THREAT-SENSITIVE LEARNING AND GENERALIZATION OF PREDATOR RECOGNITION BY AQUATIC VERTEBRATES

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Abstract

Many prey species lack innate recognition of their potential predators. Hence, learning is required for them to recognize and respond to predation threats. When wild-caught, these same species may show amazing sophistication in their responses to predator cues. They are able to adjust the intensity of their antipredator responses to a particular predator according to the degree of threat posed by that predator. This ability is therefore acquired through learning. While many studies have shown that prey can learn to respond to predator cues through different learning modes, little is known about what the prey are actually learning. The results presented in this thesis show that learned predator recognition goes beyond the simple labelling of predators as dangerous. Using fathead minnows (*Pimephales promelas*), woodfrog (*Rana sylvatica*) tadpoles and boreal chorus frog (*Pseudacris maculata*) tadpoles, I demonstrated that a one time learning event, either through pairing with alarm cues or through social learning, was enough for prey to learn the level of threat associated with the novel predator cues. I showed that the level of danger associated with the predator cues was determined by the concentration of alarm cues when learning through pairing of alarm cues, or by the intensity of antipredator response displayed by the tutors and by the tutor-to-observer ratio when learning occurred through cultural transmission. Moreover, when subsequently exposed to predator cues, prey adjusted their antipredator responses according to the change in concentration of predator cues between the learning event and the subsequent exposure. Prey displayed stronger antipredator responses when exposed to higher concentrations of predator cues and vice versa. When minnows were provided with conflicting information about the danger level associated with a predator, they displayed a safety strategy and used the most recent information available to respond to predation threats. On a longer time scale, the data also suggest that woodfrog tadpoles are able to learn to respond to predation threats according to the risk posed by the predator at different times of day. Finally, I showed that prey learn to recognize particular characteristics of predators and can generalize their antipredator responses to novel species sharing those characteristics. However, generalization of predator recognition is dependent on the level of risk associated with the predator. Threat-sensitive learning is an extremely complex process shaped by the millions of years of selection imposed by predators on prey.

Acknowledgements

Je dédie cette thèse à mes parents, qui ont supporté tous les choix que j'ai faits au cours de ma vie, incluant celui de déménager au bout du monde pour faire peur à des petits poissons.

I dedicate this thesis to my parents, who have supported all the choices I made throughout my life, including moving halfway across the world to scare little fishes.

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Chapter 1: Introduction

1.1. General information

Due to the unforgiving nature of predation, prey animals are under intense selection to detect and avoid predators (Lima & Dill 1990, Wisenden & Chivers 2006). The means by which they accomplish this task is a fundamental issue in behavioural and evolutionary ecology. Predation affects many aspects of a prey individual's life, including its life history, morphology and behaviour (Chivers & Smith 1998, Hoverman *et al.* 2005). Prey animals may adjust the timing of their life history switch points in response to predation. For example, the presence of aquatic predators induces amphibian and fish embryos to hatch earlier and at smaller sizes (Chivers *et al.* 1999, 2001a, Kiesecker *et al.* 2002, Kusch & Chivers 2005). Alterations in life history patterns due to predation also include changes in growth and reproductive patterns. For example, when exposed to crayfish predators, snails delay reproduction until they reach a larger body size. This reproductive delay results in increased longevity (Crowl & Covich 1990). Predation acts on prey morphology as well. Prey species occurring with predators often show constitutive morphological defences, such as protective armour, defensive spines, and crypsis or aposematic colouration (Edmunds 1974, Appleton & Palmer 1988). Alternatively, some species possess inducible morphological defences, in which the protective trait (e.g., presence of protective helmet or increased body depth) is reduced or absent in low or no predation situations, but is expressed in the presence of predators. Such examples are common in invertebrate taxa (Stemberger & Gilbert 1984, Kuhlman & Heckmann 1985, Appleton & Palmer 1988, Johansson 2002, Laforsch 2004, Hoverman *et al.* 2005). For vertebrates, the diversity of taxa studied to date is limited to anuran amphibians (e.g., Relyea 2004) and fishes (e.g., Brönmark & Miner 1992, Chivers *et al.* 2008). Finally, most of the literature examining prey responses to predators has documented changes in prey behaviour. Classical examples are hiding or fleeing responses to predators, but also include subtle changes in habitat choice and alterations in the timing of foraging and reproduction (reviewed in Lima & Dill 1990, Lima 1998). Responding to predators is costly but essential for prey survival.

1.2. Threat-sensitive predator avoidance

Prey animals often face an important time / energy trade-off between fitness-related activities, such as foraging or reproduction, and predator avoidance (Lima & Dill 1990). To optimize their trade-off, prey should accurately assess the level of risk associated with each predation threat and respond with an intensity that matches the threat. This hypothesis is known as the threat-sensitive predator avoidance hypothesis (Helfman 1989). It has been tested and validated many times in a wide range of taxa, including freshwater isopods (Holomuzki & Short 1990), mayflies (McIntosh *et al.* 1999), crustaceans (Wahle 1992), amphibians (Kats et al. 1994; Anholt *et al.* 1996; Puttlitz *et al.* 1999; Mathis & Vincent 2000; Amo *et al.* 2004) and fishes (Williams & Brown 1991; Hartman & Abrahams 2000; Chivers *et al.* 2001b; Golub & Brown 2003). For example, Puttlitz *et al.* (1999) showed that the intensity of antipredator responses of Pacific treefrog (*Hyla regilla*) tadpoles to caged salamanders (*Ambystoma gracile*) decreased, as tadpoles grew. Likewise, Golub & Brown (2003) demonstrated that the responses of juvenile green sunfish (*Lepomis cyanellus*) to the odour of injured conspecifics (alarm cues) depended on the ontogeny of the sunfish. When exposed to such cues, small sunfish displayed antipredator responses whilst larger sunfish showed foraging responses. While these examples illustrate variations in antipredator responses and prey vulnerability due to growth or life histories, threat-sensitive predator avoidance has also been demonstrated in moment-to-moment assessment of predation threat by prey individuals. Helfman (1989), for instance, demonstrated that three-spot damselfish (*Stegastes planifrons*) showed more intense antipredator responses to a model trumpetfish (*Aulostomus maculatus*) when the predator model was closer, larger or in a strike pose. From a chemical perspective, several studies have shown that prey use the concentration of predator odour present in the environment to assess the level of danger. For example, Kusch *et al.* (2004) demonstrated that pike (*Esox lucius*)-experienced fathead minnows (*Pimephales promelas*) increased the intensity of their antipredator responses when exposed to increased concentrations of pike odour. Moreover, fathead minnows have been shown to adjust the intensity of their antipredator response to pike odour according to pike size (Kusch *et al.* 2004) as well as pike proximity and density (Ferrari *et al.* 2006). Such sophistication reflects the importance of predation in shaping prey responses to predators.

1.3. Learned predator recognition

A prerequisite for prey to respond adaptively to predation risk is to recognize threats posed by potential predators. The first alternative is for prey to possess an innate[1] recognition of at least some of their potential predators (e.g., mammals [Fendt 2006], birds [Goth 2001], fish [Berejikian *et al.* 2003]). For example, Veen *et al.* (2000) documented that Seychelles warblers (*Acrocephalus sechellensis*) raised in a predator-free environment responded to a mounted model of an egg predator, the fody (*Foudia sechellarum*) with the same intensity as warblers raised in the presence of the fody. The responses to the predator did not differ with the warbler's age and experience with the egg predator, indicating innate recognition of the fody by the warblers. For species lacking this innate recognition of predators (e.g., invertebrates [Rochette *et al.* 1998], fishes (Mathis & Smith 1993; Chivers & Smith 1994a), birds [Curio *et al.* 1978], mammals [McLean *et al.* 1996; Griffin *et al.* 2001]), learning is a necessary step for them to recognize potential predators as threats.

Social learning

Social learning has often been defined as the transmission of information from knowledgeable tutors to naïve conspecifics. Many animals living in groups have the ability to learn novel information or behaviours by observing nearby conspecifics and most of the early studies have focused on the transmission of foraging-related information. For instance, naïve birds can learn to locate rewarding foraging areas by watching experienced conspecifics (Ward & Zahavi 1973). Social learning has also been demonstrated in the context of reproduction. Young female guppies (*Poecilia reticulata*), for example, choose mates according to the choice of older, more experienced conspecific females (Dugatkin & Godin 1992). Social learning of predators has been demonstrated in a variety of taxa. Much of the pioneer work in this field concentrated on birds (Curio *et al.* 1978, Vieth *et al.* 1980, Curio 1988). For instance, zebra finches (*Taeniopygia guttata*) and European blackbirds (*Turdus merula*) learned to mob a novel bird by observing the mobbing response of conspecifics. The conditioned response towards the novel bird was transmitted along a chain of at least six individuals (Curio *et al.* 1978). Likewise,

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[1] The use of 'innate' throughout my thesis refers to a response or behaviour that occurs in the absence of learning. It does not make any inference on the genetic basis of the response or behaviour.

Herzog & Hopf (1984) showed that juvenile squirrel monkeys (*Saimiri sciureus*) learned to avoid stuffed toy animals (snakes or tigers) when alarm calls were given in association with the stuffed toy. Griffin & Evans (2003) also demonstrated that a naïve tammar wallaby (*Macropus eugenii*) could learn to recognize a model fox (*Vulpes vulpes*) as a threat by observing a conspecific displaying a fearful response to the model fox. In aquatic systems, this mode of learning allows for visual recognition (Magurran & Higham 1988, Kelley *et al.* 2003) as well as chemical recognition of novel predators. Mathis *et al.* (1996) demonstrated that pike-naïve fathead minnows (observers) can learn to recognize the chemical cues of northern pike by observing the fright response of experienced conspecifics (tutors) in the same tank paired with pike odour. A single conditioning event was enough for the fish to learn the visual and/or chemical identity of a previously novel predator. The same phenomenon has been demonstrated in amphibians, where larval woodfrogs (*Rana sylvatica*) learn to recognize the odour of a novel predator, the tiger salamander (*Ambystoma tigrinum*), when paired with experienced conspecifics (Ferrari *et al.* 2007a).

Behavioural ecologists typically consider social learning as transmitting information from knowledgeable tutors to naïve observers of the same species. However, animals may also learn by observing the behaviour of other species. Only two studies have considered this in the context of social learning of predator recognition. Vieth *et al.* (1980) showed that European blackbirds could learn to recognize a stimulus as dangerous when the stimulus was paired with a taped heterospecific chorus of mobbing calls from chaffinch (*Fringilla coelebs*), great tits (*Parus major*) and nuthatches (*Sitta europaea*). Mathis *et al.* (1996) documented that brook stickleback (*Culaea inconstans*) could learn to recognize the odour of an unknown predatory pike by observing the response of pike-experienced fathead minnows. Cross-species cultural learning deserves more attention, both in the context of learned recognition of risk and learned recognition of food.

Social learning in a variety of contexts appears to be widespread among mammals, birds and fishes (Griffin 2004). However, the degree to which social learning plays a role in other taxa appears somewhat limited (Griffin 2004). This limitation may be due to a lack of research on those taxa. For example, only one study has considered social learning of predator recognition by larval amphibians (Ferrari *et al.* 2007a). Tadpoles are known to aggregate but the aggregations

have generally been considered in the context of foraging, where moving aggregations stir up the bottom thereby suspending particles of food (Duellman & Trueb 1994). Despite the apparent lack of complex social organization, Ferrari *et al.* (2007a) showed that larval woodfrogs could learn to recognize the odour of a novel predatory salamander (tiger salamander) when paired with an experienced conspecific. This work raised the question of the importance of social learning in amphibians and other taxa that are traditionally considered less social than most birds and mammals.

Learning through pairing of alarm cues and predator cues

Another mode of learning available for prey to learn to recognize predators is through the pairing of conspecific chemical alarm cues and stimuli from predators. Contrary to social learning, this mode of learning is thus far restricted to aquatic species and by its nature, only occurs in the context of predator recognition.

Chemical alarm cues or damage-released alarm cues have been shown in a wide variety of organisms including protozoans, flatworms, annelids, arthropods, molluscs, fishes and amphibians (reviewed by Chivers & Smith 1998, Wisenden 2003). Among fishes, members of the superorder Ostariophysi, which includes minnows, tetras and catfishes, have received the most attention. However, alarm cues are also known in salmonids, gobies, poeciliids, sticklebacks, percids, sculpins, cottids, cichlids and centrarchids (reviewed by Chivers & Smith 1998; Brown 2003). These chemicals are located in the epidermis and are released in the water column through mechanical damage of the skin, typically following a predation attempt in which the prey is injured or captured. When detected by conspecifics (and some sympatric heterospecifics), these chemical alarm cues can elicit dramatic and immediate increases in antipredator behaviours such as increased group cohesion, increased shelter use, decreased activity level and rapid escape to avoid areas where cues have been detected (reviewed by Chivers & Smith 1998). While these chemicals did not likely evolve for this signalling purpose (Chivers *et al.* 2007), there has been selection on the receiver to innately respond to these injured conspecific cues with an antipredator behaviour. In 2003 (time at which I started my research), only one study (Jachner & Rydz 2002) showed that fish increased the intensity of their antipredator responses when exposed to increased concentrations of alarm cues. In contrast, rainbow trout (*Oncorhynchus mykiss*, Mirza & Chivers 2003) and pumpkinseeds (*Lepomis*

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gibbosus, Marcus & Brown 2003) failed to show this gradation and responded to a gradient of alarm cue concentrations in an all-or-nothing fashion.

Chemical alarm cues are known to be important in facilitating learned recognition of predators in a variety of prey (e.g., flatworms [Wisenden & Millard 2001], snails [Rochette *et al.* 1998], crustaceans [Hazlett 2003], insects [Wisenden *et al.* 1997], amphibians [Woody & Mathis 1998]). Fishes acquire recognition of a novel predator based on the pairing of alarm cues with the visual and/or chemical cues of the predator (reviewed by Chivers & Smith 1998; Smith 1999). For example, European minnows (*Phoxinus phoxinus*) and fathead minnows acquire the recognition of the odour of a novel predator after a single exposure to the predator odour paired with conspecific alarm cues (Magurran 1989; Mathis & Smith 1993; Chivers & Smith 1994a). Chivers & Smith (1994b) showed that fathead minnows similarly learn to recognize the visual cues of a predator following a single conditioning experience. Assuming the prey is fully able to detect chemicals in its environment and that the alarm cues have not been chemically modified through ambient acidity for example (see Leduc *et al.* 2003), this mode of learning has never been shown to fail (however, see learned irrelevance: Hazlett 2003, Ferrari & Chivers 2006).

The content of learning

While many prey species learn to recognize their predators, little is known about the characteristics of predators that prey recognize. Wisenden & Harter (2001) showed that prey are more likely to learn to recognize moving objects than stationary objects paired with alarm cues. Karplus & Algom (1981) showed that predator facial recognition by reef fishes was mainly based on the distance between the predator's eyes and the size of its mouth. One way to uncover the specific characteristics of a predator recognized by prey as dangerous is to teach prey to recognize one predator (the reference predator) and subsequently expose the prey to different species sharing some characteristics in common with the reference predator. If the novel species is recognized as threatening, it means that the prey is likely cueing on the characteristics shared by the two species to label novel species as dangerous. Chivers & Smith (1994a) conditioned fathead minnows to visually recognize a northern pike or a goldfish (*Carassius auratus*) as a predatory threat and subsequently exposed them to the two species. They showed that minnows were only fearful of the species they were conditioned to recognize as a predator. These results are not surprising given the considerable differences in the appearance of pike and goldfish.

Griffin *et al.* (2001) showed that, although tammar wallabies do not innately recognize feral cats (*Felis catus*) or red foxes as predators, they displayed a fright response to model cats when previously conditioned to recognize a model red fox as a predator. Thus, the wallabies generalize their recognition from a fox to a cat based on the visual similarities between the two species. This area of predator recognition deserves more attention from behavioural ecologists.

1.4. Study systems

Three prey species from two taxa were used in my experiments. All are primary aquatic vertebrates.

Fathead minnows

Fathead minnows are small freshwater fish (adult length: 4-6 cm), commonly found in rivers, lakes or ponds throughout North America. Their small size makes them vulnerable to a variety of aquatic and terrestrial predators such as fishes, birds, snakes and invertebrates. Minnows belong to the order Ostariophysi, which means that they possess epidermal club cells containing the alarm cues described above. During the mating season (from April to August depending on the latitude), the males lose their club cells. Breeding males can be easily differentiated from breeding females; however, I never used breeding minnows in my experiments to avoid reproductive conflicts and to minimize mortality (mortality of male minnows increases dramatically during the breeding season).

Fathead minnows have been extensively used as test species for both toxicological and behavioural studies. Their small size and low maintenance make them a good candidate for laboratory-oriented studies. Moreover, fathead minnows have been a classic study system for chemical ecology of predator-prey interactions. It has been firmly established that minnows lack innate recognition of many predators (Chivers & Smith 1994b), that they acquire recognition of their predators through both social learning and pairing of alarm cues and predator cues after one learning trial only (see introduction), and that they are able to remember this information for more than 1 year without further reinforcement (Chivers & Smith 1994b). The antipredator response of minnows can consist of dashing (rapid bursts of apparently disoriented swimming), freezing, increased shoal coherence, decreased activity and increased shelter use.

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Woodfrog and boreal chorus frog (Pseudracris maculata) tadpoles

The two species of amphibians used in my experiments are the larval woodfrog and the larval chorus frog. The experiments involving these amphibians were performed outdoors in Alberta, under natural temperature and photoperiod.

Woodfrogs are true frogs, reaching 4 to 6 cm in body length as adults. They are largely terrestrial, but are not usually found far from water. They inhabit marshes, riparian areas, wet meadows, moist brush, and open grassy areas adjacent to such habitats. The adults are explosive breeders and usually lay their eggs in clutches of several hundred (up to 3000) within a couple of weeks in a given pond, in late April and early May at my field site. The eggs hatch after 1-2 weeks and the larvae metamorphose within a few weeks. Juvenile woodfrogs reach sexual maturity after 2 years (Duellman & Trueb 1994).

Boreal chorus frogs are the smallest frogs in western Canada, reaching less than 4 cm in body length. Chorus frogs make their home in terrestrial habitats for much of the year, particularly in damp grassy or wooded areas surrounding wetlands. They can be found in and around almost any body of water, as well as in wet meadows, moist brush, grasslands, forests, and some residential and agricultural areas. Their breeding season is longer than the one of woodfrogs, as they lay eggs from early May to late June. Each clutch contains on average 30 to 75 eggs. The adults reach maturity in only 1 year (Duellman & Trueb 1994).

At the time of my experiments, the importance of learning versus innate recognition of predators by woodfrogs or chorus frogs was unknown. This topic is particularly fascinating, as embryos of frogs and salamanders collected from certain populations occurring with predators seem to innately respond to the predator cues, whereas embryos collected from populations not occurring with predators do not (Kats *et al.* 1988, Kiesecker & Blaustein 1997). However, recent evidence would suggest that learned predator recognition occurs during embryonic development (Mathis *et al.* 2008), questioning the existence of a true innate responses to predator odours by larval amphibians.

Larval woodfrogs, like many species of larval amphibians, possess chemicals eliciting antipredator responses in nearby conspecifics (Hews & Blaustein 1985, Hews 1988, Petranka 1989, Chivers *et al.* 1999). The cues are released following mechanical damage, as would occur

during a predatory attack. Behavioural responses to alarm cues include a reduction in activity, which is the same response shown to cues from predators (Chivers *et al.* 1999, Ferrari *et al.* 2007a). In addition, woodfrog tadpoles have been shown to display threat-sensitive responses to alarm cues, increasing the intensity of their antipredator response when exposed to increased concentrations of alarm cues (Ferrari, Brown, Messier & Chivers unpublished data).

1.5. Research objectives

My overall objective is to examine the extent of the information learned by the prey during learning events. In my thesis, I present a series of experiments divided in six data chapters, showing how prey adjust the intensity of their antipredator responses to predation threats through threat-sensitive learning. I used fathead minnows, larval woodfrog and boreal chorus frog tadpoles to answer the following questions:

Can prey learn the level of risk associated with a novel predator cue? If yes, what factors affect the intensity of the learned responses by prey? Prey are known to learn to recognize predators via conditioning with chemical alarm cues paired with the odour of a novel predator. In Chapter 2, I tested whether fathead minnows learning to recognize a novel predator through the pairing of alarm cues and predator odour could also learn the level of risk associated with the novel threat using both the concentration of alarm cues and the concentration of predator odour. Chapter 3 deals with similar questions, though I used cultural transmission as the learning mode of interest. I investigated whether minnows could learn the level of risk associated with a novel predator cue using the intensity of responses displayed by the tutors or the number of tutors responding to the predator.

How do prey use information accumulated through multiple learning events to adjust responses to a predation threat? Chapters 2 and 3 showed that one learning event was enough for minnows to learn the level of risk associated with a novel predator. In Chapter 4, I investigated the responses of prey to predator cues when prey are given several learning opportunities. First, I asked how minnows would respond if given two pieces of consistent or conflicting information about the risk associated with a novel predator. Second, I investigated if prey rely more on recent and updated information when adjusting their intensity of responses to predators.

Can prey learn to incorporate temporal variability of predation risk during learning events? In Chapter 5, I investigated whether prey can adjust the intensity of their responses to predator cues according to the time of day they experienced the cues. Woodfrog tadpoles were conditioned for 9 days to one of two treatments: high risk in the morning and lower risk in the evening, or alternatively to low risk in the morning and higher risk in the evening. I then tested whether the tadpoles from both treatments would respond to the predator cues with the same intensity at a given time of day.

How specific is learned predator recognition? Chapter 6 deals with the content of learning. I was interested in knowing whether prey learn to recognize a predator in a very specific fashion, or alternatively if prey would learn some general characteristics of the predator, which they could then generalize to close relatives of that predator. To answer this question, I conditioned fathead minnows to recognize lake trout (*Salvenilus namaycush*) odour as a threat, and subsequently tested the minnows' response to the odour of lake trout, brook trout (*Salvelinus fontinalis*) and rainbow trout (close relatives of the lake trout), pike (a distantly related predator) and sucker (*Catostomus commersoni* – a distantly related non-predator). From this, I developed a theoretical framework for future research on generalization of learned predator recognition.

Is generalization of predator recognition a constant phenomenon? Chapter 7 brings together the concept of threat-sensitive learning (Chapter 2) and the concept of generalization (Chapter 6). In this chapter, I investigated possible situations under which generalization may not be occurring. More specifically, I asked whether the level of risk associated with the reference predator would influence the propensity of fathead minnow to generalize their recognition to closely related species. To answer this question, I conditioned minnows to recognize brown trout (*Salmo trutta*) odour as either a high or a low threat, and subsequently recorded their antipredator behaviour when exposed to the odour of brown trout (reference predator), rainbow trout (a closely related predator) or yellow perch (*Perca flavescens* – a distantly related predator).

1.6. Anticipated significance

My research is aimed at understanding fundamental questions about predator recognition. Many prey species require learning to recognize novel predators as dangerous. A number of studies have investigated the occurrence of and mechanisms associated with learned

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predator recognition in a variety of species. However, nobody has looked at learned predator recognition from a quantitative point of view, which is the focus of my thesis. I use a variety of study systems to understand how threat-sensitive predator avoidance develops (i.e., how fast it develops, what happens when animals get conflicting information, and how prey adjust their responses to temporal patterns of risk). Furthermore, I address the question of whether prey which learn to recognize a predator as a threat can generalize this information to other unknown predators. Apart for post-reproductive individuals, nothing reduces future individual fitness more than being consumed by a predator (but see Johns & Maxwell 1997).

Chapter 2: The development of threat-sensitive predator avoidance through pairing of predator odour and alarm cues.[2]

2.1. Introduction

Prey that fail to respond appropriately to predators may lose their life. Consequently, it is not surprising that selection pressure acts to favour prey displaying adaptive responses. Prey should be selected if they are able to optimize their fitness by matching the intensity of their antipredator response to the risk posed by their predators. This phenomenon, referred to as threat-sensitive predator avoidance (Helfman 1989), has been demonstrated in a variety of species, including fathead minnows (Kusch *et al.* 2004, Ferrari *et al.* 2006).

For aquatic species, chemicals present in the environment provide a valuable source of information, particularly in conditions in which other sensory modalities, such as vision, are limited. Such conditions may occur at night, in turbid water or in highly structured habitats. In the context of risk assessment, chemicals emanating either from the predator (predator odour) or from injured prey (alarm cues) can be used as risk assessment tools. In both cases, an increase in the concentration of chemicals likely represents a greater risk for the prey. In fact, Kusch *et al.* (2004) showed that wild-caught pike-experienced fathead minnows used the concentration of pike odour they were exposed to as a risk assessment tool, responding with a greater intensity to higher concentration of pike odour. Similarly, Jachner & Rydz (2002) observed the same phenomenon when exposing fish to increased concentrations of alarm cues. Brown *et al.* (2001a) showed that minnows increased the intensity of their antipredator response when exposed to hypoxanthine-3-N-oxide (H3NO), the putative Ostariophysan alarm cue.

While some prey possess an innate recognition of their predators, some others, like fathead minnows, require learning to be able to label predator cues as dangerous (Chivers & Smith 1994a, b). Minnows can learn to recognize a novel predator as a threat through the pairing

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[2] The content of this chapter is published in the following papers:
Ferrari, M.C.O., Trowell, J.J., Brown, G.E. & Chivers, D.P. 2005. The role of leaning in the development of threat-sensitive predator avoidance in fathead minnows. *Anim. Behav.* **70**, 777-784.
Ferrari, M.C.O., Kapitania-Kwok, T. & Chivers, D.P. 2006. The role of learning in the development of threat-sensitive predator avoidance: the use of predator cue concentration by fathead minnows. *Behav. Ecol. Sociobiol.* **60**, 522-527.

of alarm cues and predator cues (Chivers & Smith 1994a). The same learning phenomenon was obtained when conditioning minnows with a novel predator cue paired with H3NO. More interestingly, minnows have been shown to acquire the recognition of novel predators when the predator odour was paired with H3NO at concentrations well below the minimum behavioural response threshold (Brown *et al.* 2001b). A concentration of alarm cues that does not elicit any overt antipredator behaviour but is still detected by the fish, is referred to as a 'subthreshold' concentration.

In the following studies, I investigated the ability of fathead minnows to learn the intensity of response associated with the predation threat. The goal of Experiment 1 was two-fold: (1) to investigate whether minnows respond in a graded manner to a gradient of natural chemical alarm cues, and (2) to test whether the response intensity displayed by minnows in the learning phase match their subsequent response intensity to the novel predator cues. I conditioned predator-naïve minnows to recognize brook trout odour as a threat by exposing them to trout odour paired with various concentrations of conspecific alarm cues. I then compared the intensity of the minnows' response during conditioning to the intensity of their response when tested with trout odour alone in a subsequent recognition trial. I hypothesized that the intensity of minnows' response during conditioning and recognition trials would vary according to the concentration of skin extract used for conditioning, i.e., that fish conditioned with higher concentrations of alarm cues would learn to respond to trout odour alone with a higher intensity of antipredator response than fish conditioned with lower concentrations of alarm cues.

In Experiment 2, I focused on understanding the effect of manipulating predator odour concentrations on the acquisition of threat-sensitive predator avoidance in fathead minnows. I conditioned minnows with conspecific skin extract paired with either a high or a low concentration of pike odour. I subsequently tested them with either a high or a low concentration of pike odour alone and documented the intensity of their response. I predicted that minnows initially conditioned with a high concentration of pike odour should respond to high pike odour concentration with an equal intensity as those conditioned with a low concentration and tested with a low concentration. Moreover, minnows conditioned with a particular concentration of pike odour should subsequently respond with a higher response intensity to higher concentrations of pike odour and with a lower response intensity to lower concentrations of pike odour.

2.2. Methodology

Test species

  Fathead minnows used in Experiment 1 were captured from a local pond using Gee's improved minnow traps (cylindrical wire cages, 43 cm long, 22 cm diameter with inverted cones entrance at each end) in October 2003. They were housed in a 6000-L flow-through pool filled with dechlorinated tap water at 11˚C and fed *ad libitum* once a day with commercial flakes (Nutrafin basix, Rolf C. Hagen, Inc., Montreal, Quebec, Canada). The photoperiod was adjusted to 14:10 h light:dark cycle. Brook trout were obtained from the Fort Qu'Appelle fish hatchery, Saskatchewan, in October 2003 and housed in a 6000-L flow-through pool filled with dechlorinated tap water at 11˚C. Trout were fed *ad libitum* once a day with commercial trout pellets (Martin's, Elmira, Ontario, Canada).

  Fathead minnows used in Experiment 2 were captured from Feedlot Pond, located on the University of Saskatchewan campus, using minnow traps in September 2004. This population of minnows originated from the South Saskatchewan River when the pond was filled in 1959 to provide water for agricultural purposes. Intensive trapping and gill-netting over the past 10 years revealed the presence of only one other fish species in the pond, brook stickleback, and the absence of any predatory fish. Moreover, previous experiments (e.g., Chivers & Smith 1995, Brown *et al.* 1997) have demonstrated that minnows from this pond and nearby ponds do not show innate recognition of pike cues. The minnows were housed in an 18000-L flow-through pool filled with dechlorinated tap water at 12˚C and fed *ad libitum* once a day with commercial flakes. The photoperiod was adjusted to a 14:10 h light:dark cycle. Northern pike were captured in September 2004 from Eagle Creek, Saskatchewan, using seine nets. They were housed in a 6000-L flow-through pool filled with dechlorinated tap water at 12˚C. Pike were fed *ad libitum* with live fathead minnows. I ensured that some minnows were always present in the pool to avoid pike cannibalism.

Stimulus collection

Minnow skin extract

Skin extracts used in Experiment 1 were collected from six fathead minnows (fork length: mean ± S.D. = 4.62 ± 0.39 cm). Minnows were killed with a blow on the head (in accordance with the Canadian Council on Animal Care) and skin fillets were removed from either side of the body and placed in 100 mL of chilled distilled water. Skin fillets were then homogenized, and filtered through glass wool to remove remaining tissues. I collected 21.96 cm^2 of skin in a total of 439 mL of distilled water, which constituted my standardized solution containing ~1 cm^2 of skin per 20 mL of distilled water. This solution was diluted to make three experimental solutions: low (1 cm^2 of skin per 240 L), medium (1 cm^2 of skin per 120 L), and high (1 cm^2 of skin per 40 L) concentration of alarm cues. Skin extracts used in Experiment 2 were collected from six fathead minnows (fork length: mean ± S.D. = 4.76 ± 0.43 cm) following the same methodology. I obtained 19.14 cm^2 of skin in a total of 383 mL of distilled water, which constituted my standardized solution containing ~1 cm^2 of skin per 20 mL of distilled water. This solution was diluted to make the experimental solution containing ~1 cm^2 of skin per 40 L. Skin extracts were frozen in 20-mL aliquots at -20°C until required.

Trout odour

Prey animals often exhibit antipredator responses to chemical cues of predators fed conspecific of the prey, but not those fed another diet (Chivers & Mirza 2001a). Thus, two arbitrarily chosen brook trout (20.6 and 24.5 cm fork length) were kept in a 115-L tank and fed brine shrimp (*Artemia* spp) for 5 days prior to stimulus collection. Both trout were then transferred to a 72-L tank containing 60 L of dechlorinated tap water, an air stone but no filter. The trout remained in the stimulus-collection tank for 24 h, at which time they were transferred back to their initial holding pool. Water containing trout odour was frozen at -20°C in 60-mL aliquots until required.

Pike odour

Two arbitrarily chosen pike (20.0 and 22.0 cm standard length) were kept individually in two 145-L tanks for 7 days prior to stimulus collection and fed two convict cichlids

(*Archocentrus nigrofasciatus*) (standard length: mean ± S.D. = 3.93 ± 0.64 cm) once at Day 2 and once at Day 5 of the 7-day period. Both pike were then rinsed and transferred to a single 72-L tank containing 60 L of dechlorinated tap water, an air stone but no filter. The pike remained in the stimulus-collection tank for 24 h, at which time they were transferred back to their initial holding pool. Water containing pike odour was frozen at -20°C in 60-mL aliquots until required.

2.2.1. Prey learn to match their response intensity to a novel threat according to alarm cue concentrations

Experimental protocol

This experiment consisted of two phases: conditioning trials followed by recognition trials. During conditioning trials, three minnows were exposed to trout odour paired with one of the three concentrations of conspecific alarm cues (low, medium or high) or a distilled water control. The fish were exposed to trout odour alone 24 h later during recognition trials and the intensities of their responses during recognition trials were compared to the intensity of response displayed during conditioning trials.

Conditioning trials

Groups of three minnows were placed in 37-L tanks, filled with dechlorinated tap water. The tanks had a 3 x 3 grid pattern drawn on the side and contained a gravel substrate and an air stone, near to which was attached a 2-m long piece of plastic tubing used to inject test stimuli into the tanks. Prior to testing, minnows were acclimated for a 24-h period in their testing tanks (water at 12-14°C, 14:10 h light:dark cycle).

All trials were conducted between 1330 and 1600 h. Observations consisted of an 8-min pre-stimulus and an 8-min post-stimulus injection period. Prior to the pre-stimulus period, I withdrew and discarded 60 mL of water from the injection tubes (to remove any stagnant water) and then withdrew and retained an additional 60 mL. Following the pre-stimulus period, I injected either 5 mL of one of the three concentrations of skin extract or distilled water as well as 20 mL of trout odour into the tank. I used the retained tank water to slowly flush the stimuli into the tank. Once the stimuli were fully injected, I began the post-stimulus observation period.

As a measure of antipredator response, I recorded the shoaling index of the three fish every 15 sec (1: no fish within a body length of another; 2: two fish within a body length of each other; 3: all the fish within a body length of another fish). During the first 8 sec of the 15 sec periods (a stop watch was set to beep after 8 sec), the number of line crosses (using the 3 x 3 grid pattern drawn on the side of the tank) was also recorded for one of the three minnows (randomly chosen, the same fish was observed until the end of the conditioning period). An increase in shoaling index and a decrease in activity level are two typical antipredator responses in minnows (reviewed by Chivers & Smith 1998).

Recognition trials

One hour after the end of the conditioning trials, the fish were moved to a similar 37-L tank filled with clean dechlorinated tap water. After 24 h, recognition trials were performed. The protocol was the same used for the conditioning trials except that only trout odour (20 mL) was injected in the tank following the pre-stimulus period. All trials were performed blind and the order of testing was randomized. I tested 180 minnows (fork length: mean ± S.D. = 4.73 ± 0.43 cm) in total with N = 15 per treatment.

Statistical analysis

For both conditioning and recognition trials, I calculated the change in line crosses and in shoaling from the pre-stimulus baseline. My data for line crosses were parametric, but the data for shoaling index were normally distributed but not homoscedastic. I conducted Wilcoxon signed-rank tests to compare the response between conditioning and recognition trials within treatments and two-tailed Mann-Whitney tests to compare the effect of different concentrations of alarm cues during the conditioning and recognition trials. For the Mann-Whitney tests, the alpha level was set at 0.008 following a Bonferroni correction for Type I error (Higgins 2004 pp 93-4).

2.2.2. Prey learn to match their response intensity to a novel threat according to predator cue concentrations

Experimental protocol

This experiment consisted of a two by two design. Minnows were conditioned with conspecific alarm cues paired with either a high or low concentration of pike odour. During subsequent recognition trials, minnows from each group were tested for a response to either a high or low concentration of pike odour. Thus, I had four treatments: minnows conditioned with skin extract paired with low concentration of pike odour and tested with low concentration of pike odour (LL), minnows conditioned with skin extract paired with low concentration of pike odour and tested with high concentration of pike odour (LH), minnows conditioned with skin extract paired with high concentration of pike odour and tested with low concentration of pike odour (HL) and finally minnows conditioned with skin extract paired with high concentration of pike odour and tested with high concentration of pike odour (HH). To obtain the high and low concentration of pike odour, I used 60-mL syringes to inject either 20 mL (low) or 60 mL (high) of the prepared pike odour. To control for volumes injected into the tanks, the syringe containing 20 mL of pike odour was filled with an additional 40 mL of tank water. Consequently, my high concentration stimulus injected into the tanks was three times as concentrated as my low concentration of pike odour.

Due to logistic limitation, fathead minnows were held in a different room than the experimental room. To allow the fish to acclimate to the new room temperature (water temperature at 14-15°C, 14:10 h light:dark cycle), groups of about 20 minnows were transferred into a 145-L holding tank located in the experimental room 1 week before being tested.

Conditioning trials

Individual minnows were placed in 37-L tanks, filled with dechlorinated tap water. The tanks were similar to the ones used for Experiment 1. However, they were equipped with a shelter that consisted of a 10 x 20 cm ceramic tile mounted on three 3.5-cm long cylindrical glass legs. Prior to conditioning, minnows were acclimated for a 24-h period in their conditioning tanks. I fed the fish after their transfer into the tank and 1 h prior to testing (in order to reduce the potential trade-off between foraging and predator avoidance – Brown & Smith 1996). The

conditioning procedure was identical to the one used in Experiment 1. I injected 5 mL of conspecific skin extract immediately followed by 60 mL of either high or low concentration of pike odour into the tank. The injection of high or low concentration of pike odour was randomized among the tanks. No observations were made during this phase.

Recognition trials

One hour after the end of the conditioning trials, the fish were moved to identical 37-L tanks filled with clean dechlorinated tap water. After 24 h, recognition trials were performed. Observations consisted of an 8-min pre- and an 8-min post-stimulus injection period. Following the pre-stimulus period, I injected 60 mL of either high or low concentration of pike odour into the tank using the same procedure as used in the conditioning trials. Once the stimuli were fully injected, I began the post-stimulus observation period.

The most common antipredator response displayed by single minnows exposed to predator cues is shelter use, if a shelter is present, or a decrease in activity, if a shelter is not available (Chivers & Smith 1998). When sheltering, fish might still be 'active', displaying foraging behaviour for example, making the activity measure less precise when a shelter is present. In this study, I measured time spent under shelter as the primary antipredator response variable, but also recorded time spent moving as a secondary variable. Time under shelter and time spent moving were recorded during the 8-min pre- and 8-min post-injection periods. All trials were performed blind and the order of testing was randomized. I tested 30 minnows for each of the four treatments (fork length: mean ± S.D. = 5.75 ± 0.07cm).

In this experiment, I hypothesized that minnows do not innately recognize high concentration versus low concentration of predator odours (Chivers & Smith 1994b). Consequently, I predicted that HH fish should respond with the same intensity as LL fish. I tested this by comparing the intensity of response of the minnows in the LL vs. HH treatments. I then compared the LL vs. LH treatments to determine the effect of an increase in predator cue concentration between conditioning and recognition trials (predator cues represent a higher threat in recognition trials than conditioning trials). Finally, I compared the HH vs. HL treatments to determine the effect of a decrease in predator cue concentration between conditioning and

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recognition trials (predator cues represent a lower threat during recognition trials than conditioning trials).

Statistical analysis

For all behavioural measures, I calculated the change from the pre-stimulus baseline. An increase in shelter use or a reduction in time moving would indicate an increase in antipredator behaviour (reviewed by Chivers & Smith 1998). The data for change in time moving and shelter use were parametric and homoscedastic. Thus, I performed a 2 x 2 ANOVA on each of the variables, followed by post-hoc Bonferroni tests for my three pre-planned comparisons.

2.3. Results

Experiment 1

Comparisons within concentration treatments: No difference between conditioning and recognition was found for distilled water (Wilcoxon signed-ranks test: line crosses: $Z = - 1.9$, $N = 15$, $P = 0.23$; shoaling index: $Z = - 0.2$, $P = 0.83$), medium (line crosses: $Z = - 1.0$, $N = 15$, $P = 0.29$; shoaling index: $Z = - 0.1$, $P = 0.87$) or high (line crosses: $Z = - 1.1$, $N = 15$, $P = 0.28$; shoaling index: $Z = - 0.7$, $P = 0.50$) concentration of alarm cue treatments. However, differences were found for the low treatment for line crosses ($Z = - 2.2$, $N = 15$, $P = 0.027$) and shoaling index ($Z = 3.4$, $N = 15$, $P = 0.001$) (Figures 2.1a, b).

Comparisons between concentration treatments:

Conditioning trials: The intensity of the behavioural responses of minnows to alarm cues varied with alarm cue concentrations. There was no difference in the response of minnows to distilled water and low concentration of skin extract (Mann-Whitney U test: line crosses: $U = 93.5$, $N_1 = N_2 = 15$, $P = 0.44$, Figure 2.1a; shoaling index: $U = 112.5$, $P > 0.95$, Figure 2.1b). However, fish exposed to the medium concentration showed an increase in shoaling ($U = 1.5$, $N_1 = N_2 = 15$, $P < 0.001$) and a reduction in line crosses ($U = 40.5$, $P = 0.02$) compared to the distilled water control. Similarly fish exposed to high concentration of skin extract showed an increase in shoaling ($U = 3.0$, $N_1 = N_2 = 15$, $P < 0.001$) and a reduction in line crosses ($U = 20.5$, $P < 0.001$). The low concentration treatment was significantly different from the medium ($U = 27.5$, $P < 0.001$; $U= 4.5$, $P < 0.001$) and high ones ($U = 2.5$, $P < 0.001$; $U = 1.0$, $P < 0.001$) for

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line crosses and shoaling respectively. For change in line crosses, the response to medium concentration of skin extract was significantly different from the high (U = 54.5, P = 0.015). However, no difference was found for shoaling index (U = 64.5, P = 0.045) but a trend is clearly observable on the graph.
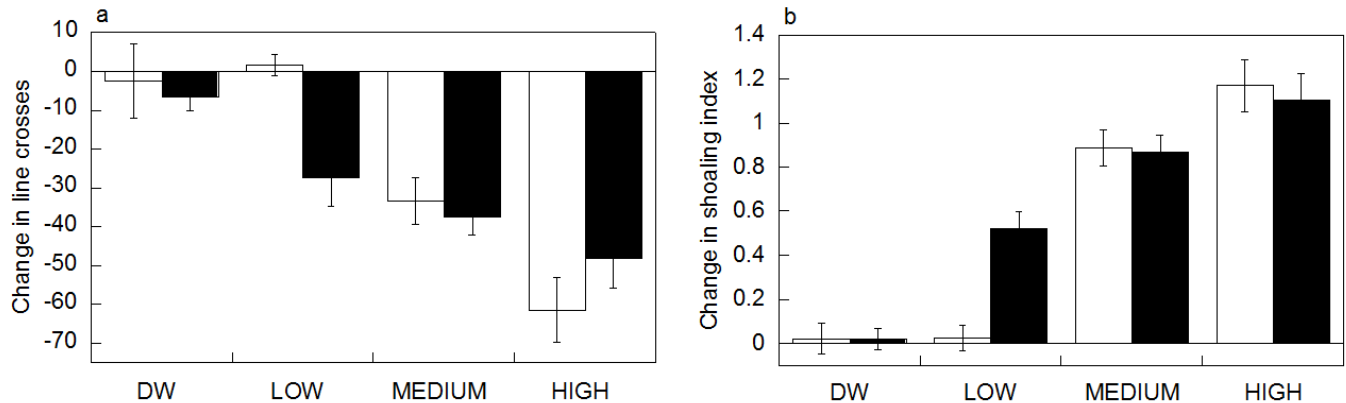
Figure 2.1: Mean (± S.E.) change from the pre-stimulus baseline in (a) number of line crosses and (b) shoaling index for fathead minnows exposed to a low, medium or high concentration of conspecific skin extract or distilled water (DW) paired with trout odour during conditioning trials (empty bars), or exposed to trout odour alone during recognition trials (solid bars) (N = 15/treatment).

Recognition trials: The responses of minnows to trout odour in the recognition trials was influenced by the cues the fish were exposed to during the conditioning trials. Minnows initially exposed to the low concentration of skin extract paired with trout odour displayed a significant fright response to trout odour when compared to those initially exposed to distilled water combined with trout odour (Mann-Whitney U test: line crosses: U = 41.5, $N_1 = N_2 = 15$, P = 0.002; Figure 2.1a; shoaling index: U = 16.0, P < 0.001; Figure 2.1b). Moreover, responses to trout odour for fish in the control treatment were significantly lower than those of fish initially exposed to trout odour paired with medium and high concentrations for both line crosses (U = 17.0, $N_1 = N_2 = 15$, P < 0.001; U = 16.5; P < 0.001) and shoaling (U = 2.5, P < 0.001, U = 0.0, P < 0.001). These results demonstrate that minnows have learned to recognize trout odour as a threat when conditioned with each of the three concentrations of skin extract. For change in shoaling index, the responses of minnows in the low concentration treatment were significantly lower than the ones of fish in the medium (U = 44.0, $N_1 = N_2 = 15$, P = 0.004) and high treatments (U = 35.5, P = 0.001; Figure 2.1b). Responses of minnows conditioned with a high concentration were not significantly different from those of minnows conditioned with the

medium concentration of alarm cues (U = 79.5, P = 0.17). For change in line crosses, responses of minnows in low and medium, medium and high, low and high treatments were not significantly different from each other (U = 93.0, P = 0.44; U = 78.0, P = 0.16; U = 63.5, P = 0.041 respectively; Figure 2.1a), however a trend is clearly observable on the graph.

Experiment 2

The analysis showed a significant effect of conditioning and testing concentrations on both shelter use (for conditioning: $F_{1,113}$ = 4.9, P = 0.029, for testing: $F_{1,113}$ = 19.9, P < 0.001) and time spent moving (for conditioning: $F_{1,113}$ = 18.5, P < 0.001, for testing: $F_{1,113}$ = 13.6, P < 0.001). However, no interaction between conditioning and recognition concentrations was found for either shelter use ($F_{1,113}$ = 0.05, P = 0.82) or time moving ($F_{1,113}$ = 1.2, P = 0.27). The post-hoc comparisons showed that LL minnows did not respond with a different intensity than than HH minnows for change in shelter use (P = 0.68; Figure 2.2a) or time moving (P = 1.00; Figure 2.2b).
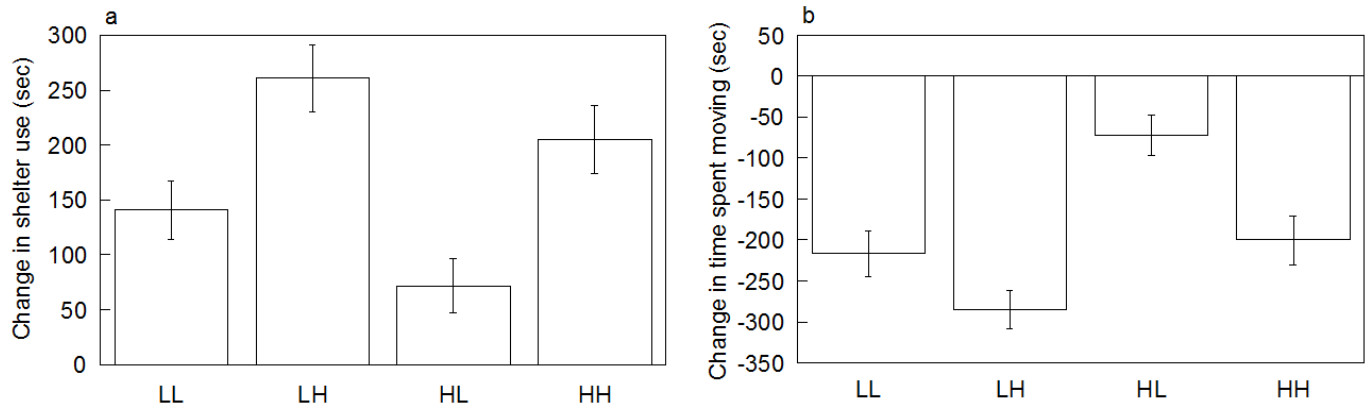
Figure 2.2: Mean (± S.E.) change from the pre-stimulus baseline in (a) shelter use and (b) time spent moving for minnows conditioned with skin extract paired with either a low or a high concentration of pike odour and tested with a low or a high concentration of pike odour. The first letter of the treatment indicates the concentration of pike odour received during conditioning (L = low; H = high) and the second letter indicates the concentration received during testing (N = 30/treatment).

LL minnows responded with a lower intensity than LH minnows for change in shelter use (P = 0.020). However, I did not find a significant difference for time spent moving (P = 0.43). HL minnows responded with a lower intensity than HH minnows for change in shelter use (P = 0.007) and time moving (P = 0.006).

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2.4. Discussion

The results of my experiments demonstrate that minnows can learn to recognize the identity of unknown predators through conditioning with alarm cues in a threat-sensitive manner. Minnows indeed match the intensity of their behavioural response in the conditioning and recognition trials (Experiment 1). Minnows can also adjust the intensity of response to novel predator cues according to the concentration of predator odour experienced during the learning phase. When conditioned with skin extract and a given concentration of pike odour, minnows increased the intensity of their antipredator response when subsequently exposed to higher concentrations of pike odour and decreased their intensity of response when subsequently exposed to lower concentrations of pike odour (Experiment 2).

In Experiment 1, fish increased their intensity of response when exposed to increased concentrations of alarm cues. This result suggests the existence of a graded response for minnows exposed to a concentration gradient of natural alarm cues. I use the term "graded" to express the existence of a correlation between the intensity of response and the concentrations used. Graded does not necessarily mean that the relationship between the two factors is linear. Similar graded responses were later found in redbelly dace (*Phoxinus eos* – Dupuch *et al.* 2004) and goldfish (*Carassius auratus* – Zhao & Chivers 2005) exposed to a concentration gradient of conspecific alarm cues, and also in larval mosquitoes (Kesavaraju *et al.* 2007, Ferrari *et al.* 2008) and larval amphibians (Ferrari, Brown, Messier & Chivers unpublished data). This type of response appears to be adaptive as it allows prey to match the intensity of antipredator response to the predation risk they are exposed to, if the concentration indeed reflects predation risk. In contrast, Brown *et al.* (2001a) exposed fathead minnows to a concentration gradient of H3NO, and found a non-graded response. This could be explained by the difference existing between the natural and the artificial alarm cue, or simply by difference between populations, body conditions or parasite load of the fish, for instance.

During recognition trials, fish initially conditioned with higher concentrations of alarm cues displayed stronger fright responses to trout odour than fish conditioned with lower concentrations. These data suggest that, in general, the graded response is conserved when minnows are subsequently exposed to predator cues alone. This demonstrates that minnows acquire more than predator recognition during conditioning trials, but also the intensity of

response displayed, that is, the level of risk associated with a given concentration of predator cues. Similar results were found in goldfish (Zhao *et al.* 2006) and larval and embryonic amphibians (Ferrari & Chivers unpublished data, Ferrari, Brown, Messier & Chivers unpublished data). Interestingly, minnows exposed to the lowest alarm cue concentration in the conditioning trials did not show overt responses but still acquired the recognition of trout as a predator. A similar learning effect with a sub-threshold concentration of H3NO was demonstrated by Brown *et al.* (2001b). Even if fish do not display overt antipredator behaviour, this sub-threshold concentration increases their vigilance and reliance on secondary visual cues (Brown & Magnavacca 2003; Brown *et al.* 2004).

The results of Experiment 2 confirm that minnows learn to recognize predator odour based on a single conditioning event and that they acquire this recognition in a threat-sensitive manner. Interestingly, no statistical difference was found between LL and HH treatments. These results suggest that the initial intensity of response to a novel predation threat is determined primarily by the concentration of alarm cue and not the concentration of predator odour. I acknowledge the fact that only one concentration of skin extract was used in this experiment, limiting my power of conclusion with this data set only. However, the results of Experiment 1 demonstrated that the intensity of response of naïve minnows to novel predator odour paired with alarm cues is determined primarily by alarm cues concentration and the intensity of antipredator response in the conditioning trials matched the intensity of response during the recognition trials when fish were exposed to the same concentration of predator odour. Taken with the results of this experiment, I can conclude that the concentration of a novel predator odour does not affect the intensity of response of naïve fish, since no difference was found between the LL and HH treatments. In addition, no interaction was found between the concentration of predator odour used during conditioning and testing. If the predator odour concentration was driving the intensity of response, I would expect to have an interaction between conditioning and recognition factors. Put together, this strongly supports the fact that naïve minnows do not have *a priori* knowledge that higher concentrations of novel predator odours represent bigger threats and they respond to changes in relative concentration rather than the actual concentration of predator odours.

Helfman (1989) showed that three-spot damselfish exposed to a predatory model increased the intensity of their antipredator response when the model was larger, closer or in a strike pose. Similarly, cueing on chemical concentrations to respond to a predation threat can be seen as an adaptive way to assess the level of risk to which the prey is exposed. Indeed, stronger concentrations of predator odours could be indicative of the close proximity of the predator or a larger number of predators. Moreover, prey living in complex or murky habitats should rely heavily on chemical cues since visual cues are limited in these types of environments. However, prey living in moving water might deal with chemical cues in a slightly different way. Depending on the type of current, odours are more or less diluted as they travel downstream. Light counter currents might also contain low concentrations of the odour of a predator located in close proximity downstream (Dahl *et al.* 1998). For these reasons, it might be possible that prey living in different habitats rely differently on chemical cue concentrations to which they are exposed to assess the risk of predation.

Chapter 3: The development of threat-sensitive predator avoidance through social learning.[3]

3.1. Introduction

Fathead minnows have been shown to learn to recognize a novel predator through different modes of learning, including social learning. For instance, Mathis *et al.* (1996) placed individual pike-naïve minnows (observers) in tanks with individual pike-experienced minnows (tutors) and exposed the pairs to the odour of pike. When the observers were then placed in a tank alone and subsequently exposed to the pike odour, they displayed a fright response to the previously unknown stimulus. Social learning has also been demonstrated to occur between different species of birds (Vieth *et al.* 1980) and between two species of fish (Mathis *et al.* 1996). It is unknown whether such interspecific social learning occurs in other taxa.

Only one study has examined factors affecting the transmission of social information regarding predation risk. Vilhunen *et al.* (2005) tested the effect of tutor-to-observer ratio on the transmission of recognition of pikeperch (*Sander lucioperca*) by artic charr (*Salvelinus alpinus*). With a greater ratio of tutors to observers, one could predict an increase in the transmission of information due to the increase in opportunities for the observers to learn. Contrary to predictions, they found that an increase in the number of tutors reduced the ability of naïve charr to learn the novel predator. They argued that groups with a large number of knowledgeable individuals reduced the intensity of response of the tutors, due to the dilution effect.

The purpose of this chapter was to investigate in more detail the development of threat-sensitive social learning of predator recognition. In Experiment 3, I investigated whether 1-fathead minnows could learn to recognize brook trout as a threat by observing the fright response of conspecifics placed in an adjacent tank (i.e., if social learning could occur through transmission of visual cues only) and 2- whether there is a correlation between the intensity of

[3] The content of this chapter is published in the following papers:
Ferrari, M.C.O., Trowell, J.J., Brown, G.E. & Chivers, D.P. 2005. The role of leaning in the development of threat-sensitive predator avoidance in fathead minnows. *Anim. Behav.* **70**, 777-784.
Ferrari, M.C.O. & Chivers, D.P. 2008. Cultural learning of predators in mixed species assemblages: the effects of tutor-to-observer ratio. *Anim. Behav.* **75**, 1921-1925.

response displayed by the tutors in the learning phase and the intensity of response displayed by the observers when exposed to predator cues alone. I hypothesized that observers learning from tutors displaying high intensity of antipredator responses would learn to respond to the brook trout odour with a higher intensity than that of observers learning from tutors displaying lower intensities of responses.

The purpose of Experiment 4 was 2-fold: 1- to test whether or not cross-species cultural transmission of predator recognition occurs in larval amphibians, i.e., whether chorus frog tadpoles could learn to recognize novel tiger salamanders as a threat from woodfrog tadpoles; and 2- to test if the tutor-to-observer ratio would have an effect on learning efficacy. I conducted this experiment in three phases: 1- I obtained salamander-naïve and salamander-experienced woodfrog tadpole tutors, 2- I paired experienced and naïve tutors with naïve chorus frog tadpoles (observers) and exposed them to salamander odour, and 3- I subsequently tested the chorus frog observers for a response to salamander odour or a water control. To test for the effect of tutor-to-observer ratio, I paired either two woodfrog tutors with five chorus frog observers, or alternatively five woodfrog tutors with two chorus frog observers. The ability of prey to learn to recognize novel predators has far-reaching implications for individual survival.

3.2. Methodology

Test species & stimulus collection for Experiment 3

Experiment 3 was performed simultaneously with Experiment 1 (see Chapter 2). Thus, the origin of the minnows and the trout and the stimulus preparations are identical to that of Experiment 1.

Water, predators and test subjects for Experiment 4

Five weeks prior to starting the experiment, a 1900-L tub was filled with well water and left outdoors. The tub was enriched with aquatic plants (sedges: *Carex* spp, slough grass, horsetail: *Equisetum* spp.), zooplankton and phytoplankton from a local pond using a fine mesh dip net. This ensured that the holding and testing water contained a full array of algae and plankton but no salamander cues that could possibly be present in any local pond water. This water is hereafter referred to as 'well water'.

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Two tiger salamanders (snout-vent length: ca. 18 cm) were caught from Feedlot pond on the University of Saskatchewan campus in April 2007, using minnow traps. The salamanders were kept in a 30-L plastic tub filled with 15 L of well water and fed earthworms.

Four freshly laid woodfrog egg clutches and 20 freshly laid boreal chorus frog egg clutches were collected from a pond in central Alberta on April 27, 2007. Field research for the past 3 years has demonstrated that no salamanders were present in this pond, even though they are present in this region of Alberta. Moreover, Ferrari *et al.* (2007a) demonstrated that woodfrog tadpoles from this population did not have an innate recognition of salamander cues. No studies have considered whether chorus frogs showed antipredator responses to salamander predators in the absence of experience.

I held the tadpoles of the two species separately in two pools (60-cm diameter) containing pond water and aquatic plants. The pools were positioned on the pond, to equalize the temperature of the pool water with the pond water. After hatching, the tadpoles were raised for 2 weeks. I provided the tadpoles with rabbit food to supplement the algae already present in the pools.

3.2.1. The response intensity of observers correlates with that of their tutors

Experimental protocol

This experiment consisted of two phases: conditioning trials followed by recognition trials. During the conditioning trials, a single observer minnow was exposed simultaneously to trout odour and to the sight of three conspecific tutors in an adjacent tank displaying different intensities of antipredator response. The tutors used in this experiment were the fish that were exposed to the various concentrations of alarm cues paired with trout odour in Experiment 1. The conditioning trials for both experiments were performed simultaneously.

Experimental setup

The experimental set up consisted of paired 37-L tanks put side by side and separated with a one-way mirror (Figure 3.1). The observer tanks were identical to the tutors' tanks; they were equipped with a gravel substrate, an air stone and injection tube. A light source was placed

above the tutor tanks, with a black plastic partition preventing the light from reaching the observer tank. The difference in luminosity between the two tanks made it easier for the observer fish to look at the tutors through the one-way mirror. Moreover, the tutors could not see the observer so their behaviours were not influenced by the observer fish. I chose to set up a single observer to maximize the effect of the tutors on the observer.
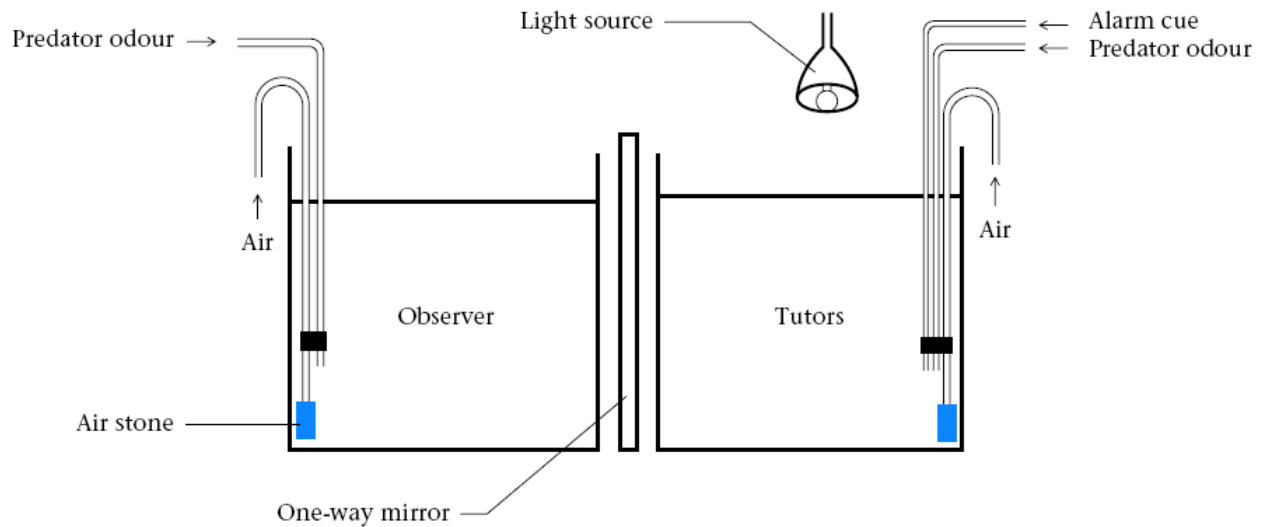
Figure 3.1: Schematic diagram (side view) of test tanks used in Experiments 1 and 3.

Conditioning trials

Prior to conditioning, observers were acclimated for a 24-h period in their respective tanks (water at 12-14°C, same photoperiod as mentioned before). I conditioned 60 observers (fork length: mean ± S.D. = 4.73 ± 0.54 cm), each of which was paired with a group of three tutor fish from Experiment 1. During the conditioning trials, I introduced 20 mL of trout odour at the same time as the skin extract and trout odour were added to the tutor tanks. I did not quantify the behaviour of the observer fish during the conditioning trials. Indeed, any behavioural responses from the observer at that time would be due to social facilitation and not a result of learning. To ensure that the observers had acquired recognition of trout odour as a threat, I needed to subsequently test each minnow for its response to trout odour alone.

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Recognition trials

One hour after the end of the conditioning trials, the observers were transferred into similar 37-L tanks filled with clean dechlorinated tap water. The tanks contained a gravel substrate, an air stone, an injection tube and a shelter that consisted of a 10 x 20 cm ceramic tile mounted on three 3.5-cm long cylindrical glass legs. After 24 h, recognition trials were performed. The same experimental procedure as the conditioning trials was used. After the pre-stimulus period, 20 mL of trout odour were injected in the observer tank. The behavioural measures recorded were time spent moving (in sec) and time spent under shelter (in sec). All trials were performed blind and the order of testing was randomized.

Statistical analysis

For both conditioning and recognition trials, I calculated the change in time spent moving and in time spent under shelter from the pre-stimulus baseline. A reduction in time spent moving and an increase in shelter use indicate an antipredator response. The data did not meet parametric assumptions. Thus, I analysed the effect of treatment on each of the two response variables using a Kruskal-Wallis test followed by two-tailed Mann-Whitney U tests. To correct for type I error, the level of rejection was set at 0.008 following a Bonferroni adjustment.

To test whether the intensity of response displayed by the tutors was correlated with the intensity of response displayed by the observers during the recognition trials, I ranked the tutor responses from 1 to 60 (1 = lowest response; 60 = highest response) for both change in line crosses and shoaling index, and the observer responses from 1 to 60 for change in time spent moving and shelter use. The degree of association between tutor and observer responses was then analyzed using Spearman correlations.

3.2.2. Learning efficacy increases when the tutor-to-observer ratio increases

Experimental protocol

Training of woodfrog tutors

Following the methodology of Ferrari *et al.* (2007a), two groups of ca. 400 woodfrog tadpoles were placed into two tubs (56 x 42 cm) containing 47 L of well water and rabbit chow.

A tiger salamander was added to one of the tubs and the two tubs were left undisturbed for 3 days. I did not quantify the predation rate on tadpoles in the tub. However, I observed the salamander feeding on tadpoles numerous times and the tadpoles avoiding the area of the tub containing the salamander. After 72 h, the salamander was removed from the tub and a 100% water change was performed on both tubs. I considered as salamander-experienced tutors the tadpoles from the tub containing the salamander, and as salamander-naïve tutors the tadpoles from the tub without the salamander. I also considered the chorus frog tadpoles to be naïve to salamander cues.

Conditioning of chorus frog observers

I placed groups of either two or five chorus frog observers in 3.7-L plastic pails containing 3 L of well water and added either experienced or naïve woodfrog tutors to obtain a total of seven tadpoles per pail. Due to the size difference between the tadpoles of the two species, I could always identify the woodfrog tadpoles from the chorus frog tadpoles (mean ± S.D. total length of woodfrog tutors: 1.60 ± 0.07 cm; total length of chorus frogs: 1.37 ± 0.01 cm). I let the tadpoles acclimate for 5 h. I then injected 20 mL of salamander odour in each pail, and performed a 100% water change 2 h after the injection of the stimulus in the pails. The salamander odour was obtained by placing a salamander in a plastic tub (56 x 42 cm) containing 15 L of well water for 3 days. The soaking tub water was used as salamander odour. The salamander used for odour collection was maintained on an earthworm diet and was never fed tadpoles. This ensured that the response of the tadpoles to salamander odour was not confounded by cue emanating from the predator's diet (reviewed by Chivers & Mirza 2001a)

Testing of chorus frog observers

After the conditioning trials, I placed two chorus frog tadpoles from each bucket in individual 0.5-L plastic cups filled with well water and left them to acclimate for 1 hr. I then exposed one of the two chorus frog observers to 5 mL of salamander odour while exposing the other tadpole to 5 mL of well water. I recorded their behavioural responses using the methodology described below.

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Behavioural assay

Numerous studies have established that frog tadpoles decrease activity when exposed to predation risk (e.g., Hokit & Blaustein 1995, Kiesecker & Blaustein 1997, Chivers & Mirza 2001b). To quantify activity, a diameter line was drawn on the bottom of my testing cups and the number of line crosses was counted. A line cross occurred when the entire body of the tadpole crossed over the line. My testing protocol consisted of quantifying line crosses for 4 min prior to and 4 min following the injection of the stimulus (5 mL of either salamander odour or well water) in the cup. The stimulus was injected gently on the side of the cup to minimize disturbance. I recorded the antipredator behaviour of 126 tadpoles in a 3-way design testing the effect of tutor experience (naïve vs. experienced), tutor-to-observer ratio (2 to 5 vs. 5 to 2) and cue (water vs. salamander odour). All trials were performed blind and the order of testing was randomized.

Statistical analysis

I analyzed the change in number of line crosses from the pre-stimulus baseline using parametric tests, as the data was normally distributed and homoscedastic. As the two tadpoles from one pail were not independent, the effect of pail was included in the analysis. I analyzed the change in the tadpole responses using a 3-way mixed ANOVA model, using tutor experience, tutor-to-observer ratio and cue as fixed factors and pail as a random factor. Due to a significant 3-way interaction, I subsequently performed two 2-way ANOVAs on the response of tadpoles to further investigate the nature of the interaction.

3.3. Results

Experiment 3

Comparisons between treatments

The Kruskal-Wallis tests revealed that minnows exposed to the different groups of tutors differed in their subsequent responses to trout odour alone (time spent moving: $\chi^2_3 = 30.1$, P < 0.001; shelter use: $\chi^2_3 = 18.8$, P < 0.001). For both change in time moving (Figure 3.2a) and shelter use (Figure 3.2b), minnows having the opportunity to learn from tutors exposed to low concentrations of alarm cues did not respond differently from fish having the opportunity to learn

from tutors exposed to distilled water (U = 99.0; P = 0.60; U = 100.0; P = 0.62). However, response of minnows having the opportunity to learn from tutors exposed to medium concentrations (U = 29.5; P < 0.001; U = 52.0; P = 0.011) and high concentrations of skin extract (U = 3.0; P < 0.001; U = 29.5; P < 0.001) were significantly different from those having the opportunity to learn from tutors exposed to distilled water.
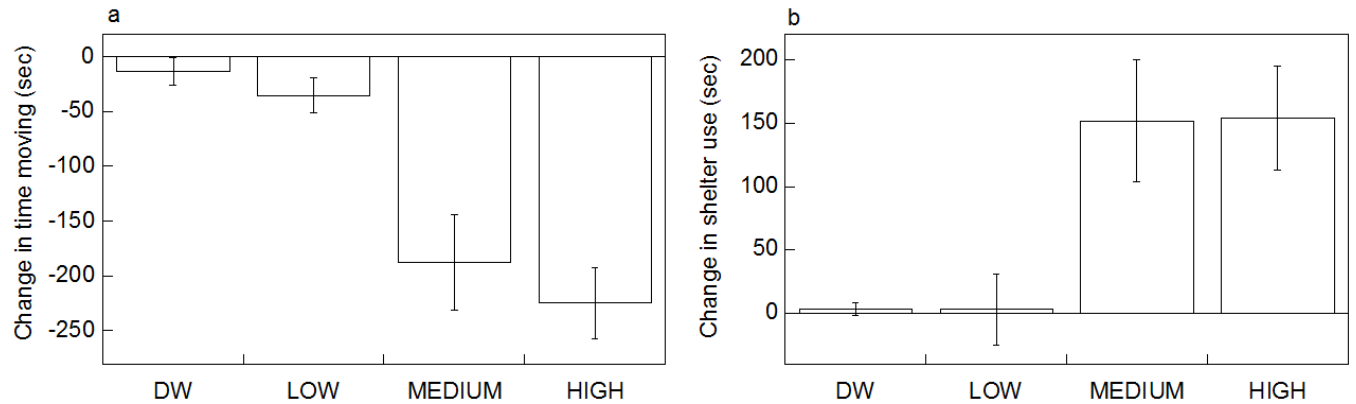
Figure 3.2: Mean (± S.E.) change from the pre-stimulus baseline in (a) time spent moving and (b) shelter use for observer fathead minnows conditioned with tutors exposed to different concentrations of skin extract or a distilled water (DW) control (N = 15/treatment).

Spearman correlations

I found significant correlations (all P < 0.001) between the responses of the tutors during conditioning trials and the responses of the observers during recognition trials for each of the measured response variables. The correlation coefficient (r_s) equals 0.688 for change in shoaling index of the tutors and change in time moving of the observers (Figure 3.3a). Similar correlation coefficients for other pairs were observed (0.546 for shoaling index vs. shelter use [Figure 3.3b], 0.708 for line crosses vs. time moving [Figure 3.3c] and 0.567 for line crosses vs. shelter use [Figure 3.3d]).
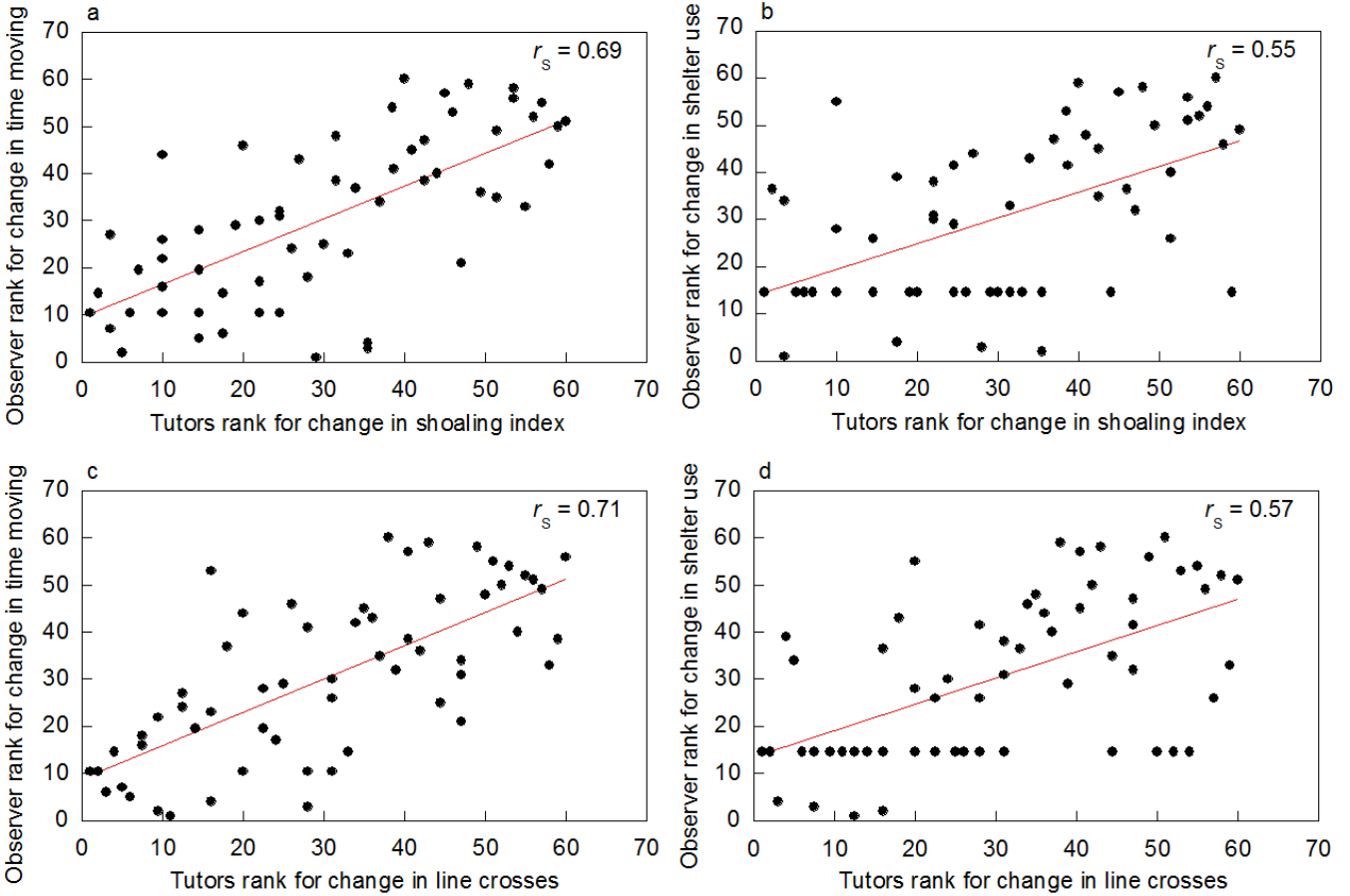
Figure 3.3: Correlation between tutor rank during conditioning and observer rank during recognition trials for (a) change in shoaling versus change in time spent moving, (b) change in shoaling versus change in shelter use, (c) change in line crosses versus change in time spent moving and (d) change in line crosses versus change in shelter use (N = 60).

Experiment 4

The result of the 3-way ANOVA revealed a 3-way interaction ($F_{1,59.6} = 5.0$, P = 0.028, Figure 3.4) on the responses of tadpoles. The 2-way ANOVA performed on the responses of chorus frog tadpoles initially paired only with naïve tutors revealed no effect of cue ($F_{1,30.9} = 0.2$, P = 0.69), no effect of tutor-to-observer ratio ($F_{1, 30.9} = 0.4$, P = 0.53) and no interaction between the two factors ($F_{1,30.9} = 0.4$, P = 0.52). This means that chorus frog tadpoles did not display fearful behaviours to salamander odour without prior experience with it as they responded with the same intensity to water and salamander odour when initially paired with predator-naïve tutors regardless of the tutor-to-observer ratio. For chorus frog tadpoles initially paired with predator-experienced tutors, the 2-way ANOVA revealed a significant interaction between cue and tutor-

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to-observer ratio ($F_{1,29.3} = 6.1$, P = 0.020, Figure 3.4). Subsequent LSD post-hoc comparisons revealed that chorus frog tadpoles responded with the same intensity to water when initially paired with either two or five tutors (P = 0.66). However, I found that tadpoles initially paired with five experienced tutors responded to salamander odour with a greater intensity than tadpoles initially paired with only two experienced tutors (P < 0.001).
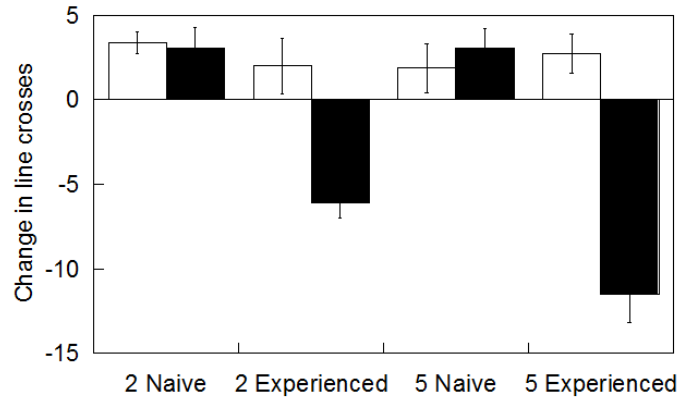
Figure 3.4: Mean (± S.E.) change in line crosses from the pre-stimulus baseline of boreal chorus frog tadpoles responding to well water (empty bars) or salamander odour (solid bars). The chorus frog tadpoles were previously paired with two or five woodfrog tutors that were either salamander-naïve or salamander-experienced (N = 25-30/treatment).

3.4. Discussion

The results of these experiments demonstrate that threat-sensitive learning of predator recognition can occur through cultural transmission and that the intensity of the response to the predator odour is determined by the intensity of response displayed by the tutors (Experiment 3) as well as the number of tutors displaying a response (Experiment 4).

The results of Experiment 3 demonstrate that minnows can learn to recognize the identity of unknown predators through cultural transmission of visual information only. More interestingly, minnows also learn predator recognition in a threat-sensitive manner. Observers simultaneously exposed to trout odour and tutors given distilled water or sub-threshold concentrations of alarm cues (see Experiment 1 for details) did not show fright responses when subsequently exposed to trout odour alone. This suggests that observers do not seem to learn from tutors that do not display any overt antipredator responses. The correlations clearly

demonstrate that observers appear to match their intensity of response to the intensity of response displayed by their respective tutors. This is the first experiment demonstrating that the intensity of a fright response of fish can be culturally transmitted. Similar to the results of Experiment 1, minnows seem to acquire the recognition of predator as well as the level of risk associated with the predator cues. Although this phenomenon has not been investigated in predator avoidance learning in fish, a positive correlation between the acquired alarm behaviour of observers and that of tutors during training was demonstrated in other taxonomic groups, including birds and mammals (reviewed by Griffin 2004).

The results of Experiment 4 provide clear evidence that salamander-naïve chorus frog tadpoles do not show antipredator behaviour to salamander odour without prior experience with it and can learn to recognize salamander cues as a threat when paired with salamander-experienced woodfrog tadpoles. These results raise the possibility that cultural learning in amphibians is indeed widespread. This is only the second species of amphibians for which cultural learning of predator recognition has been considered (see also Ferrari *et al.* 2007a). Further work should test for social learning of predators in this and other taxa that have traditionally been considered much less social. Even more interesting, researchers should examine cross-species cultural learning. My experiment clearly shows that larval chorus frogs that naturally co-occur in the same pond and share similar predators as larval woodfrogs can learn to recognize predators through social learning from each other. This is the first documentation of this phenomenon in amphibians. Many groups of animals show mixed-species aggregations (e.g., mammals, birds, amphibians, fishes). Information transfer regarding predation risk may be a prime factor leading to the evolution of multi-species assemblages.

In Experiment 4, I documented that chorus frog tadpoles responded with a higher intensity of response when they were paired with five experienced tutors, as opposed to only two experienced tutors. Naïve observers have, on average, more opportunities to learn from the experienced tutors, resulting in better information transmission. Alternatively, it might be possible that individual tadpoles use an averaging process to set the intensity of the response that should be associated with a given threat. In one case, each observer is faced with one non-responding conspecific and five responding heterospecifics. In the other case, each observer is faced with four non-responding conspecifics and two responding heterospecifics. It would be

interesting to know whether conspecific chorus frogs and heterospecific woodfrogs are equally reliable from the perspective of a naïve chorus frog. Reliability across the two species could be adaptive, as they do in fact share the same predators (salamanders, diving beetles, larval dragonflies etc.).

An unexplored aspect of social learning in amphibians is the exact mode of transmission of the information. In fishes for example, it has been established that the transmission of the information could be purely visual as observers can learn from watching tutors in an adjacent tank (Experiment 3). Likewise, cultural learning of predator recognition by birds is based on the sight of conspecifics mobbing the unknown predator. In larval amphibians, however, visual or mechanical transmissions are both probable mechanisms for learning. If the transmission is based on mechanical stimuli, then I would predict that the higher the number of tutors, the better the rate of transmission. It is important to realize that a tadpole's antipredator response is to reduce activity. Consequently, the decrease in mechanical disturbance that is associated with the antipredator response of the tutors will be linked to the ratio of active vs. non-active tadpoles. Moreover, in my case, woodfrog tadpoles were larger than the chorus frog tadpoles, and it is likely that larger individuals create more disturbances, and thus induced a higher decrease in disturbance following the injection of the cues.

Social learning is categorized into several types of cognitive mechanisms, ranging from stimulus enhancement (increase in attention of an individual for an object because another individual pays attention to this object) to imitation (an individual imitates the exact behaviour of another individual to achieve a desired goal). The social learning process occurring in this experiment is likely observational conditioning. Observational conditioning, often associated with social learning of predator recognition, is a form of Pavlovian conditioning in which the response of the demonstrator acts as an unconditioned stimulus that elicits a matching response on the part of the observer (Emery & Clayton 2005). However, more testing is needed to ascertain this hypothesis.

Studying learned predator recognition is of particular importance in amphibian species. One of the reasons put forwards for the global decline of amphibian populations is their inability to cope with introduced competitive or predatory species of fish and amphibians (Gamradt & Kats 1996, Blaustein & Kiesecker 2002, Blaustein & Bancoft 2007). More work is needed to

investigate whether amphibians have similar predator learning abilities as other vertebrates. In particular, researchers should focus on the limitations (both spatial and temporal) of learned predator recognition in amphibians, which could partly explain why some species seem to be particularly vulnerable following the introduction of new predators.

Chapter 4: How do prey adjust their response intensity after several learning opportunities?[4]

4.1. Introduction

Even though threat-sensitive predator avoidance appears widespread in a diversity of taxa, very little is known about how such responses develop. This chapter is aimed at understanding the role of learning in the development of threat-sensitive predator avoidance. In a social context, predator-naïve prey (fish, birds and mammals) that encounter a predator often match the intensity of their antipredator behaviour to that of predator-experienced prey in the vicinity (reviewed by Griffin 2004). The obvious question that arises from a threat-sensitive predator avoidance perspective is whether the naïve prey learn to recognize the predator in this social context, and if so, whether the intensity of the learned responses matches that of the predator-experienced prey; in other words: can prey learn the level of risk associated with a particular predation threat? In Experiment 3 (Chapter 3), I exposed naïve fathead minnows to brook trout odour paired with the sight of experienced conspecifics. I showed that if the experienced individuals showed a strong response, then the naïve individual learned to respond strongly. If the experienced individuals responded weakly, then the naïve individual learned to respond weakly. This research shows that minnows acquire predator recognition in a threat-sensitive manner. In a similar experiment (Experiment 1, Chapter 2), I conditioned naïve minnows with different concentrations of alarm cues paired with the odour of brook trout and subsequently tested them for recognition of trout odour alone. During conditionings, I found that minnows responded with stronger intensities when exposed to increased concentrations of alarm cues. I also showed that the intensity of the response during the conditioning phase was retained during subsequent recognition trials, again demonstrating threat-sensitive learning.

Even though a single event is enough to learn the recognition of a novel predator as well as the intensity of response associated with the predation risk, learning should occur continuously. Indeed, as predation level is not fixed and fluctuates in space and time (Lima &

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[4] The content of this chapter is published in the following paper:
Ferrari, M.C.O. & Chivers, D.P. 2006. Learning threat-sensitive predator avoidance: how do fathead minnows incorporate conflicting information? *Anim. Behav.* **71**, 19-26.

Dill 1990), a continuous learning process could keep prey "up-to-date" regarding the actual threat of a given predator. Thus, the purpose of this study was to understand the development of such threat-sensitive responses through multiple conditioning events. In Experiment 5, trout-naïve fathead minnows were conditioned twice with either a high or low concentration of skin extract (low/low, low/high, high/low, high/high) paired with trout odour. Minnows were subsequently tested for recognition of trout odour alone. For each phase, the intensity of minnows' behavioural response was recorded and compared between treatments. In Experiment 6, I investigated the importance of the conditioning sequence, testing if the sequence of conditioning has a significant effect on the intensity of antipredator behaviours displayed during the recognition trial. I wanted to examine if more recent experiences played a key role in the intensity of response displayed by minnows.

4.2. Methodology

Test fish

Fathead minnows used in Experiment 5 were captured from a local pond using minnow traps in October 2003. They were housed in a 6000-L flow-through pool filled with dechlorinated tap water at 13-15°C and fed *ad libitum* once a day with commercial flakes. The photoperiod was adjusted to 14:10 h light:dark cycle. Minnows used in Experiment 6 were caught in May 2004 from the same pond and housed in the same conditions. These minnows were kept 1 month in captivity prior to the experiment to ensure all of them were out of breeding condition (minnows in breeding condition stop breeding within 3 weeks in captivity in my laboratory conditions, pers. obs.). Brook trout were obtained from the Fort Qu'Appelle fish hatchery, Saskatchewan, in October 2004 and housed in a 6000-L flow-through pool filled with dechlorinated tap water at 13-15°C. Trout were fed *ad libitum* once a day with commercial trout pellets.

Stimulus collection

Minnow skin extract

I collected skin extract from six donor fathead minnows (fork length: mean ± S.D. = 4.65 ± 0.34 cm) and obtained 19.2 cm^2 of skin in a total of 484 mL of distilled water, which

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constituted my standardized solution containing ~1 cm^2 of skin per 20 mL of distilled water. This solution was diluted to make two experimental solutions: low (1cm^2 of skin per 240 L) and high (1cm^2 of skin per 40 L) concentration of alarm cues. Skin extracts were frozen in 20-mL aliquots at -20°C until required. The high and low concentrations used in this experiment are the same high and low concentrations used in Experiment 1. The skin extracts for Experiment 6 was obtained from six minnows (fork length: mean ± S.D. = 5.33 ± 0.37 cm). I collected 30.61 cm^2 of skin in a total of 612 mL of distilled water. This solution was diluted to obtain the same low and high concentrations of alarm cues mentioned above.

Trout odour

Prey animals often exhibit antipredator responses to chemical cues of predators fed conspecifics of the prey, but not those fed another diet (Chivers & Mirza 2001a). Thus, two arbitrarily chosen brook trout (20.6 and 24.5 cm fork length for Experiment 5, and 23.5 and 25 cm fork length for Experiment 6) were kept in a 115-L tank and fed brine shrimp for 5 days prior to stimulus collection. Both trout were then transferred to a 72-L tank containing 60 L of dechlorinated tap water, an air stone but no filter. The trout remained in the stimulus-collection tank for 24 h, at which time they were transferred back to their initial holding pool. The trout were not fed during this 24-h period. Water containing trout odour was frozen at -20°C in 400-mL aliquots until required.

4.2.1. Prey use a safety strategy to respond to predation threats

Experimental protocol

This experiment consisted of three phases: two conditioning trials followed by a recognition trial. During the first conditioning trial, groups of three minnows were exposed to trout odour paired with a high or low concentration of conspecific alarm cues. During the second conditioning trial, the same fish were exposed again to one of two concentrations of alarm cues paired with trout odour. Consequently, I have four treatments depending upon the concentrations used for the first and second conditioning respectively: low/low, low/high, high/low or high/high. During the recognition trials, fish were exposed to trout odour only.

Conditioning trials

Twenty four hours prior to the first conditioning event, groups of three minnows were placed in 37-L tanks (50.3 cm x 25.3 cm x 30 cm) filled with dechlorinated tap water (~15°C, same photoperiod as mentioned before) for acclimation. Fish were fed twice a day: 1 h after being transferred into their tank and 1 h prior to conditioning. Each tank contained a gravel substrate, an air stone and a 2-m piece of plastic tubing used to inject stimuli in the tank. There were 20 replicates of each treatment, with three fish per replicate (fork length: mean ± S.D. = 4.92 ± 0.36 cm).

All trials were conducted between 1300 and 1900 h. Observations were conducted following the same protocol as used in Experiment 1. Following the pre-stimulus period, I injected 5 mL of either the high or low concentration of skin extract as well as 20 mL of trout odour into the tank.

As a measure of antipredator response, I recorded an estimated shoaling index of the three fish every 15 sec as well as the number of line crosses (see methodology for Experiment 1). An increase in shoaling index and a decrease in activity level are two typical antipredator responses in minnows (reviewed by Chivers & Smith 1998).

One hour after the end of the first conditioning trial, the fish were moved to an identical 37-L tank filled with clean dechlorinated tap water. After 24 h, the second conditioning trial was performed following the same procedure described above. After the pre-stimulus period, I also injected 20 mL of trout odour and 5 mL of a high or low concentration of alarm cue.

Recognition trials

One hour after the end of the second conditioning trial, the fish were moved to an identical 37-L tank filled with clean dechlorinated tap water. After 24 h, the recognition trials were performed. The protocol used was the same used for the conditioning trials with 20 mL of trout odour injected in the tank after the pre-stimulus period. All trials were performed blind and the order of testing was randomized.

Statistical analysis

For both conditioning and recognition trials, I calculated the change in shoaling index and in line crosses from the pre-stimulus baseline. The data were normal and homoscedastic. I conducted an independent sample *t*-test to compare the responses of minnows to the two concentrations of alarm cues during the first conditioning. For response to the second conditioning, paired *t*-tests were used to compare the means between low/low and low/high, as well as between high/low and high/high treatments. Independent sample *t*-tests were used to compare the means between low/low and high/low, and between low/high and high/high treatments. For recognition trials, independent *t*-tests were used to compare the overall response of minnows between the four treatments for each of the two behavioural measures. The alpha value for these comparisons was set to 0.008 following a Bonferroni adjustment.

4.2.2. Prey use updated information to respond to predation threats

Experimental protocol

The experiment consisted of six conditioning events followed by a recognition trial. Fish were conditioned once a day with trout odour paired with either one of the two concentrations of minnow alarm cues. Four conditioning sequences, constituting my four treatments, were used: fish were either conditioned six times with the low concentration of alarm cues only (6L), conditioned the first time with a high concentration followed by five conditionings with the low concentration of alarm cues (1H + 5L), conditioned five times with the low concentration and the last time with the high concentration of alarm cues (5L + 1H), or conditioned six times with the high concentration of alarm cues only (6H). During the recognition trials, fish were given trout odour only and their antipredator responses were recorded.

Conditioning

The experimental set-up and protocol were the same as used in Experiment 5 except that no behavioural measures were recorded during the conditioning trials. Twenty groups of three fish were used for each of the four treatments (fork length: mean ± S.D. = 5.90 ± 0.27 cm). All conditionings were performed between 1200 and 1500 h.

Recognition trials

One hour after the end of the last conditioning, fish were transferred into an identical 37-L tank. Twenty four hours later, recognition trials were performed. Observation consisted of an 8-min pre-stimulus and an 8-min post-stimulus injection period. The same recording method and behavioural responses used in Experiment 5, i.e., change in line crosses and shoaling index, were used for this experiment. All trials were performed blind and the order of testing was randomized.

Statistical analysis

For both conditioning and recognition trials, I calculated the change in shoaling index and in line crosses from the pre-stimulus baseline. The data were normally distributed but the variances among treatments were not all homogenous. For this reason, I used either independent *t*-tests or Welch's *t'*-tests (for comparing heteroscedastic samples - Zar 1999 pp 128-9) to perform all six possible comparisons between treatments. The alpha level was set at 0.008 following a Bonferroni correction for type I error.

4.3. Results

Experiment 5

First conditioning trials

Fish exposed to trout odour paired with the high concentration of skin extract exhibit a significantly stronger antipredator response than fish exposed to trout odour paired with the low concentration of skin extract for change in line crosses ($t_{78} = 12.2$; $P < 0.001$; Figure 4.1a) and shoaling index ($t_{78} = -9.9$; $P < 0.001$; Figure 4.1a').

Second conditioning trials

Fish having the same first conditioning: When fish initially conditioned with a low concentration of skin extract plus trout odour were given low skin extract plus trout odour, they responded significantly less than the same fish given high concentration of skin extract with trout odour for change in line crosses ($t_{19} = 4.9$; $P < 0.001$; Figure 4.1b) and shoaling index ($t_{19} = -3.5$; $P = 0.002$; Figure 4.1b'). When fish initially conditioned with the high concentration of skin

extract plus trout odour were exposed to a low concentration of skin extract and trout odour, they responded less than the same fish given the high concentration of skin extract with trout odour for both change in line crosses ($t_{19} = 5.3$; $P < 0.001$) and shoaling index ($t_{19} = -3.6$; $P = 0.002$).
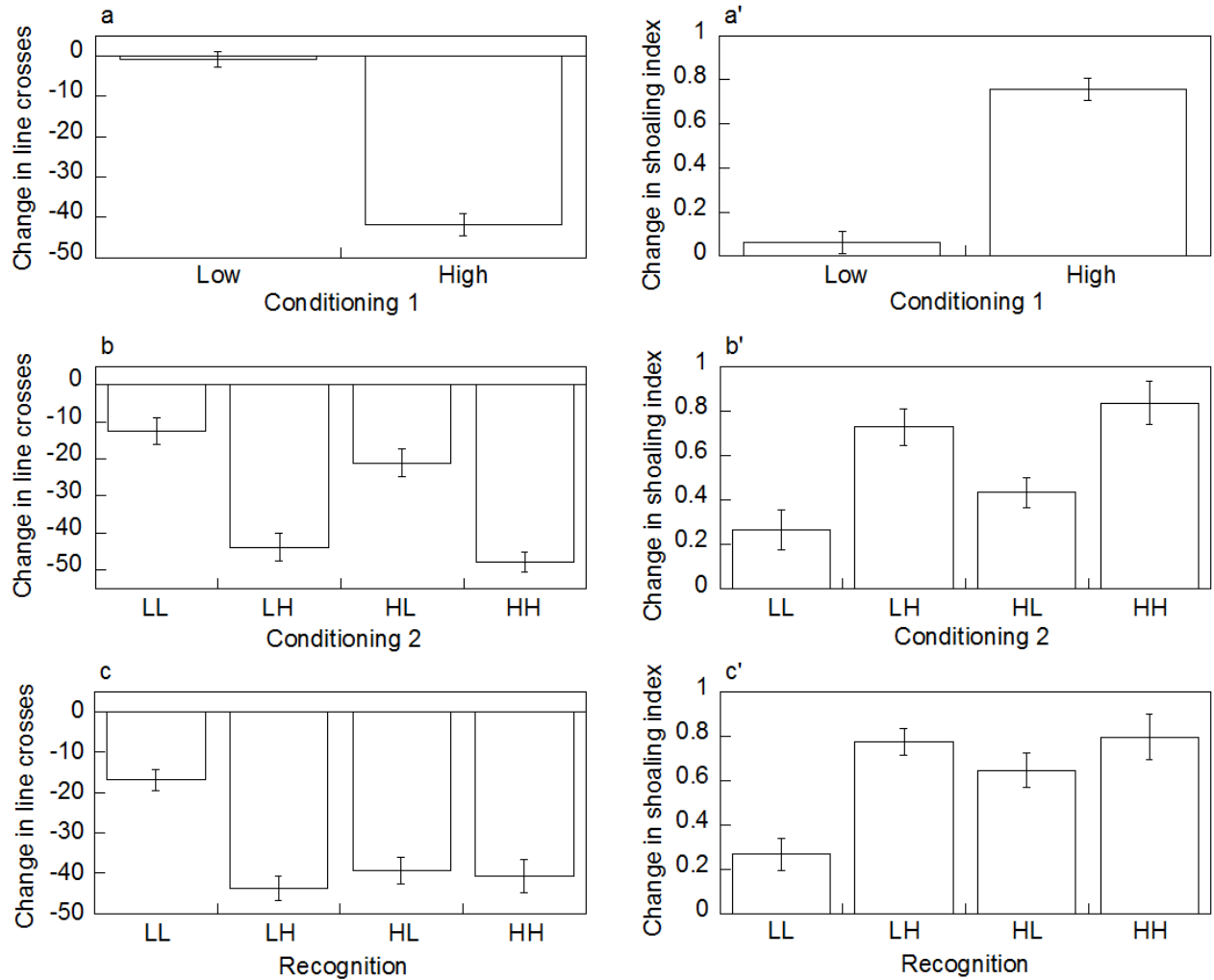
Figure 4.1: Mean (± S.E.) change from the pre-stimulus baseline in line crosses (left panels) and shoaling index (right panels) for minnows exposed to (a) a high or low concentration of skin extract paired with trout odour during the first conditioning, (b) a high or low concentration of skin extract paired with trout odour during the second conditioning, and (c) trout odour only during recognition trials. The first letter of the treatment indicates the concentration of skin extract received during the first conditioning (L = low; H = high), the second letter indicates the concentration received during the second conditioning (N = 20/treatment).

Fish having different first conditioning: Fish initially conditioned with high or low concentration of skin extract did not respond with significantly different intensities to a low concentration of skin extract paired with trout odour during the second conditioning for both

change in line crosses (t_{38} = 1.6; P = 0.11; Figure 4.1b) and shoaling index (t_{38} = -1.5; P = 0.13; Figure 4.1b'). A similar result was found for fish initially conditioned with a low or high concentration of skin extract and trout odour, responding to a high concentration of skin extract paired with trout odour (change in line crosses: t_{38} = 0.8; P = 0.42; change in shoaling index: t_{38} = -0.9; P = 0.39).

Recognition trials

For line crosses and shoaling index respectively, fish given the low/low treatment responded less to trout odour than fish responding to low/high (t_{38} = 6.6, P < 0.001, Figure 4.1c; t_{38} = -5.4, P < 0.001, Figure 4.1c'), high/low (t_{38} = 5.4, P < 0.001; t_{38} = -3.5, P = 0.015) or high/high (t_{38} = 4.9, P < 0.001; t_{38} = -4.1, P < 0.001). Fish did not respond differently when given low/high vs. high/low (t_{38} = -1.0, P = 0.32; t_{38} = 1.3, P = 0.20), low/high vs. high/high (t_{38} = -0.6, P = 0.56; t_{38} = -0.2, P = 0.86) and high/low vs. high/high (t_{38} = 0.3, P = 0.79; t_{38} = -1.2, P = 0.25).

Experiment 6

When tested for recognition of trout odour as a predation threat, minnows' responses varied among treatments. Minnows in the 6L and 1H+5L treatment did not respond differently to trout odour (shoaling index: t_{28} = 0.3, P = 0.78, Figure 4.2a; line crosses: $t'_{20.2}$ = 0.9, P = 0.38, Figure 4.2b). Minnows in the 5L+1H treatment responded stronger to trout odour than minnow in the 6H treatment when looking a line crosses (t_{28} = -2.9, P = 0.007) but this difference was not found when looking at shoaling index (t_{28} = 1.5, P = 0.14).

Minnows in 5L+1H and 6H treatments responded stronger to trout odour than minnows in the 6L treatments for both shoaling index ($t'_{20.2}$ = -5.4, P < 0.001; $t'_{21.2}$ = -3.67, P = 0.001) and line crosses ($t'_{16.8}$ = 6.4, P < 0.001; $t'_{20.5}$ = 4.5, P < 0.001). Minnows in the 5L+1H and 6H treatments responded stronger to trout odour than minnows in the 1H+5L for shoaling index (t_{28} = -5.1, P < 0.001; t_{28} = -3.5, P < 0.001) and line crosses (t_{28} = 5.1, P < 0.001; t_{28} = 2.8, P = 0.008).
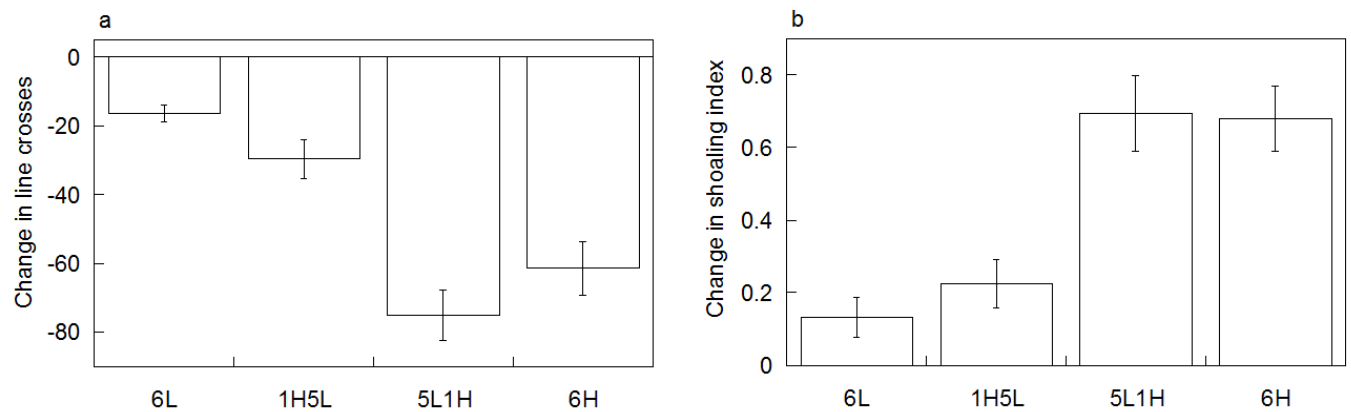
Figure 4.2: Mean (± S.E.) change from the pre-stimulus baseline in (a) shoaling index and (b) line crosses for minnows exposed to trout odour only during recognition trials. Each fish was conditioned six times with trout odour paired with either a high (H) or low (L) concentration of skin extract (N = 20/treatment).

4.4. Discussion

My results demonstrated once again the role of learning in the development of threat-sensitive predator avoidance. Experiment 5 showed that over a short period of time (i.e., 2 days), fish learn to respond to predator cues with a response intensity that matches the highest risk associated with the alarm cues used in the conditioning. The order of conditioning with low vs. high risk cues does not appear to influence the intensity of the learned response. Experiment 6 conducted over a longer period of time (6 days) showed that minnows seem to rely primarily on the latest of a series of conditioning events to respond to a predator threat. Fish responding to a threat with an intensity that matches the present level of risk associated with the threat should be at a selective advantage. Since predation fluctuates in space and time, it would appear adaptive for a prey to use the most updated information regarding the level of threat associated with a given predator.

During the first conditioning event in Experiment 5, fish exposed to the low concentration of alarm cues paired with trout odour responded less than those exposed to the high concentration, as expected (Experiment 1, Chapter 2). During the second conditioning, fish given trout odour paired with different concentrations of alarm cues responded differently even if having undergone the same first conditioning (low/low and low/high as well as high/low and high/high). The results of Experiment 1 (Chapter 2) demonstrated that fish conditioned with the

high concentration of alarm cues displayed significantly stronger antipredator responses when exposed to trout odour alone than those conditioned with low concentration of alarm cues. However, during the second conditioning of this experiment, fish given low concentration of alarm cues paired with trout odour (low/low and high/low) did not display different intensities of response. Both of these responses were significantly weaker than the response of fish given high concentration (low/high and high/high), regardless of the concentration of alarm cues used during the first conditioning. This suggests that when exposed to both known stimuli (at this point, minnows have already been conditioned to recognize trout odour as a predation threat), fish rely primarily on alarm cues to adjust the intensity of their response rather than relying on predator odour. This raises the question of the reliability of predator cues over alarm cues. It is possible that fish simply respond preferentially to the latest alarm cue, because it might represent the most updated information they can get about predation threat. Responding to alarm cues rather than predator odour can also decrease their chance of mispairing a neutral and non-dangerous stimulus as a threat, or allow them to isolate the relevant cue associated with their initial fright response. Indeed, natural habitats are quite complex. Identifying an unknown dangerous stimulus when detecting alarm cues can be challenging in an environment filled with multifaceted biotic and/or abiotic elements. If this is the case, the intensity of response to predator odour alone should depend on the frequency of previous encounters of this odour in the presence of higher or lower concentrations of alarm cues. This computation of previous experiences could be one of the mechanisms used by the fish to display threat-sensitive responses (Lima & Bednekoff 1999, Sih *et al.* 2000). However, a small trend is observable on figures 4.1b and b' suggesting that fish initially conditioned with a high concentration of alarm cues respond slightly more strongly than those initially conditioned with the low concentration. This could also suggest an additive effect of the alarm cues and predator odour on the intensity of response displayed.

During the recognition trials, fish conditioned with trout odour and low concentration of alarm cues only (low/low) displayed significantly lower antipredator responses than fish that had been conditioned at least once with high concentration of alarm cues. Moreover, the intensity of responses of fish conditioned once with high concentration of alarm cues (low/high and high/low) was not significantly different from the intensity of responses of fish conditioned twice with high concentration of alarm cues (high/high). This suggests that the number of conditioning

events does not seem to affect the intensity of response, at least with a double conditioning. The conclusion that can be drawn from this experiment is that minnows learn to recognize predators in a threat-sensitive manner. They seem to use a "safety" strategy, responding to the predator cues with an intensity that matches the highest risk associated with the alarm cues used in the conditioning. The fish do not simply average the two pieces of information to determine the intensity of response. However, 2 days might not be enough for the fish to incorporate a time component to the intensity of response displayed.

This experiment was repeated with more conditioning events to investigate if minnows' intensity of response during recognition trials was determined by a computing of past experiences and/or by the sequence of conditioning. The results of Experiment 6 indicated that the last conditioning event plays a major role in the intensity of response displayed by minnows. Indeed, in my four treatments, fish receiving a high concentration of alarm cues during their last conditioning responded significantly more to predator cues alone, than fish having received a low concentration. There was no significant difference between fish receiving a high concentration for their last conditioning and fish having been conditioned six times with a high concentration. Fish do not seem to use an average of their previous experiences to respond to predator odour alone.

These experiments provide considerable insights into how minnows incorporate past information to update their responses to predators. In Experiment 5, some fish were provided with conflicting information on the risk associated with the predator. The fish were given a high then a low concentration of alarm cues paired with trout odour. Alternatively, they were given a low then a high concentration of alarm cues paired with trout odour. In both of these cases, the fish subsequently responded to the trout odour with a high intensity response regardless of the order of conditioning. These results somewhat contrast with the results of Experiment 6. When the fish were given contradictory information over a longer period of time they only seem to take into account the most recent information. The question that I cannot yet answer is how much successive consistent information is necessary to override past conflicting information. My results show that they will not ignore conflicting information when it is most recent (Experiment 5), but they will ignore it when the conflicting information is followed by at least four pieces of consistent information (Experiment 6).

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These two experiments tell us that prey fish use updated information to respond appropriately to a predation threat. When given both alarm cues and the odour of a known predator, fish rely primarily on the alarm cues to decide at which intensity they will respond to the threat. Once again, the alarm cues seem a best way to get updated information about a given predator. Threat-sensitive predator avoidance implies that the intensity of antipredator behaviours displayed by the prey matches the level of the threat they are exposed to and it seems that continued learning about a given predator is the best way to display threat-sensitive predator avoidance. More work needs to be done to examine the importance of the ratio of high vs. low conditioning, i.e., how previous experience can act as a fine adjustment for the intensity of response to a known predator.

Chapter 5: Temporal aspects of predator learning by prey.[5]

5.1. Introduction

The importance of spatial variability in predation pressure in driving the behaviour of animals has been a cornerstone of much of the past research in ecology and behavioural ecology (Lima & Dill 1990). Variation in risk among different habitats drives many of the decisions animals make including where they forage and reproduce (Werner *et al.* 1983, Magnhagen 1988), what food items they eat (Lima & Valone 1986) and which mate they select (Kelly *et al.* 1999). In contrast, the importance of temporal variability of predation risk in decision making by animals has receiving surprisingly little attention and has only recently come to the forefront of behavioural ecology (Brown *et al.* 2006, Ferrari *et al.* 2008). In one influential paper, Lima & Bednekoff (1999) provided a theoretical model, the Risk Allocation Hypothesis, which forced behavioural ecologists to move away from the static approach of predator-prey interactions and to consider how the frequency of risk over ecological time scales influences behavioural decision making. Intuitively, ecologists know that predation risk can vary from moment to moment and over daily and seasonal cycles. However, the degree of predictability in risk that predators pose to prey is not well studied. We know that some predators feed at night, others are active at dawn and dusk, and others are diurnal. Can prey learn to recognize the temporal frequency of risk to which they are exposed and respond to reduce their risk of predation?

There are some great examples of the importance of temporal variability of risk in driving behavioural patterns. For example, the change in luminosity associated with lunar cycles affects the foraging and activity patterns of many rodents (Clarke 1983, Bowers 1988, Wolfe & Summerlin 1989). Rodents often avoid foraging during periods of full moon light, presumably to avoid nocturnal predators. Little Blue Herons (*Egretta caerulea*) are known to switch their foraging to safer times, such as rainfalls or dusk, when under intense hawk predation (Caldwell 1986). Sullivan *et al.* (2005) demonstrated that red-backed salamanders (*Plethodon cinereus*) respond more strongly to the odour of garter snakes (*Thamnophis sirtalis*) early at night than late

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[5] The content of this chapter is published in the following paper:
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night, likely because thermal constraints restrict the activity of predatory snakes later at night, making them less of a threat for the salamanders. As well, copepods exhibit diel vertical migration as an antipredator response to escape from predators (Neill 1990). While these behavioural responses might be a result of selective removal through natural selection, i.e., the individuals failing to exhibit this behavioural pattern become depredated, an alternative explanation is that prey learn to respond to predation risk in a temporal threat-sensitive manner.

Many prey species have been shown to respond to predators in a threat-sensitive manner, i.e., with an intensity that matches the level of threat to which they are exposed (Helfman 1989). For example, Pacific treefrog tadpoles increased the intensity of their antipredator response to cues from caged North-Western salamander larvae when their vulnerability to the predators increased (Puttlitz *et al.* 1999). Similarly, Mathis & Vincent (2000) showed that larval Central newts (*Notophthalmus viridescens*) responded less to tiger salamander larvae as the newt size increased. Recent work on fathead minnows has shown that minnows adjust the intensity of their antipredator response to predatory pike odour on the basis of the pike size (Kusch *et al.* 2004), and pike density and proximity (Ferrari *et al.* 2006). The level of sophistication exhibited in response to predator cues reflects highly developed predator learning abilities, as minnows are known to lack a response to pike without prior experience with them (e.g., Chivers & Smith 1994b, see previous experiments). Given the sophistication of predator learning abilities of certain prey species and the widespread occurrence of threat-sensitive predator avoidance, it would be surprising if natural selection does not favour individuals that have the ability to learn to respond appropriately to predator cues on a temporal basis, at least in response to predators with predictable diel cycles.

Here, I investigated whether a larval anuran amphibian, the woodfrog, has the ability to learn to respond to novel predation cues in a temporally threat-sensitive manner. I tested whether tadpoles could associate a new threat with the time of day they encounter it, hence responding more during the periods of day when the predator was more likely to be present and feeding, than during periods of day when the predator was non-threatening. The ability of prey to exhibit more intense antipredator responses to periods of the day when they are more vulnerable should allow the prey to maximize trade-offs between predator avoidance and other activities such as foraging. In addition, if prey can learn the periods of days when they are most vulnerable and combine this

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with information about location of danger, then prey may be able to exhibit time/place learning of predation risk (Reebs 2002).

Amphibian tadpoles, like many species of aquatic organisms, have been shown to acquire predator recognition through the pairing of alarm cues with novel predator cues (Chivers & Smith 1998, Woody & Mathis 1998, Mirza *et al.* 2006). Thus, I used this mechanism to teach naïve woodfrog tadpoles to learn to recognize the odour of tiger salamanders as a threat. For several days, I exposed groups of tadpoles to alarm cues paired with salamander cues in the morning, and salamander cues alone in the evening, thus indicating to them that the salamander was feeding and hence dangerous in the morning, while present but not feeding in the evening. Another group was given the opposite treatment, for which the salamander was more dangerous in the evening than in the morning. After the treatment period, I planned to test both groups of tadpoles for their response to salamander cues in the morning and the evening. Several predictions can be made. First, tadpoles might respond equally to salamander cues in the morning and in the evening regardless of treatments, as the treatment period might not be long enough for the tadpoles to learn the predator's foraging cycle. Moreover, exposing the tadpoles to salamander odour alone still indicates the presence of the predator in the vicinity and prey might not take a chance of being depredated by lowering their intensity of response. Alternatively, tadpoles might show a different intensity of antipredator response according to the threat posed by the salamander. Prey should be at a selective advantage if they adjust their antipredator response to match their vulnerability. In this way, they have the opportunity to maximize foraging while not over-responding to predators. Predators feeding on larval amphibians include many ectotherms that have thermal constraints limiting their effectiveness as predators at specific times (Sullivan *et al.* 2005). The predators I used in my experiment, the tiger salamander, are known to exhibit diel patterns of activity and movement (Holomuzki & Collins 1983). Consequently, they should be a predator that tadpoles could learn represents different levels of risk during different periods of the day. Woodfrog tadpoles provide a great study model to address temporal aspects of predator recognition.

5.2. Methods

Water, predators and test species

Four weeks prior to starting the experiment, a 1900-L tub was filled with well water and seeded with zooplankton, phytoplankton and aquatic plants using a fine mesh dip net. This was done to ensure that my holding and test water did not contain any cues from salamanders. Tiger salamanders occur in the region of my field site but research from the past 3 years indicates that no salamanders inhabit my study pond and that woodfrog tadpoles do not show any innate recognition of salamander cues (Ferrari *et al.* 2007a, Experiment 4). This water is hereafter referred to as well water.

Two tiger salamanders (snout-vent length: ~18 cm) were caught from a pond in Saskatoon, Saskatchewan, in April 2007 using minnow traps. The two salamanders were kept in a plastic tub containing 30 L of well water and fed earthworms.

Woodfrog egg clutches were collected in late April 2007 from a pond in central Alberta. Four clutches were transferred into a plastic pool filled with pond water and left floating on the pond to equalize the temperature of the pool water with the temperature of the pond water. After hatching, the tadpoles were provided with rabbit chow to supplement the algae already present in the pool. The tadpoles were raised for 2 weeks before being used in my experiments.

Experimental protocol

The goal of this experiment was to test whether tadpoles could learn to recognize a novel predator and subsequently respond to it with an intensity that reflects the risk posed by the predator at different times of day. The experiment was performed outdoors. My conditioning protocol consisted of exposing a group of tadpoles to alarm cues paired with salamander odour in the morning and water paired with salamander odour in the evening (morning risk treatment), while exposing other tadpoles to water paired with salamander odour in the morning and alarm cues paired with salamander odour in the evening (evening risk treatment). Initially, I planned to condition the two groups of tadpoles for 9 consecutive days and subsequently test tadpoles from each group in the morning and in the evening for response to salamander odour and well water. However, the cool temperatures of early spring nights (water temperature of 2°C or below)

prevented me from testing the tadpoles in the morning, as tadpole activity remained close to nil in these conditions. Thus, I decided to test the tadpoles in the evening only (after the water temperature increased to 12-15°C). I treated the tadpoles for 9 days, left them untreated for 4 days (Days 10 – 13) and tested them the following two evenings (Days 14-15).

Conditioning procedure

Groups of six tadpoles were randomly assigned to each of 48 3.7-L plastic pails filled with 3 L of well water and provided with rabbit chow. Twenty four pails were then randomly assigned to the morning risk treatment, while the remainder of the pails were assigned to the evening risk treatment. The "alarm cues paired with salamander odour" stimulus consisted of injecting 5 mL of a solution of crushed tadpoles paired with 20 mL of salamander odour in each pail. The "water paired with salamander odour" stimulus consisted of injecting 5 mL of well water paired with 20 mL of salamander odour in each pail. The solution of crushed tadpoles was obtained by grinding 48 tadpoles using a mortar and pestle and suspending the cues in 120 mL of well water. A new solution was made fresh, twice a day, just prior to treating the pails. The salamander odour was obtained by soaking two tiger salamanders in a plastic tub containing 15 L of well water. I removed 3 L of soaking water (referred to as salamander odour) twice a day for treating the tadpoles and immediately added 3 L of fresh well water in the tub. The salamanders were fed three earthworms each, twice, during the conditioning phase of my experiment. The stimuli were gently injected on the side of the pails, to minimize disturbance to the tadpoles. I treated the pails between 0800 and 1000 h each morning and 2000 and 2200 h each evening for the duration of the conditioning phase. The sun rose at ~ 0450 h and set at 2140 h at my field site during this period. After treating the tadpoles for 9 days, I performed a 100% water change on all the pails and provided the tadpoles with rabbit chow. The tadpoles were then left undisturbed for 4 days.

Testing procedure

As explained above, the testing procedure took place in the evening only. The tadpoles were tested between 2000 and 2145 h during two consecutive evenings, 24 pails tested during the first evening (12 from each treatment) and the remaining 24 tested the following evening. Two

tadpoles from each pail were tested, one being exposed to salamander odour and the other one being exposed to a control of well water and the behaviour of tadpoles was recorded.

Twenty minutes prior to testing, individual tadpoles were placed in 0.5-L plastic cups filled with well water. The tadpole behaviour was recorded for 4 min prior to and 4 min following the injection of the stimulus in the cup. During the injection period, 5 mL of either well water or salamander odour was gently injected on the side of the cup to minimize disturbance to the tadpoles. The number of line crosses was the behavioural measure recorded for this experiment. The behavioural assay was similar to the one used for Experiment 4 (Chapter 3). All trials were performed blind and the order of testing was randomized.

Statistical analysis

I used the difference in activity from the pre-stimulus baseline for analysis. The data followed the assumptions of normality and homoscedasticity, allowing me to perform parametric analyses. The response of the two tadpoles coming from the same pail were not considered independent and thus, I tested for the effect of time of day treatment (morning vs. evening), cue (well water vs. salamander odour) as fixed factors and pail effect as a random factor using a 2 x 2 mixed ANOVA model, followed by LSD pairwise comparisons.

5.3. Results

The 2 x 2 ANOVA revealed a significant interaction between time of day treatment and cue ($F_{1,77} = 9.9$, P = 0.002, Figure 5.1). Post-hoc comparisons revealed that the responses of tadpoles conditioned in the morning or in the evening to water did not differ (P = 0.64). However, tadpoles exposed to salamander odour always displayed greater antipredator responses than tadpoles exposed to water only (all P < 0.001). In addition, the tadpoles conditioned in the evening responded with a greater intensity to salamander odour than did the tadpoles conditioned in the morning (P < 0.001, Figure 5.1).
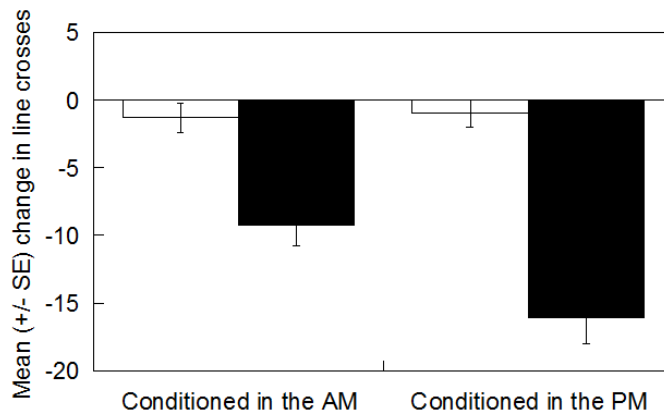
Figure 5.1: Mean (± S.E.) change from the pre-stimulus baseline in line crosses for tadpoles from the morning risk or the evening risk treatments, exposed to water (empty bars) or salamander odour (solid bars) in the evening (N = 19-22/treatment).

5.4. Discussion

My results clearly demonstrate that woodfrog tadpoles have the ability to develop threat-sensitive responses to salamander odour based on the temporal pattern of risk they experience. Indeed, I found that woodfrog tadpoles that were exposed to higher risk in the evening (evening risk treatment) responded with a greater intensity of response to salamander odour in the evening than did the tadpoles exposed to higher risk in the morning (morning risk treatment). Unfortunately, due to inclement weather, I could not test the tadpoles in the morning to verify that the opposite was true, i.e., the tadpoles exposed to higher risk in the morning responding with a greater intensity in the morning than the tadpoles exposed to the higher risk in the evening. However, data collected in my subsequent field season indicate that the evening pattern is indeed reversed from that in the morning (Ferrari & Chivers unpublished data).

The marked temperature difference that tadpoles experience in early spring at northern latitudes provides a confounding factor to the conclusion that time of day *per se* is driving the temporal pattern of learned response intensities. Are prey that are conditioned in the evening, learning to respond with a greater intensity of response to a specific time of day that they were conditioned or alternatively, to temperature conditions during the day that match those under which they were conditioned. I know of no studies that show that learning ability is impaired by low temperature and I found evidence of learning for tadpoles conditioned in the morning and evening. Thus, it seems more likely that the cues the animals cue on, are related to time as

opposed to temperature. This is an important proximate distinction for future researchers to consider in other systems. However, the distinction is somewhat ecologically irrelevant in my study system. Tadpoles in early spring at my latitude undergo a regular temporal pattern that is associated with very cold overnight temperatures followed by considerable daytime warming. Temperature and time of day are intimately linked. The novelty of this research is my demonstration that tadpoles quickly develop differential responses to temporal patterns of predation risk. This learning occurs after the prey experience the predator for only 9 days. One other study has examined whether amphibians exhibit temporal variability in the intensity of their antipredator responses. Sullivan *et al.* (2005) demonstrated that red-backed salamanders respond more strongly to the odour of garter snakes early at night than late night. Low temperatures restrict the activity of predatory snakes later at night, making them less of a threat for salamanders. Whether the temporal patterns of responses of salamanders tested by Sullivan *et al.* (2005) are innate or result from learning is unknown. In my study system, I am confident that the temporal pattern of antipredator responses are due to learning, as woodfrog tadpoles from this exact pond have been shown to lack an innate recognition to salamander cues (Ferrari *et al.* 2007a, Experiment 4). This is the first study providing evidence for temporal threat-sensitive learning of predators by prey animals.

My results raise several interesting proximate and ultimate questions. If prey can learn to recognize the risk associated with a predator at a specific time of day, can they also learn to recognize the risk at a specific location? Even more interesting, can they match both the time and location and thereby exhibit time/place learning of predation risk? Such higher order learning of risk (Reebs 1999, 2002) has not been documented, but clearly deserves consideration. The importance of spatial variability in driving predator-prey interactions is well established in ecology. In contrast, temporal variability in predation pressure has received much less attention (Lima & Bednekoff 1999, Brown *et al.* 2006, Ferrari *et al.* 2008). The development of theoretical models, such as the Risk Allocation Hypothesis, has led me to the realization that, in most predator-prey systems, we know little information about the predictability of risk that prey experience throughout daily, seasonal or yearly cycles. Theory dictates that prey have the opportunity to avoid times at which predators are active; however, predators could counter by matching the activity of prey. I encourage future work to specifically address the issue of predictability. My work suggests that future work should specifically address whether the speed

to which temporal learning occurs is related to the degree to which the predators exhibit temporal variability in their foraging patterns.

Chapter 6: Generalization of learned predator recognition.[6]

6.1. Introduction

A prerequisite for prey to respond adaptively to predation risk is to recognize threats posed by potential predators. The first alternative is for prey to possess an innate recognition of at least some of their potential predators (e.g., mammals [Fendt 2006], birds [Goth 2001], fish [Berejikian *et al.* 2003]). Some other species, however, require experience to respond to predation (learning). Learned predator recognition has been demonstrated in a wide variety of taxa, for both invertebrates (Rochette *et al.* 1998) and vertebrates (fishes [Chivers & Smith 1998], amphibians [Woody & Mathis 1998; Mirza *et al.* 2006], birds [Curio *et al.* 1978], mammals [McLean *et al.* 1996; Griffin *et al.* 2001]).

For many aquatic species, one mode of learning is through the pairing of cues (either chemical or visual cues) from a novel predator with the odour of injured conspecifics (reviewed by Wisenden 2003). For many species of fish, chemicals present in the epidermis, commonly referred to as 'alarm cues', have been demonstrated to elicit a dramatic increase in antipredator responses upon detection. Those chemicals are usually released upon damage of their skin, which usually occurs when a fish is either captured or injured by a predator (Chivers & Smith 1998).

From a phylogenetic perspective, predators that are closely related will generally share similar foraging habits. For example, carnivorous species will require specific behavioural, morphological and physiological adaptations to capture, handle, eat and digest their prey. While these adaptations are diverse among taxa, closely related species will usually share similar adaptations. Thus, prey should have an advantage if they can generalize the recognition of a specific predator to closely related novel predators. This phenomenon, which I refer to as 'generalization of predator recognition' has surprisingly not received much attention from behavioural ecologists. Only two studies have empirically tested for visual generalization of predator recognition. In a landmark study, Griffin *et al.* (2001) demonstrated that tammar

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[6] The content of this chapter is published in the following paper:
Ferrari, M.C.O., Gonzalo, A., Messier, F. & Chivers, D.P. 2007. Generalization of learned predator recognition: an experimental test and framework for future studies. *Proc. R. Soc. B* **274**, 1853-1859.

wallabies conditioned to recognize a red fox, subsequently displayed an antipredator response when exposed to a red fox and generalized their antipredator response to a feral cat, but not to a juvenile goat (*Capra hircus*). Chivers & Smith (1994a) conditioned fathead minnows to visually recognize either a northern pike or a goldfish as a predatory threat. Subsequent testing demonstrated that minnows displayed an antipredator response to the fish they were conditioned to, but did not generalize the fear response to the other species. Perhaps this is not surprising given the considerable differences in the appearance of pike and goldfish. Only one study indirectly tested for the possibility of chemical generalization of predator recognition. Darwish *et al.* (2005) conditioned juvenile glowlight tetras (*Hemigrammus erythrozonus*) to recognize a cocktail of odours containing cues from largemouth bass (*Micropterus salmoides*), convict cichlids and comet goldfish. The tetras displayed an antipredator response when subsequently exposed to each of the predator odours separately, but not when exposed to the novel odour of yellow perch. Again, this may not be surprising given that perch belong to a different family (Percidae) than all of the other fishes (Centrarchidae, Cichlidae and Cyprinidae). The studies completed so far indicate generalization of predator recognition via visual cues by mammals but not other vertebrates, and none demonstrated generalization of predator recognition by chemical cues for any species. These results raise the questions of whether generalization is an ability that is restricted to the most advanced vertebrates and whether it is restricted to visual modalities.

Here, I tested whether a prey fish has the ability to generalize its antipredator response to predator odours of closely related predator species. I conditioned fathead minnows to recognize the odour of lake trout as a predation threat, and I subsequently tested them for a response to lake trout (reference predator), brook trout (same genus as the reference predator), rainbow trout (same family but different genus), northern pike (distantly related predatory fish) or white sucker (distantly related non-predatory fish). An underlying assumption of my work is that taxonomic relatedness will be reflected in the odour signatures of the fishes.

6.2. Methods

To investigate whether fathead minnows could generalize the recognition of potential predators based on predator odours, I first conditioned naïve fathead minnows to recognize the odour of lake trout as a predatory threat. Naïve minnows learn to recognize the odour of a novel predator (including other salmonid fishes) based on the pairing of alarm cues and predator odour.

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Thus, I exposed naïve minnows to lake trout odour paired with either alarm cues (to obtain a group of minnows displaying a fright response when exposed to lake trout odour) or water (control – to obtain a group of minnows solely exposed to lake trout odour without any risk association). The second phase consisted of recording the intensity of antipredator responses displayed by the minnows when subsequently exposed to the 'reference predator' (lake trout) odour, or to the odour of one of the other four fishes (see Table 6.1).

```
Division Teleostei
        Subdivision Euteleostei
                Superorder Ostariophysi
                        Order Cypriniformes
                                Family Catostomidae - white sucker
                Superorder Protacanthopterygii
                        Order Salmoniformes
                                Family Salmonidae
                                        Genus Salvelinus - lake trout, brook trout
                                        Genus Oncorhynchus - rainbow trout
                        Order Esociformes
                                Family Esocidae - northern pike
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Table 6.1: Simplified representation of the taxonomic relationship between the five fish species used in Experiment 8.

Predictions

The minnows used in this experiment were collected from a body of water lacking other fish species. Fathead minnows are known to lack innate predator recognition of the predators used in this experiment (trout: see previous experiments, pike: Chivers & Smith 1994b). Consequently, I predicted that water-conditioned minnows should fail to exhibit antipredator responses to any of the five fishes.

Several predictions are made regarding the responses of alarm cue-conditioned minnows. First, because minnows have been conditioned to recognize the odour of lake trout as a threat, I predicted that minnows should display their highest intensity response to lake trout odour. I could not standardize the diet of all the fish I used, as pike are exclusively piscivorous and do not eat trout pellets, the food which was provided to the four other fishes. I tried to minimize potential diet effects by eliminating the remnants of the last meal of all fishes (see below). If the

generalization of the response of minnows was based on the diet of the reference predator (i.e., the lake trout's diet), I predicted that minnows should show an antipredator response to all fishes but pike (Scenario 1). If the antipredator response of minnows to lake trout odour is not generalized to the odour of other fishes, I predicted that minnows should show an antipredator response when exposed to the odour of lake trout only, and not when exposed to the odours of other salmonids, pike or suckers (Scenario 2). However, it is possible that minnows display partial or total generalization to other salmonid fishes. As brook trout belong to the same genus as lake trout, I predicted that if generalization occurs, minnows should generalize their antipredator responses to brook trout more than to rainbow trout (Scenario 3). It might be possible that minnows generalize their response to all predatory fishes and would display an antipredator response to the odour of all fishes but suckers (Scenario 4), or they might even generalize their responses to all large fishes (Scenario 5). The last two scenarios are less likely, given the knowledge of fathead minnow's response to the odour of unknown predators.

Test fish

Fathead minnows were captured from a local pond using minnow traps in September 2006. They were housed in a 6000-L flow-through pool filled with dechlorinated tap water at 11ºC and fed *ad libitum* with commercial fish flakes.

The brook trout and rainbow trout were obtained from the Fort Qu'Appelle fish hatchery, Saskatchewan, in October 2004, and the lake trout were obtained from the same place in April 2006. The three species were housed separately in 6000-L flow-through pools filled with dechlorinated tap water and fed daily with commercial trout pellets. The three species were kept under the same conditions for at least 5 months. Juvenile pike were captured from Pike Lake, Saskatchewan, in October 2005, using a seine net. They were housed in a 6000-L flow-through pool and fed live minnows and dace. The white suckers were caught using a seine net in Katepwa Lake, Saskatchewan, in April 2006, kept in a 6000-L pool and fed trout pellets. All the fish were kept under a 14:10 h light:dark cycle.

Stimulus collection

Minnow skin extract

I collected skin extract from five fathead minnows (fork length (FL): mean ± S.D. = 5.66 ± 0.46 cm) and obtained 25.9 cm^2 of skin in a total of 518 mL of distilled water. This solution was diluted to obtain a final solution containing ~ 1 cm^2 of skin per 40 L. This concentration has been shown to elicit overt antipredator responses in fathead minnows (see Chapters 2 and 4). Skin extracts were frozen into 20-mL aliquots at -20ºC until required.

Fish odour

The three species of trout and the suckers were kept on a diet of trout pellets. However, pike are strictly piscivorous and thus, could not be fed trout pellets. Furthermore, fish can respond to predators based on the presence of conspecific alarm cues in the diet of the predator (Chivers & Mirza 2001a), thus I had to remove any remnants of fathead minnow or dace alarm cues in the diet of the pike. According to Bevelhimer *et al.* (1985), the gut evacuation of juvenile pike takes 5 days at 5ºC. Thus, 8 days prior to stimulus collection, two arbitrarily chosen juvenile pike (FL = 32 and 38 cm) were transferred into two 74-L tanks, containing a corner filter and an air stone and maintained at 18ºC. The pike were not fed for 4 days and each pike received two adult green swordtails (*Xiphophorus helleri*, ca. 4.5 cm standard length) per day for the next 2 days. Swordtails were fed to the pike, as they are known to lack the alarm substances recognized by fathead minnows (Mathis & Smith 1993, Brown *et al.* 1995).

Three days prior to stimulus collection, two lake trout (FL = 25 and 26 cm), two brook trout (FL = 34 and 35 cm), two rainbow trout (FL = 39 and 40 cm), two suckers (FL = 38 and 38 cm) and the two juvenile pike were placed individually in tanks containing 74 L of clean dechlorinated tap water. The fish were arbitrarily chosen so as to minimize the size difference between all five species. The fish were kept in these individual tanks to allow the elimination of remnants of their last meal, to minimize the potential effect of diet and maximize the effect of species' odour on the response of minnows.

For stimulus collection, the two fish from each species were placed in a 74-L tank containing 50 L of dechlorinated tap water and were left to soak for 24 h. The fish were then

removed, returned to their original holding facility and fed. The fish-conditioned water was stirred and frozen in 60-mL aliquots until required.

Experimental procedure

Conditioning phase

Twenty four hours prior to being conditioned, groups of three fathead minnows were placed in 37-L tanks (50 x 25 x 30 cm) containing 30 L of dechlorinated tap water and a gravel substrate. The tanks were also equipped with an air stone to which was attached a 2-m long piece of tubing used to inject the stimuli into the tanks. Minnows were fed after being transferred and 1 h prior to being conditioned the next day. The conditioning protocol was identical to the one used for previous experiments (see Chapters 2 and 4). The conditioning consisted of injecting sequentially 5 mL of either alarm cues or dechlorinated tap water and 20 mL of lake trout odour, followed by 60 mL of the retained tank water. On each conditioning day, half the tanks received the alarm cue treatment and the other half the water treatment, and the treatments were randomly assigned to the conditioning tanks within the experimental room. At least 1 h after being conditioned, the groups of three minnows were randomly transferred to identical 37-L tanks (used for testing) containing clean dechlorinated tap water and were fed.

Testing phase

The testing phase took place 24 h after the conditioning phase. Minnows were fed 1 h prior to being tested. During this phase, groups of minnows were randomly exposed to 20 mL of the odour of lake trout, brook trout, rainbow trout, pike or sucker, and their behaviour was recorded. The protocol for the stimulus injection followed the same protocol as used in the conditioning phase. As behavioural responses, I recorded both shoaling index and line crosses. The behavioural assay used was identical to the one used in Chapters 2 and 4. I tested 194 minnows. All trials were performed blind and the order of testing was randomized.

Statistical analysis

The data used for the analysis were the difference in behavioural measures between the pre- and post-injection periods. The data were normally distributed but the variance was not homogenous among treatments.

I first investigated potential interactions between the effect of fish species and conditioning on the responses of minnows by performing a two-way Scheirer-Ray-Hare extension of the Kruskal-Wallis test (Sokal & Rohlf 2003 pp 446-7), which is an ANOVA design for ranked data. I then investigated the effect of conditioning on the responses of minnows to each fish odour by performing five independent Welch's t'-tests on the five odour treatments (the alpha level was not modified as the 5 tests use 10 different samples). I then analyzed the effect of fish separately by performing two Kruskal-Wallis tests on the responses of minnows conditioned with water, and minnows conditioned with alarm cues, followed by Mann-Whitney post-hoc tests to investigate the difference between the groups of interests. Due to drastic loss of power related to the number of comparisons, only three Mann-Whitney tests were performed to compare the difference in response of minnows exposed to lake trout, brook trout and rainbow trout odour (the comparisons of interests). For these tests, the alpha level was set to 0.016 following the Bonferroni correction to minimize the likelihood of type I error.

6.3. Results

The results of the multifactorial ANOVA revealed a significant interaction between fish and conditioning for both shoaling index ($H_{4, 188} = 7.8$, P < 0.001, Figure 6.1a) and the number of line crosses ($H_{4, 188} = 5.8$, P < 0.001, Figure 6.1b). The t-tests showed no significant differences in the intensity of response of minnows conditioned with water or alarm cues when the minnows were exposed to the odour of either pike or sucker (shoaling index: both P > 0.15, line crosses: both P > 0.23). However, minnows conditioned with alarm cues displayed significantly higher antipredator responses than water-conditioned minnows, when exposed to the odour of lake trout, brook trout or rainbow trout (shoaling index: all P < 0.039, line crosses: all P < 0.002). The Kruskal-Wallis test on the responses of water-conditioned minnows revealed no significant effect of fish on either change in shoaling index ($\chi^2_4 = 3.7$, P = 0.44) or number of line crosses ($\chi^2_4 = 0.5$, P = 0.97). However, the Kruskal-Wallis test on the responses of minnows conditioned with alarm cues revealed a significant effect of fish on both shoaling index ($\chi^2_4 = 28.5$, P < 0.001) and number of line crosses ($\chi^2_4 = 39.3$, P < 0.001). These results, in conjunction with the results of the t-tests, show that minnows conditioned with alarm cues display antipredator responses when exposed to the odour of the three trout only. The post-hoc Mann-Whitney U tests on those three groups revealed that minnows did not display statistically different intensity

responses to the odour of lake trout and brook trout (shoaling index: U = 162.5, P = 0.063, line crosses: U = 163.0, P = 0.065) but minnows did display a higher response intensity to lake trout odour than rainbow trout odour (shoaling index: U = 124.0, P = 0.006, line crosses: U = 114.0, P = 0.003). When comparing the intensity of response to brook trout versus rainbow trout, minnows did not significantly differ in their shoaling index (U = 179.0, P = 0.30) but they decreased activity significantly more when exposed to brook trout than rainbow trout (U = 116.0, P = 0.009).
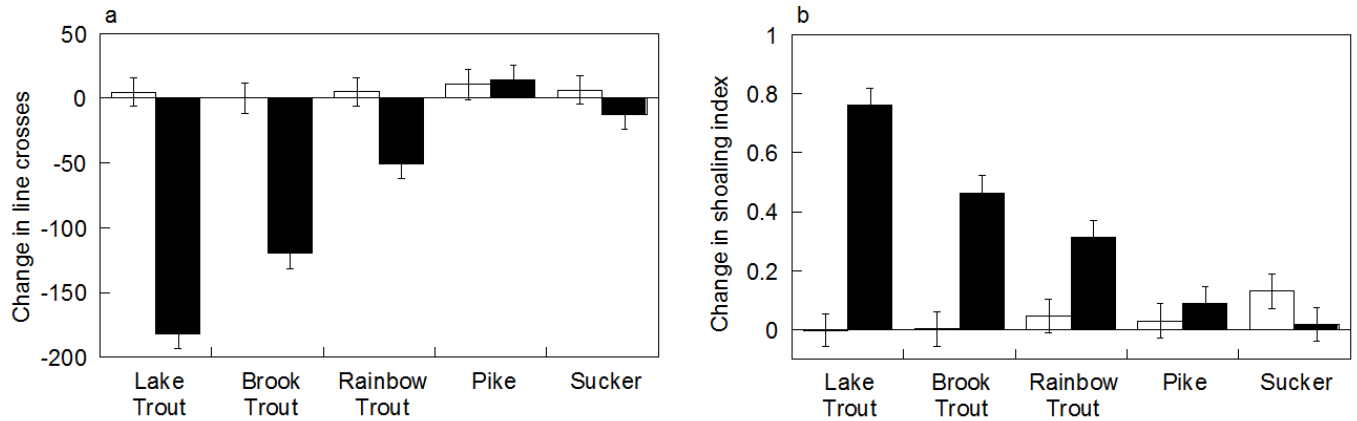
Figure 6.1: Mean (± S.E.) change from the pre-stimulus baseline in (a) shoaling index and (b) line crosses for minnows conditioned with lake trout odour paired with either water (empty bars) or conspecific alarm cues (solid bars), and tested for a response to the odour of either lake trout, brook trout, rainbow trout, northern pike or white sucker (N = 20-23/treatment).

6.4. Discussion

These results suggest that fathead minnows conditioned to recognize the odour of lake trout generalized their recognition to closely related species, the brook trout and rainbow trout, but not to distantly related predatory (pike) or non-predatory (sucker) fish. The absence of response to the sucker odour indicates that minnows did not rely on diet cues to generalize their recognition. The absence of a response to pike odour indicates that the generalization is limited to trout only, and not all fish predators. As expected, minnows responded with the highest response intensity to the odour of lake trout, the species they were conditioned to recognize as a threat. The level of generalization was dependent to some extent, on the degree of relatedness of the other potential predators to the reference predator. Minnows did not respond differently to lake trout and brook trout, but the P-values for both behavioural measures (0.065, 0.067) indicate

that I may have weak support to say that minnows chemically differentiated the two species. I also have evidence suggesting that minnows responded with less intensity to rainbow trout odour than to brook trout odour, hence displaying a graded response to other trout odour, reflecting the taxonomic closeness of these trout species to the reference predator.

The proximate mechanism behind this response pattern may be a difference in the suite of molecules that form the trout odour. In this case, odour molecules among the trout species are likely similar as they are recognized by the minnows, but are not identical as the minnows clearly differentiate the odours. Alternatively, the graded responses could be explained by the existence of a concentration gradient of specific chemicals. Fathead minnows have been demonstrated to adjust the intensity of their antipredator response according to the concentration of predator odour they are exposed to (Experiment 2, Chapter 2). Here, minnows may have learned to recognize specific chemicals from lake trout that are present in high concentration, and may have adjusted the intensity of their antipredator response during subsequent exposures to other trout odours according to the concentration of these particular chemicals.

Plasticity of Generalization of Predator Recognition

Taxonomy of generalization

This chapter presents evidence that fathead minnows are able to chemically generalize the antipredator responses from lake trout to closely related salmonid fishes. Griffin *et al.* (2001) demonstrated that tammar wallabies have the ability to visually generalize their antipredator response from a red fox to a cat. Stankowich & Coss (2007) used felid predator models and showed that Columbian black-tailed deer (*Odocoileus hemionus columbianus*) exhibited a strong antipredator response to a model puma, their current predator, an intermediate response to a novel tiger model but did not differ in their responses to a model jaguar or a model mule deer. Whether the ability of black-tailed deer to generalize resulted from learning or whether it represents generalization from an innate recognition template deserves further consideration. Both fish and mammals have the ability to generalize their recognition of predators to closely related novel predators, consequently it is not unreasonable to think that other vertebrates may also possess this ability. Given the considerable implications of these findings for prey risk

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assessment, I strongly encourage additional work by researchers studying both vertebrates and invertebrates.

When to generalize?

I hypothesize that the degree of flexibility in generalization of predator recognition is dependent on the evolutionary history of predation experienced by each prey species. Species living in relative 'isolation' for long evolutionary periods might have a limited ability to learn and generalize predator recognition. For example, if a prey species is always exposed to the same species of predators over long periods of evolutionary time, then it is likely that animals evolving an innate recognition of those predators will be at a selective advantage, as they do not require the first 'learning trial' to identify the predator as a threat. However, prey species do not always possess innate recognition of predators, implying that there has not been enough time to genetically fix the response and/or there is a cost to genetically fixing such a response.

Two factors affecting generalization in a given environment may be predictability of predation and predator diversity. I hypothesize that it would be beneficial for prey to have innate predator recognition in environments where predictability of attack from a given predator is high and in environments where predator diversity is relatively low. Conversely, it would be beneficial for prey to base their responses on learned predator recognition and have predator generalization abilities in environments where predation predictability is low and predator diversity is high. An unknown aspect of innate predator recognition is whether prey are cuing on specific or general characteristics of the predators they respond to, i.e., the extent to which they can generalize a response to a novel predator.

Imagine a prey animal living in an environment where the ratio of 'predators to non-predators' is high (e.g., a rodent exposed to 10 species of birds, 9 being predatory and 1 not). The rodent would likely benefit from generalizing its predator recognition to all birds as it would do better if it was always scared of a bird, given the probability that failing to respond will result in death. Conversely, if the ratio of 'predators to non-predators' is low (e.g., a rodent exposed to 10 species of birds, 1 being predatory and 9 not), the prey may do better if it specifically learned to recognize the only predatory species and hence not be scared of the non-predatory ones. Keep in mind that responding to predators is costly as it takes time and effort away from fitness-related

activities such as foraging or reproduction (Lima & Dill 1990). Hence, the ability of generalize predator recognition is likely to be directly related to the predation history experienced by prey species in a given habitat. Consequently, it is likely that prey species may have innate recognition of some predators and learned recognition and generalization of some others. Perhaps the best way to approach predator recognition is to think of it as a continuum from "innate predator recognition" to "learned predator recognition without generalization" and finally to "learned predator recognition with generalization". I refer to this as the 'Predator Recognition Continuum Hypothesis'.

Fathead minnows used in my study are common through most of central North America. They inhabit ponds, lakes and rivers and can easily move from one to another during floods. Hence, as a species, their small size and wide distribution will likely result in exposures to a wide variety of predators, particularly when considered over an evolutionary time scale. Hence it might be adaptive for a species like fathead minnows, to be able to have flexibility (or plasticity) in the recognition pattern of potential predators.

What to generalize?

Prey animals likely cue on some specific characteristics of the predators, such as shape, colour or odour. Stankowich & Coss (2007) showed that black-tailed deer do generalize their visual recognition of a puma to a tiger, but not to a jaguar. While all of these felids have the same general shape, they differ in their coat pattern. In this case, deer generalize from a felid with a uniform coat (a cougar) to a felid with a stripped-coat (a tiger), which implies that deer do not cue solely on coat colour to recognize predators. However, the camouflage spotted coat pattern of the jaguar seems to deceive the prey, as the deer are not able to recognize the jaguar as a predator.

In any theoretical consideration of the generalization of predator recognition, I need to consider what cues the prey should use to generalize the predators. A predator's diet has been demonstrated to be an important factor in predator labelling in many species. For example, many fish have been demonstrated to label a novel fish as a predator when detecting conspecific alarm cues in the fish's diet (Chivers & Mirza 2001a). Likewise, rodents cue in on the breakdown of sulphur products in the diet of their predators (Fendt 2006). Thus, one can make the argument

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that diet plays a role in generalization of predator recognition. Here, I argue that diet is a labelling tool, which allows prey to label a novel species as predatory. This phenomenon does not require any true 'recognition' of the predator, but instead the recognition of cues indicating risk. In contrast, true predator generalization requires the ability of prey to use specific characteristics of already known predators to respond to somehow similar unknown species, and thus, should be independent of diet effects.

Blumstein (2002) discussed the effect of relaxed predation pressure on predator recognition in tammar wallabies. He argues that while visual predator recognition could be retained for several thousands of years of predator relaxation, chemical and acoustic predator recognition needed to be learned. Similarly, it may be possible that prey may generalize predator recognition using one type of stimulus but not another. Further research needs to address the use of different stimulus types in generalization of predator recognition. Such work may reveal fascinating taxonomic predispositions towards particular sensory systems.

Generalization of non-predator recognition

A thorough consideration of generalization should include not only what predators prey can generalize to recognize as a threat but also what non-predators prey can generalize to recognize as not a threat. In one study, Griffin *et al.* (2002) tried to condition tammar wallabies to recognize a juvenile goat as a threat, but wallabies did not acquire a fear response to the goat. Three scenarios could explain these results. It is possible that wallabies had previous experience with goats in their environment and had previously learned that goats were not a threat, as goat cues were never associated with risk. Thus, learning to recognize the predator failed due to latent inhibition (Acquistapace *et al.* 2003, Ferrari & Chivers 2006). The second explanation could be that wallabies were previously exposed to a close relative of the goat and thus, as before, did not learn to associate the danger with the sight of the goat due to generalization. Thirdly, it is possible that wallabies innately recognize goats as a non-predator. It is not unrealistic to imagine that the costs associated with responding to non-predators may be high enough for prey to genetically fix the recognition of non-predator characteristics. Thus, there may be a generalization of non-predators. This is an exciting topic that deserves further consideration.

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More studies on the topic of generalization would allow us to answer questions such as: How specific or general is predator recognition? What types of information are prey using to recognize predators? Which factors affect the specificity of learned predator recognition and how does the evolutionary history of predation drive these differences? Factors limiting generalization of predator recognition might be of prime important for endangered species that are translocated in new habitats and exposed to new predator communities. Moreover, the propensity of some species to rapidly and adaptively respond to new communities of predators might help us predict the level of invasiveness of those species.

Chapter 7: Threat-sensitive generalization of predator recognition.[7]

7.1. Introduction

A pre-requisite for prey to show adaptive responses to predation threats is that prey actually recognize the predator as dangerous. Some prey have been shown to display antipredator responses to novel predators upon their first encounter (i.e., innate predator recognition – birds: Veen *et al.* 2000, fishes: Hawkins *et al.* 2004). However, many other species require learning to recognize novel predators as threats (mammals: McLean *et al.* 1996, birds: Curio *et al.* 1978, fishes: Mathis *et al.* 1993). Although many studies have investigated the existence of predator recognition in a variety of species, very little is known about the specific characteristics of the predator that the prey learn to recognize. Three recent studies have revolutionized the way in which ecologists view predator recognition (Griffin *et al.* 2001, Stankowich & Coss 2007, Chapter 6). These studies revealed that prey animals have the ability to display an antipredator response to a novel predator if it is closely related to a predator they recognize. I refer to this phenomenon as generalization of predator recognition (see Chapter 6). These results indicate that learned predator recognition requires labelling of specific characteristics of predators and that predatory traits shared by closely related species of predators can be used by prey to label them as dangerous, prior to any experience with the novel threats. The extent of generalization of predator recognition is unknown. Presumably, generalization of predator recognition would be beneficial for prey, as it would increase their chances of surviving their first encounter with unknown predators, similar to the benefits that innate predator recognition represents. Responding to novel and potentially non-threatening species would, on the other hand, represent a waste of time and energy, which could have been allocated to other fitness-related activities. This paradox raises the question of whether generalization is a rigid phenomenon or whether it is only expressed in situations that would likely benefit the prey.

In the present study, I investigate whether generalization of predator recognition is dependent on the level of risk associated with the known predator, i.e., if there is a threat-

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threshold associated with the known predator that would determine whether or not closely-related species should be labelled as dangerous. Keeping with the example from Chapter 6 of Griffin *et al.* (2001), I could ask whether wallabies would still be scared of cats if the red foxes only represented a mild threat. To answer the question of whether the level of threat of the predator influences the generalization to other predators by the prey, I used fathead minnows as my test subject. In Chapter 6, I showed that fathead minnows conditioned to recognize lake trout odour as a threat, generalize their recognition to novel brook trout and rainbow trout, but not to distantly-related predatory pike or non-predatory white suckers. Using the same system, I conditioned fathead minnows to recognize the odour of predatory brown trout as a high or low risk. I then tested the minnows for a response to the odour of brown trout (reference predator), closely related rainbow trout (same family) or distantly related yellow perch (Table 7.1). I hypothesized that if generalization of predator recognition is a constant phenomenon, then the minnows should display antipredator behaviour when exposed to both brown and rainbow trout, regardless of the level of threat associated with the brown trout (as long as the brown trout represent a threat). Alternatively, if generalization of predator recognition is dependent on the level of risk associated with the reference predator, I hypothesized that minnows should recognize rainbow trout as dangerous only when brown trout are already labelled as highly threatening and minnows should not respond to the odour of rainbow trout when the brown trout represent a low threat. In all cases, minnows are not expected to respond to the odour of yellow perch, as they are distantly related.

Division Teleostei
Subdivision Euteleostei
Superorder Protacanthopterygii
Order Salmoniformes
Family Salmonidae
Genus Salmo - *brown trout*
Genus Oncorhynchus - *rainbow trout*
Superorder Acanthopterygii
Order Perciformes
Family Percidae – *yellow perch*
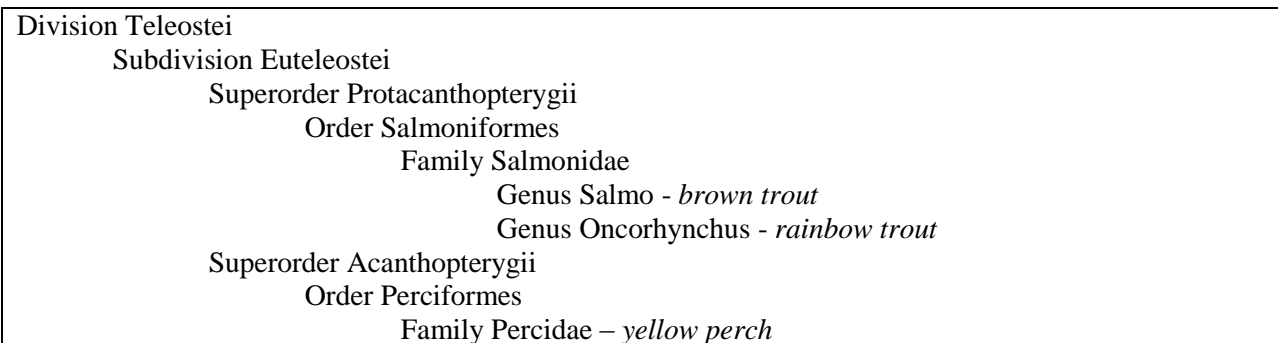
Table 7.1: Simplified representation of the taxonomic relationship between the three fish species used in Experiment 9.

7.2. Methods

Test fish

Fathead minnows were captured from Feedlot pond, a pond located on the University of Saskatchewan campus, using minnow traps in October 2007. Feedlot pond contains minnows and brook stickleback, but lacks any fish predatory species. The minnows were housed in a 6000-L flow-through pool filled with dechlorinated tap water at 11ºC and fed *ad libitum* with commercial fish flakes.

Brown trout and rainbow trout were obtained from the Fort Qu'Appelle fish hatchery, Saskatchewan, in July 2006 and April 2007 respectively. The two species were housed separately in 6000-L flow-through pools filled with dechlorinated tap water and fed daily with commercial trout pellets. Yellow perch were captured from Blackstrap Lake, Saskatchewan, in July 2005 using seine nets. They were similarly housed in a 6000-L flow-through pool filled with dechlorinated tap water and fed live prey (minnows, dace, stickleback or goldfish). All fish were kept under a 14:10 h light:dark cycle.

Stimulus collection

Minnow skin extract

I used a high and a low concentration of conspecific alarm cues to mediate the differential learning of predatory brown trout by the minnows (see Chapter 2). Minnows conditioned with high concentrations of alarm cues recognize the trout as a high-level threat while those conditioned with a low concentration of alarm cues recognize the trout as a low-level (see Chapters 2 and 4).

To produce alarm cues, I collected skin extract from four fathead minnows (fork length (FL): mean $\pm$ S.D. = 5.50 $\pm$ 0.18 cm). I collected 13.9 cm^2 of skin in a total of 278 mL of distilled water. This solution was diluted to obtain a final solution containing ~ 1 cm^2 of skin per 40 L. This concentration has been shown to elicit overt antipredator responses in fathead minnows (see previous chapters). Skin extracts were frozen into 20-mL aliquots at -20ºC until required.

Fish odour

Prey animals often respond to predators based on the presence of conspecific alarm cues in the diet of the predator (Mathis & Smith 1993, Chivers & Mirza 2001a). Thus, the perch were deprived of food for 5 days prior to stimulus collection. After this period, two perch (FL: 17.3 and 17.4 cm), two rainbow trout (FL: 16.0 and 19.1 cm) and two brown trout (FL: 17.4 and 17.6 cm) were removed from their holding pool and placed in pairs in three 74-L tanks filled with dechlorinated tap water at 18ºC. The fish were chosen so as to minimize the difference in size between the three species. To control for the effect of diet in my experiment, all fishes were fed two earthworms (obtained from a local bait store) the following day. The earthworms were cut in ~1-cm long pieces to facilitate feeding. Two days after feeding, the two fish of each species were rinsed and placed in a 74-L tank filled with 50 L of dechlorinated tap water and left to soak for 24 h. Each tank was equipped with an air stone but no filter. After this period, the fishes were returned to their original holding pool and fed. The fish-conditioned water was stirred and frozen in 60-mL aliquots until required.

Experimental procedure

Conditioning phase

Twenty four hours prior to being conditioned, groups of three minnows were placed in 37-L tanks (50 x 25 x 30 cm) containing 30 L of dechlorinated tap water and a gravel substrate. The tanks were also equipped with an air stone to which was attached a 2-m long piece of tubing used to inject the stimuli into the tanks. Minnows were fed after being transferred and also 1 h prior to being conditioned, the next day. Prior to injecting the stimuli in the tank, I withdrew and discarded 60 mL of water from the injection tubes (to remove any stagnant water) and an additional 60 mL of water was withdrawn and retained to flush the stimuli into the tank.

The conditioning consisted of injecting sequentially 5 mL of a high or low concentration of alarm cues or dechlorinated tap water, followed by 20 mL of brown trout odour, and finally 60 mL of the retained tank water. For the high concentration of alarm cues, I injected 5 mL of the prepared solution of alarm cues (1 cm^2 of skin per 40 L - see above). For the low concentration

of alarm cues, I withdrew 1 mL of the prepared solution of alarm cues and 4 mL of dechlorinated tap water in a 5 mL syringe and injected the content of the syringe (equivalent to 1 cm^2 of skin per 200 L) into the tank. Previous work (see Chapter 2) showed that fathead minnows acquire recognition of the odour of a novel predator through similar conditioning using alarm cues at a concentration as low as 1 cm^2 of skin per 240 L. On each conditioning day, a third of the tanks received the high concentration of alarm cue treatment, a third received the low concentration of alarm cue treatment and the last third received the water treatment. The three treatments were randomly assigned to the conditioning tanks in the experimental room. At least 1 h after being conditioned, the groups of three minnows were randomly transferred to identical 37-L tanks (used for testing) containing clean dechlorinated tap water and were fed.

Testing phase

The testing phase took place 24 h after the conditioning phase. Minnows were fed 1 h prior to testing. During this phase, minnows were randomly exposed to 20 mL of the odour of brown trout, rainbow trout or perch. The behaviour of the minnows were recorded prior to and following the injection of the stimulus into the tank. The protocol for the stimulus injection followed the same protocol as used in the conditioning phase. As behavioural responses, I recorded both shoaling index and line crosses. The behavioural assay used was identical to the one used in Chapters 2 and 4. I tested 17 minnows in each of the nine treatments. All trials were performed blind and the order of testing was randomized.

Statistical analysis

The data used for the analysis were obtained from the difference in behavioural measures between the pre- and post-injection periods. The data were normally distributed and homoscedastic. Thus, I performed a 2-way ANOVA on the change in shoaling index and line crosses to investigate the effect of cue (water, low or high concentration of alarm cues) and the effect of predator (brown trout, rainbow trout or yellow perch) on the responses of minnows. To verify the existence of differential learning of brown trout odour by the minnows, a 1-way ANOVA was performed to test the effect of cue on the responses of minnows to brown trout. To further investigate the existence of generalization of predator recognition, subsequent tests (one for each cue) were performed to investigate the responses of minnows to different predators.

Generalization could result from an additive or synergistic effect of combining threat-sensitive learning with generalization. In the case of an additive effect, there is some point where the combined effect of reduced risk and reduced specificity would fall below the threshold for evoking a significant antipredator response. In the case of a synergistic effect, the difference in intensity of response between the high alarm cue conditioning group and low alarm cue conditioning group exposed to the reference predator should be smaller than the difference in intensity between the high alarm and low alarm cue conditioning groups in response to the closely related predator (i.e., the response to the low-risk closely-related predator should be lower than expected in the additive scenario). Consequently, to test for a possible interaction between the intensity of threat associated with the known reference predator and the response to the closely related predator, I performed a partial 2-way ANOVA, comparing only two levels of threat (high and low) and two predators (brown and rainbow trout).

7.3. Results

The results of the ANOVA revealed a statistically significant main effect of predator (shoaling index: $F_{2,144} = 36.9$, P < 0.001; line crosses: $F_{2,144} = 49.9$, P < 0.001), a significant effect of cue (shoaling index: $F_{2,144} = 33.2$, P < 0.001; line crosses: $F_{2,144} = 51.3$, P < 0.001) and a significant interaction between the two factors (shoaling index: $F_{4,144} = 13.6$, P < 0.001; line crosses: $F_{4,144} = 13.0$, P < 0.001 – Figure 7.1a,b).

The 1-way ANOVA investigating the effect of cue (high or low concentrations of alarm cues or water) on the responses of minnows to brown trout only revealed threat-sensitive learning by minnows (shoaling index: $F_{3,48} = 113.1$, P < 0.001; line crosses: $F_{3,48} = 91.6$, P < 0.001 – Figure 7.1a,b), i.e., minnows conditioned with a high concentration of alarm cues responded to brown trout odour with a greater response intensity than the ones conditioned with a low concentration of alarm cues (both P < 0.001) or with the water control (both P < 0.001). Moreover, minnows conditioned with a low concentration of alarm cues showed a higher response intensity to brown trout odour than did the water control ones (both P < 0.001).

When investing the effect of predator (brown or rainbow trout or perch) by cue, I found that, as expected, minnows conditioned with water did not differ in their responses to the odour of brown trout, rainbow trout or yellow perch (shoaling index: $F_{2,48} = 0.6$, P $= 0.566$; line

crosses: $F_{2,48} = 1.0$, $P = 0.377$ – Figure 7.1), indicating that minnows did not innately respond to any of the fishes. Consistent with previous results, minnows conditioned with the high concentration of alarm cues paired with brown trout odour showed generalization of predator recognition, i.e., responded to both brown and rainbow trout odour with an antipredator response (all $P < 0.001$). Moreover, minnows responded with a greater response intensity to the brown trout odour than the rainbow trout odour (both $P < 0.001$). Interestingly, when conditioned to recognize brown trout odour with a low concentration of alarm cues, minnows showed an antipredator response to brown trout odour (both $P < 0.001$), but failed to show a response to the odour of rainbow trout (both $P > 0.4$ – Figure 7.1).

The results of the partial 2-way ANOVA indicated no significant interaction between predator and cues for either shoaling index ($F_{1,64} = 0.4$, $P > 0.5$) or line crosses ($F_{1,64} = 0.04$, $P > 0.7$).
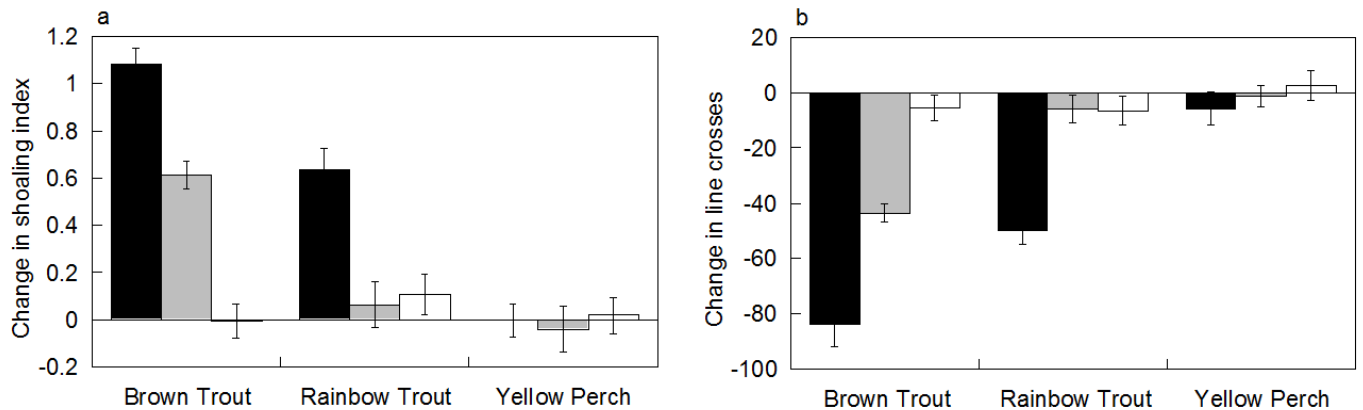
Figure 7.1: Mean (± S.E.) change in (a) shoaling index and (b) line crosses for minnows responding to the odour of brown trout, rainbow trout or yellow perch, but initially conditioned with brown trout odour paired with high concentration (black bars) or a low concentration (grey bars) of alarm cues or a water control (white bars) (N = 17/treatment).

7.4. Discussion

Consistent with previous results (see Chapters 2, 4), I showed that the level of risk associated with a new learned threat is dependent on the concentration of alarm cues that prey experienced during the conditioning event. In this case, minnows conditioned with the high concentration of alarm cues labelled brown trout as high-risk predators whilst minnows

conditioned with the low concentration of alarm cues labelled brown trout as lower risk predators. Moreover, my results clearly suggest that generalization of predator recognition is not a fixed phenomenon, but depends on the level of risk associated with the reference predator (the brown trout in this case). When brown trout are labelled as high-risk predators, minnows responded to closely related rainbow trout but not to distantly related yellow perch. These results are consistent with the results of Chapter 6. Most interestingly, minnows failed to recognize rainbow trout as threatening when brown trout are labelled as low-risk predators. In this study, I combined threat-sensitive learning (see Chapter 2) with the concept of generalization of predator recognition (see Chapter 6) and refer to the phenomenon as threat-sensitive generalization of predator recognition. Similar results were found in larval (Ferrari, Brown, Messier & Chivers unpublished data) and embryonic amphibians (Ferrari, Messier & Chivers unpublished data). Future studies examining this phenomenon should test whether threat-sensitive generalization results from additive or synergistic effects of combining these two phenomena. In the additive scenario, there is some point where the combined effect of reduced risk and reduced specificity falls below the threshold for evoking a significant antipredator response. In the synergistic scenario, the difference in intensity of response between the high alarm cue conditioning group and low alarm cue conditioning group exposed to the reference predator should be smaller than the difference in intensity between the high alarm and low alarm cue conditioning groups in response to the closely related predator. In my experiment, I observed an additive effect. However, it is important to note that I could have missed a synergistic effect due to a zero-truncation problem (i.e., the response of the minnows to the low risk closely related predator was not different than the control and hence any additional decrease would not have been observable).

In Chapter 6, I presented the Predator Recognition Continuum Hypothesis, highlighting some of the situations that would lead prey to display either innate or learned recognition of predators. Intuitively, innate predator recognition represents a great advantage to prey, as it likely dramatically increases the prey chances of survival upon their first encounter with a novel predator. Prey showing learned predator recognition need the first encounter with a novel predator to label it as dangerous. The fact that many prey do not show innate predator recognition indicates that either predator and prey did not co-occur for a long enough period of time to allow the genetic fixation of the recognition, and/or that innate predator recognition is

costly. In the Predator Recognition Continuum Hypothesis (Chapter 6), I hypothesized that recognition of predators is dependent on (but not limited to) the temporal and spatial predictability of predation and the diversity of the predators. Indeed, prey would likely benefit from innate predator recognition in environments where the predictability of attack from a predator is high and the diversity of predators is low (i.e., a few but constant predators). Alternatively, prey exposed to a great variety of predator species that are unpredictable in their probability of attack (e.g., due to seasonal diet switches) should benefit more from learned predator recognition, which allows a case-by-case learning of potential threats. In addition, prey exposed to the greatest variability of predation contexts should display the greatest plasticity in their responses to predators, of which generalization of predator recognition is included. This would allow the prey to increase their chances of survival from the first 'learning trial' by using their knowledge on close relatives of the novel predator. My present results refine this aspect of generalization. Prey animals seem to generalize their recognition to close relatives of known predators only for highly threatening species and not for those that represent a low threat. Put back in the context of optimizing trade-offs, differential generalization should allow the prey to be able to match the intensity of their antipredator response to the threat posed by the predator.

My results indicates that if a predator represents a high level threat, then prey should exhibit antipredator responses to close relatives of that predator, as closely related species usually share similar foraging habits. When predators are only mildly threatening however, prey seem to restrict their antipredator responses to the specific species of the predator that they learned. While initially counterintuitive, these results may indicate that the more dangerous the predator, the less specific its recognition. It may be interesting to consider the phenomenon of generalization in the context of recognition templates. While both groups of minnows have the opportunity to acquire the same amount of information regarding the predator characteristics, it may be that the degree of matching of the predator characteristics to the template varies for the two groups of minnows. When a predator represents a mild threat, minnows might respond to any predators, which characteristics match exactly the template used for recognition, i.e., species-specific recognition. As the level of threat associated with the learned predator increases, the window of matching necessary to elicit a fright response might become wider and wider, allowing prey to generalize their recognition to all species that fit more or less the characteristics possessed by the reference predator.

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In this experiment, I investigated generalization from a chemical perspective. I conditioned minnows to recognize the brown trout as a threat by using a constant concentration of trout odour paired with different concentrations of alarm cues. When the minnows recognize the brown trout as a high-level threat, they generalize this threat to rainbow trout. The reduction in intensity of response of these minnows to rainbow trout indicates that the rainbow trout odour does not match the brown trout odour exactly (i.e., there are fewer chemicals in common or the concentrations of specific chemicals are different). When the brown trout is recognized as a low-level threat the mismatch between the rainbow trout odour and brown trout odour is the same. However, given that the level of threat of the brown trout is lower, the reduction in the intensity of antipredator response as a result of the mismatch is enough to eliminate the response to rainbow trout. From a proximate perspective, this could be interpreted as an effect of diluting the concentration of the specific chemicals or suite of chemicals that elicit the response. Future researchers should use this framework to address how the specific visual characteristics of predators are likewise diluted to eliminate the recognition in a generalization context. This would allow us to address the specific characteristics that prey use to recognise predators.

The ability of prey to avoid predators is a fundamental issue in biology. The specific ecological and evolutionary pressures that lead to learning versus fixed recognition have received surprisingly little attention (but see Blumstein 2002, 2006, Chapter 6). My results expands on the theoretical framework of the Predator Recognition Continuum Hypothesis demonstrating that the ability of prey to generalize their recognition of predators is dependent on the relative threat posed by the predator.

Chapter 8: General discussion

8.1. Threat-sensitive predator learning

At the time I started my research, many studies had focused on the occurrence of and mechanisms associated with learned predator recognition, but little information was available regarding the quantitative nature of predator learning, i.e., how threat-sensitive predator avoidance develops in prey species. My thesis work represents the first attempt to link the basic concept of learned predator recognition to dynamic threat-sensitive predator avoidance displayed by wild-caught prey. Moreover, my research on generalization provides insights into how prey that lack innate predator recognition may receive similar benefits upon detecting novel predators as prey showing innate recognition of predators.

Upon encountering a predator for the first time, prey have an opportunity to learn to recognize it as threatening through one of several learning mechanisms. For aquatic prey, these learning mechanisms include pairing of predator cues with the cues of damaged conspecifics, pairing of predator cues with frightened conspecifics or direct encounters with predators. However, prey need to be able to adjust their responses to predation threats to optimize the trade-off between predator avoidance and other activities. Put together, the results of my thesis indicate that the process that prey use to adjust the intensity of their antipredator response to a given predation threat is extremely complex.

To understand how prey adjust their response to predators, I first investigated the informative value of the cues prey use to learn to recognize predators as threatening. When prey learn from the pairing of predator cues and chemical alarm cues, they are exposed to known (alarm cues) and unknown (predator cues) stimuli. Thus, any information regarding the level of threat of a novel predator can only be conveyed through alarm cues. In Experiment 1, I showed that, after a single pairing event, minnows can learn to respond with a specific intensity to a given predation threat using the concentration of alarm cues as an indicator of the riskiness of the predator. Indeed, higher concentrations of alarm cues could suggest that the killing site is in closer proximity or could indicate that the predator is more dangerous (i.e., more successful at killing multiple prey). In addition, I showed that once learning occurs, predator cues become known stimuli and prey use the change in predator cue concentrations during subsequent

encounters to further adjust their response intensity to the threat (Experiment 2). In an attempt to generalize these findings, I looked at similar questions using social learning as the learning mode of interest. In this case, prey learn through simultaneous pairing of frightened conspecific(s) (known stimuli) and predator cues (unknown stimuli). In concurrence with my previous results, I found that, after a single pairing event, minnows learn to respond with a specific intensity to predator cues using the antipredator response intensity displayed by conspecifics as an indicator of the riskiness of the predator (Experiment 3). To further investigate the nature and reliability of the information passed on by the conspecifics, I performed an experiment (Experiment 4) manipulating the ratio of naive 'observers' (chorus frog tadpoles) and heterospecific tutors (woodfrog tadpoles). I found that observers learn to recognize the predator as more dangerous when the tutor-to-observer ratio (ratio of responding to non-responding prey) was higher, indicating that prey individuals may even use the group consensus on a predator riskiness to further adjust their response intensity to a given predator.

My results indicate that prey can gather quite an impressive amount of information regarding a predator's threat after only one learning opportunity. In nature, prey will likely encounter numerous situations that would allow them to gather additional information about the level of risk of predators. An intriguing aspect of this concept is to investigate how prey deal with conflicting information. My results suggest that when exposed to two pieces of conflicting information regarding the level of threat of a predator, minnows respond to the predator using a safety strategy, which consists of responding with the strongest, most conservative response intensity (Experiment 5). However, when information is accumulated over a longer timeframe (6 days, Experiment 6), minnows seem to also rely on the most recent and consistent piece of information to keep up-to-date regarding the risk that a predator represents. While yet to be tested, I predict that it would take longer (in terms of time or number of consistent pieces of information) for prey to decrease the risk associated with a predator than to increase it. Although the previous rules would allow prey to respond to predators in an absolute fashion, processes that are more complex are in place to allow prey to adjust their response to predators according to the temporal variability in predation risk. Prey experiencing predators being risky at one point in time and less risky later in the day are exposed to somewhat conflicting information at first. However, the repetition of conflicting but consistent information seems to override the 'safety' strategy and allows for a temporal adjustment of the prey's response intensity that matches the

risk posed by the predator throughout the day. Indeed, I have strong indications that woodfrog tadpoles can learn to adjust their responses to predators throughout a daily cycle (Experiment 7). The mechanism that allows the prey to slowly override the 'safety' strategy is not known.

The ability of prey to learn to recognize predators has been well established; however, little is known on the specific characteristics that prey use to identify species as threatening. One way to uncover the specific characteristics of a predator recognized by prey as dangerous is to teach prey to recognize one predator and subsequently expose the prey to different species sharing some characteristics in common with the reference predator. In Experiment 8, I showed that minnows that learn to recognize a specific predator subsequently respond to that predator, but also to species closely related to that predator. The response intensity seems to correlate with the degree of relatedness of the novel species to the reference predator. These results are the first to indicate that prey that do not have innate predator recognition may have benefits similar to those prey showing innate predator recognition. Indeed, generalization of predator recognition would allow prey to display an antipredator response to a predator without having any prior experience with it. This description is, in fact, often used by behavioural ecologists to define innate predator recognition. While generalization may confer an advantage to the prey when the related species is predatory, the uncertainty regarding the predation status of a novel species is higher when close relatives are only mildly threatening. In the last experiment of my thesis (Experiment 9), I showed that minnows exhibit generalization of predator recognition when the reference predator is highly threatening, but in contrast, do not exhibit generalization when the reference predator represents a low risk. An exciting area for future researchers is to expand the context of generalization of predator recognition to include generalization of non-predator recognition. Ferrari & Chivers (2006) showed latent inhibition of predator recognition by fathead minnows. Minnows exposed to brook trout 1 h per day for 5 consecutive days failed to learn to recognize trout odour as a threat when it was subsequently paired with alarm cues. Can minnows generalize this recognition of non-predators to other trout in the same way they generalize recognition of predators to other trout?

The series of experiments presented in this thesis illustrate many different ways that prey can adjust their response intensities to predators. While I have not tested the outcome of all the combinations of assessment tools in one experiment, it is easy to imagine how these tools would

allow prey to slowly adjust and fine-tune their responses to predators to reach the amazing level of sophistication in predator risk assessment seen in wild-caught prey. The amazing sophistication of predator recognition that I observed highlights the importance of predation as a selection pressure over evolutionary time.

8.2. Chemosensory assessment of risk

Throughout my thesis, I used chemosensory information to investigate learned predator recognition in aquatic prey. Contrary to visual information, which is spatially and temporally reliable (i.e., the prey can see the exact location of the predator in real time), chemical cues indicating predation risk may not reliably indicate the true threat that the prey are exposed to if chemicals persist in the environment after the predator has left the area. The disconnection between perceived and actual risk has received little attention from chemical ecologists. Different concentrations of cues could indicate that the threat is either closer or further away (distance indicator – Ferrari *et al.* 2006) or that the stimulus is fresh or partially degraded (temporal indicator – Ferrari *et al.* 2007b). This time-space dichotomy becomes more problematic in terms of risk assessment as the chemicals persist for longer times in the environment. Given the ubiquitous nature of chemosensory risk assessment by prey, it is surprising that very little is known regarding the persistence of cues under natural conditions. Ferrari *et al.* (2007b) showed that chemical alarm cues of woodfrog persist in a natural pond for less than 2 h. Other studies (e.g., Hazlett 1999) have shown that predator odours can persist for at least 6 h. Unfortunately, these experiments were done in clean water in the laboratory. Hence, the lack of biofauna and photodegradation that would occur under natural conditions, likely makes these estimates ecologically unrealistic.

My thesis work focused on the ability of aquatic prey to assess risk using chemosensory information. However, additional sensory inputs, such as visual, mechanical and electrical stimuli, are also involved in risk assessment, and these multiple cues may work to enhance risk recognition through sensory complementation (Lima & Steury 2005, Ferrari *et al.* in press). The relative importance of a given sensory modality in the process of decision making is likely to be dependent on the species, its habitat and local conditions (e.g., day versus night). Hartman & Abrahams (2000) developed a sensory compensation model, in which they predict that prey should rely more on their chemical senses than visual senses in turbid water. In clear water,

chemical cues may be less important if they are not associated with visual cues. All my work was done in clear dechlorinated tap water or in the field using pond water with low turbidity. If Hartman & Abrahams' (2000) model is correct, then prey may be able to assess even more information than I demonstrated.

8.3. Conservation applications of threat-sensitive learning of predator recognition

My thesis extends the quantitative nature of predator learning. Indeed, I showed that some prey species can quickly adjust the intensity of antipredator responses to a specific threat through repetitive learning. My test species (fathead minnows, woodfrogs and chorus frogs) are all widespread in North America, despite being small prey for many predators. Their sophisticated abilities to learn to recognize predators are likely not unrelated to their widespread distribution and success. It remains unknown whether my test species are exceptional in their learning abilities compared to other species, but this deserves consideration in future analyses of predator learning.

The ability of prey species to learn to recognize novel threats has important implications for conservation biologists. Indeed, a common method for the recovery of locally extinct populations consists of obtaining individuals through captive breeding followed by the re-introduction of these individuals to the wild (e.g., Griffin *et al.* 2000). A problem with such a methodology is that individuals are kept in predator-free environments with constant food supplies, which excludes any selection by predation or learning opportunities from predation events against risky foraging and excessive aggressive behaviour (Johnsson *et al.* 1996). Thus, as predicted, the efficacy of this method is limited, as many individuals are depredated shortly after being re-introduced (e.g., Brown & Laland 2001). Consequently, techniques allowing naïve individuals to be trained to recognize potential predators provide conservationists with a tool to improve the success of their re-introduction efforts.

The ability of prey to deal with novel predation threats has importance in the context of invasive species' biology. A greater ability for exotic species to cope with predators compared to native species has often been correlated with their colonization success. For example, Pennuto & Keppler (in press) showed that the invasive amphipod *Echinogammarus ischnus* displaced the native amphipod, *Gammarus fasciatus* shortly after its introduction to the Great Lakes. Short-

term behavioural experiments on the two species demonstrated that, while both species responded to a variety of predator cues, the invasive amphipods learn to respond to a larger range of predators and with a greater intensity of response than the native amphipods, which may partly explain their success. Similarly, Hazlett (2000) and Hazlett *et al.* (2003) showed a wider use of chemical information regarding predation threats by invasive species of crayfish compared to native species. Thus, learning to simply respond to predation threats is often not enough to guarantee survival of native or reintroduced species. Their success will also depend on the ability of competitive species to deal with predators.

A thorough understanding of predator learning has important conservation implications as anthropogenic changes affect aquatic ecosystems. For example, if global warming allows for the expansions of fish and amphibian populations, we could predict that species with superior predator learning abilities may be able to colonize new areas and start to compete with native species. Invading species bring with them new disease and parasites that may dramatically influence native species. Predators, competitors and diseases are often density-dependent factors that ultimately influence population sizes and distributions (Messier 1991, 1994, Joly & Messier 2004).

8.4. The allocation of risk: do controlled experiments reflect reality?

All of the experiments I presented in my thesis were conducted in the laboratory or under controlled field conditions, in which the prey were held for considerable time under conditions of low predation risk and were fed *ad libitum* prior to testing. However, under natural conditions, prey are likely exposed to frequent risk and often do not have unlimited food. Lima & Bednekoff (1999) developed a theoretical model, the Risk Allocation Hypothesis (RAH), in which they propose that prey adaptively allocate their foraging efforts, and therefore, their exposure to predation across high-risk and low-risk situations. Prey behaviour in any situation should depend on the overall risk experienced by prey, more specifically the duration of high-risk versus low-risk situations and the relative level of risk associated with each of them. The RAH predicts that as the duration of exposure to risk increases, prey should decrease their antipredator response, as long periods of sustained vigilance may result in unacceptable decreases in energy intake. Moreover, animals exposed to long periods of high risk should forage particularly actively during brief periods of safety, compared to prey exposed to infrequent risk. The model also

predicts that as the risk associated with high-risk situations increases, prey should increase their antipredator response, but will consequently increase their foraging effort in low-risk situations to compensate for the loss of foraging opportunities. The RAH has been tested in many systems and there is some support for this hypothesis (e.g., Hamilton & Heithaus 2001, Sih & McCarthy 2002, reviewed by Ferrari, Sih & Chivers - unpublished review).

The question that naturally follows from studies on risk allocation is whether all of the experiments that I conducted can be validated under natural conditions. When a prey animal detects a predation event on a nearby conspecific or heterospecific prey guild member, can it simultaneously process all of the pieces of information that I showed are possible. Can the prey determine the alarm cue concentration, the predator odour concentration, the relative fright level of other prey in the vicinity and subsequently respond appropriately. Can prey determine all of the characteristics of a novel predator on their first encounter and be able to generalize (or not generalize) this information in subsequent encounters. I do not know if this is possible, but I do know that wild-caught prey demonstrate amazing sophistication in their ability to differentiate levels of risk. For example, Ferrari *et al.* (2006) showed that fathead minnows could differentiate pike proximity and density based on odour alone. Kusch *et al.* (2004) demonstrated that minnows could differentiate pike size based on odour alone. Moreover, minnows can determine the diet of predators based on the predator's odour (Mathis & Smith 1993, Ferrari *et al.* 2007c). Such responses are likely only possible if minnows have an amazing ability to assess risk under natural conditions, but under these conditions, the number of learning opportunities may be immense due to the frequency of predation.

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Curriculum Vitae

VITAL STATISTICS

Date of birth: June 5, 1981
Place of birth: Lyon, France
Citizenship: French, Permanent Resident of Canada

EDUCATION

2003 – present   **Ph.D. candidate**, University of Saskatchewan, Saskatoon, Canada. Tentative thesis title: Threat-sensitive learning and generalization of predator recognition by aquatic vertebrates. Supervisor: Dr. François Messier

2002 – 2003   "**Maitrise**" (equivalent to a B.Sc.) **in Biology of Populations and Ecosystems**, Foreign Studies Program, Université Joseph Fourier, Grenoble, France. Host University: Concordia University, Montreal, Canada.

2001 – 2002   "**Licence**" **in Biology of Organisms**, Université Joseph Fourier, Grenoble, France.

2000 – 2001   **General University Studies Diploma in Sciences of Life**, Université Joseph Fourier, Grenoble, France.

1999 – 2000   Veterinary Preparatory School, Lycée Champollion, Grenoble, France.

WORK / CAREER RELATED EXPERIENCE

2008 – present   **Statistical and Writing Consultant**
Working with Dr. Gary Bortolotti, University of Saskatchewan.

May-Aug. 2003   **Aquatic Ecosystems Consultant**
Working with Dr. Daniel Boisclair, University of Montreal.

2002 – 2003   **Research Assistant**
Supervisor: Dr. Grant Brown, Concordia University, Montreal.

June-Aug. 1995   **Veterinary Assistant**
Working for Dr. P-J. Charvier, DVM in Sète, France.

PROFESSIONAL AFFILIATIONS

Animal Behaviour Society
Ecological Society of America
International Society of Chemical Ecology

PROFESSIONAL TRAINING

2007   The Experimental Fish – Aquatic Animal Care User Training
Canadian Aquaculture Institute, University of Prince Edward Island

2006   Biosafety Certification
Department of Health, Safety and Environment, University of Saskatchewan

2004   Laboratory Animal Care Certification
Animal Resource Center, University of Saskatchewan

TEACHING EXPERIENCE

University of Saskatchewan:

BIO. 880.3 – Applied Statistics in Ecology (graduate level course)
Teaching Assistant: 2006-2007, 2008-2009

BIO. 472.3 – Animal Behaviour
Teaching Assistant: 2007-2008

BIO. 211.3 – Genetics: from Genes to Genomics
Laboratory Demonstrator: 2004-2005

BIO. 110.6 – General Biology
Head Laboratory Demonstrator: 2005-2006
Laboratory Demonstrator: 2003-2004, 2004-2005, 2005-2006

Université Joseph Fourier:

Tutor for second year biology, biochemistry, genetics, chemistry, physics and mathematics for physically disabled students: 2002-2003.

GRANTS AND AWARDS

2009-2011   Australian Research Council Grant DP0985015: "Catch me if you can: predator recognition and anti-predator behaviour in marine fishes" (AU$ 115,000). Co-PI with M.I. McCormick, D.P. Chivers & L.A. Fuiman.

2008   College of Graduate Studies & Research Travel Award ($550)

2007   Malcolm A. Ramsay Memorial Award ($3,000)

2007   Outstanding Young Scientist Travel Award from the International Society of Chemical Ecology (US$1,100)

2007   University of Saskatchewan Graduate Teaching Fellowship ($19,000)

2007   College of Graduate Studies & Research Travel Award ($550)

2006   University of Saskatchewan Graduate Scholarship ($18,500)

2005   R. Jan F. Smith Memorial Research Grant ($2,000)

2005   University of Saskatchewan Graduate Scholarship ($18,000)

2005   College of Graduate Studies & Research Travel Award ($500)

2004   University of Saskatchewan Graduate Scholarship ($15,000)

2003   University of Saskatchewan Graduate Scholarship ($15,000)

PROFESSIONAL SERVICE

Committee member: development of guidelines and policies for animal transportation, University Committee on Animal Care and Supply (UCACS), University of Saskatchewan.

Reviewer of the Aquatic Training Module for the Education and Training Committee of the UCACS. Ensured University compliance with the Canadian Council of Animal Care researcher training guidelines.

Reviewer of 3 grant proposals for: National Science Foundation (USA)
Netherlands Organisation for Scientific Research
M.J. Murdoch Charitable Trust (USA)

Reviewer of 46 papers for:

Animal Behaviour (4)	Chemical Senses	Journal of Ethology
Animal Cognition (2)	Chemoecology (2)	Journal of Exp. Biology
Aquatic Ecology (2)	Coral Reefs (3)	Journal of Fish Biology (2)
Behavioral Ecology (2)	Ecology (2)	Journal of Herpetology
Behav. Ecol. Sociobiol. (3)	Ecol. Freshwater Fishes	Limnology & Oceanography
Behaviour (4)	Ethology	Oecologia (2)
Behavioural Processes	Evolutionary Ecology	Oikos (2)
Biological Conservation	Freshwater Biology	PLoS One
Canadian J. Zoology (2)	Journal of Animal Ecology	Proc. Royal Society B

RESEARCH PUBLICATIONS AS PRIMARY AUTHOR

Ferrari, M.C.O. & Chivers, D.P. In press. Latent inhibition of predator recognition by embryonic amphibians. ***Biology Letters*** (Journal Impact Factor of 2.716; Ranked 17th out of 70 Biology journals)

Ferrari, M.C.O., Vavrek, M.A., Elvidge, C.K., Fridman, B, Chivers, D.P. & Brown, G.E. In press. Sensory complementation and acquired predator recognition by salmonid fishes. ***Behavioral Ecology and Sociobiology*** (JIF: 2.754; 9/124 Zoology)

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Ferrari, M.C.O. & Chivers, D.P. 2008. Cultural learning of predators in mixed species assemblages: the effects of tutor-to-observer ratio. ***Animal Behaviour*** 75: 1921-1925 (JIF: 2.752; 10/124 Zoology)

Ferrari, M.C.O., Rive, A.C., MacNaughton, C.J., Brown, G.E. & Chivers, D.P. 2008. Fixed vs. random temporal predictability of predation risk: an extension of the Risk Allocation Hypothesis. ***Ethology*** 14: 238-244 (JIF: 2.245; 13/114 Zoology)

Ferrari, M.C.O., Messier, F. & Chivers, D.P. 2008. Degradation of alarm cues under natural conditions: risk assessment by larval amphibians. *Chemoecology* 17: 263-266 (JIF: 1.439; 53/112 Ecology)

Ferrari, M.C.O., Messier, F. & Chivers, D.P. 2008. Variable predation risk and the dynamic nature of mosquito antipredator responses. *Chemoecology* 17: 223-229 (JIF: 1.439; 53/112 Ecology)

Ferrari, M.C.O., Gonzalo, A., Messier, F. & Chivers, D.P. 2007. Generalization of learned predator recognition: an experimental test and framework for future studies. ***Proceedings of the Royal Society of London B 274: 1853-1859*** (JIF: 3.510; 6/65 Biology)

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RESEARCH PUBLICATIONS AS CONTRIBUTING AUTHOR

Mathis, A., **Ferrari, M.C.O.**, Windel, N., Messier, F. & Chivers, D.P. 2008. Learning by embryos and the ghost of predation future. *Proceedings of the Royal Society of London B* 275: 2603-2607 (JIF: 4.112; 7/70 Biology)

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RESEARCH PUBLICATIONS CURRENTLY UNDER REVIEW

Ferrari, M.C.O., Brown, G.E., Messier, F. & Chivers, D.P. Under review. Threat-sensitive assessment of risk through chemical alarm cues by woodfrog tadpoles. ***Chemoecology*** (submitted May 15, revised Oct. 22)

Ferrari, M.C.O., Sih, A. & Chivers, D.P. Under review. The paradox of Risk Allocation: a review and prospectus. ***Animal Behaviour*** (submitted July 15, in revision)

Brown, G.E., Harvey, M.C., Leduc, A.O.H.C., **Ferrari, M.C.O.** & Chivers, D.P. Under review. Social context, competitive interactions and the dynamic nature of antipredator responses of juvenile rainbow trout (Oncorhynchus mykiss). ***Journal of Fish Biology*** (submitted Aug. 16)

Ferrari, M.C.O. & Chivers, D.P. Under review. How risky is the ghost of predation future? Threat-sensitive and temporal assessment of risk by embryonic woodfrogs. ***Behavioral Ecology*** (submitted Sept. 30)

Ferrari, M.C.O., Brown, G.E., Messier, F. & Chivers, D.P. Under review. Threat-sensitive generalization of predator recognition by amphibians. ***Behavioral Ecology and Sociobiology*** (submitted Nov. 6)

Ferrari, M.C.O. & Chivers, D.P. Under review. Temporal variability, threat-sensitivity and conflicting information about the nature of risk: understanding the dynamics of tadpole antipredator behaviour. ***Animal Behaviour*** (submitted Dec. 12)

RESEARCH PUBLICATIONS CURRENTLY IN PREPARATION

Ferrari, M.C.O., Elvidge, C.K., Jackson, C.D., Chivers, D.P. & Brown, G.E. In prep. Temporal variation in responses to predation risk: habituation or risk allocation? ***American Naturalist***

CONFERENCE PRESENTATIONS (* denotes presenting author)

Ferrari, M.C.O.* Time to be scared yet? Temporal threat-sensitive learning of predators by larval amphibians.Oral presentation (Allee Competition) at the 45th Annual Meeting of the Animal Behavior Society (Snowbird, Utah, August 16-20, 2008).

Chivers, D.P.*, Messier, F. & **Ferrari, M.C.O.** Threat-sensitive generalization of predator recognition by fish and amphibians. Oral presentation at the 45th Annual Meeting of the Animal Behavior Society (Snowbird, Utah, August 16-20, 2008).

Ferrari, M.C.O., Messier F. & Chivers, D.P.* Social learning in aquatic amphibians: the effect of tutor-to-observer ratio. Poster presentation at the 4th European Congress on Behavioural Biology (Dijon, France, July 18-20, 2008).

Chivers, D.P., Messier, F. & **Ferrari, M.C.O.*** Generalization of predator recognition. Oral presentation at the 4th European Congress on Behavioural Biology (Dijon, France, July 18-20, 2008).

Ferrari, M.C.O.*, Messier F. & Chivers, D.P. Degradation of alarm cues under natural conditions: risk assessment by larval amphibians. Oral presentation (Outstanding Young Scientists section) at the 23rd Annual meeting of the International Society of Chemical Ecology (Jena, Germany, July 22-26, 2007).

Chivers, D.P.*, Wisenden, B.D., Hindman, C.J., Michalak, T.A., Kusch, R.C., Kaminskyj, S.J., Jack, K.K., **Ferrari, M.C.O.**, Pollock, R.J., Halbgewachs, C.F., Pollock, M.S., Alemadi, S., Clayton, J.T., Savaloja, R.K., Goater, C.P., Corwin, A., Mirza, R.S., Kiesecker, J.M., Brown, G.E., Adrian, Jr.J.C., Krone, P.H., Blaustein, A.R. & Mathis, A. Epidermal 'alarm substance' cells of fishes are maintained by non-alarm functions: defence against pathogens, parasites and ultraviolet radiation. Oral presentation at the 23rd Annual meeting of the International Society of Chemical Ecology (Jena, Germany, July 22-26, 2007).

Ferrari, M.C.O.*, Messier F. & Chivers, D.P. The nose knows: minnows determine predator proximity and density through detection of predator odours. Poster presentation at the 11th Congress of the International Society of Behavioural Ecology (Tours, France, July 23-29, 2006).

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, Jr.J.C., Mathis, A., Brown, G.E., Krone, P.H., & Blaustein, A.R. The ecology of fear: predators, pathogens, parasites and pollutants. Oral presentation at the 11th Congress of the International Society of Behavioural Ecology (Tours, France, July 23-29, 2006)

Ferrari, M.C.O.*, Messier F. & Chivers, D.P. The development of threat-sensitive predator avoidance in fishes. Oral presentation at the 29th International Ethological Congress (Budapest, Hungary, August 20-27, 2005)

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, Jr.J.C., Mathis, A., Brown, G.E., Krone, P.H., & Blaustein, A.R. Manipulating predation risk and pathogens to understand the evolution of Schreckstoff. Oral presentation at the 29th International Ethological Congress (Budapest, Hungary, August 20-27, 2005)

Ferrari, M.C.O.*, Messier F. & Chivers, D.P. The role of learning in development of threat-sensitive predator avoidance in fathead minnows. Poster presentation at 90th Ecological Society of America Annual Meeting (Montreal, QC, August 7-12, 2005)

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, Jr.J.C., Mathis, A., Brown, G.E., Krone, P.H., & Blaustein, A.R. Manipulating predation risk and pathogens to understand the evolution of Schreckstoff. Oral presentation at the 90th Ecological Society of America Annual Meeting (Montreal, QC, August 7-12, 2005)

Ferrari, M.C.O.* & Chivers, D.P. Development of threat sensitive predator avoidance in fathead minnows. Oral Presentation at the 39th Prairie Universities Biological Symposium (Saskatoon, SK, February 10-12, 2005)

POPULAR MEDIA COVERAGE

2006   Fox News – Minnows quickly learn to sniff out predators (click for link)
2006   Live Science – Tiny fish learn to sniff out predators (click for link)
2008   Natural history Magazine – Early life lessons (click for link)
2008   New Scientist – Frogspawn learn the smell of death (click for link)
2008   Research News, Univ. SK – Frogs smell death before birth (click for link)