.1. Introduction

Chapter 2 demonstrated that minnows and stickleback must co-occur for minnows to learn to recognize stickleback damage-released cues as an indication of predation threat. Chapter 3 showed that minnows and stickleback must co-exist in a ratio high enough as to afford minnows enough ‘chances’ to associate known indications of danger (i.e. conspecific damage-released cues or predator odours) with the novel damage cue. This chapter examines the effects of habitat complexity on a minnow’s ability to learn to recognize stickleback damage-released cues.

Despite the importance of habitat characteristics in driving differences in predation rate (Lima & Dill 1990, Gazdewich & Chivers 2002), we know little about how habitat characteristics influence learning in animals. The goal of the current research was to place learned recognition of heterospecific alarm cues in an ecological context by testing the effects of habitat complexity on learning. Minnows and stickleback are known to co-occur in a diversity of environments from small streams to large rivers and lakes. They often co-occur in highly structured habitats (i.e. in areas with dense vegetation and benthic structure) but also co-occur in areas with minimal habitat complexity. Given this fact, I pose the question: Does habitat complexity (i.e. the

presence of structure that obscures the visual environment) play a factor in mediating learning of heterospecific alarm cues? I propose two mechanisms by which structure may play a role in the learning process.

First, the presence of structure may influence the relative predation on the two species and hence influence the potential number of opportunities to learn. If structure directs a greater proportion of predator attacks towards stickleback, then minnows will have greater opportunities to learn to correlate stickleback damage-released cues with known odours. In contrast, if shelter directs more predation towards minnows, then there will be fewer opportunities for minnows to learn to recognize stickleback damage-released cues. Similarly, the presence of shelter could influence the spatial relationship between the two species and thus influence the probability that an individual would be within the appropriate distance to visually or chemically detect a predator attacking a stickleback and therefore the opportunity to associate predation with the smell of the heterospecific alarm cue.

4.2. General methods

4.2.1. Collection and maintenance

As in Chapter 3, all minnows used in this experiment were collected from Briarwood pond and all stickleback from Lakeview pond; they were collected and placed into the outdoor conditioning ponds on the same day. Conditioning lasted only eight days after which time the surviving minnows were brought into the laboratory and held under the same condition as Chapter 2 until behavioural assays began. Similar to Chapters 2 and 3, minnows were transferred to the observation tanks 48 hrs prior to the

start of the trials to allow for acclimation. The photoperiod in the testing tanks was also 14:10 h light:dark cycle.

4.2.2. General set-up and conditioning regime

The pools were 18,000 l, circular in shape (~ 1 m deep X 4.6 m across) and had a flow through rate of 5 l/min. A white, semi-transparent tarp was suspended over the pools to avoid predation by birds. The pools representing the highly complex habitat contained nine 60 X 60 X 3 cm Styrofoam blocks under which hung 81 pieces of twine (60 cm in length) held down with flat nickel-plated washers. The pools also contained five circular plumbing coils (2.5 cm width X 16.6 m length) suspended in the water. The coils and the suspended twine visually obscured the pools. For ground cover, 15 cinder blocks were placed along the bottom of the pools. The cinder blocks were rectangular in shape (20 X 20 X 40 cm), and had two holes passing completely through them (15 X 15 cm).

The pools representing low habitat complexity were of the same size, with the same flow rate and same semi-transparent tarp. They also had nine 60 X 60 X 3 cm Styrofoam blocks, however, the blocks lacked the suspended twine. The Styrofoam was added to the pools to keep the light levels in the two pool types the same. The pools representing the low complexity environment also lacked cinder blocks. Consequently, the only visual obstruction resulted from the plumbing coils. Each of the pools (four with high complexity and four with low complexity) contained 400 minnows and 100 stickleback, with one pike (~ 1 kg) as a predator. During the conditioning phase the

fishes were supplemented with commercial flake food and frozen brine shrimp (*Artemia* sp.)

4.2.3 General set-up and behavioural assays

I used a similar experimental procedure as in Experiment 2 of Chapter 1. Treatments included skin extracts of fathead minnow, brook stickleback and swordtail and each fish was tested only once. My sample size was 25 for each of the three treatments, though my sampling unit was the pool itself which was replicated three times.

Behavioural observations were completed in 37 l glass aquaria filled with dechlorinated tap water as in previous chapters. All materials and methods were similar to Chapter 3 with the exception of a decrease in the sub-sample size taken from each pool (sub-n = 12) and the deletion of fathead minnow skin extract as a treatment. Fathead minnow extract was deleted as a treatment because Chapters 2 and 3 had demonstrated that minnows which had learned to respond to stickleback cues responded with equal intensity when compared with the conspecific cue, thus a conspecific was deemed redundant.

4.3. Preparation of stimulus

The same methods were used to create the skin extracts as in previous chapters. To prepare the stickleback skin extract, 28 adult stickleback (SL = mean \pm SD = 4.7 \pm 0.5 cm) and 29 adult swordtails (4.4 \pm 0.3 cm) were used.

4.4. Statistical analysis

As in Chapter 3, a subset of minnows and stickleback were measured prior to the conditioning phase to be sure all pools started with fishes of similar mean length. As there were only two pool types in the current experiment's conditioning phase, a t-test was used. A two-way ANOVA was used to calculate a change in shelter use, using habitat type and damage-released cue as factors. Therefore, my pools were the sampling unit and all means from the pools were compared. To calculate the difference in survival rate a two-way ANOVA was used with habitat type and species as factors.

4.5. Results

4.5.1. Conditioning and survival rate

A sample of 40 minnows and 25 stickleback were measured (SL) before being added to the pools to ensure there was no difference in the size of fishes between treatments (t-test, minnows: $t = -0.035$, $n = 4$, $df = 6$, $p = 0.973$ (two-tailed); stickleback: $t = 0.146$, $n = 4$, $df = 6$, $p = 0.889$ (two-tailed)).

A two-way ANOVA revealed that the survival rate was not influenced by shelter ($F_{1,12} = 0.867$, $p = 0.370$) or species ($F_{1,12} = 1.44$, $p = 0.252$), nor was there a significant interaction between shelter and species ($F_{1,12} = 0.824$, $p = 0.382$) (Table 4.1).

Table 4:1 Mean survival rate (percent survived \pm 1 SD) of minnows (FHM) and stickleback (SB) from the two habitat types (n = 4).

	Habitat type	
	Structured	Non-structured
SB Survival	29.0 \pm 8.6 %	29.5 \pm 24.2 %
FHM Survival	32.1 \pm 21.3 %	51.8 \pm 26.8 %

4.5.2. Behavioural assay

Both dashing and freezing behaviours were rare; consequently I did not conduct statistical tests on these data (but see Table 4.2). Prior to analyses of shelter use data, I ensured all the assumptions of an ANOVA were met. I observed that minnows showed a general increase in shelter use in response to all of the test stimuli (Figure 4.1). However, the intensity of the antipredator response of minnows from the low complexity habitat to stickleback alarm cues was considerably greater. The results of the two-way ANOVA revealed that there was an interaction between the two factors ($F_{1,12} = 10.296$, $p = 0.008$). From these data we can conclude that fathead minnows learned to recognize stickleback damage-released cues in the low complexity habitat but not in the high complexity habitat.

4.6. Discussion

The importance of habitat characteristics in driving different predation rates is well established in ecology (e.g. Lima & Dill 1990; Gazdewich & Chivers 2001). In structurally complex habitats, prey generally benefit because they have the opportunity to increase refuge use (Petersen et al. 1999). Moreover, structure increases the search time of predators (Kaiser 1983). However, little is known about how habitat characteristics influence the propensity of animals to learn to recognize predation risk cues. My results demonstrate that habitat complexity can be an important factor mediating the learning of heterospecific damage-released cues. Structure inhibited the ability of minnows to learn to recognize stickleback cues. My results may provide important insights into ecological constraints on learned recognition in other systems. I speculate that other habitat characteristics may similarly influence learned recognition in fishes. For example, an increase in turbidity, while increasing the probability that a minnow will respond to an alarm cue (Hartman & Abrahams 2000), will decrease the transmission of visual cues (as does structure) and hence may reduce propensity for learning. However, this reduction in learning may be offset if animals in the turbid environment are more likely to respond to chemical cues (Hartman & Abrahams 2000).

Table 4.2. Percentage of occurrence of dashing and freezing by minnows exposed to stickleback (SB) and swordtail (SWT) cues following the conditioning phase (n = 4).

Habitat type	Cue	Percentage of dashes	Percentage of freezes
Structured	SB	0 %	0 %
	SWT	2.0 %	2.0 %
Non-structured	SB	4.2 %	0%
	SWT	2.0 %	4.2 %

There are several potential reasons why I observed differences in learning between the two habitat types. Initially, I hypothesized that the presence of structure could influence the relative predation on the two species and hence any differences in learning could result from differences in opportunities to learn. Likewise, the presence of structure could influence the spatial relationship between the two species and this could influence the opportunities to learn. The fact that I observed no differences in relative survival rate between the two conditioning types means that differences in learning, in my experiment, is likely a result of differences in spatial distribution between the two species in the different habitats. Although not quantified, my casual observations indicate that minnows were more likely to be associated with the structure than were stickleback.

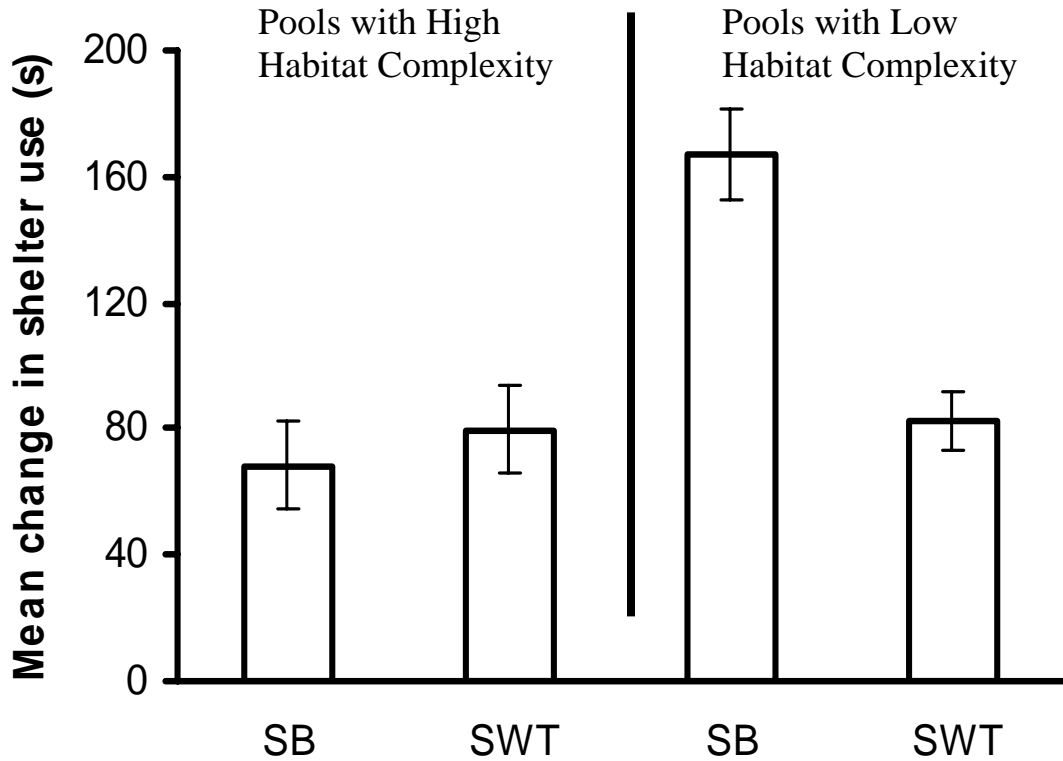


Figure 4.1. Mean (\pm SE) change in time spent under shelter by minnows exposed to stickleback (SB) and swordtail (SWT) cues following the conditioning phase where they were held in habitats with low or high complexity ($n = 4$).

So, while equal numbers of stickleback were being preyed upon in either habitat types, the stickleback may not have been in the immediate area to chemically or visually “witness” the event. To better address this question future research designed to examine ecological constraints on learning should consider the influence of habitat on spatial segregation, as well as the benefits of visual aids in learning chemical cues.

Chapter 5: Individual variation in the intensity of antipredator responses to damage-released cues: effects of body size, body condition and breeding state.

5.1. Introduction

While several species responses to damage-released cues have been documented in the literature (for review see Chivers & Smith 1998), few studies have examined the factors that may affect the intensity of the response to damage-released cues. The few studies examining factors affecting the use of chemical cues have concentrated on factors external to the subject. For example, it has been well established that fishes will change their response to correlate with the concentration of predator cues, as well as size and presumably relative danger of a known predator (Kusch et al. 2004). Studies have also indicated that the condition of the victim releasing the cue will also affect the behaviour of cue recipients. Brown et al. (2004) found that both adult and juvenile convict cichlids (*Cichlasoma nigrofasciatus*) responded stronger to damage-released cues from donors with a high body condition versus individuals with a low body condition. Further studies examining the external factors affecting a fish's response to damage-released cues found that visual cues can mediate antipredator responses. Hartman and Abrahams (2000) found that in the presence of threatening visual cues, fathead minnows responded to conspecific damage-released cues with greater intensity when compared to minnows exposed to the same cues paired with "safe" visual information.

Despite the numerous studies examining the threat-sensitive nature of fishes' responses in relation to external factors, as seen in the above examples, few studies have examined the threat-sensitive nature of a minnow's response as it relates to factors intrinsic to the fish. One of the few studies that has examined such factors demonstrated that minnows deprived of food for 24 hours will not respond to damage-released cues (Brown & Smith 1996). However, in the same study, Brown and Smith noted that minnows are capable of learning to recognize a new cue as a predation threat during this time, if the novel odour is paired with a known odour (i.e. conspecific damage-released cue), but will not respond until they are satiated. Studies of this type demonstrate that the learning and use of chemical alarm cues is not a simple on/off switch, but in reality animals may be taking several factors into consideration before responding to a predation risk.

The purpose of the current chapter is to expand on this knowledge and determine if body condition, experience, or breeding condition will affect the intensity or presence or absence of a response to damage-released cues in fathead minnows. I hypothesize that fishes in poor body condition, similar to fishes deprived of food (Brown & Smith 1996), may take greater risks and demonstrate a decreased antipredator response. Conversely, emaciated individuals may not have the energy reserves to risk confrontation with a predator and may view exposure to damage-released cues as a riskier event than an individual with an abundance of energy; thus, we may see individuals with higher body conditions taking greater risks.

I further hypothesize that longer, and presumably older, individuals will have more experience with predation and should be more likely to respond to damage-released

cues requiring learning (i.e. damage-released cues from unrelated known heterospecifics). Aside from the learned cues, I predict that minnows of all lengths should respond to the conspecific cues as well as the controls with equal intensity.

Finally I hypothesize that fishes in an advanced state of breeding may take greater risks when given an indication of predation, particularly the males as they claim and defend territories at these times. Conversely, both genders may be more cautious, demonstrating increased antipredator responses with increased state of primary and secondary sexual characteristics (advanced gonad development, tubercles, etc.). This may be due mostly to the inability to respond quickly to a more immediate predation threat (decreased mobility due to increased gonad size), thus, they should treat an earlier warning, such as a chemical cue, with more gravity.

5.2 General methods

5.2.1. Collection and maintenance of fish

Trials were conducted in two chronologically separate, but otherwise equal parts. In part one, fishes were collected prior to the breeding season (December 2003) for the purposes of ascertaining the responses of non-breeding individuals. In part two, individuals were collected in the middle of the breeding season (June 2004) to record their responses while in an advanced reproductive state. In both cases fishes used in the study were collected from Feedlot pond. Stickleback and minnows have co-occurred at this pond for the past six years (see Chapter 2) and are known to respond to the damage-released cues of one another (Chapter 2, Wisenden et al. 2003). Fishes were collected using Gee's improved minnow traps and immediately transported to the laboratory and

maintained in a 151 l glass tank at approximately 18 °C on a 14:10 h light:dark cycle, and fed daily with commercial fish food.

5.2.2. General set-up and behavioural assays

Behavioural assays were conducted as in Chapters 2-4. Following trials, minnows were removed from the observation tanks and given a lethal dose of MS-222 (methane tricaine sulfonate). Data including subject weight and length was recorded, as well as gonad weight for fish in the breeding season, as fish gonads regress during the non-breeding season. All raw data were used to calculate age (a correlate of standard length: length from the tip of nose to the last body scale), body condition (calculated by weight/standard length³) and gonadosomatic index (calculated by gonad weight/body weight).

5.3. Preparation of skin extracts

The same methods were used to create the skin extracts as in previous chapters. To prepare the stickleback skin extract, nine adult stickleback (SL = mean \pm SD = 4.74 \pm 0.26 cm), seven adult minnows (4.47 \pm 0.56 cm) and eight adult swordtails (4.98 \pm 0.05 cm) were used.

5.4. Statistical analysis

Prior to the calculation of the correlations, the difference in shelter use was compared using a Kruskal-Wallis test followed by Dunn's paired comparisons. This was done for two reasons, first to be sure the minnows were responding in a manner similar to

previous studies (i.e. see previous chapters); second, to note any changes in their overall response between the breeding and non-breeding periods.

Correlations were derived by plotting the difference in shelter use by treatment with each factor. Prior to statistical analysis, I first found the model that best fit the data. In all but one case (Figure 5.7B, Logistic model) a linear model (Model 1) was the best fit, thus a Pearson correlation was used for analysis. For the non-linear case, a Spearman rank correlation was used. All statistics were calculated using SPSS version 13.0.

5.5. Results

5.5.1 Experiment 1 - Global analysis

Kruskal-Wallis tests revealed a significant difference in shelter use among treatments ($X^2 = 8.336$, $df = 3$, $p = 0.04$, $n = 36$). Subsequent Dunn's paired comparisons revealed the only significant difference to be between minnow damage-released cues and the control of swordtail ($n = 36$, $p < 0.05$, Figure 5.1). All other paired comparisons proved to be non-significant.

5.5.2 Experiment 1 – Correlations

Pearson correlations conducted on part one of the study (non-breeding individuals) revealed a correlation between standard length and a minnow's response to fathead minnow and stickleback damage-released cues (Figures 5.2A and 5.2B). Longer, and presumably older and more experienced minnows, demonstrated an increase in shelter use in responses to both conspecific cues ($R^2 = 0.12$, $p = 0.021$, $n = 36$) and known heterospecific cues ($R^2 = 0.16$, $p = 0.048$, $n = 36$). While a statistically similar

trend was noted in response to the unknown swordtail cues, little of the variation was represented ($R^2 = 0.05$, $p = 0.072$, $n = 36$, Figure 5.2C). There was no correlation in response to distilled water (see Figure 5.2D).

Correlations between body condition index and any of the four treatments failed to produce any significant results (see Figures 5.3A, B, C and D).

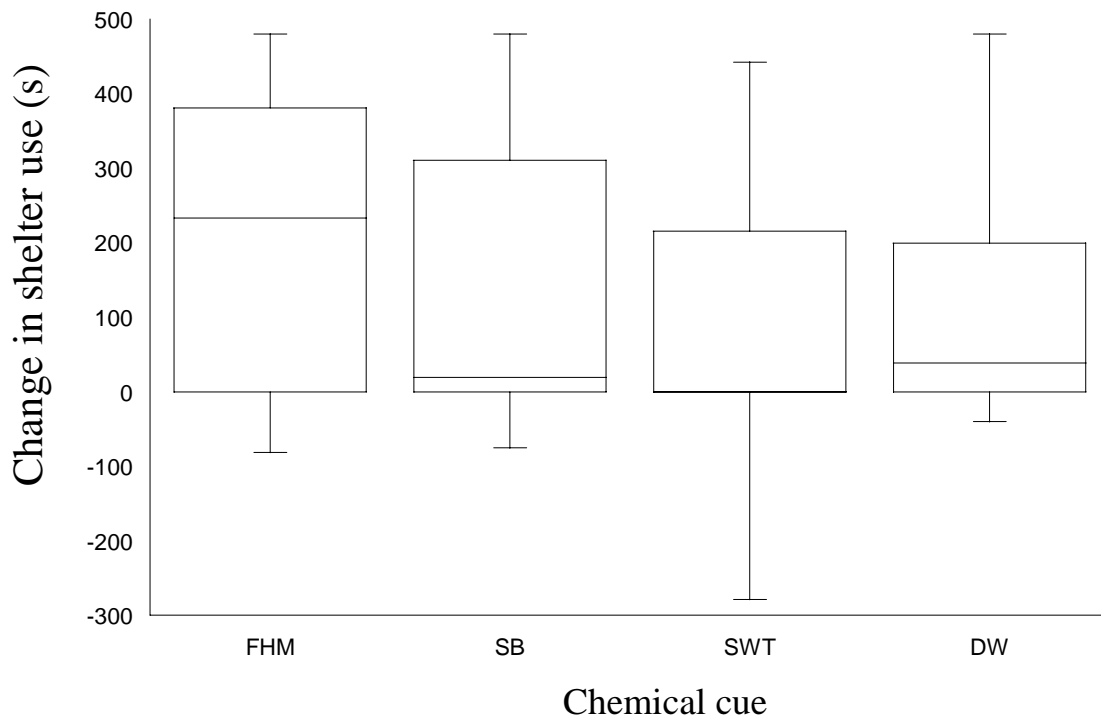


Figure 5.1. Difference in shelter use by non-breeding fathead minnows exposed to the skin extracts of fathead minnows (FHM), brook stickleback (SB), swordtails (SWT) and a control of distilled water (DW) (Median value within box with 25th and 75th percentile on either side surrounded by the range (error bars)) ($n = 36$).

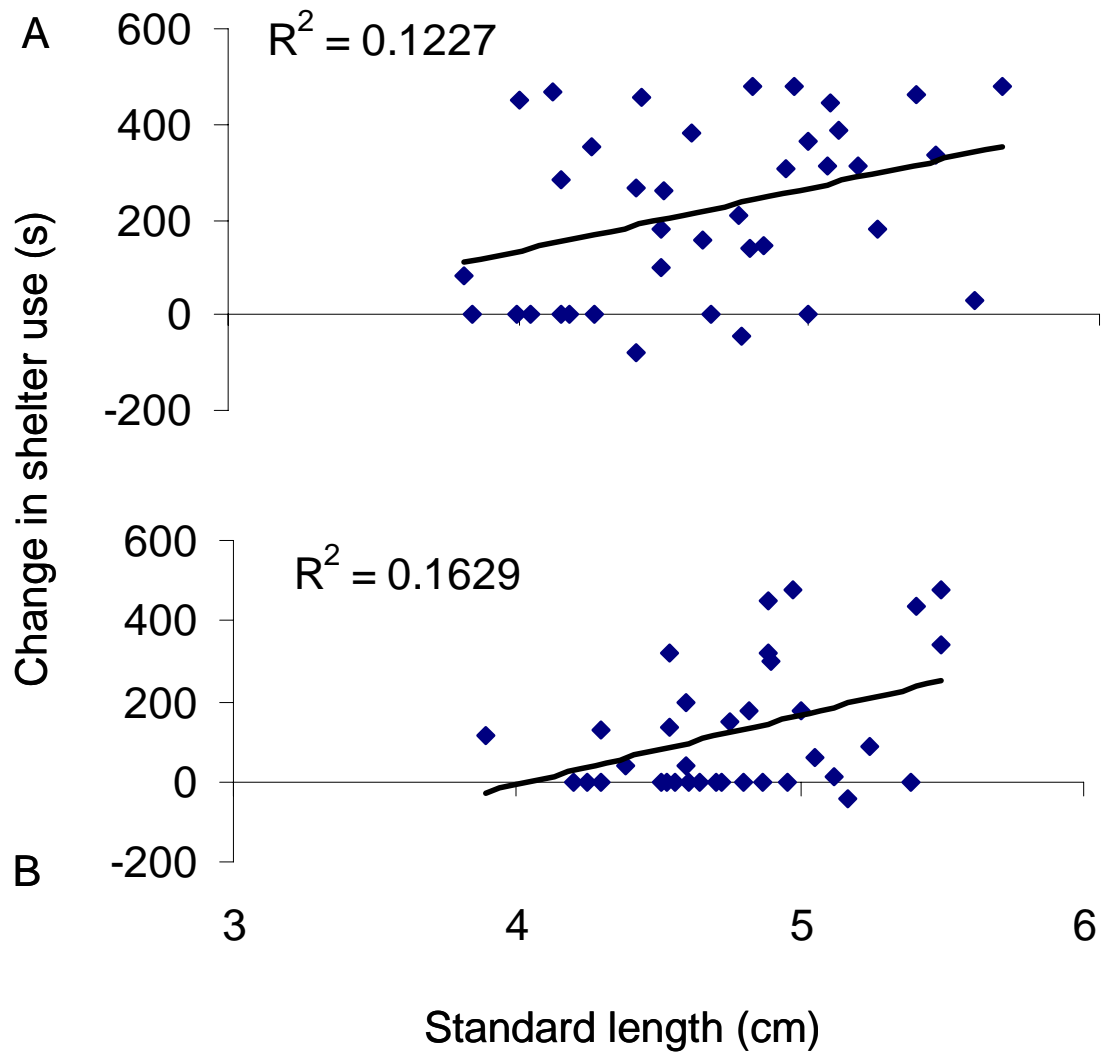


Figure 5.2A. Correlation between non-breeding fathead minnow standard length (SL) and change in shelter use in response to fathead minnow damage-released cues ($n = 36$).

Figure 5.2B. Correlation between non-breeding fathead minnow standard length (SL) and change in shelter use in response to stickleback damage-released cues ($n = 36$).

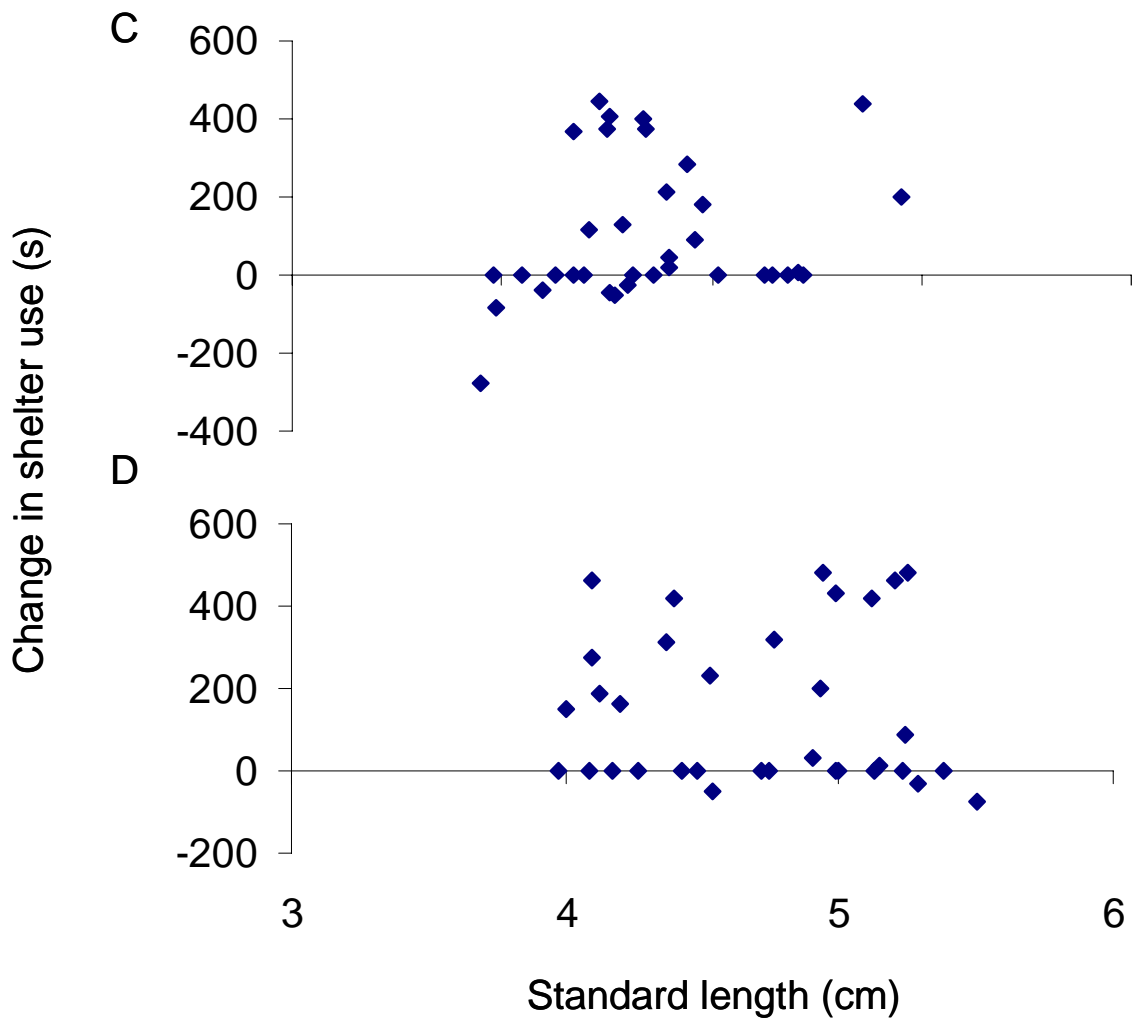


Figure 5.2C. Correlation between non-breeding fathead minnow standard length (SL) and change in shelter use in response to swordtail damage-released cues (n = 36).

Figure 5.2D. Correlation between non-breeding fathead minnow standard length (SL) and change in shelter use in response to distilled water (n = 36).

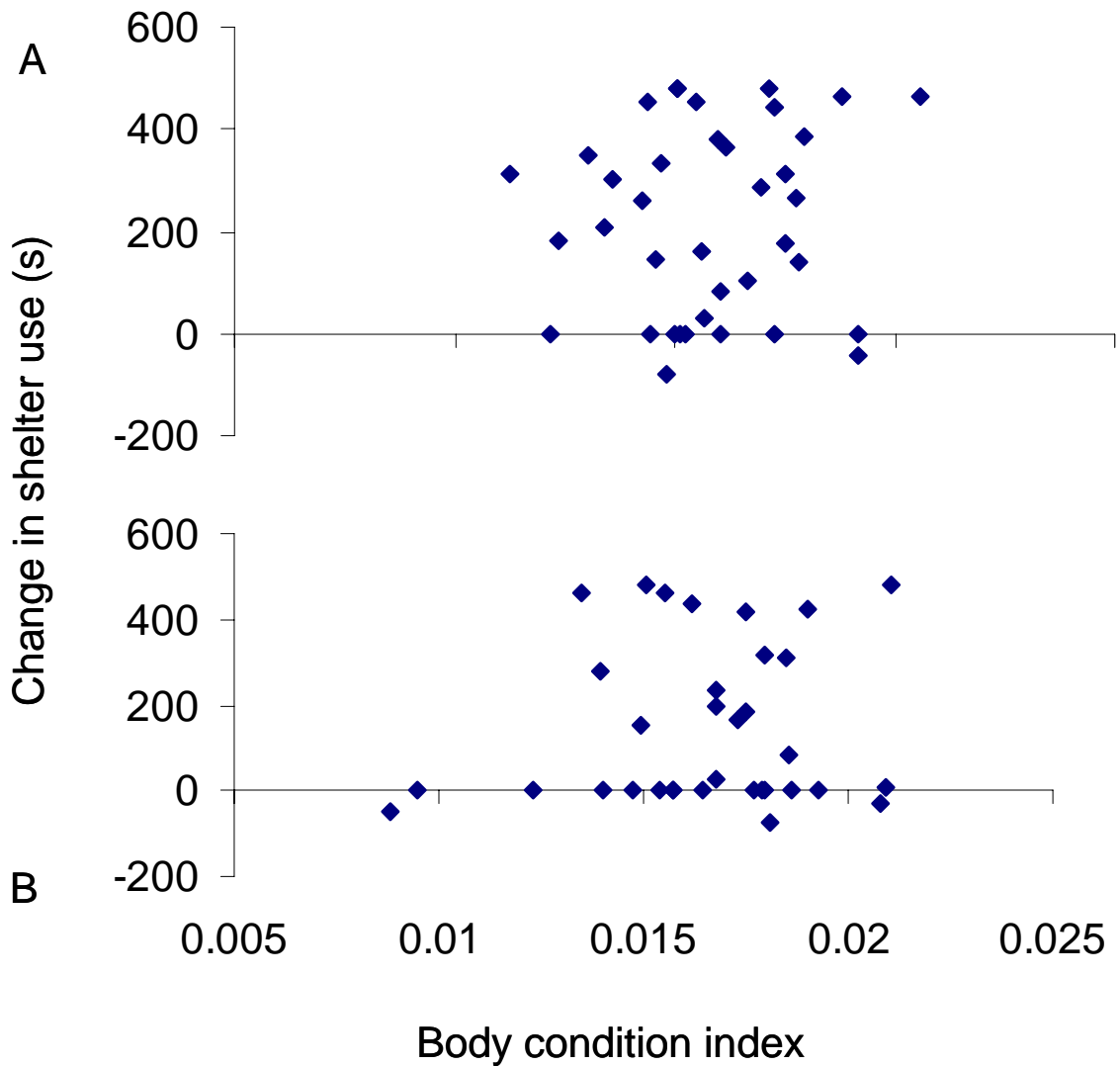


Figure 5.3A. Correlation between non-breeding fathead minnow body condition index (BCI) and change in shelter use in response to fathead minnow damage-released cues (n = 36).

Figure 5.3B. Correlation between non-breeding fathead minnow body condition index (BCI) and change in shelter use in response to stickleback damage-released cues (n = 36).

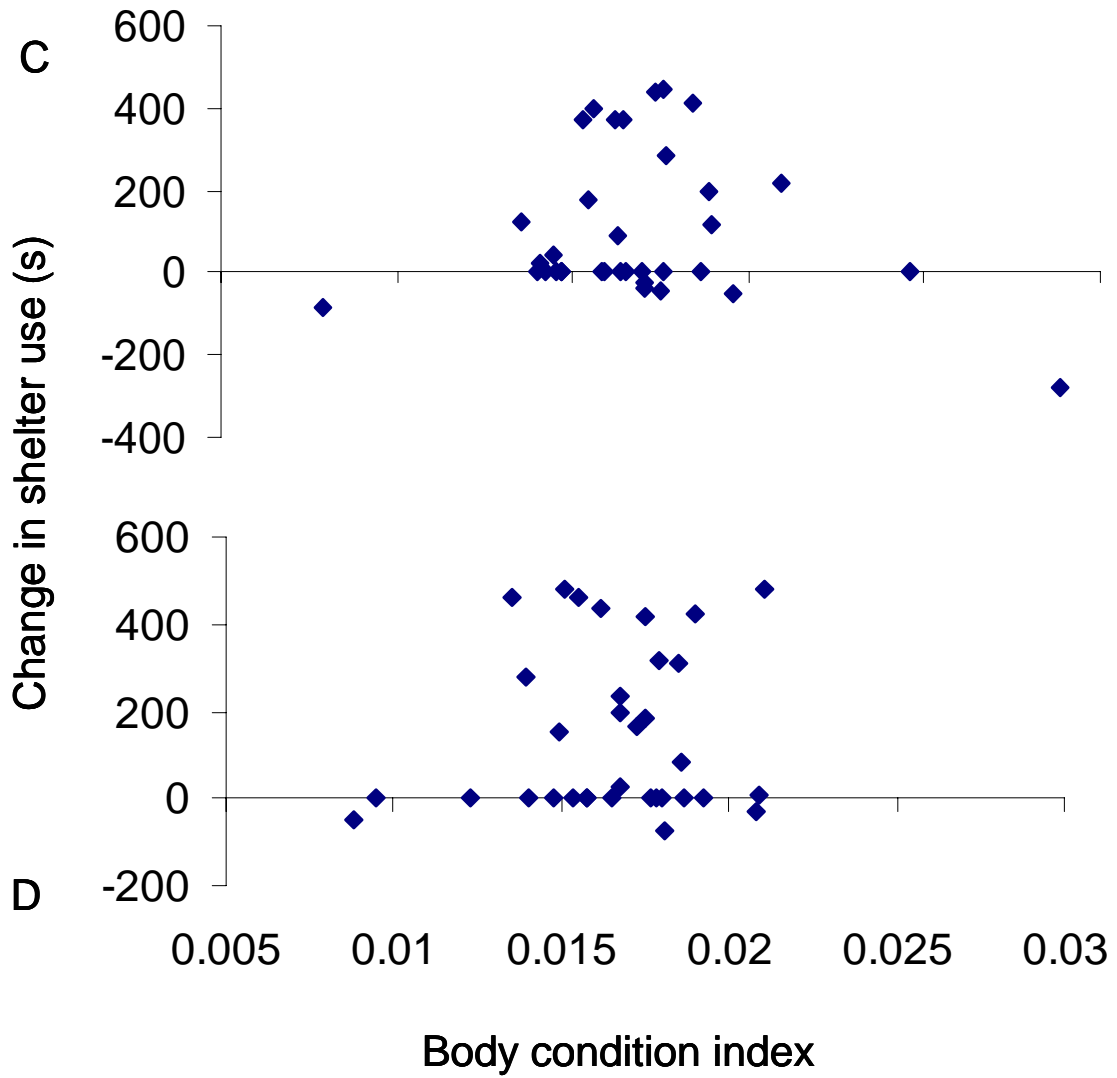


Figure 5.3C. Correlation between non-breeding fathead minnow body condition index (BCI) and change in shelter use in response to swordtail damage-released cues (n = 36).

Figure 5.3D. Correlation between non-breeding fathead minnow body condition index (BCI) and change in shelter use in response to distilled water (n = 36).

5.5.3 Experiment 2 - Global analysis

Kruskal-Wallis tests in Experiment 2 also revealed a significant difference in shelter use among treatments ($X^2 = 11.86$, $df = 3$, $p = 0.008$, $n = 38$, Figure 5.4). The response by fathead minnows to minnow damage-released cues were significantly higher than both swordtail and distilled water cues ($p < 0.05$, Figure 5.4). Also significant was the difference in response to stickleback and distilled water cues ($p < 0.05$, Figure 5.4). All other paired comparisons proved to be non-significant.

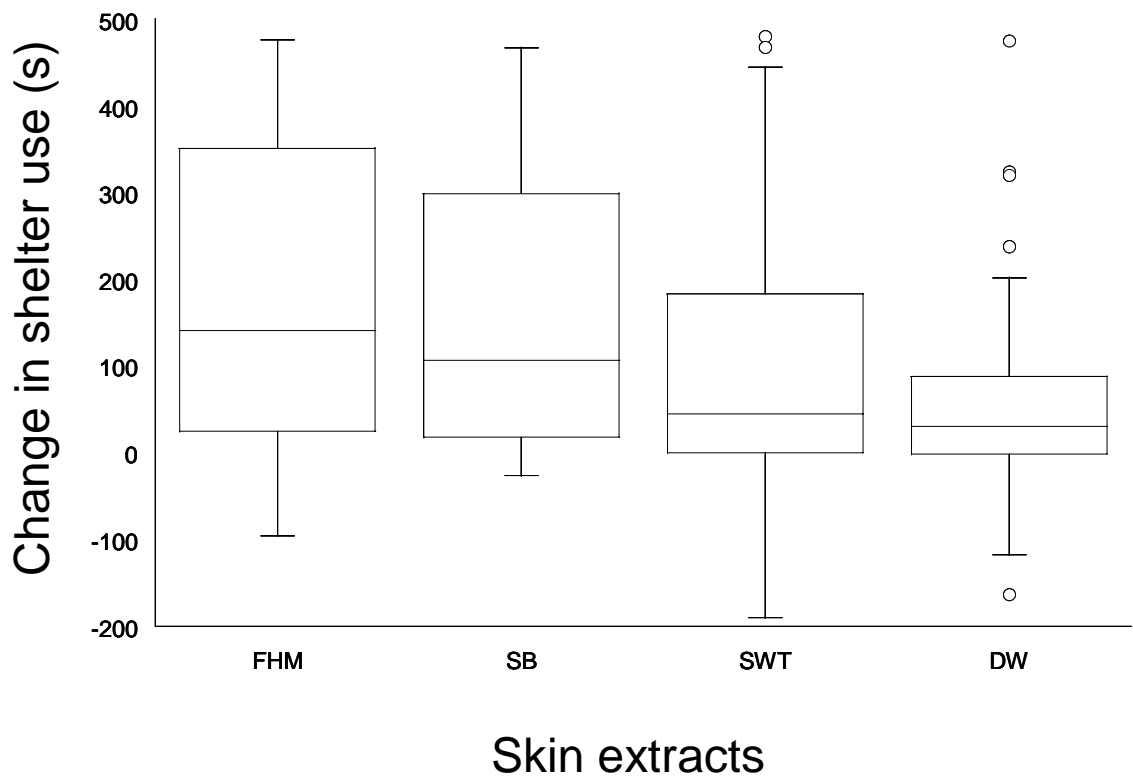


Figure 5.4 Difference in shelter use by breeding fathead minnows exposed to the skin extracts of fathead minnows (FHM), brook stickleback (SB), swordtails (SWT) and a control of distilled water (DW) (circles are outliers greater or less than one and a half times the interquartile distance) ($n = 38$).

5.5.4 Experiment 2 - Correlations

Correlations between antipredator responses and both standard length and body condition during the breeding season failed to produce any significant results (Figures 5.5A, B, C, and D and 5.6A, B, C and D). Correlations between gonadosomatic index and any of the four treatments failed to produce any significant results (see Figures 5.7A, B, C and D). However, a strong statistical trend was noted in the minnow's response to stickleback cues (Spearman rank correlation $R = 0.42$, $p = 0.055$, $n = 18$, Logistical model fit $F = 5.038$, $p = 0.04$, Figure 7B).

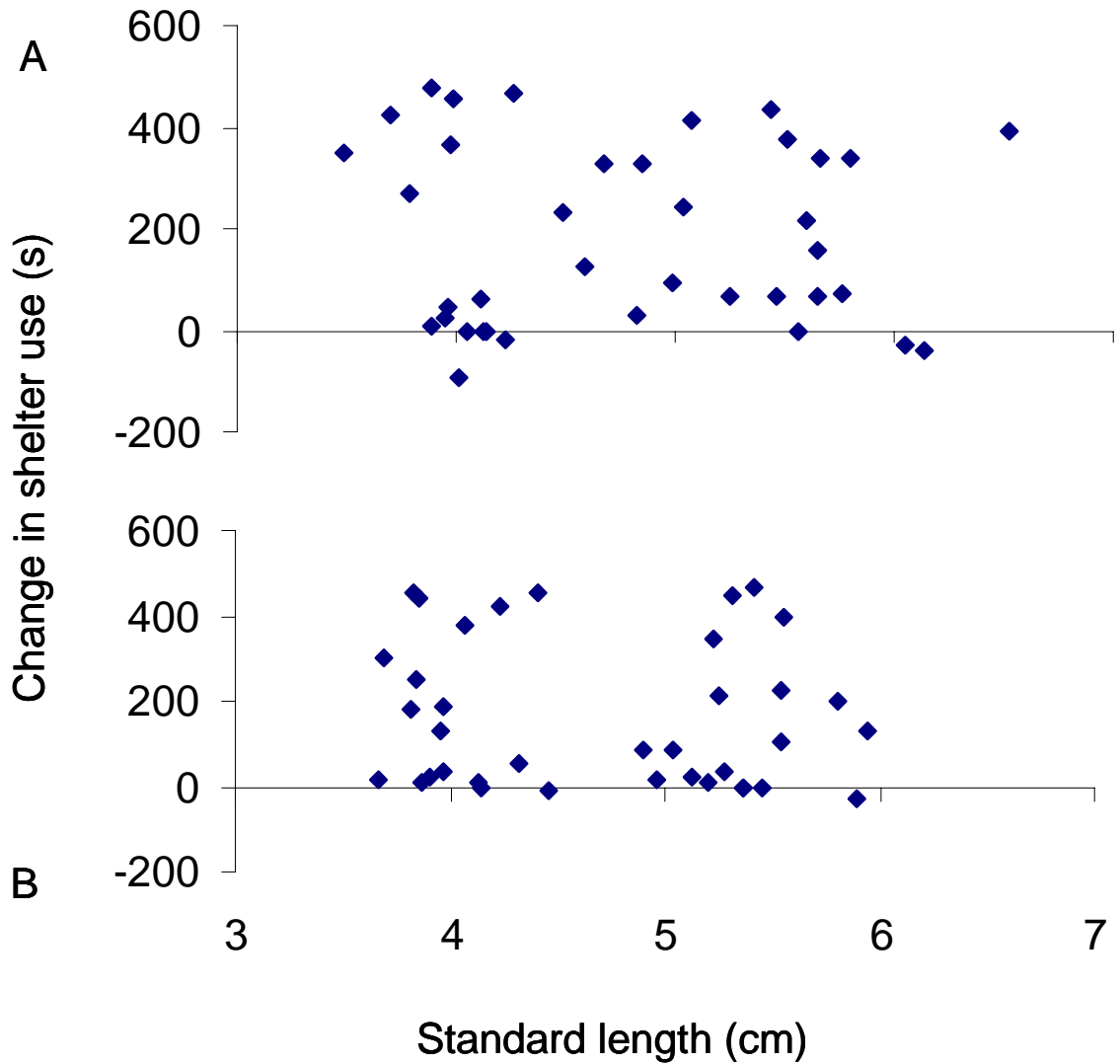


Figure 5.5A. Correlation between the standard length (SL) of fathead minnows in breeding condition and change in shelter use in response to fathead minnow damage-released cues (n = 38).

Figure 5.5B. Correlation between the standard length (SL) of fathead minnows in breeding condition and change in shelter use in response to stickleback damage-released cues (n = 38).

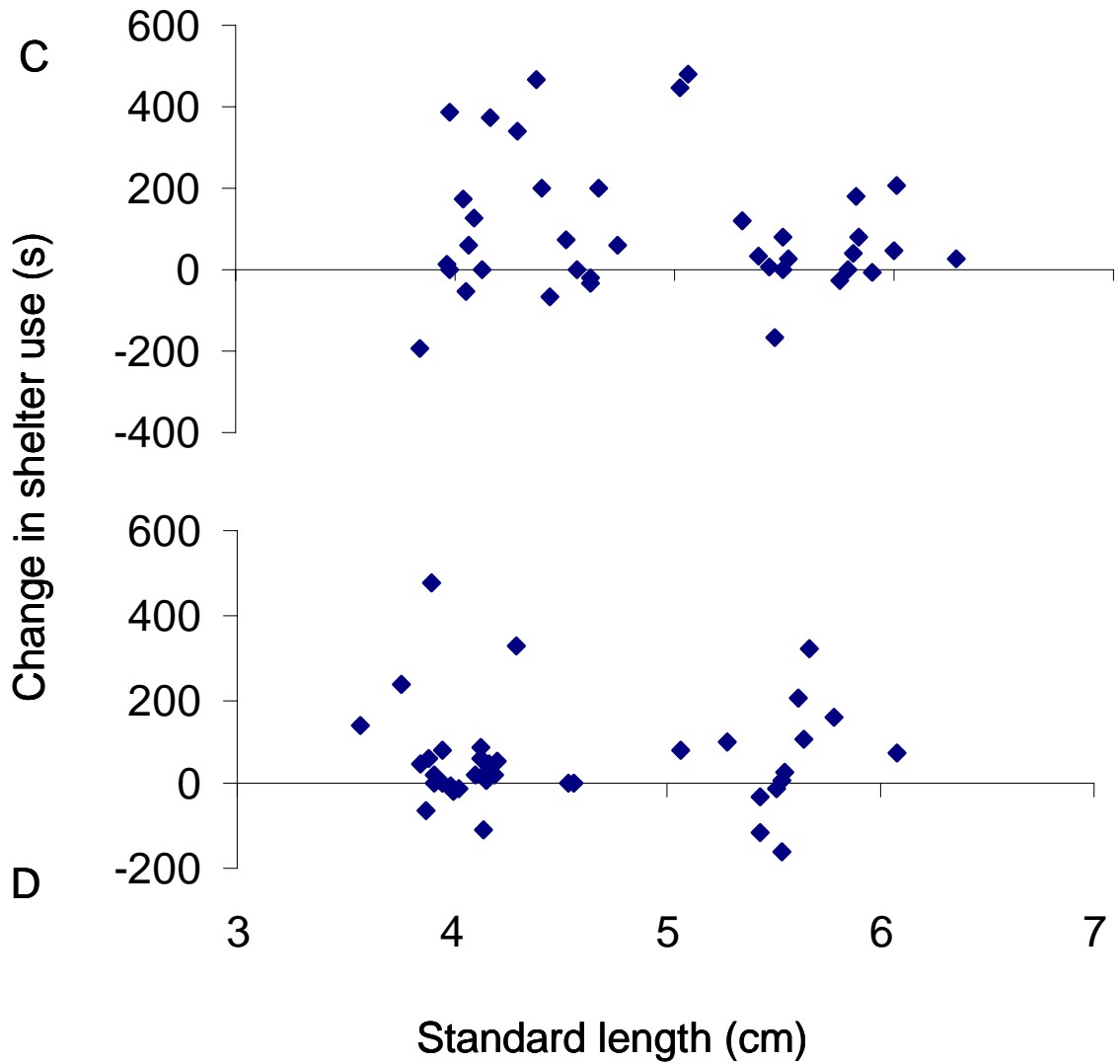


Figure 5.5C. Correlation between the standard length (SL) of fathead minnows in breeding condition and change in shelter use in response to swordtail damage-released cues (n = 38).

Figure 5.5D. Correlation between the standard length (SL) of fathead minnows in breeding condition and change in shelter use in response to distilled water (n = 38).

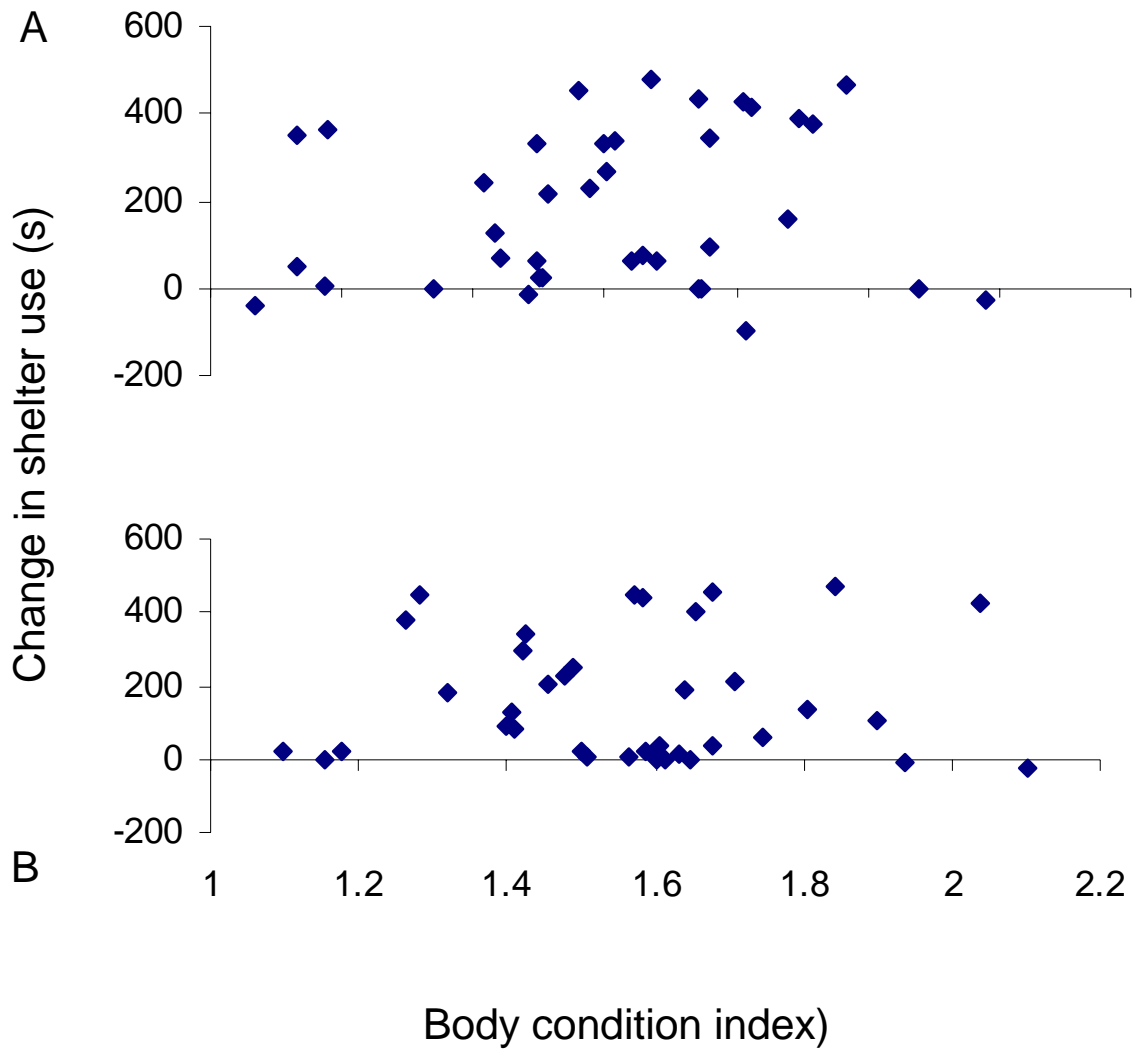


Figure 5.6A. Correlation between fathead minnow body condition index (BCI) and change in shelter use in response to fathead minnow damage-released cues (n = 38).

Figure 5.6B. Correlation between fathead minnow body condition index (BCI) and change in shelter use in response to stickleback damage-released cues (n = 38).

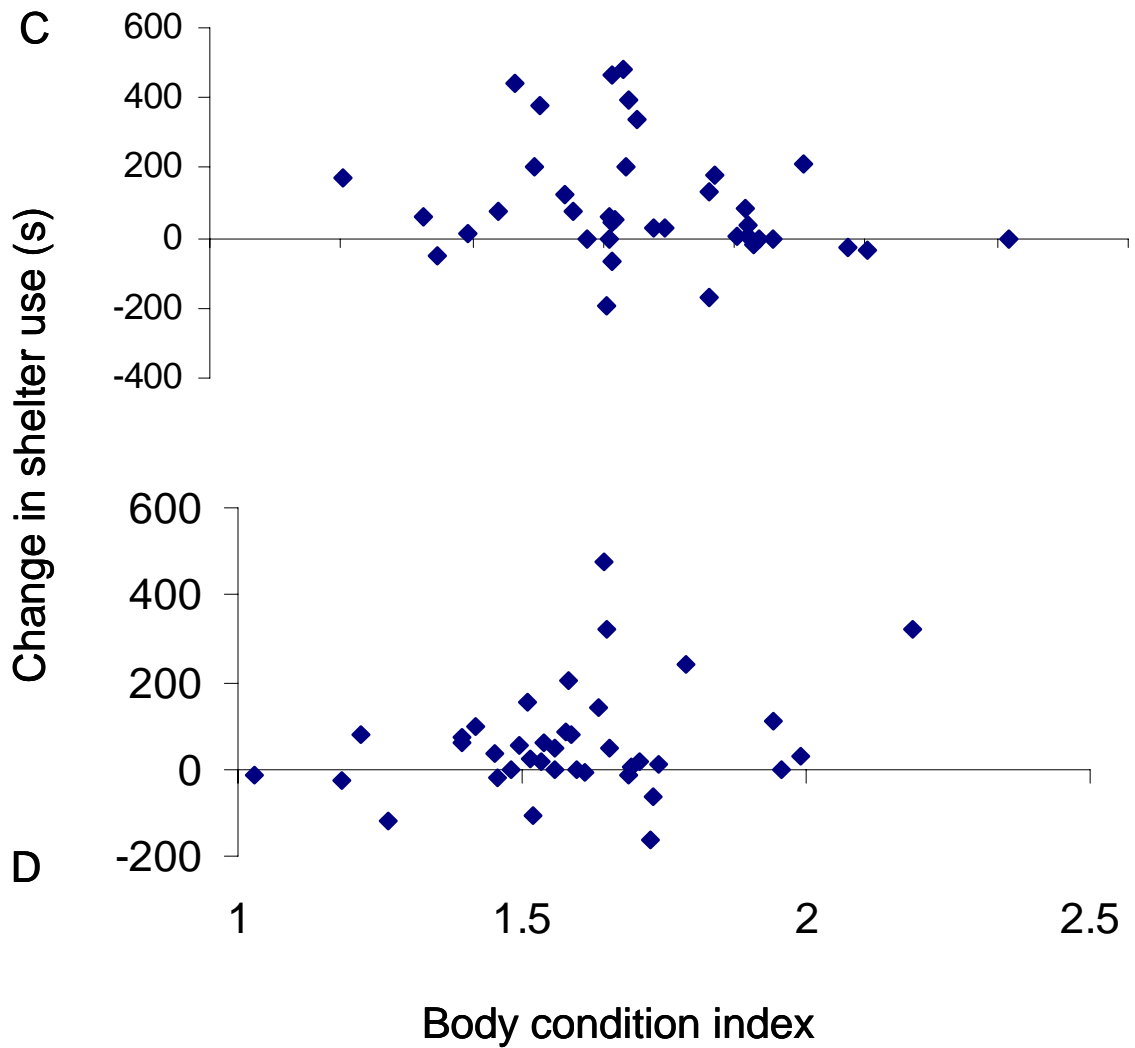


Figure 5.6C. Correlation between fathead minnow body condition index (BCI) and change in shelter use in response to swordtail damage-released cues (n = 38).

Figure 5.6D. Correlation between fathead minnow body condition index (BCI) and change in shelter use in response to distilled water (n = 38).

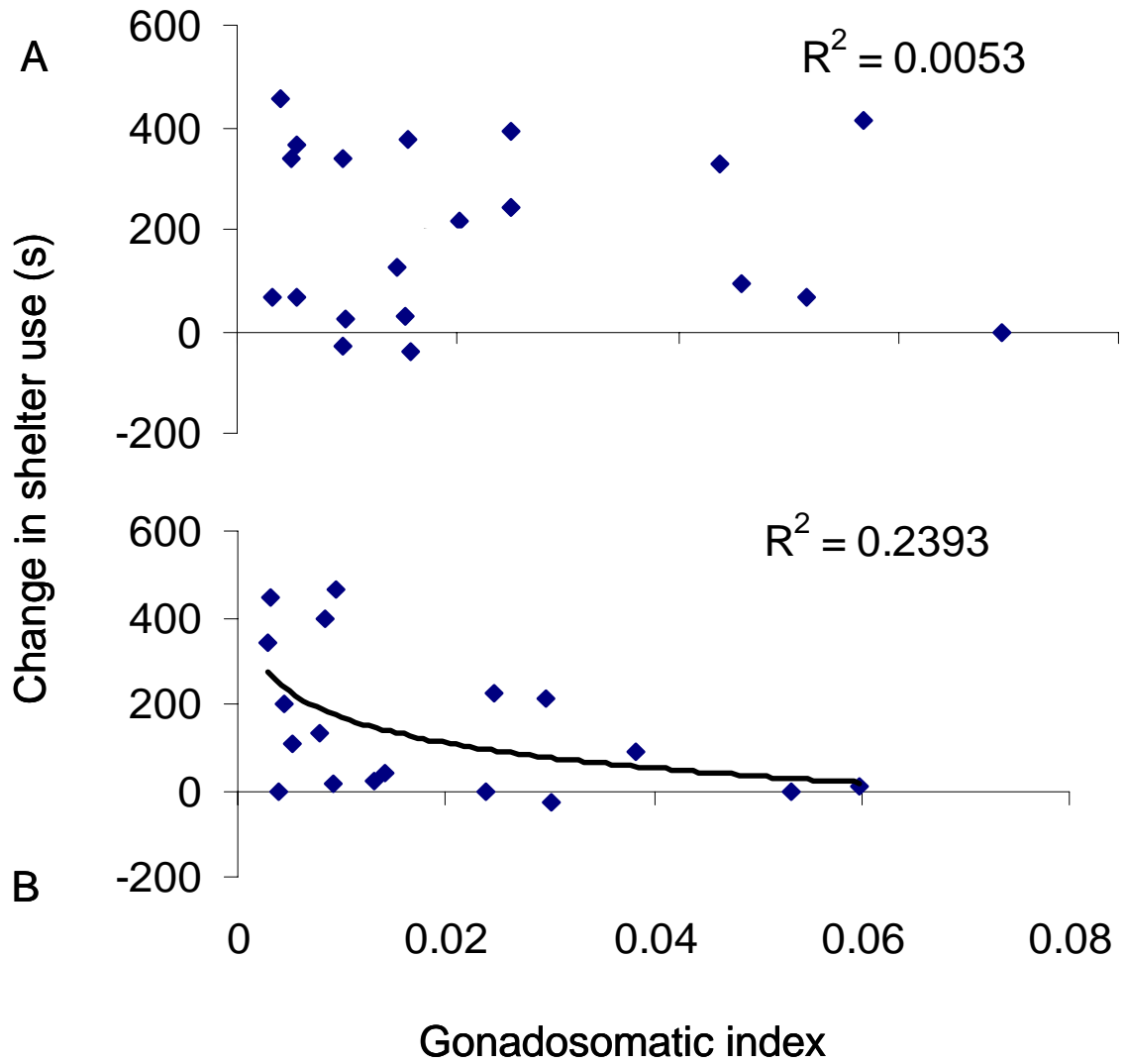


Figure 5.7A. Correlation between fathead minnow gonadosomatic index (GSI) and change in shelter use in response to fathead minnow damage-released cues (n = 20).

Figure 5.7B. Correlation between fathead minnow gonadosomatic index (GSI) and change in shelter use in response to stickleback damage-released cues (n = 18).

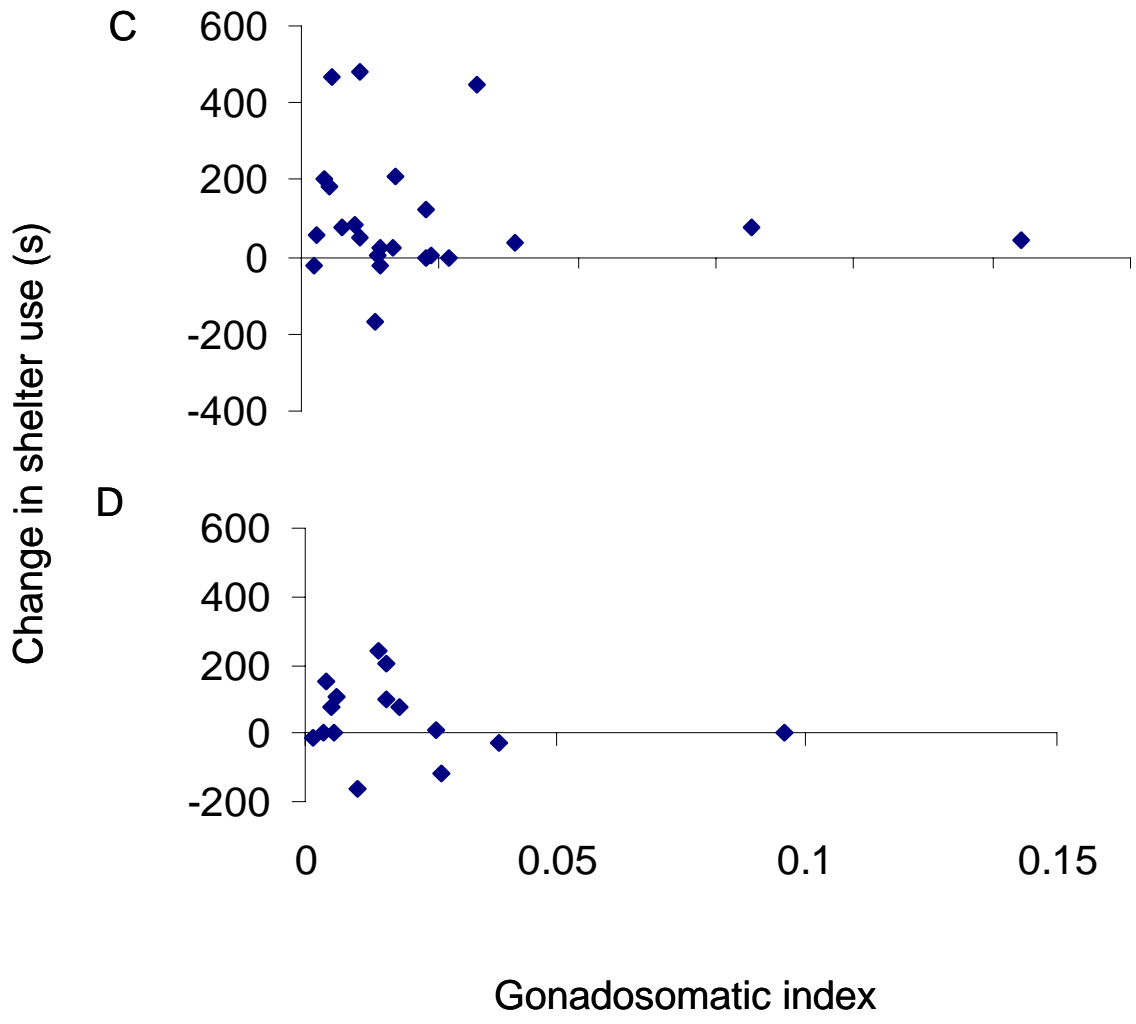


Figure 5.7C. Correlation between fathead minnow gonadosomatic index (GSI) and change in shelter use in response to swordtail damage-released cues (n = 23).

Figure 5.7D. Correlation between fathead minnow gonadosomatic index (GSI) and change in shelter use in response to distilled water (n = 17).

5.6 Discussion

Results of the current chapter brought forth several interesting and novel findings. First, the more stereotypical responses by minnows to the various damage-released cues (i.e. see previous chapters) were noted only during the breeding season with a weaker response noted in the winter months (Figures 5.1 and 5.4). It may be that during the breeding season minnows were unwilling to take additional risks and thus displayed an increased fear response. This may also explain the lack of significant correlation between experience (a correlate of standard length) and the antipredator response seen in breeding minnows versus the significant correlation noted in non-breeding individuals, with the overall increased level of fear in breeding individuals nullifying the correlation.

Despite my hypothesis that breeding index may significantly correlate with a change in antipredator response, none of the damage-released cues caused either an increase or decrease in minnow antipredator behaviour. However, in response to the stickleback cues, minnows in a relatively low breeding state tended to produce a higher response than those in a higher breeding state. Although only a trend, this may be evidence that as minnows advance in breeding condition they decrease their response due to either energy required to respond (reduced mobility) or guarding behaviour. As for the response to the remainder of the cues, particularly the conspecific cue, it may simply be that any state of breeding is enough to create an increase in antipredator behaviour as noted in the overall response (Figure 5.2) and may not increase and correlate with an increase in vigilance as gonadosomatic index increases.

Similarly, the correlations between body condition and an increase in shelter use failed to produce significant results in either breeding or non-breeding minnows. While this study is not the first to produce negative results when attempting to link body

condition and an antipredator response in fishes (Mathis & Smith 1993), there may be reasons for the failure of such studies. It may be that fishes used in the study were all in a relatively homogenous body condition. In a natural setting, several factors are known to affect body condition in fishes such as seasonal food availability, reproductive status, or location in a pond (Songguang et al. 1999). For example, Songguang et al. (1999) found the mean total length of *Hypseleotris swinhonus* and body condition of *Ctenogobius giurinus* were significantly higher in samples taken at the lake centre than in the near shore areas. However, fishes used in laboratory experiments, as in the current study, are commonly captured at one point in time and usually from relatively close areas which may negate factors influencing body condition. Furthermore, the fishes are then maintained in aquaria for several days or weeks in the absence of disease and predation and fed with high quality commercial food, thus creating an unnaturally homogenous body condition within the population.

Therefore, a better test of the effects of body condition on antipredator behaviour may be to artificially create individuals with low and high body condition in the laboratory (see Brown et al. 2004) and conduct a comparative study similar to the current design. Alternatively, a field study utilizing underwater cameras (see Wisenden et al. 2004) could be conducted on various points in a water body, assuming one could control for potential confounds between sites and could document a statistical difference in body condition between locations.

Evidence for the effects of body condition on an animal's antipredator behaviour has been noted in several species. For example, Bachman (1993) found that Belding's ground squirrels (*Spermophilus beldingi*) in poor body condition were less vigilant and

foraged longer when exposed to conspecific alarm calls than squirrels with only a moderately better body condition. I believe the lack of similar evidence for the effects of body condition on a fish's antipredator response has yet to be fully tested and should be addressed in future studies.

The final, and perhaps most important results of my study, are the implications that time of year and minnow length can have on antipredator behaviour. Commonly used behavioural subjects, such as the fathead minnow, are caught throughout the year and used in various behavioural studies in laboratories throughout North America. If time of year (Figures 5.1 and 5.4) and standard length (Figures 5.2A, B, C, D, and 5.5 A, B, C, D) can have such a marked effect on the outcome of an experiment, such factors must be taken into account before conducting future studies. For example, a researcher may record an exaggerated response if he/she were to use large minnows for a study in the summer months versus the winter or vice versa. Researchers should keep such factors in mind when interpreting results and be sure the standard length of fish is equal across treatments within any one study. Further work should be conducted to better understand the effects of time of year on fish antipredator behaviour, not only to re-test the current hypothesis, but to add clarity and understanding to the interpretation of future experiments.

Chapter 6. The effects of social context on the response of fathead minnows to damage-released cues

6.1. Introduction

In recent years, researchers have spent an increasing amount of time attempting to determine which factors are important in predicting fishes' responses to chemical cues. For example, Mirza and Chivers (2002) found that body size of brook char (*Salvelinus fontinalis*) influenced the intensity of antipredator responses to damage-released cues. Likewise, Brown et al. (2004) showed that body condition was a factor influencing the intensity of responses of convict cichlids (*Archocentrus nigrofasciatus*) to damage-released cues. In another study, Wisenden et al. (2003) found that minnow traps labelled with fathead minnow skin extract (odour of a damaged conspecific) caught more minnows if the trap contained a shoal of minnows (contained within a jar) than traps containing the same skin extract and no minnows, or traps containing a shoal of minnows labelled with no skin extract. Wisenden et al. (2003) concluded that the antipredator benefits of being in a shoal at a time of high predation threat were greater than the benefits of leaving a dangerous area. The purpose of the current experiment was to further explore the relationship between multiple information sources, and hence I ask: does the presence of fish shoals influence the intensity of antipredator responses to damage-released cues?

I hypothesize that the presence of a fish shoal should act not only to attract frightened individuals as Wisenden et al. (2003) report, but should also function to mediate their response. For example, if a fish detects a damage-released cue in the

presence of conspecifics or heterospecifics that do not appear to be frightened, this lack of a shoalmate response should serve to reduce the intensity of the response of the individual. I also attempted to determine if minnows would reduce the intensity of their response to alarm cues more in the presence of conspecifics than in the presence of known heterospecifics. Finally, I tested whether the source of alarm cues from conspecifics or heterospecifics would interact with the presence of a fish shoal to mediate the intensity of the response of the test fish. For example, if a fathead minnow is exposed to a stickleback damage-released cue in the presence of a shoal of unexposed “calm” stickleback, is it less likely to respond with an antipredator response than if it was in the presence of a shoal of unexposed “calm” minnows?

6.2. General Methods

6.2.1. Collection and maintenance of animals

Minnows and stickleback, used for both observation and the production of skin extract, were collected using Gee’s improved minnow traps from Feedlot pond. Fishes were collected in January of 2004, a time at which neither species is breeding. The species were collected and held separately in 1000 l flow-through stream tanks with a flow rate of approximately 1 l/minute on a 14:10 h light:dark cycle. Minnows were fed Tetramin flake food and the stickleback were fed previously frozen brine shrimp (*Artemia franciscana*).

6.2.2. General set-up and behavioural assays

Behavioural assays consisted of minnows being exposed to one of four chemical cues: 1) stickleback (known heterospecific), 2) swordtail (unknown heterospecific control), 3) minnow (conspecific) skin extracts or, 4) a control of distilled water. Minnows were provided one of these four cues in the presence of a minnow shoal (4 individuals), a stickleback shoal (4 individuals) or nothing. I conducted a total of 20 replicates of each of the 12 treatments for a total of 240 trials.

Observations were conducted using paired 37 l aquaria separated by a one-way mirror. The mirror was positioned such that the solitary minnow was able to see the shoal in the neighbouring tank, but fish in the shoal were unable to see the solitary minnow. This arrangement ensured that any fright response of the test minnow would not evoke a similar response in the shoaling fish. We ensured a one-way exchange of visual information by placing a light over the neighbour's tank, while keeping the subject tank dimly lit.

The observation tank was set-up as in previous studies and trials conducted in the same manner. Besides the addition of the neighbour tank and one-way mirror, a vertical line was drawn down the middle of the observation tank which was used to record the difference in area use (time spent near neighbour versus away from neighbour) between pre- and post-stimulus periods. As in previous studies the difference in shelter use between the post- and pre-trial periods was also recorded. Due to the infrequent nature of dashing and freezing in previous studies these data were not recorded.

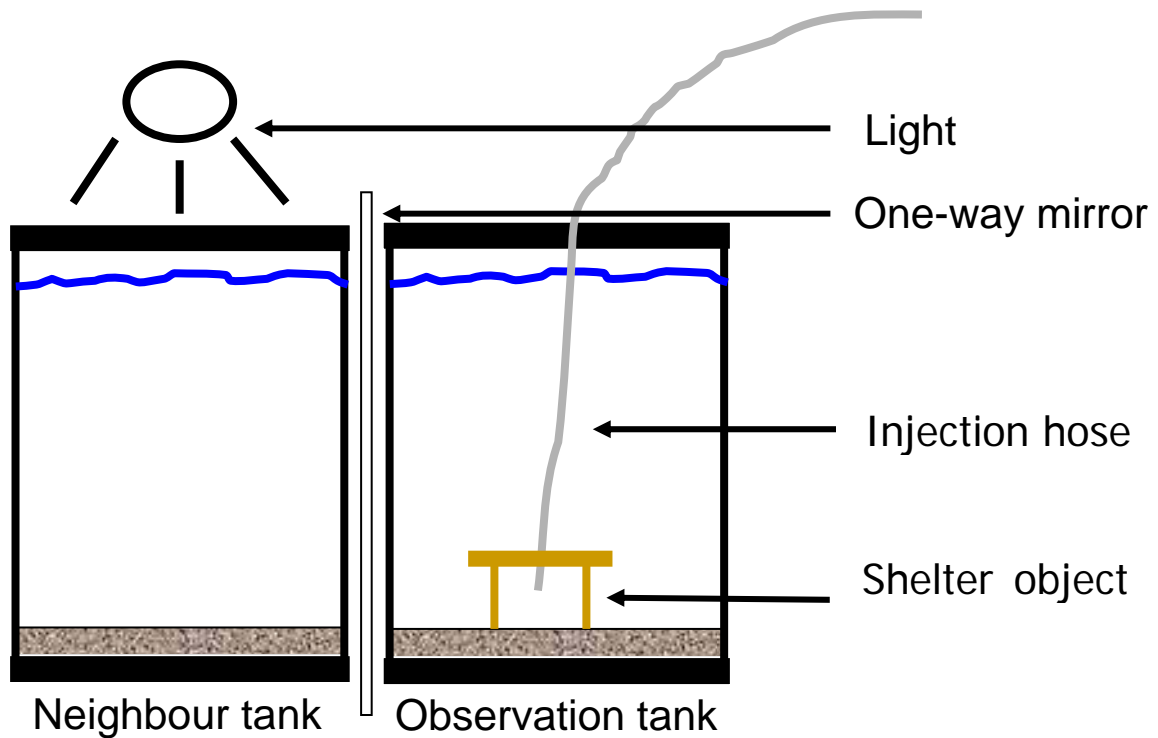


Figure 6.1 Experimental set-up for Experiment 6-1.

6.3. Stimulus preparation

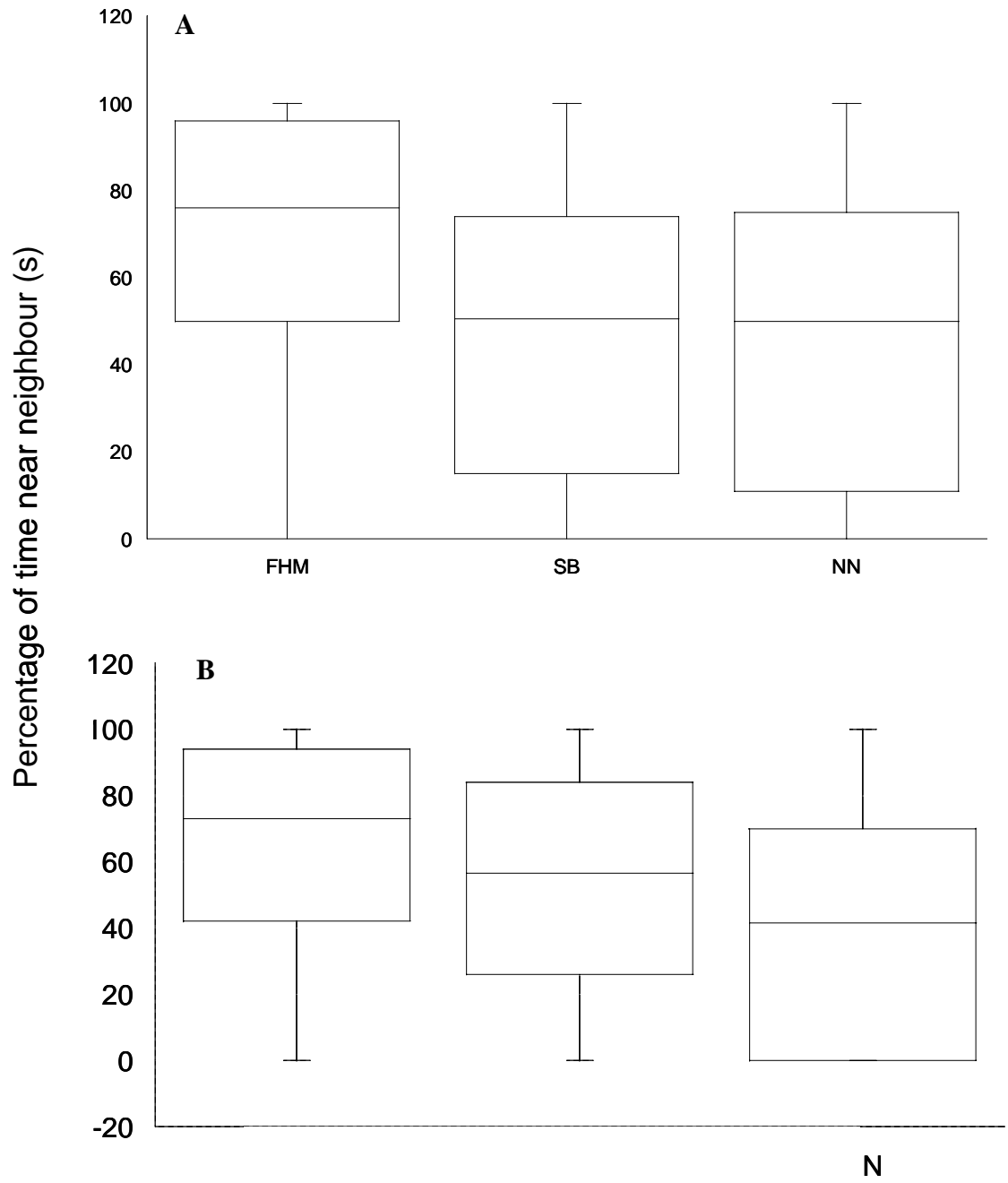
The same methods were used to create the skin extracts as in previous chapters. I used 12 adult stickleback ($SL = \text{mean} \pm 1 \text{ SD} = 4.56 \pm 0.85 \text{ cm}$), 14 adult minnows ($4.69 \pm 0.49 \text{ cm}$) and 16 adult swordtails ($3.53 \pm 0.23 \text{ cm}$).

6.4. Results

Data pertaining to pre-stimulus area use was analyzed by grouping all minnows with the same species of neighbour into three groups ($n = 80$). As the following data failed to meet the assumptions needed for parametric analysis, a Kruskal-Wallis one way analysis of variance by ranks was used, followed by Dunn's paired comparisons (Sokal & Rohlf 1995). Results indicated a significant difference in the time spent near the various

neighbours in the pre-stimulus portion of the trial ($X^2 = 20.70$, $df = 2$, $n = 80$, $p < 0.001$). Subsequent paired comparisons indicated that minnows prefer to be near conspecifics more than either stickleback ($p < 0.05$) (Fig. 6.2A) or the tank with no neighbour ($p < 0.05$) (Fig. 6.2A). However, the minnows show no preference between being near stickleback versus no neighbour ($p > 0.05$) (Fig. 6.2A). Taken together these data indicate that the minnows not only prefer to shoal with minnows in times of no predation, but also were able to see the fishes in the neighbouring tank and differentiate species.

Data pertaining to the post-stimulus area use were analyzed in a similar fashion to pre-stimulus area use except minnows were initially divided into 12 groups; the type of neighbour present crossed with one of four chemical cues ($n = 20$). As the data in the following section again failed to meet the standards of normality, and because I now had a two factor design, a two-way ANOVA design for ranked data was initially used, specifically the Scheirer-Ray-Hare extension of the Kruskal-Wallis test (Sokal & Rohlf 1995). Results indicated no effect of cue ($H = 2.67$, $df = 2$, $n = 60$, $p > 0.25$), nor was there an interaction between cue and neighbour ($H = 3.100$, $df = 6$, $n = 60$, $p > 0.75$). However, fish in the post-stimulus portion of the study did demonstrate a significant preference for the type of neighbour they chose to be near ($H = 13.67$ $df = 2$, $n = 80$, $p < 0.001$).



FHM

SB

NN

Figure 6.2A. Pre-stimulus median preference of shoal type by fathead minnows [fathead minnow shoal (FHM), stickleback shoal (SB), no shoal (NN)] (n = 80).

Figure 6.2B. Post-stimulus median preference of shoal type by fathead minnows [fathead minnow shoal (FHM), stickleback shoal (SB), no shoal (NN)] (n = 80).

To determine which of the neighbour types was preferred, a Kruskal-Wallis test followed by Dunn's multiple comparisons was performed (as in the pre-stimulus portion). Results of the Kruskal-Wallis test indicated that, indeed, fish demonstrated a preference for neighbour type ($X^2 = 15.806$, $df = 2$, $p < 0.001$) (Figure 6.2B). Multiple comparisons revealed that following exposure to the neighbours, minnows prefer to be near minnows and stickleback over no neighbour ($p < 0.05$ in both cases) (Figure 6.2B) and no longer distinguish between stickleback and minnows as in the pre-stimulus portion of the study ($p > 0.05$) (Figure 6.2B). These data would appear to indicate that under the increased levels of predation threat, minnows abandon their preference for conspecifics over heterospecifics and are willing to shoal equally with both. However, many of the minnows chose to spend much of their post-stimulus time under the shelter object, at which time area use was not recorded. For these reasons, readers are cautioned on interpretation of the post-stimulus area use results as they may be potentially confounded by the data pertaining to shelter use.

Data pertaining to shelter use were analyzed in a fashion similar to post-stimulus area use. As the data failed to meet the standards of normality, a two-way ANOVA design for ranked data was used, again in the form of the Scheirer-Ray-Hare extension of the Kruskal-Wallis test (Sokal & Rohlf 1995) with chemical cue and type of neighbour as the factors and change in shelter use as the variable.

Results indicate significant effects for both chemical cue ($H = 11.56$, $df = 3$, $p < 0.01$, Figure 6.3) and type of neighbour ($H = 9.77$, $df = 2$, $p < 0.01$, Figure 6.3), as well as a significant interaction ($H = 12.98$, $df = 6$, $p < 0.05$, Figure 6.3). Subsequent Kruskal-Wallis tests analyzing the effect of cue within a neighbour type indicated that when minnows were coupled with either a minnow or stickleback neighbour they treated controls and known cues equally ($X^2 = 1.120$, $df = 3$, $p = 0.772$, $X^2 = 6.341$, $df = 3$, $p = 0.096$ respectively, Figure 6.3). However, when minnows were tested with no neighbour present significant differences in fear responses between cues were noted ($X^2 = 14.041$, $df = 3$, $p = 0.003$). Subsequent Dunn's paired comparisons indicated that minnows differentiated between minnow cues, and both swordtail cues ($p < 0.05$) and distilled water ($p < 0.05$).

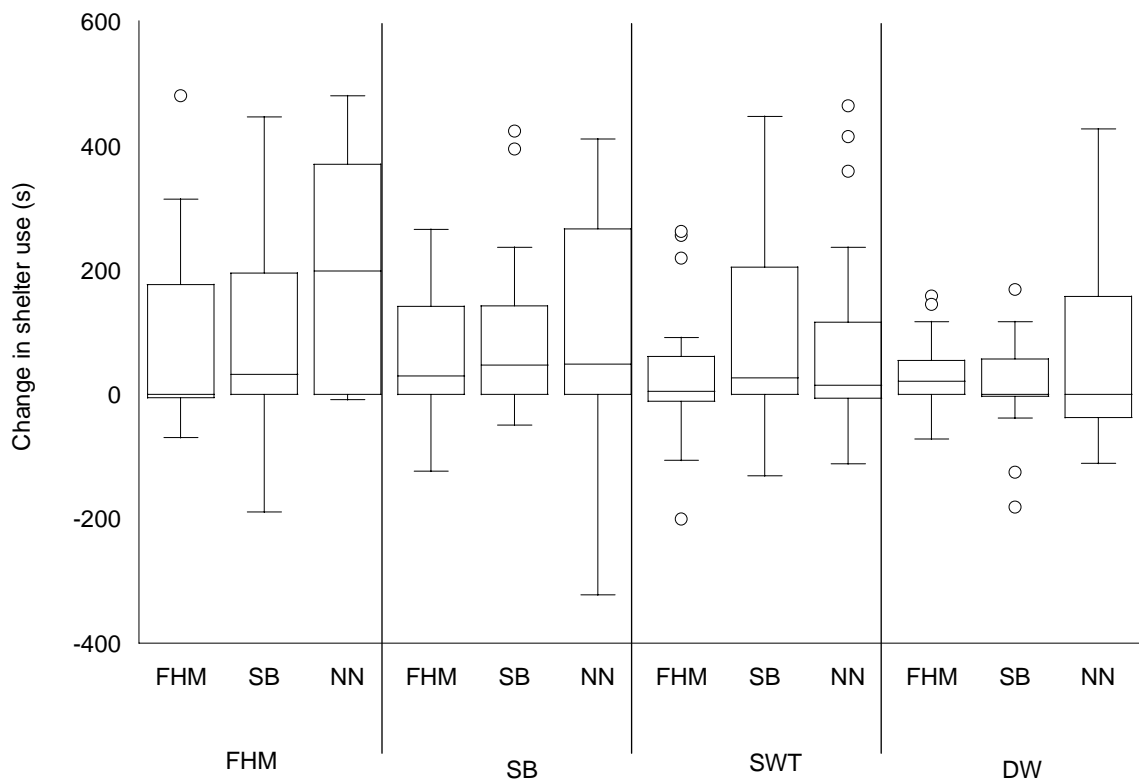


Figure 6.3. Median change in shelter use by fathead minnows exposed to fathead minnow (FHM), stickleback (SB), swordtail (SWT) and distilled water (DW) cues in the

presence of either a fathead minnow shoal (FHM), stickleback shoal (SB) or nothing (NN) (circles are outliers greater or less than one and a half times the interquartile distance) (n = 20).

6.5. Discussion

The results of this study indicate that social context significantly influences the intensity of antipredator behaviours of prey fishes. Minnows exposed to conspecific and heterospecific damage-released cues demonstrated a response equal to that of the controls when paired with a shoal of minnows or stickleback versus nothing (Figure 6.3). I also noted a preference for minnows to shoal with minnows over stickleback in the pre-stimulus period of the study with no such preference existing in the post-stimulus period, resulting in equal schooling with both species.

Due to the potential confound of the increase in shelter use, I was not able to directly compare the pre- and post-stimulus area use; however, no statistical difference between a minnow's choice to school with conspecifics over heterospecifics existed in the post-stimulus period as it had in the pre-stimulus period. Magurran and Pitcher (1987) found that European minnows (*Phoxinus phoxinus*) often show increased shoaling under low to moderate predation risk. It may be that the minnows in the current study, under increased predation pressure (post-stimulus period), had a greater desire to shoal and were less discerning with regard to the species it was with.

Also significant in my study is that the shoal of stickleback appeared to have a similar mediating effect on the minnows' responses when compared with the shoal of conspecifics (Figure 6.3). Minnows appeared to treat all cues equally in terms of a fear response when they were placed next to either a shoal of stickleback or minnows. This study is the first to demonstrate that minnows will depend not only on conspecifics, but

also on known heterospecifics, over their own senses when determining an appropriate antipredator strategy. Considering the negative effects predation has on fitness, the “trust” that minnows place on risk assessment by other prey fishes cannot be underrated.

Our data strengthen the findings of Wisenden et al. (2003). In their study, minnows were significantly more likely to enter traps containing a jar of minnows and conspecific extract than when the traps had no minnows and conspecific extract. Wisenden et al. (2003) concluded that the minnows’ advantage to being in a group at a time of predation threat was greater than the benefit of leaving the area. The results of the current study indicate that minnows do increase shoaling more often in the presence of minnow cue than cues deemed less dangerous. However, as Wisenden et al. (2003) correctly pointed out, the assessment of predation risk is not completely species stereotypical, but rather, it is influenced by social environment and context (Wisenden et al. 2003).

Numerous studies have documented reasons why prey animals should prefer to be in herds or shoals, both at times of high and low predation, and why such situations may serve to mediate an antipredator response. In the presence of predators, individuals in groups often experience higher survival than solitary individuals (Mathis & Chivers 2003), for reasons including dilution of predation risk, increased predator detection, increased efficiency of evasive manoeuvres, the ability to hide behind group mates (selfish herd effects), transmission of information among group members, and predator confusion (reviewed in Godin 1997, Pitcher & Parrish 1993). The current study adds to the literature, demonstrating the transmission of information in shoals and potential importance of social factors in mediating antipredator responses.

Further research examining the role of the shoal's effect on a subject's behaviour is needed. For example, if the shoal was frightened, how would the solitary individual respond? Similarly, will a solitary minnow place equal trust in the fright response of a single minnow versus the fright response of a shoal of stickleback? Considering that a large percentage of studies examining the antipredator behaviour of social animals (particularly fishes) are conducted on single individuals that normally occur in large numbers, such studies are needed to comprise an understanding of social information and its effects on antipredator behaviour. In fact, there is an ongoing controversy regarding the importance of fish damage-released cues in mediating antipredator responses in the field (e.g. Magurran et al. 1996, Smith 1997), which may be partly explained by the differences in laboratory and field testing paradigms. I caution that researchers need to carefully consider the social context in which these experiments take place.

Chapter 7. Discussion

The results of the studies presented in this thesis outline ecological and social factors which affect the learning and use of heterospecific damage-released cues. I first demonstrated that minnows allopatric with stickleback are not able to use stickleback damage-released cues as an indicator of predation risk (Chapter 2). However, once stickleback are introduced into the population, minnows respond to cues as an indication of danger. Further evidence for the hypothesis that learning was the mechanism behind the newly acquired response was found in the observation that laboratory-reared minnow fry, originating from a population known to respond to stickleback cues, do not respond to stickleback cue, unlike wild-caught cohorts. These results may explain discrepancies in the literature in which minnows from populations sympatric with stickleback will at times respond while at other times fail to respond (Mathis & Smith 1993, Brown et

al.1995). If learning is the mechanism behind the response, then it may be that some populations are better able to learn to use stickleback alarm cues than others. To test this hypothesis I tested factors known to vary between populations which may affect learning including the relative density of stickleback to minnows and the type of habitat in which the two species occur.

If some populations of minnows sympatric with stickleback fail to respond, it may be that such a low number of stickleback occur in the population as to offer few opportunities to learn. It is assumed that learning occurs in natural populations as it occurs in laboratory populations when a known negative stimulus (conspecific damage-released cue, predator odour, etc.) is paired with an unknown stimulus (novel heterospecific damage-released cue) (Mirza & Chivers 2001b, Chivers et al. 2002). If the numbers of stickleback were low enough in a population such that minnows are rarely afforded the opportunity to make the association between the known and novel odour, the response may be non-existent. The results of Chapter 3 indicate that it is possible for minnows to co-occur with stickleback while not acquiring the ability to recognize stickleback damage-released cues as a threat, which again, could explain the occurrence of a response by some minnows that occur with stickleback and not others. It may also explain the lack of a response in other studies, including the lack of response in some species to conspecific damage-released cues (Chivers et al. 1997).

In a study by Chivers et al. (1997) it was demonstrated that Dunn's salamanders failed to respond to the damage-released cues as seen in other plethodontid salamanders. Chivers et al. (1997) speculated that the conspicuous absence of alarm responses in Dunn's salamanders (*Plethodon dunni*) but not other plethodontid salamanders may be a

consequence of low density. If the density of a given species was so low that conspecific contact was rare, selection for the maintenance of a response to conspecific cues may not occur in the population. Results from Chapter 3 support such a hypothesis.

When examining ecological factors which affect learning, I also examined the effect of habitat type on the ability of fathead minnows to learn to use stickleback damage-released cues. As minnows and stickleback occur over a large geographic range it would be ecologically relevant to determine if certain habitats enhance or inhibit learning. For example, are minnows from large wide open lakes and ponds with little shelter more able to learn to use stickleback cues than minnows found in complex cluttered habitats? Results from Chapter 4 indicate that minnows from open habitats are better able to learn to use stickleback damage-released cues than minnows from complex habitats. If equal ratios of minnows and stickleback occur, and are being consumed, in each habitat type, several hypotheses must be tested to better explain the differences in learning. It may be that one species prefers to use shelter in complex environments more often than the other, causing social segregation of the two species. So, although stickleback are being consumed, the minnows may not be within the immediate vicinity and less likely to make the association between the known and novel cue. In addition, the importance of visual cues in the learning of novel chemical cues has not been addressed.

It is known that learning can occur in the complete absence of visual cues. In one of the few papers to examine the mechanism of learning, Chivers et al. (2002) demonstrated that minnows can learn to identify stickleback cues when they are associated with conspecific damage-released cues in the diet of a predator. However, it may be that the visual stimuli of a known predator consuming a stickleback in addition to

the chemical association may lead to a comparably rapid learning response. As the conditioning phase in Chapter 4 was eight days in length, I cannot conclusively say learning would not have occurred in the complex habitat. Rather it may just occur at a slower rate. To better address this question a similar experiment of longer length would be required. To further refine the understanding of learned cues, an experiment allowing the chemical association between a known and unknown cue should be conducted in the absence and presence of visual cues. These studies would not only shed light on the data reported in Chapter 4 but also reveal the importance of visual cues in learning.

Many more ecological variables exist that may enhance or inhibit learning. For example, minnows and stickleback co-occur in various water bodies with different predators. It may be that different types of predators offer a variety of learning opportunities. Different predators may release more or less alarm cue during a predation event. They may also release varying amounts of predator odour including varying degrees of diet cues. Several factors similar to the ones tested and proposed should be examined to broaden the understanding of factors that affect the learning of novel chemical cues.

My thesis also tested factors affecting a minnow's response to heterospecific cues once the cue was successfully learned. Results from Chapter 5 indicate that the length of a minnow (a correlate of age) and the time of year both affect the response of minnows not only to heterospecific cues but also to conspecific cues. These data suggest that younger fish may be more at risk for predation due to inexperience. To fully explore this hypothesis, live predation trials, such as the ones conducted by Mirza and Chivers (2002), should be conducted testing the survival rate of fishes correlated with experience.

Data from the same chapter revealed that minnows tested in the summer months failed to show a correlation between experience and an antipredator response, but those in the winter months did. This may be a result of the demands associated with breeding condition, although breeding condition itself failed to produce a response. More studies must be done to fully explain this discrepancy. However, the observation that length correlates with an antipredator response in one part of the year but not another should be of interest to researchers as time of year and minnow length may bias their results.

Taken together, data from Chapters 2 through 5 can contribute to the survival of hatchery-reared and released fishes. For example, we know fishes must learn to use novel heterospecific cues. If fishes reared to be released were taught to use the damage-released cues of other prey species with which they will co-occur, their chance of survival should increase as demonstrated by Chivers et al. (2002). When training fishes to respond we now know a wide open training environment would be more conducive to learning than a structured one. We also know what kind of ratio of each species would be required for learning to occur most efficiently. In hatchery situations, a caged predator with access to the novel heterospecific and not the desired learner would be more ideal to curtail target species mortality. Further, as we know smaller, less experienced fishes may be more vulnerable to predation due to a decreased response, exposure to multiple predation events before release may further add to the survival rate of released fish.

To add relevance to the above results, a study in which hatchery-reared fishes were trained, tagged and released into test ponds with un-trained individuals should be conducted to determine if the results of Chivers et al. (2002) can be applied to a natural setting. Should such a study demonstrate a significant increase in survival for trained

fishes, the results and protocols of Chapters 2 through 5 should be considered as part of the release strategy for stocked fish species.

The final data chapter of my thesis indicates that minnows tested alone will have an exaggerated fear response when given both learned and innate cues. This experiment demonstrates, as does Chapter 5, that a minnow's response to chemical cues is not a simple on-off switch. In fact, Chapter 6 demonstrates that both conspecific and heterospecific damage-released cues will be "ignored" under the proper social situations. Data from the current study also demonstrated a minnow's antipredator response is mediated not only by a shoal of conspecifics but also heterospecifics. Much like a minnow's ability to respond to ecologically similar heterospecific damage-released cues, the mediation of antipredator behaviour by a shoal of heterospecifics should increase the fitness of the minnow. If stickleback and minnows share the same suite of predators (Mathis & Smith 1993), minnows may gain twice as much knowledge on their current predation risk level, assuming equal numbers of both species exist in a population, by watching a shoal of minnows or stickleback.

The results of Chapter 6 have equally important ramifications for future experimental design and reinterpretation of past results. In recent years much debate has been raised regarding the apparent heightened response of minnows in the laboratory setting versus the decreased response seen in field studies (i.e. Magurran et al. 1996, Smith 1997). Although many differences exist between laboratory and field experiments (i.e. water chemistry, competing chemical cues, etc.), this study provides direct evidence for potential differences between laboratory and field experiments, and more specifically potential problems with laboratory studies. As minnows are social animals that naturally

occur in large shoals, one must carefully consider the potential usefulness of an experiment in which minnows are tested alone. If the response of solitary minnows is exaggerated, as the data from Chapter 6 indicates, one must question the ecological relevance of the results. Future researchers must therefore not only carefully design studies and cautiously consider results, but also re-examine and perhaps reinterpret data from previous experiments.

Taken together, the chapters of my thesis show the importance of learning in the use of heterospecific damage-released cues. Given the usefulness of heterospecific damage-released cues in mediating predation risk (Mirza & Chivers 2001b), understanding the manner in which heterospecific alarm cue responses originate is not only novel but timely. Further, given the importance of predation as both a selective force (Lima & Dill 1990) and a potential problem for hatchery-released fishes, the results of my work are useful to both understanding predatory/prey dynamics within water bodies and also to mediate predation losses seen in hatchery-reared and released fishes.

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Pollock, M.S., Pollock, R.J. & Chivers, D.P. (In press) Social context influences the antipredator behaviour of fathead minnows to chemical alarm cues. *Ethology*.

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CONFERENCE PRESENTATIONS

- Chivers, D.P., Hindman, C.J., Michalak, T.A., Jack, K.K., Ferrari, M.C.O., **Pollock, M.S.**, Pollock, R.J., Kusch, R.C., Kaminskyj, S.J., Wisenden, B.D., Alemadi, S., Mirza, R.S., Kiesecker, J.M., Adrian J.C. Jr., Mathis, A., Brown, G.E., Krone, P.H. and Blaustein, A.R. Manipulating predation risk and pathogens to understand the evolution of Schreckstoff. International Ethological Conference 29th, Budapest, Hungary. 2005 (oral).
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- Pollock, M.S.**, Pollock, R.J., & Chivers, D.P. Know thy neighbour: the importance of public information in predation assessment. Prairie Universities Biological Symposium, University of Saskatchewan, Saskatoon, Canada, 2005 (oral).
- Pollock, R.J., **Pollock, M.S.**, & Chivers, D.P. Anti-predator responses of freshwater snails to chemical cues. Prairie Universities Biological Symposium, University of Saskatchewan, Saskatoon, Canada, 2005 (oral).
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- Chivers, D.P. & **Pollock, M.S.** Learned recognition of chemical alarm cues: understanding the importance of habitat characteristics and density. 13th Biennial Conference on the Ecological and Evolutionary Ethology of Fishes. Laval University, Quebec City, Quebec, 2002 (oral).
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