THE ROLE OF EARLY LIFE HISTORY IN LEARNING AND RETENTION OF PREDATOR RECOGNITION BY FISH AND AMPHIBIANS

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ABSTRACT

Predation risk is a major driver of evolution and ecology of animals. Predator avoidance is essential because failing to evade threats will result in injury or death. However, avoiding predators is costly because it means missing out on foraging and mating opportunities. To maximize opportunity, organisms must be able to perceive and assess threat levels, allowing them to avoid reducing activity when risk is minimal. Many aquatic organisms use injury-released "alarm" cues from conspecifics to detect a predation threat. They may learn to recognize predators from simultaneous exposures to alarm cues and a novel predator odour. Any other information that they can incorporate as they are learning may improve the accuracy with which they can predict the degree of threat in subsequent exposures. In this thesis, I explore embryonic and early learning of predator recognition by aquatic organisms. Using paired exposure to alarm cues and novel predator odours, I show that embryonic fish are not only able to learn to recognize predators, but can also do so in a threat-sensitive manner and from cues of closely-related heterospecific fish. I also demonstrate that the effect of a difference in age between the alarm cue producer and receiver varies between species and is situation-dependent. Specifically, newly-hatched rainbow trout respond equally to alarm cues from newly-hatched and six-month-old individuals at various concentrations. In contrast, although the scientific record shows that embryonic minnows hatch early in response to embryonic alarm cues, I found that they do not modify their hatching time in response to adult alarm cues. I also use learning exposures of paired alarm cues and predator odour at different early life stages to demonstrate that ontogeny has significant impacts on fish and tadpoles' retention of learned predator information, whereby embryonic learners forget more slowly than slightly older larval conspecifics. I propose that embryonic learners may have increased cognitive plasticity as a result of a sensitive period in their development.

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DEDICATION

I dedicate this thesis to my wonderful children, Marie and Nathan.

May you never stop asking questions.

I love you always.

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CHAPTER 1: Introduction

1.1 Predation risk and antipredator responses

1.1.1 Predation risk versus reward

Predator-prey interactions are a well-known driver of ecological and evolutionary change across the animal kingdom (Lima & Dill, 1990). Prey species must constantly weigh the rewards of fitness-promoting activities, such as foraging and mating, against the risks of being injured or even killed by a predator (Pfeiffer, 1977; Lima & Dill, 1990; Sih, Ziemba, & Harding, 2000). The costs of missed mating or foraging opportunities may carry over multiple generations. However, because failure to respond to novel predators can be very costly (Sih, 2013), sometimes even resulting in death, maintaining an appropriate level of caution is imperative for survival (Bouskila & Blumstein, 1992).

Prey species have a variety of defense mechanisms that may improve their chances of survival in face of an enemy, including morphological, life-history and behavioural modifications (Chivers & Smith, 1998). Individuals that are exposed to risk for extended periods may also develop a fear of the unknown, a phenomenon known as neophobia (Brown *et al.*, 2013b) which has been observed in a wide range of taxa including birds, mammals, fishes and amphibians (reviewed in Crane & Ferrari, 2017b; Crane *et al.*, 2020). Exposure to alarm cues as early as in the embryo has been shown to induce neophobia in tadpoles (Ferrari, Brown, & Chivers, 2015).

Another effect of constant or prolonged exposure to a threat of predation may be physical alteration of phenotypes to decrease the risk of death or severe injury. A classic example of this is the crucian carp (*Carassius carassius*, Linnaeus 1758) which increases the depth of its body in the presence of northern pike (*Esox lucius*, Linnaeus 1758) in order to prevent these gape-limited predators from being able to swallow it. This phenotypic change comes at the cost of slower, less agile movement (Brönmark & Miner, 1992). These types of induced morphological changes to reduce predator impacts are also observed in tadpoles (Relyea, 2004) and a wide variety of invertebrate species (Stemberger & Gilbert, 1987; Pettersson & Brönmark, 1997).

Species may also alter their life-history, by shifting the timing of reproduction or life stage transitions, to avoid predation risk. Early hatching to avoid egg predators has been observed in amphibians and fish (Mirza, Chivers, & Godin, 2001; Kusch & Chivers, 2004; Touchon *et al.*, 2013), delayed hatching to avoid larval predators has been observed in salamanders (Sih & Moore, 1993) and delayed reproduction occurs in snails in response to predators (Crowl & Covich, 1990).

Behavioural modifications are perhaps the most immediate manner by which to address the presence of predators. Antipredator behaviours such as avoidance of high-risk areas, refuge seeking, reduced movement and decreased foraging are common throughout the animal kingdom (Lima & Dill, 1990; Chivers & Smith, 1998; Sih *et al.*, 2000). Fish are known to reduce their movement and foraging behaviours and increase shoaling and shelter use (Chivers & Smith, 1998). Shelter use may be a last, drastic resort because it is the most costly behaviour (Magurran & Pitcher, 1987). These antipredator behaviours and adaptations have been shown to increase survival in predator encounters (Chivers & Smith, 1994a; Mirza & Chivers, 2000, 2001a; Lönnstedt *et al.*, 2012).

1.1.2 Perception of risk

In order for some antipredator mechanisms to be effective, an organism needs to be able to identify its predators. The more promptly and accurately an animal can identify risk, the more rapidly it can implement antipredator mechanisms, thus significantly improving its chances of survival (Pfeiffer, 1977; Hews, 1988; Mathis & Smith, 1993a; Wisenden, Cline, & Sparkes, 1999). The primary mechanisms for identifying predators include visual (Chivers & Smith, 1994b), auditory (Kelley & Magurran, 2003) and olfactory identification (Chivers & Smith, 1998). Prey may also make assumptions about their threat level by observing experienced conspecifics (Griffin, 2004) and may learn to recognize predators from social cues (Ferrari, Messier, & Chivers, 2007c). Furthermore, they may be able to extrapolate to predict the threat level of novel predators, or generalize that closely related species may also be predators. For example, fathead minnows (*Pimephales promelas*, Rafinesque 1820) trained to recognize lake trout (*Salvelinus namaycush*, Walbaum 1792) generalize that brook char (*Salvelinus fontinalis*, Mitchill 1814) and rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) also represent a threat, but do not generalize that distantly-related pike present a threat (Ferrari *et al.*, 2007b).

In aquatic systems, olfactory cues are extremely important for predator recognition (Chivers & Smith, 1998; Wisenden, 2000), as they allow observation of predators even in low light, high turbidity and at long distances. Fish have highly acute olfactory receptor cells which allow them a keen and nuanced perception of predator odour (Laberge & Hara, 2001). In some species, such as salamanders, there is innate recognition of a limited number predator species. There is little evidence of this in tadpoles or fish (Brown, 2003; Mathis *et al.*, 2008), but even in organisms with innate recognition of some species of predators, learned predator recognition offers greater versatility for prey, allowing them to learn to respond to the wider guild of relevant predators that awaits them. Predator recognition can take place through exposure to a novel predator odour concurrent with a predation event (Chivers & Smith, 1998). One of the most precise indicators of a predation event that can be used for this type of learning is injury-released alarm cues (Lawrence & Smith, 1989; Chivers & Smith, 1998; Wisenden, 2015).

1.1.3 Injury-released alarm cues

Injury-released alarm cues, originally referred to as "Schreckstoff" by von Frisch (1938), are kairomones released from damaged cells. Variations in the chemistry of alarm cues between species allow for species-specific responses to alarm cues, such that conspecific alarm cues consistently elicit dramatic, innate antipredator responses in a wide range of aquatic organisms from coral to vertebrates (Chivers & Smith, 1998; Wisenden, Vollbrecht, & Brown, 2004b; Ferrari, Wisenden, & Chivers, 2010c), including in wild studies (Wisenden *et al.*, 2004b). In fish, these cues are released from damaged cells, typically epidermal cells, and provide very reliable information about the threat of physical harm (Pfeiffer, 1977). They can also be released in the fecal matter of predators who have consumed an organism whole, and the cues are sufficiently persistent that prey can learn from these dietary cues even after digestion (Mathis & Smith, 1993b; Ferrari *et al.*, 2007a; McCormick *et al.*, 2019). These cues are potent enough that the alarm cues released from a single square centimetre of fathead minnow skin is enough to alert any minnow in a volume greater than 58 000 L (Lawrence & Smith, 1989).

There is much discussion and little consensus throughout the literature on the evolution and chemistry of alarm cues (Chivers & Smith, 1998; Meuthen, Baldauf, & Thünken, 2012). There has been speculation that alarm cues are actually pheromones evolved to warn kin, a suggestion which has been disputed by Meuthen *et al.* (2012). Other evidence suggests that alarm cue

chemicals may provide protection against UV, pathogens or parasites (Chivers *et al.*, 2007), or that they may disrupt predation events by attracting other predators (Mathis & Chivers, 1995).

Numerous studies have pointed to hypoxanthine-3(N)-oxide as a component of alarm cue in fish, as it elicits an innate antipredator response in a variety of species (Pfeiffer *et al.*, 1985; Parra, Adrian, & Gerlai, 2009). More recent research has pointed to chondroitins as a possible chemical signature within the alarm cues of zebrafish as it also elicits a fright response (Mathuru *et al.*, 2012; Faulkner *et al.*, 2017). Despite some homology, the chemistry of alarm cues varies enough between species that while some species may generalize from conspecifics to heterospecifics, their response is typically weaker to heterospecific cues (Mirza & Chivers, 2001b; Mitchell, Cowman, & McCormick, 2012). However, in special circumstances, including sympatry and shared predators, unrelated species may in fact learn to respond to each other's cues (Chivers, Mirza, & Johnston, 2002; Pollock *et al.*, 2003; Pollock & Chivers, 2004).

1.2 Learned predator recognition and retention

Prey species often learn to recognize their predators by observing a predation event. Exposure to the alarm cues released during a predation event concurrent with exposure to a novel predator odour triggers what is known as "releaser-induced recognition learning," a variant of classical Pavlovian learning (Suboski, 1990). Subsequent to such a learning event, the individual will respond to the formerly novel predator in the same manner that it would innately respond to alarm cues, allowing it to avoid the threat of the predator. Organisms from flatworms to vertebrates (reviewed in Ferrari *et al.*, 2010c) are capable of learning to recognize predators through the association of predator odours and alarm cues. This learning can take place after only one concurrent exposure (Magurran, 1989; Mathis & Smith, 1993b; Chivers & Smith, 1994b) and can result in greatly increased survival in subsequent encounters (Mirza & Chivers, 2000; Lönnstedt *et al.*, 2012).

1.2.1 Contextual learning

Organisms may also be capable of assessing the context in which a predation event takes place (Chivers & Smith, 1995b; Sih *et al.*, 2000; Ferrari & Chivers, 2009b). Context allows organisms to make more accurate inferences about degree of risk (Helfman, 1989; Lima & Dill, 1990; Brown, Ferrari, & Chivers, 2011a). Many aquatic organisms can detect variance in the

concentration of cues (reviewed in Brown *et al.*, 2011a). Using the assumption that concentration represents extent of damage, number of predators, or relative proximity of these predators, individuals can vary their antipredator responses accordingly, saving extreme (and costly) antipredator defenses for greater, more urgent threats (Helfman, 1989; Kusch, Mirza, & Chivers, 2004; Ferrari *et al.*, 2005). However, some fish, such as rainbow trout and pumpkinseeds (*Lepomis gibbosus*, Linnaeus 1758), do not appear to show a graded response (Mirza & Chivers, 2003; Marcus & Brown, 2003).

Organisms may also include this contextual information in their learned antipredator responses. Threat-sensitive learning has been observed in fish (Ferrari *et al.*, 2005; Zhao, Ferrari, & Chivers, 2006), amphibians (Ferrari & Chivers, 2009c) and even insects (Kesavaraju *et al.*, 2007; Ferrari, Messier, & Chivers, 2008a). Fathead minnows, for example, can assess the proximity, density and size of predators based on odour (Kusch *et al.*, 2004; Ferrari, Messier, & Chivers, 2006). Prey may also gather environmental information, such as the time of day, and grade their responses to future events based on temporal risk (Ferrari & Chivers, 2010). They may use predator cues to generalize other potential predators. The more closely related a predator is to the one that they recognize, the greater their antipredator response will be (Mirza & Chivers, 2001b; Mitchell *et al.*, 2012).

1.2.2 Adaptive forgetting

At a certain point, some organisms stop responding to predator odours that they have learned. There is a cost associated with retaining obsolete information; foraging or mating opportunities may be missed due to perceived threat when there is no actual risk. For this reason, we refer to this termination of response as "adaptive forgetting" (Bouton, 1994; Kraemer & Golding, 1997; Ferrari *et al.*, 2010a). There is little information regarding the period of time for which an organism will retain what it has learned, most likely due the highly dynamic nature of learning. For example, rainbow trout may stop responding to conditioned predator odours after eight days (Brown *et al.*, 2011b) and yet salmonids retain homing cues for their entire four-year life cycle (Cooper *et al.*, 1976). Furthermore, individuals may retain information about a predator without using it, as demonstrated in wood frogs (*Lithobates sylvaticus*, LeConte 1825) that ceased to respond to certain predator information, yet continued to adjust their behavioural responses to the predator (Chivers & Ferrari, 2013). Specifically, tadpoles that stopped responding to a predator

odour subsequently increased their responses to it when re-exposed, as compared to those that had never learned, indicating that although they were not responding to it, they recalled its significance.

A variety of environmental and intrinsic factors affect an organism's retention of learned predator information, including both environmental factors, such as the presence and appetite of the predator, and intrinsic factors, such as changes in body size or habitat shifts (reviewed in Ferrari *et al.*, 2010a). For example, tadpoles and fish may retain predator recognition longer in the presence of increased risk of predation (Ferrari *et al.*, 2010a,b). Shifts in size may cause constant changes in predators over time, as gape limited predators can only eat smaller individuals (Paine, 1976; Persson *et al.*, 1996) and therefore organisms may grow through various predator guilds (Werner & Gilliam, 1984). Indeed, rainbow trout growing at a higher rate retained information for shorter time than their slow-growing conspecifics (Brown *et al.*, 2011b).

Finally, information may also gain different contexts over the course of an organism's life. Carnivorous fish such as the largemouth bass (*Micropterus salmoides*, Lacépède, 1802) may demonstrate cannibalistic tendencies on smaller individuals of their species, and thus alarm cues may switch from being a threat or deterrent in their youth to a foraging cue or attractant as adults (Brown *et al.*, 2011c).

1.3 Ontogeny and risk

Ontogeny may have a large impact on how an organism interacts with its environment. "Sensitive periods" refer to periods of development in which the environment may have a more intense impact on an organism (Westeberhard, 1989; Travis, 1994; Panchanathan & Frankenhuis, 2016; Arnett & Kinnison, 2017). For example, phenotypic plasticity and morphological changes that may protect an organism from predators may need to be induced during certain developmental stages, often before the organism reaches adulthood (Brönmark & Miner, 1992; Januszkiewicz & Robinson, 2007). Another example of an ontogenetically regulated environmental interaction is the imprinting of salmonids on natal streams. Salmonids imprint on natal streams so that they may return to spawn, sometimes after years in the ocean, but they are only capable of this imprinting during early developmental stages (Cooper *et al.*, 1976; Hasler & Scholz, 1983; Dittman, Quinn, & Nevitt, 1996). It is also possible that the longevity of memory

is affected by ontogeny. Indeed, the effects of age on learning capacity have been studied extensively (Campbell & Spear, 1972; Wyss *et al.*, 2000; Madsen & Kim, 2016). Although learning and memory generally decrease with age (Wyss *et al.*, 2000), there are exceptions in phenomena such as infantile amnesia, where restructuring of the brain is believed to alter retention of early-acquired information, suggesting complex dynamics in age-related information retention (Campbell & Spear, 1972; Madsen & Kim, 2016).

1.3.1 Ontogeny and fright responses

In addition to gathering contextual information from the environment, animals may also gain contextual information from the cues themselves. Some fish may be able to discern the ontogeny of the conspecifics that produced the alarm cues (Mirza & Chivers, 2002; Lönnstedt & McCormick, 2011; Mitchell & McCormick, 2013). Furthermore, fish may respond more strongly to cues from conspecifics of the same age or size, presumably based on the assumption that these cues are the most relevant (Mirza & Chivers, 2002), or in fish such as damselfish, they may even completely neglect to respond to cues from other ontogenetic groups (Lönnstedt & McCormick, 2011; Mitchell & McCormick, 2013). This is not true of all species, however; adult fathead minnows responded equally to larval and adult cues, and sunfish respond equally to conspecific alarm cues regardless of donor size (Golub & Brown, 2003; Carreau-Green *et al.*, 2008). This discrepancy may be a result of different life histories, as ontogeny-specific cues may be more beneficial for species whose predator profiles and niches change greatly over their lifespans, than for those who maintain the same predator species throughout their lives.

Additionally, ontogeny may have an impact on antipredator behaviour in general, such that the age of an individual may affect how it responds, or even if it responds at all. Crane and Mathis (2013) showed that hellbenders (*Ciyptobrancims alleganiensis*) have completely inverted behavioural threat responses, with younger individuals reducing activity in contrast to older individuals that increase their activity in response to predators. Waldman (1982) and Pfeiffer (1963) found that during the first month post-hatch, zebrafish (*Danio rerio*, Hamilton, 1822) did not respond with typical antipredator behaviours. Carreau-Green *et al.* (2008) found same to be true of fathead minnows. Marcus and Brown (2003) found that a lower concentration of conspecific alarm cue from intermediate-sized donors was required to elicit a fright response in

juveniles than in sub-adults, suggesting that older fish associated a lower risk with the same concentration of cues.

Not seeing an overt response, however, does not necessarily mean that an organism does not perceive the risk, or that it is failing to gather the information. It is possible for the individual to take in and acknowledge information but opt not to act on it. Brown *et al.* (2001a) showed that in spite of not responding behaviourally to an exposure of a low concentration of alarm cue, still learned to recognise the predator odour that they encountered concurrently, demonstrating that learning is possible even below the behavioural modification threshold. Indeed, there is strong evidence to show that although embryos have limited means by which to respond to threats, they are keenly aware that a threat is present (Chivers *et al.*, 2001; Mathis *et al.*, 2008). For example, Atherton and McCormick (2015) showed that the heart rate of embryonic damselfish increased with exposure to alarm cues.

1.3.2 Embryonic defences against risk

Although their means of responding to threats as embryos are limited, aquatic organisms may be capable of responding by altering the timing of their hatching (Chivers *et al.*, 2001; Mathis *et al.*, 2008; Atherton & McCormick, 2015). Either premature or delayed hatching times may occur in response to abnormal conditions or cues (Warkentin, 2011a). Temperature (Jungwirth & Winkler, 1984), levels of oxygen (Czerkies *et al.*, 2001), risk of desiccation (Wedekind & Müller, 2005), presence of pathogens (Warkentin, Currie, & Rehner, 2001; Pompini, Clark, & Wedekind, 2013), high population densities or predation pressure (Sih & Moore, 1993; Warkentin, 1995; Kusch & Chivers, 2004) may all impact hatching times. Early hatching is a common response to physical or chemical threats of predation in both amphibians (Chivers *et al.*, 2001; Touchon *et al.*, 2013) and fish, including brook char and fathead minnows (Mirza *et al.*, 2001; Kusch & Chivers, 2004).

1.3.3 Embryonic learning

Newly-hatched organisms are often at their most vulnerable (Stangel, 1988; Sogard, 1997). The transition from an embryonic membrane to the unprotected world is inherently drastic. Although embryonic organisms use their muscles to move within their membranes, and innately know how to swim, with any new activity there is a period of learning and improvement. As they

learn to control their movements, they begin to search for food and shelter. There are typically very high mortality rates in the wild among newly hatched fish (Caley, 1998; Almany & Webster, 2006), an inevitability which is evolutionarily managed by the parental strategy of releasing high numbers of eggs to ensure that at least a few survive. Nevertheless, a newly-hatched fish will also engage in risk-mitigation strategies to try to increase its individual probability of surviving this highly dangerous period. Some fish, such as trout, hatch out into a semi-embryonic state in which they lose their egg membranes but maintain yolk sacs, allowing them to live hidden for up to several weeks without needing to forage as they acclimate to their new environment (Behnke, 2002). Others, such as reef fish, adopt different strategies, where the newly-hatched larvae undergo an open-ocean pelagic phase to avoid benthic predators (Almany & Webster, 2006). Others hatch out in the same microhabitat they will inhabit for their entire lifespan and must therefore rely heavily on behavioural antipredator strategies early in life or be protected by their parents.

One way in which animals may mitigate risk in early life stages is to learn about predators before they hatch. The capacity for embryonic learning has been observed in many contexts across many species, ranging from kin recognition (Hepper, 1987) to food preferences to predator recognition (Mathis *et al.*, 2008; Nelson, Alemadi, & Wisenden, 2013; Atherton & McCormick, 2015). Amongst aquatic organisms, embryonic learning of predator recognition has been observed in amphibians and fish, including wood frogs (Mathis *et al.*, 2008), convict cichlids (*Amatitlania nigrofasciata*, Gunther 1867) (Nelson *et al.*, 2013) and cinnamon clownfish (*Amphiprion melanopus*, Bleaker 1852) (Atherton & McCormick, 2015). Indeed, embryonic wood frogs have been demonstrated to possess a high sophistication of learning, including the ability to incorporate contextual information such as temporal factors and threat-sensitivity (Ferrari & Chivers, 2010).

1.4 Study systems

My research focuses on freshwater prey species common to Saskatchewan. Two species of fish with highly contrasting life histories were selected to allow consideration of the implications of life history on learning and memory: fathead minnows and rainbow trout. I also used wood frogs to test the pervasiveness of sensitive periods of learning across aquatic organisms.

1.4.1 Fathead minnow (Pimephales promelas, Rafinesque 1820*)*

Fathead minnows are small, social, omnivorous Ostariophysi fish (4 – 6 cm) that spend their lives in the shallow areas of pond, lakes, rivers and streams across North America. Females lay their eggs on the undersides of hard surfaces, which are typically defended by the male until they hatch approximately five days later. They are tolerant of a wide range of conditions and typically do not travel far from their natal area over the course of their lives. Minnows are prey to a wide range of predators, both aquatic and terrestrial, including invertebrates, larger fish, birds and snakes (Matity, Chivers, & Smith, 1994).

Minnows are commonly used for toxicological research and their behaviour has been extensively studied in the field of chemical ecology. Fathead minnows do not innately recognize predators, such as pike, but can learn to recognize predators through a single pairing of predator odour with alarm cues or dietary cues and are known to retain this information for at least a year (Mathis & Smith, 1993b; Chivers & Smith, 1994a). Brown, Chivers and Smith (1997) found that the entire population of minnows in a 4 ha pond (estimated at 78 000 individuals) came to recognize pike as predators within two to four days of pike being released into their ecosystem. Minnows typically decrease activity (movement and foraging), increase shoaling behaviour, and increase shelter use in the presence of predators.

1.4.2 Rainbow trout (Oncorhynchus mykiss, Walbaum 1792*)*

Rainbow trout are large, carnivorous Salmonid fish (50 – 75 cm) found in cooler lakes, river and streams across North America. Females travel up to their natal streams to deposit their eggs in rocky substrates to mature. Egg development rates vary greatly depending on temperature. After hatching out of their egg membrane, rainbow trout enter an intermediate developmental stage (alevin) with their yolk sacs still outside of their bodies. During this stage, they typically remain well hidden amongst the rocks. The yolk sac absorbs over the course of a few weeks, and the alevin becomes a fry and begins to swim up and forage for food. Fry eat voraciously and grow rapidly, allowing them to move quickly between life stages and prey guilds. Juveniles begin to explore and may move away from their natal regions. In fact, in some subgroups of rainbow trout, such as steelheads, the fish will slowly migrate all way to the ocean, where they will remain until they return to their natal stream to spawn before dying (Behnke, 2002).

Trout are highly-prized game fish and are regularly farmed to stock water bodies, or to sell as food. This public interest has led to rainbow trout being well-studied, including in terms of antipredator responses to alarm cues (Brown & Smith, 1997; Mirza & Chivers, 2003). Like minnows, trout do not innately recognize predators but may learn through pairing of predator odour and alarm cues. Interestingly, trout have higher response threshold than some species, such as minnows (Mirza & Chivers, 2003). They typically reduce activity and foraging, or use shelter in response to predators.

1.4.3 Wood frogs (Lithobates sylvaticus, LeConte 1825*)*

Wood frogs are small, omnivorous amphibians (4 – 6 cm) that spend their embryonic and larval stages primarily in ephemeral wetlands and their adult lives in the terrestrial environments that surround their aquatic birth places, including riparian areas, marshes and other damp habitats. Females lay large clutches of hundreds to thousands of eggs in ponds and marshes in mid-spring. Embryos take one to two weeks to hatch, depending on temperature, and larvae develop within a few weeks depending largely on food availability.

The responses of wood frogs to alarm cues have been extensively studied (Chivers & Ferrari, 2013). Wood frogs do not typically demonstrate innate predator recognition, but are capable of predator recognition-learning as early as in the embryonic stage (Mathis *et al.*, 2008). They are, in fact, capable of both threat-sensitive and temporally-regulated predator recognition from learning as embryos (Ferrari & Chivers, 2010). Typically, wood frog larvae dramatically reduce their activity in response to alarm cues. The larvae are extremely delicate after hatching so care must be taken to avoid handling them until they are slightly more developed.

1.5 Research objectives and hypotheses

My objective in this thesis was to explore the capacity for embryonic learning in two species of fish with very different life histories: fathead minnows and rainbow trout. I also explored the sophistication of learning demonstrated by embryonic fish, and the impacts of ontogeny on early-life response in these species. These studies led to questions about the impact of ontogeny on adaptive forgetting, which I investigated using rainbow trout and wood frogs. My experiments addressed the following three questions:

How does ontogeny impact early life-stage responses to alarm cues?

In Chapter 2, I investigated whether the ontogeny of the cues affects the response of juvenile trout to alarm cues, by comparing responses to cues from alevins and six-month-old juveniles. I hypothesize that ontogeny of donor cues will not affect the responses of juvenile trout. In Chapter 3, I tested the effect of ontogeny on the early hatching response, by investigating whether minnows also demonstrate an early hatching response to adult alarm cues as they are known to do to embryonic cues, and if they do whether that response is threat-sensitive. I hypothesize that, because fathead minnows hatch early in response to embryonic cues, they will also hatch out early in response to adult alarm cues.

Are fish capable of sophisticated embryonic learning?

At the time that I started my thesis, there was no scientific record of embryonic learning of predator recognition in fish. After I started my thesis, two articles (Nelson *et al.*, 2013; Atherton & McCormick, 2015) were published demonstrating the capacity for embryonic learning in fish. I tested two species from different orders than those previously published: minnows and rainbow trout. I also did in-depth exploration of the sophistication of learning in embryonic fish. In Chapter 4, I investigated the capacity of embryonic rainbow trout (Order: Salmoniformes) to learn to recognize predators using both conspecific and heterospecific cues paired with novel predator odour. I hypothesize that trout embryos will be able to learn predator recognition using either conspecific cues or the heterospecific cues of brook trout. In Chapter 5, by exposing embryonic fathead minnows (Order: Cypriniformes) to different concentrations of conspecific alarm cues in combination with predator odour, I tested whether they are capable of learning to recognize predators and whether this learning has a threat-sensitivity component. I hypothesize that embryonic fathead minnows will learn in a threat-sensitive manner.

Does ontogeny impact retention of learned predator recognition?

In light of the great variability of retention of learned predator recognition, I was curious about the impact that the precise stage of development might have on the rate of adaptive forgetting. In Chapter 4, I tested this by exposing three different early-life stages of rainbow trout (embryos, alevins and fry) to pairs of alarm cues and novel predator odour to induce learning. The fish were then tested over time for responses to predator odour. I hypothesize that the

retention of learned predator recognition in trout will be dependent on ontogeny at the time of learning. In Chapter 6, I tested the same question using tadpoles to ascertain whether the phenomenon of sensitive learning periods in early development of amphibians reflects that of trout. I hypothesize that the retention of learned predator recognition in wood frogs will be dependent on ontogeny at the time of learning.

1.6 Anticipated significance

The questions posed in my thesis address a fundamental understanding of learning and memory retention at early stages of development. In this thesis, I explore the capacity of embryonic fish to learn to recognize predators and demonstrate a sophistication of learning at the embryonic stage that has not been previously been investigated in fish. I test different ways in which ontogeny impacts responses to alarm cues in early-life stages in fish and address novel questions about the effects of the timing of learning on retention of learned predator recognition.

1.7 Ethical statement

The following research was approved by the University of Saskatchewan Committee on Animal Ethics under the protocol number 20070083. Fish were humanely euthanized after use by either a blow to the head or with an overdose of tricaine methanesulfonate (MS-222).

1.8 Thesis format

This thesis is presented in manuscript format. The contents of each chapter have been prepared as an independent manuscript for the purpose of publication and therefore there is some redundancy in content of the introductions within individual chapters. The corresponding publishing body is listed as a footnote at the start of each chapter along with the contributions of each published author.

CHAPTER 2: Alarm cue specificity and response ontogeny in juvenile rainbow trout[1]

2.1 Introduction

To avoid being eaten, prey must constantly evaluate the predation risk they face (Lima & Dill, 1990). Aquatic organisms can identify predation risk through recognition of the release of alarm cues (AC) from damaged skin of conspecifics (Lawrence & Smith, 1989; Chivers & Smith, 1998; Wisenden, 2015). Recognition of these cues gives animals the opportunity to respond promptly and avoid predation by hiding or decreasing conspicuous behaviours such as foraging (Pfeiffer, 1977).

While being insufficiently careful may result in predation, being overly cautious may result in missed opportunities to improve fitness by mating or foraging (Lima & Dill, 1990). Therefore, an individual that can evaluate the degree of predation risk to which it is exposed may ultimately achieve greater fitness. One mechanism for evaluating levels of risk is a threat-sensitivity to alarm cues, where the strength of the fright response of an individual is proportional to the concentration of alarm cues present, as concentration is often representative of either the extent of the damage, or the relative distance of the predation event (Helfman, 1989; Mirza & Chivers, 2003).

Ontogeny may also play a role in response to conspecific alarm cues. Waldman (1982) and Pfeiffer (1963) found that zebra fish do not respond to alarm cues for at least a month after hatching and suggest that a developmental milestone must be reached before this behavioural shift occurs. Carreau-Green *et al.* (2008) found the same lack of fright response to alarm cues during the first month in fathead minnows. Marcus and Brown (2003) found that a lower concentration of conspecific alarm cue from intermediate-sized donors was required to elicit a fright response in juveniles than in subadults, which suggests that the older fish are associating a

[1] The content of this chapter is published in the following publication. For this publication, I designed and executed the experiment, did the analyses and wrote the first draft of the manuscript. DP Chivers contributed to the final draft. Changes have been made to avoid redundancy with other chapters and for consistency among chapters.

Horn ME, Chivers DP. (2017) *Alarm cue specificity and response ontogeny in juvenile rainbow trout (Oncorhynchus mykiss)****. Behaviour, 153(3): 377-385.***

lower risk with the same concentration of alarm cue. Some fish, such as the largemouth bass, may even shift completely from exhibiting fright responses to alarm cues to using the alarm cues as foraging cues (Brown, LeBlanc, & Porter, 2001b).

Many fish grow immensely over the span of their lives and may therefore move through various prey guilds (Werner & Gilliam, 1984). Because the gape-limited predators which forage on small juveniles typically differ from those which forage on large adults, it would be detrimental for a large adult to respond to alarm cues released by smaller juvenile conspecifics; the risk to them is small and therefore restricting foraging behaviour would be costly rather than beneficial. Some studies have found that individuals exhibit a stronger fright response when exposed to alarm cues from conspecifics of the same age. Both Lönnstedt and McCormick (2011) and Mitchell and McCormick (2013) found that juvenile or new recruit damselfish responded most strongly to alarm cues from conspecifics of the same age, with no response to alarm cues from adults. Mirza and Chivers (2002) found that even within the same age group, size affected the strength of response. Small juvenile brook char responded more strongly to alarm cues from other small juveniles than they did to alarm cues from larger juveniles and vice versa. However, size- or age-dependent responses are not universal. Carreau-Green *et al.* (2008) found that adult fathead minnows responded equally to larval and adult alarm cues, and Golub and Brown (2003) found equal responses in sunfish to conspecific alarm cues regardless of donor size.

Research to date portrays a complex picture of varied responses based on the ontogeny of the alarm cue donor and receiver. Further research studying fish with different life histories is necessary to understand potential patterns in the association between ontogeny and response to conspecific cues. Rainbow trout provide an interesting model in questions of ontogeny because of their rapid growth, the large size discrepancy between life stages and their migration behaviours (Behnke, 2002). Antipredator responses to conspecific alarm cues have been well-documented in rainbow trout (Brown & Smith, 1997; Mirza & Chivers, 2003). They have a threshold for fright response to conspecific alarm cue that is higher than some other species such as minnows (Mirza & Chivers, 2003). Here, we investigate the complex role of ontogeny in fright behaviour by evaluating the response of newly-hatched rainbow trout to cues from

conspecifics of the same age, and cues from older, larger juvenile conspecifics at three concentrations.

2.2 Methods

2.2.1 Holding conditions

Embryonic rainbow trout were acquired from Troutlodge Inc. of Sumner, WA. The embryos were held in one-L hatching tanks with a bottom air supply and water supply, with a flow rate of approximately 0.5 L / min. After hatching, they were transferred to baskets made of mosquito netting in 500-L flow-through tanks with dechlorinated tap water and maintained at 10 °C with a flow rate of approximately four L / min and a light:dark cycle of 16:8 h. Each tank had a large Aquaclear water filter (Hagen, Montreal, QC) with a charcoal filter, a Biomax filter and a foam insert that were rinsed daily and changed as needed. Trout were fed six times daily with commercial floating trout feed.

2.2.2 Collection of alarm cues

Juvenile conspecific alarm cues (JAC) were collected from the skin of ten randomly selected six-month-old juvenile rainbow trout (fork length: mean ± SE: 19.3 cm ± 0.5). Larval conspecific alarm cues (LAC) were collected from 70 newly-hatched rainbow trout (fork length: 21.7 mm ± 0.1) six days post hatch, when they still had their yolk sacs. The fish were euthanized with a blow to head (as per the guidelines from the Canadian Council on Animal Care). A layer of skin is removed from each side of the body of the six-month-old juveniles. The same was attempted with the larval samples, but due to their fragility the larvae were ultimately homogenized whole. The skin was homogenized in chilled, distilled water and then filtered through cotton batting to remove tissue fragments leaving a concentrated solution of 0.1 cm^2 / mL water. Thirty-mL aliquots of the concentrated solution were frozen at −20 °C and preserved. Prior to use, the solution was thawed overnight at room temperature, and then diluted into concentrations of 0.001 (Low), 0.01 (Med) and 0.1 cm^2 / mL (High).

2.2.3 Testing conditions

Juvenile trout were tested as soon as they absorbed their yolk sacs and became free swimming, at 12–28 days post hatch (fork length: 29.5 mm ± 0.3). Based on Chivers and Smith's

(1998) review of antipredator responses, observations of fright response focused on decreases in activity by measuring the number of line crossings. Testing of responses to alarm cue took place in 745-mL round plastic containers (no name brand, Loblaws, Brampton, ON) marked externally with perpendicular lines that divided the bowl into quarters. A line crossing was counted when 3/4 of the body of the fish had crossed the line. Individual fish were placed into testing bowls at least one hour before testing to allow acclimation to the tank. The number of line crossings was assessed for five min before the stimulus injection. One mL of test cue was injected followed by 30 mL of water to flush the line. Line crossings were then assessed for another five min. The test cue used was either a water control (DW) or one of three different concentrations of either larval or juvenile alarm cues. The final concentrations of alarm cue in the testing bowls were 1 cm^2 / 745 L (Low), 1 cm^2 / 74.5 L (Med) or 1 cm^2 / 7.45 L (High).

2.2.4 Statistical analysis

Proportional change in line crossings was calculated ((poststimulus − prestimulus) / prestimulus) and data analyses were performed using RStudio, v.1.3.1073 (n = 20 for alarm cue recipients and n = 10 for controls). Because the data were non-normally distributed, a Scheirer-Ray-Hare extension of the Kruskal-Wallis test was performed, which is analogous to a 2-way ANOVA performed on rank-transformed data (Sokal & Rohlf, 1995).

2.3 Results

The analysis showed a significant difference between the responses of the fish to different concentrations ($H_{1,132}$ = 13.31, p = 0.004, Figure 2.1) but there were no significant differences in the response to larval versus juvenile alarm cues ($H_{1,132}$ = 0.705, p = 0.401) and no interactions between age cues and concentration ($H_{1,132}$ = 1.29, p = 0.731). A Tukey's HSD post-hoc analysis on the concentration response showed that the fish responded significantly more strongly to the two higher concentrations of alarm cue than to the distilled water control (Med AC: p = 0.004; High AC: p = 0.004), but not the lowest concentration (p = 0.091) and showed no significant differences, between responses to low and medium (p = 0.576), medium and high (p = 1.00) and high and low (p = 0.567) concentrations of alarm cue.

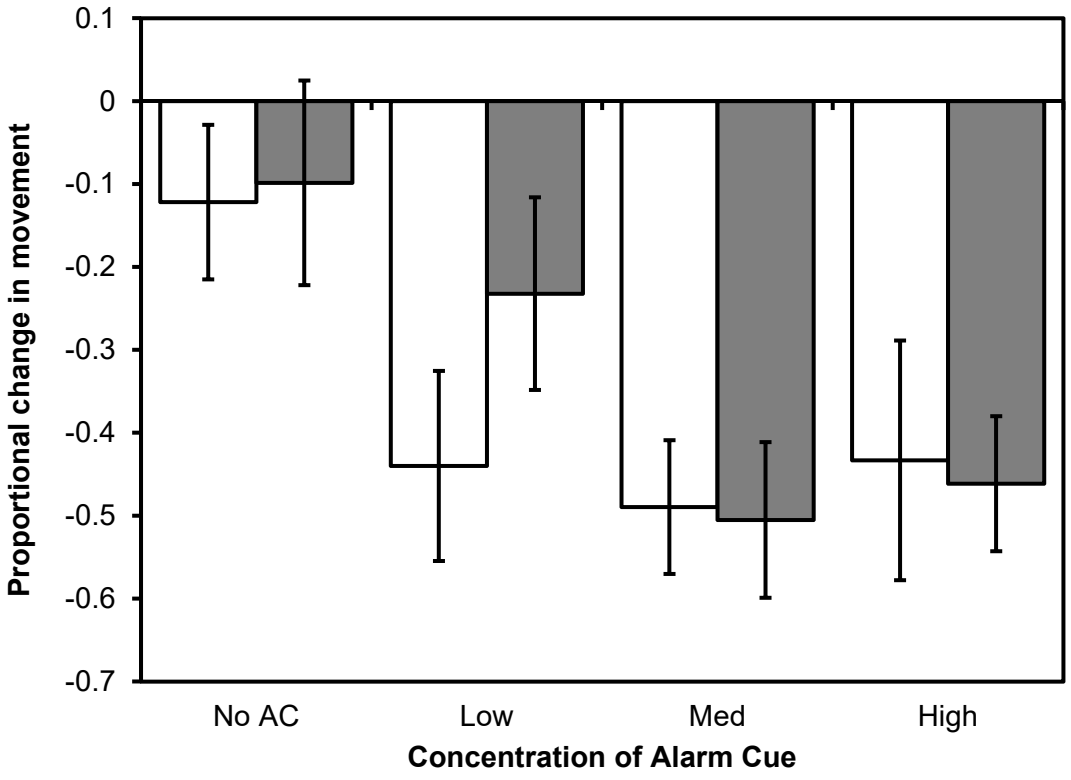

Figure 2.1. Mean (± SE) proportional change in movement measured by line crosses by newly-hatched rainbow trout in response to distilled water (no AC) or alarm cue from newly-hatched (open bars) or six-month-old (grey bars) conspecifics at concentrations of 0.001, 0.01 or 0.1 cm^2 / mL.

2.4 Discussion

I found that juvenile trout responded to alarm cues as early as twelve days after hatching, unlike zebra fish and minnows, which fail to respond to alarm cue for at least 32 days after hatching (Pfeiffer, 1963; Waldman, 1982; Carreau-Green *et al.*, 2008). My findings in trout are similar to the findings of Lönnstedt and McCormick (2011) who looked at newly settled damselfish as young as sixteen days old. Phylogenetic differences may explain this difference in early response to alarm cues, but testing method may also play a role, as the behavioural fright responses of a small juvenile may vary from those of the more developed juveniles that are more frequently studied. Future studies might benefit from the use of different behavioural metrics, such as time spent moving or use of vertical space.

The newly free-swimming juveniles in this experiment exhibited a fright response to the higher two concentrations of alarm cues. A lack of significant difference in the intensity of fright response between the three concentrations or between the responses to larval and juvenile alarm cues indicates that these fish are responding to threats with equal intensity. The fright response observed here (reduction of movement by 40–50%) is not so dramatic that one would assume that the concentrations were too high for differentiation. Different species of fish have different degrees of threat-sensitivity in their fright responses (Helfman & Winkelman, 1997; Brown *et al.*, 2006) and some, including rainbow trout, tend to respond with a relatively non-graded response (Mirza & Chivers, 2003). The lack of variation between fright responses to the different concentrations of alarm cue can be directly explained by this supposition, but it may not explain the lack of differentiation between age groups observed by studies using similar species (Mirza & Chivers, 2002) or comparable concentrations (Lönnstedt & McCormick, 2011; Mitchell & McCormick, 2013). Either the juvenile trout failed to respond differently because they cannot differentiate between the cues from different age groups, or because, although they observe the difference, they assess the threats as equal.

These newly hatched trout, while free swimming, do not have the mobility of older, larger fish. Gape-limitation protects larger juveniles from being eaten by some predators that can prey on small juveniles, but there is no gape-limitation protecting smaller fish from predators of the larger juveniles (Paine, 1976; Persson *et al.*, 1996) and predatory fish may consume prey fish more than 50% of their body length (Popova, 1978). Consequently, there should be greater

benefit in a small fish responding to alarm cues from larger conspecifics than vice versa. In general, limited mobility and smaller size puts these fish at higher risk of predation than their larger conspecifics. Additionally, Brown *et al.* (2006) demonstrate that single fish, as tested here, may show a more dramatic fright response than their conspecifics tested in groups, as a result of the higher predation risk they face alone. It is conceivable that because of the greater risk that they face, these younger, less mobile individual fish attribute an equally strong risk to all potential threats.

Given the general sensitivity of fish to different concentrations of alarm cues, as well as the variety of studies demonstrating ontogeny-based cue response, it seems unlikely that the newly-hatched trout are unable to differentiate between cues from conspecifics of the same age and those of older individuals. It is far more plausible that because these individuals are at sufficiently high risk of predation, they exhibit fright responses to even the lowest signs of risk. Generalization of learned predator responses is more common in individuals conditioned with higher risk (Ferrari, Messier, & Chivers, 2008b).We propose that here, because smaller juveniles are at a higher risk in general, they too will take a more generalized approach to their perceived predation risk, and generalize that even cues which are not age-specific may be relevant. Further research into the importance of predation risk in ontogenetic response to alarm cues across different taxa is warranted.

CHAPTER 3: Hatch time in embryonic fathead minnows exposed to predation risk[2]

3.1 Introduction

Animals facing predation must continually weigh the gains of activities such as foraging and mating against the risk of being injured or killed by a predator. Predation risk has a large impact on behaviour, morphology and life-history. The behavioural impacts are well studied; animals typically exhibit fright behaviours, such as reduced movement and foraging (Lima & Dill, 1990), and in the case of fish, increased shelter use and shoaling (Chivers & Smith, 1998). These behaviours may significantly decrease their risk of predation (Mirza & Chivers, 2000, 2001a). Individuals that are under a constant threat of predation even begin to demonstrate neophobia – a fear of all novel stimuli (Brown *et al.*, 2013b). More drastic behavioural responses may come at a greater cost, as exhibiting fear of everything may prevent low risk gains (Lima & Dill, 1990). Individuals that experience extended exposure to predation risk at certain life stages may also change their morphology to decrease their risk of predation. A classic example is the crucian carp, whose body depth increases in the presence of pike predation (Brönmark & Miner, 1992). This morphology is advantageous in the presence of the gape-limited pike, which cannot open their mouths wide enough to consume the taller morphotype. This morphological variation is not without cost as it slows the swimming of the carp, but in a setting where the predation risk is great, this cost is negligible compared to the decreased risk of predation. Fathead minnows likewise change their morphology in response to risk, but this trait is restricted to males. Females appear to have a consistent morphology, whereas males exhibit considerable variation, with early alarm cue exposure inducing deeper head and body structures, as well as shorter caudal penduncles and fins and longer dorsal fins (Meuthen *et al.*, 2019). As much as morphological changes may help animals avoid predation, so too can life-history shifts such as timing of hatching. Because some predators forage selectively on specific life stages of prey, shifting

[2] This chapter has been prepared for submission to PLOS ONE. For this publication, I designed and executed the experiment, did the analyses and wrote the first draft of the manuscript. DP Chivers contributed to the final draft. Changes have been made to avoid redundancy and for consistency among chapters.

Horn ME, Chivers DP. *Embryonic exposure to predation risk and hatch time variation in fathead minnows.* ***PLOS ONE.*** *(Forthcoming)*

stages early or late may help prey reduce their predation risk. For example, newly-hatched salamander larvae are at high risk of predation by flatworms. Sih and Moore (1993) found that in the presence of flatworms, salamanders delayed hatching to postpone their encounters with these predators until they were larger and better able to withstand the attacks.

Newly-hatched organisms are commonly considered to be at their most vulnerable due to their size, their naivety and their sudden loss of their egg membrane (Sogard, 1997). However, being an embryo is not without risk either, as their immobility makes it impossible for embryos to escape danger. Risk-induced hatching variation is one of the few mechanisms by which an embryo can mitigate risk. Embryos typically hatch spontaneously at a certain stage under typical conditions, but may hatch prematurely or delay hatching in the presence of higher-than-normal cue levels or extreme conditions (Warkentin, 2011a). Threats such as low oxygen levels (Czerkies *et al.*, 2001), pathogen presence (Warkentin *et al.*, 2001; Pompini *et al.*, 2013), elevated temperature (Jungwirth & Winkler, 1984; Réalis-Doyelle *et al.*, 2016), risk of desiccation (Wedekind & Müller, 2005), high population densities or predation pressure (Sih & Moore, 1993; Warkentin, 1995; Kusch & Chivers, 2004) may all impact embryonic hatching times (Martin *et al.*, 2011; Warkentin, 2011a). In some cases, the organisms can actually speed up their growth within the egg in order to hatch out earlier but at the same developmental stage, yet in other cases they simply escape their egg membrane at an earlier developmental stage (Warkentin, 2011b).

For some organisms that are under threat of predation, early hatching may afford them the ability to escape their predators. This phenomenon, has been observed across a wide range of taxa, including arthropods, amphibians and fish (Chivers *et al.*, 2001; Mirza *et al.*, 2001; Kusch & Chivers, 2004; Touchon *et al.*, 2013). Kusch and Chivers (2004) showed that when fathead minnow embryos were subject to predation risk, they increased the speed with which they hatched out of their egg membranes, affording them the opportunity to seek shelter, while costing them developmental time within the egg. Individuals that hatched early due to predation pressure on the embryos had shorter fork lengths than their unthreatened conspecifics, the lifetime consequences of which are unstudied in fish. Studies in amphibians show a wide range of consequences, ranging from changes in hatching time, to morphological changes to differences in growth rates, all varied by species (Relyea *et al.*, 2018). Another study shows

effects of predation-induced hatching that carried through two subsequent life stages in red-eyed tree frogs (Touchon *et al.* 2013). It is possible that while embryos that hatch early may have the ability to escape whatever immediate predation pressure they incur, it may come at a significant cost over their lifespan, depending on what physiological changes are needed to induce early hatching. However, other changes may be easily reversible, as in wood frog tadpoles.

The ability to respond to a threat is predicated on the ability to detect said threat. It has long been established that embryos are capable of identifying predation risk, usually via olfactory or mechanical cues. Mechanical cues may simply be direct physical contact. Chivers *et al.* (2001) found that Pacific tree frogs (*Hyla regilla*) subject to direct mechanical contact from both predatory leeches (families Glossiphonidae and Erpobdellidae) and non-predatory worms hatched early. These responses were intensified by the combined presence of olfactory cues. Olfactory cues are often innately recognized cues from injured conspecifics in the form of alarm cues (Chivers & Smith, 1998). They may also come from innately recognized predators (Mathis *et al.*, 2008), but this is not typically the case in fish (Brown, 2003). The cues could also theoretically be learned predator cues, based on recent evidence that embryos can learn to identify predators in the embryonic stages (Mathis *et al.*, 2008; Nelson *et al.*, 2013; Atherton & McCormick, 2015; Horn, Ferrari, & Chivers, 2019, Chapter 4). Interestingly, some fish are capable of discerning the ontogeny of the conspecifics that produced the alarm cues (Mirza & Chivers, 2002; Lönnstedt & McCormick, 2011; Mitchell & McCormick, 2013). Fish that can differentiate between cues from different aged conspecifics may have the benefit of being able to only respond to relevant cues, rather than responding to cues from a life stage that experiences different predation risk due to size, habitat or behaviour.

In this experiment I investigate whether alarm cues from adult minnows induce a change in hatching time in embryonic minnows, and whether the concentration of the alarm cue impacts the hatch time.

3.2 Methods

3.2.1 Holding conditions

One hundred and twenty-five mating pairs of adult fathead minnows were obtained from Osage Catfisheries Inc. of Osage Beach, MO. Each mating pair was placed in an individual 10-L glass tank (Hagen, Montreal, QC) filled with dechlorinated water and an airstone in a light:dark cycle of 16:8 h at 25.8 °C (±2.8). A piece of polyvinyl chloride (PVC) pipe cut in half was added to serve as a shelter for mating and egg-laying. Minnows were fed a combination of dried flakes and fresh brine shrimp. The adult minnows were removed from the tanks as soon as the eggs were deposited to prevent a parental care bias. Any pair that hatched out a particularly low viability set of eggs (less than ten percent hatch rate) was replaced with a different partner and allowed to have a second discrete mating event.

3.2.2 Collection of cues

Four adult fathead minnows were sacrificed via a blow to the head (as per guidelines from the Canadian Council on Animal Care) for alarm cue collection. A thin layer of skin was removed from each side of each fish (1 cm^2 per fish) and was homogenized in 40 mL of chilled, distilled water before being filtered through cotton batting to remove any remaining tissue. The solution was diluted to produce three concentrations of alarm cue stock: high, medium and low (1 cm^2 of skin / 40 L, 120 L or 240 L respectively). Thirty-mL aliquots of skin solution were stored at –20ºC and thawed in a water bath prior to use. These concentrations are known to be sufficient to induce behavioural changes in minnows (Ferrari *et al.*, 2005).

As these fish were going to be used for another experiment post hatch (see Chapter 5), I also included predator odour in four of our conditioning treatments. The predator odour is not expected to have impacted this experiment because many experiments with minnows have established that upon hatching minnows lack recognition of pike as a predator. However, for the sake of thoroughness, we explain the process of predator odour collection. Predator odour was collected from three pike (fork length: mean ± SE: 19.1 cm ± 0.2) starved for one week prior to cue collection to prevent the presence of dietary alarm cues (Mathis & Smith, 1993b). The pike were placed in individual 60-L glass collection tanks with only an airstone and clean dechlorinated water for 24 h to produce the predator odour. After the fish were returned to their

regular tanks, the water was filtered through polywool. Bags of 125 mL of predator odour were stored at –20ºC and thawed just before use.

3.2.3 Conditioning

Starting 24 – 36 h after deposition, the clutches of eggs were exposed to the conditioning cues for one hour each morning and one hour each afternoon for two days. We did not believe there would be any gain to beginning conditioning prior to this time because the fundamental neural structure development would be incomplete (US EPA, 1996). The egg surface (PVC pipe) was transferred into a 1.5-L bucket of water which also contained one of six sets of conditioning cues: DW (25 mL distilled water); PO (20 mL predator odour + 5 mL DW); AC (5 mL high conspecific alarm cue control + 20 mL distilled water); LO (5 mL low concentration AC + 20 mL predator odour); MED (5 mL medium concentration AC + 20 mL predator odour); HI (5 mL high concentration AC + 20 mL predator odour). As mentioned, these conditioning treatments were designed for another experiment, but for our purpose they provide three concentrations of alarm cues, and a control to ensure that our addition of predator odour had no impact on hatching. The final concentrations of alarm cues in the treatment buckets were as follows: 1 cm^2 / 73 200 L in the LO, 1 cm^2 / 36 600 L in the MED, and 1cm^2 / 12 200 L in the AC and HI, with none in the DW and PO buckets. After exposure, the pipe with the eggs was removed from the cue and placed in a clean water bath for two min before it was returned to its holding tank.

3.2.4 Testing conditions

Egg clutches were checked every 4 h during the day (0600h, 1000h, 1400h, 1800h, 2200h) for signs of hatching. I considered clutches rather than individual eggs due to the large number of eggs (almost 17 000). I noted the temperature at each 4 h interval, the times at which the eyes appeared, the first fish hatched, approximately 90% had hatched, and the last viable (not discoloured) egg hatched, and recorded if there were any signs of fungal infection (common in eggs lacking parental care). Unfortunately almost all clutches suffered from fungal infection, so it was not possible to exclude pathogen-infected clutches from our analysis. Any infected eggs were carefully excised from the rest of the clutch to prevent pathogen spread.

3.2.5 Statistical Analyses

Because I was considering clutches rather than individual eggs, I used several different measures for hatching time, all recorded in hours. I used two starting points: time from deposition, and time from eye development, and three different end points: first hatch, 90% hatch, and final hatching of all viable eggs, for a total of 6 measures of hatching time. I included the time from eye development measure to prevent bias from our treatments not beginning until 24 hours (shortly before eye development at around 43 h). The appearance of eye spot pigmentation also lines up with developmental milestones including completion of fundamental structural neural development, which may be important for cue detection (US EPA, 1996).

I used a correlation-based principal component analysis (PCA) to combine the six measures of hatching time into a single synthetic variable. I used this variable in an analysis of variance (ANOVA) to evaluating the effect of conditioning treatments with temperature as a covariate. No interaction was found between temperature and treatment ($F_{5,77} = 1.330$, $p = 0.261$). Clutches with hatching success rates of less than ten percent were excluded due to their low viability, leaving 118 clutches of eggs (subgroup of clutches with and without outliers, n = 18 – 22). I also looked at temperature across treatments to make sure there were not categorical differences.

3.3 Results

Clutch hatch rates (mean ± SE) from egg deposition ranged from first hatch at 79 h ± 1.7, to 90% hatch at 111 h ± 1.8, to final hatch at 122 h ± 1.8. From the appearance of eye spots the first hatch was at 35 h ± 1.3, 90% hatch at 67 h ± 1.2, and final hatch at 79 h ± 1.3. The first eigenvector of the PCA captured 73% of the variance and had correlation coefficients with the original response variables that ranged from 0.63 to 0.92. Clutch hatch rate was unaffected by treatment ($F_{5,110} = 0.927$, $p = 0.467$, Figure 3.1), but was affected by temperature ($F_{9,110} = 1.330$, $p < 0.001$). Temperature did not vary significantly across treatments ($F_{5,119}= 0.196$, $p = 0.963$).

3.4 Discussion

Our results provide strong evidence that hatching rates were not affected by the presence of adult alarm cues. Given our design, unequivocal results and large sample sizes, we are confident that our results are robust and reflect a true non-significant effect of risk on hatching time. The

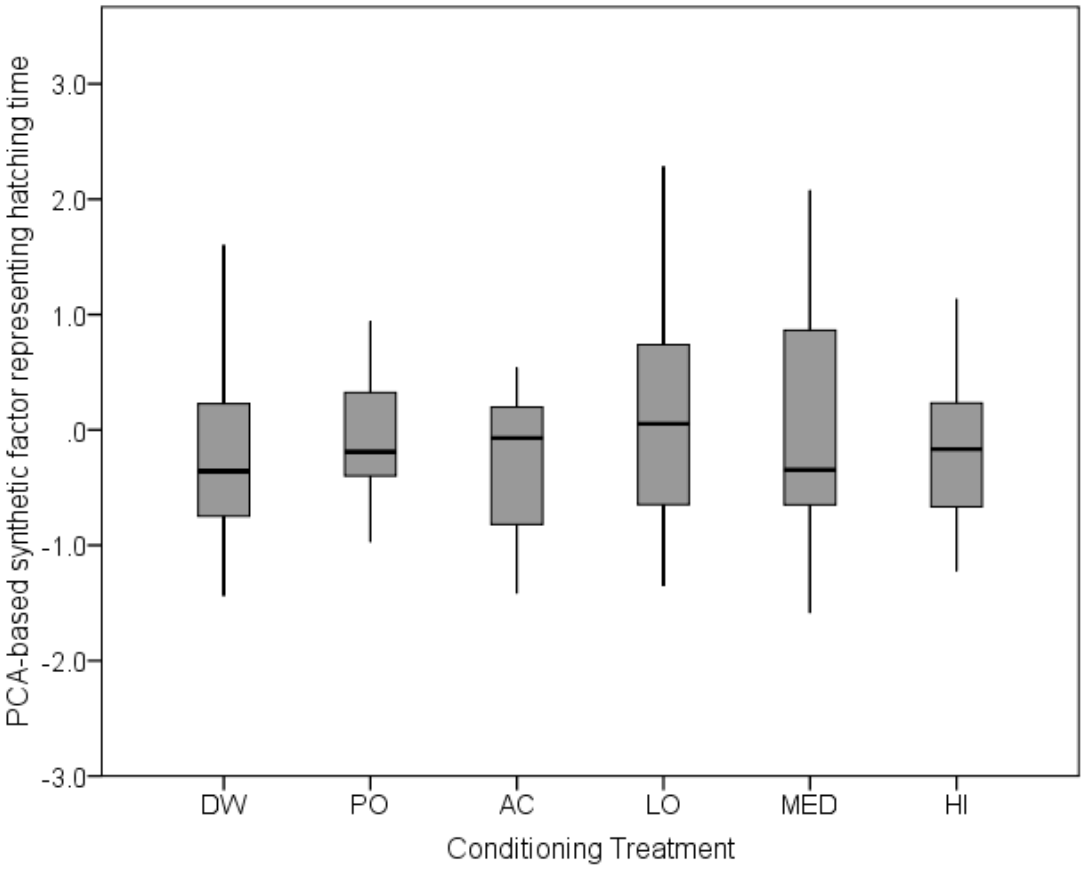

Figure 3.1. Variation in clutch hatching times following embryonic exposure to different treatment cues as represented by a PCA correlation-based synthetic factor that combines six measures of hatching time. DW = water, PO = predator odour, AC = alarm cue, LO = PO + low concentration AC, MED = PO + medium concentration AC, HI = PO + high concentration AC.

only significant differences in hatching time observed in this experiment occurred as a result of temperature, which is a known phenomenon across various species of fish (Brungs, 1971a).

As mentioned, a myriad of factors can affect the hatching time of embryos. In consideration of those many factors, it may seem superficially inconsequential to have stumbled across a factor that is not incurring an effect. However, what makes this observation interesting is the fact that the minnows may be differentiating between this and other highly similar information. Kusch and Chivers (2004) showed earlier hatching in minnows exposed to alarm cues from crushed conspecific embryos combined with feeding cues from virile crayfish fed embryos. In our experiment, we tested whether embryos exposed to adult alarm cues might also hatch prematurely, but found they did not. The concentration of alarm cues from embryos and adults cannot be directly compared as it is measured by the numbers of eggs or the cm^2 of skin per volume (respectively). However, we do know from a separate subsequent experiment that the concentration of adult cues to which the minnows were subjected was enough to elicit a learned antipredator response (see Chapter 5), which clearly indicates that the concentration was adequate to indicate a threat. In spite of the recognized threat to adults, however, these embryos did not hatch early. Although it is theoretically possible that ability to hatch early is not present in this genetic lines of fathead minnows, as it was in the minnows used by Kusch and Chivers (2004) many studies have demonstrate condition-dependent hatching rates across genetically independent fathead minnow populations (Brungs, 1971a,b; Sargent, 1989; Kusch & Chivers, 2004). I believe it is far more plausible that the minnow embryos are responding differently to ontogenetically distinct cues.

Werner (1986) suggests that shifts in the timing of a life-history switch point should only occur in instances when the mortality to growth ratio of the current life stage is greater than that of the subsequent stage. Warkentin (1995) expands this idea to propose that if embryos are in danger but juveniles are successful, early hatching would be favoured, and conversely, high juvenile mortality and safe embryos would favour a delay in hatching. The findings of Kusch and Chivers (2004) follow this trend, with high embryonic risk incurring early hatching. Our experiment tests the reverse – a situation in which the embryos are not at risk, but the adults are. We did not observe early hatching in this scenario, but neither did we observe delayed hatching. Nevertheless, when considered alongside Kusch and Chivers' (2004) work demonstrating early

hatching in embryos following exposure to embryonic cues, our results suggest that the minnows may be able to discern the ontogeny of the cues and use the information accordingly. Several species of fish are known to discern the ontogeny of conspecific alarm cues, and to put more value in the cues from individuals of the same age. For example, Lönnstedt and McCormick (2011) show a clear trend in the response of newly hatched damselfish to alarm cues of different aged conspecifics: response weakens as the age difference increases. Responses range from strong threat-sensitive responses to ontogenetically similar recruit aged fish, to lower and non-threat dependent responses to juvenile cues, down to a complete lack of response to adult cues. Our findings suggest that minnows perceive the presence of a threat to a different life-stage (their free-swimming conspecifics) and recognize that this does not indicate a current threat to their safety. Because hatching early would not help them avoid this threat, they have no cause to hatch early. Indeed, in this instance, early hatching in the presence of a predator to adults would prove to be mismatched as it would increase their risk of mortality rather than decreasing it, not only through increased predator exposure, but also through the long term costs associated with hatching at a less developed stage (Touchon *et al.*, 2013). A sophisticated level of perception of risk could provide advantages to the current life stage by not inducing ill-advised premature hatching, while still allowing collection of information regarding future predation threats.

CHAPTER 4: Retention of learned predator recognition in embryonic and juvenile rainbow trout[3]

4.1 Introduction

Animals must constantly weigh the risk of predation against the rewards of behaviours such as foraging or mating (Lima & Dill, 1990). Although increased foraging maximizes growth and future reproductive success, death negates any such benefits; therefore, living with a degree of caution is paramount (Bouskila & Blumstein, 1992). Animals may mitigate predation risk through a variety of antipredator behaviours (Lima & Dill, 1990), including avoiding high-risk areas, seeking refuge, decreasing movement and reducing foraging behaviours, all of which are most effective when predation risk is detected early (Pfeiffer, 1977). For aquatic organisms, for instance, chemicals present in the environment may provide information regarding imminent predation risk. It is, therefore, not surprising that risk-mediated alarm cues elicit dramatic and consistent antipredator responses in many aquatic species, ranging from coral to vertebrates (Ferrari *et al.*, 2010c).

Damage-released chemical cues, commonly referred to as alarm cues, are an effective warning system for aquatic organisms (Chivers & Smith, 1998). These cues can only be released when epidermal cells are damaged (Pfeiffer, 1977), making them a highly reliable indicator of risk. Unsurprisingly, these alarm cues elicit an innate antipredator response in nearby conspecifics. Although each species possesses its own alarm cues, individuals can sometimes respond to alarm cues from another species via one of two mechanisms: phylogenetic relatedness or sympatry. First, if the responders are phylogenetically closely related to the cue donor, then the alarm cue homology between the species will allow the responders to innately recognize the cues as risky; such heterospecific cues will typically elicit a weaker antipredator response than

[3] The content of this chapter is published in the following publication. For this publication, I designed and executed the experiment and wrote the first draft of the manuscript. I worked with MCO Ferrari to perform the analyses and all three authors contributed to the final draft. Changes have been made to avoid redundancy with other chapters and for consistency among chapters.

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would their own alarm cues (Mirza & Chivers, 2001b; Mitchell *et al.*, 2012). Second, two unrelated species may respond to one another's alarm cues if they are sympatric and share predator threats. Such responses to heterospecific alarm cues increase the likelihood of early predator detection and may be the result of learning (Chivers *et al.*, 2002; Pollock *et al.*, 2003; Pollock & Chivers, 2004).

Although some prey species innately recognize cues from certain predators (Göth, 2001), most prey fish do not (Brown, 2003). Instead, they must learn to recognize the sight or smell of such potential predators. Alarm cue learning is a widespread and efficient learning mechanism in aquatic species, known to occur in a variety of species, from flatworms to aquatic vertebrates (reviewed in Ferrari *et al.*, 2010a). After simultaneous exposure to a novel predator cue (sight, smell, or sound) and alarm cues, fish learn to recognize novel threats (Suboski, 1990), thus increasing their chances of survival (Mirza & Chivers, 2000; Lönnstedt *et al.*, 2012). This type of learning, called releaser-induced recognition learning, is a variant of classical Pavlovian conditioning (Suboski, 1990). Several studies have demonstrated successful learned predator recognition after a single conditioning event (Magurran, 1989; Mathis & Smith, 1993b; Chivers & Smith, 1994b).

Alarm cue learning has been shown to occur as early as the embryonic stage. For instance, wood frog embryos exposed to a novel predator odour in combination with alarm cues responded to predator odour when subsequently tested as tadpoles, demonstrating learning in the embryonic stage (Mathis *et al.*, 2008). Embryonic learning has also been demonstrated in convict cichlids (Nelson *et al.*, 2013). Atherton and McCormick (2015) also showed that alarm cue-mediated embryonic learning in cinnamon clownfish was accompanied by an increase in embryonic heart rates at the time of conditioning. The capacity to learn to recognize predators while in the embryonic stage may provide an advantage for new hatchlings living in high-risk environments.

In spite of abundant research focusing on the acquisition of novel information via learning, few studies have investigated the retention of such information. After a certain period of time, fish stop responding to their conditioned stimuli. This phenomenon, often referred to as adaptive forgetting (Bouton, 1994; Kraemer & Golding, 1997; Ferrari *et al.*, 2010a), is essential for an animal to maintain accurate responses in an environment where cues only predict environmental

conditions for short periods of time. Such "forgetting" has been used to describe situations where an individual does not demonstrate the response it has been conditioned to display. However, a lack of response does not necessarily indicate that the learned information is no longer available to, or retrievable by, the individual. Wood frogs, for instance, can adjust their behavioural response based on information to which they have ceased to respond (Chivers & Ferrari, 2013), demonstrating that even if the information is not necessarily acted on, it still exists in their memories. In this paper, we use the term "forgetting" to refer to the phenomenon whereby previously learned information is not actively used for subsequent decision-making, without making inference as to whether or not the information could be retrieved by the individual.

Little is known about the period for which individuals retain a response to their conditioned behaviours, in part because it varies so greatly depending on ecological context. Rainbow trout may cease to respond to conditioned predator odours in as little as eight days (Brown *et al.*, 2011b) but retain homing cues for more than a year and a half (Cooper *et al.*, 1976). Additionally, within ecological contexts, a number of factors may affect the longevity of such a memory, including the duration or intensity of learning. Ferrari *et al.* (2010a) propose a number of different factors, both environmental and intrinsic, which may affect how long an individual retains the information about a predator. Environmental factors, such as the presence and appetite of the predator, may reinforce learned predator information. Each encounter, dangerous or safe, adds to the available information about potential predators and increases its reliability for use in future threat assessment. For instance, with increased risk of predation, tadpoles and fish retained their learned predator recognition longer, whereas in cases where the tadpoles were less certain of the predator risk, they stopped responding to the predator cues after a shorter period of time (Ferrari *et al.*, 2010a,b). Intrinsic factors include a shift in the risk posed by a predator due to either changes in body size (growth) or habitat shifts. Rainbow trout maintained on a higher growth rate trajectory while they underwent conditioning retained the information for a shorter time than those growing more slowly (Brown *et al.*, 2011b). Physiological and behavioural changes that occur as the individual grows may also increase the likelihood of successful escape from predators. As a result of a decreased growth rate, adult prey may maintain a relatively constant community of predators and may, consequently, benefit from maintaining their responses to predation threats for longer than embryos or juveniles whose predator risk is changing more rapidly.

Another hypothesis is that the longevity of the memory is affected by the ontogeny of the learner. Sensitive periods in development have been observed in many species (Westeberhard, 1989; Travis, 1994; Panchanathan & Frankenhuis, 2016; Arnett & Kinnison, 2017). Sensitive periods refer to periods of development which are particularly susceptible, or plastic, to environmental or experiential effects. These periods of plasticity allow organisms to change or adapt more rapidly than they might at other times. Such adaptations may be physical, such as the alteration of morphological traits to reduce predator damage (Januszkiewicz & Robinson, 2007), or behavioural, such as adaptive antipredator behaviours (Dill, 1983; Robinson, Januszkiewicz, & Koblitz, 2008). The extent to which an individual can learn and retain information may, thus, be impacted by these sensitive periods. A prime example of a sensitive period of learning is observed in salmonids. During a sensitive period early in their lives, these fish imprint on their home streams so that they may travel great distances and ultimately return to their natal sites to spawn (Cooper *et al.*, 1976; Dittman *et al.*, 1996). Streams and lakes visited later in life do not make this same indelible mark as those visited during the sensitive period.

The goal of this study was two-fold, with each goal tested in a separate experiment. First, we investigated whether rainbow trout are capable of embryonic learning from either conspecific or heterospecific alarm cues and tested the duration of information retention. Second, we investigated whether learning at different life stages, specifically as an embryo, newly hatched larvae, or free-swimming juveniles, would lead to different retention of learned information.

4.2 Methods

4.2.1 Holding conditions

Fertilized rainbow trout eggs were purchased from Troutlodge Inc., Sumner, WA. Embryos take approximately a month to hatch at a temperature of 10ºC and were shipped to us three weeks postfertilization. Embryos were held in groups of approximately 60 in 140 hatching tanks equipped with an airstone and filled by flow-through dechlorinated water (0.5 L / min, 10ºC). After hatching, the trout alevins remain as larvae for a few weeks, during which time they continue to rely on an external yolk sac for nutrition. At this time, they were transferred into mosquito netting baskets in four large flow-through tanks (500-L, flow rate 4 L / min), with airstones and Hagen Aquaclear water filters with charcoal, Biomax, and foam inserts. The filters

were cleaned daily and replaced as needed. Temperatures in the holding tanks varied seasonally between 8 and 16°C, but all experimental tanks were the same temperatures at the same time. The light:dark cycle was 16:8 h. After two to three weeks, the yolk sacs were absorbed. The now-juvenile trout began to swim up to feed and were ready for behavioural testing. The trout were fed floating commercial trout food six times daily.

4.2.2 Collection of cues

Ten six-month-old juvenile rainbow trout (fork length: mean ± SE: 19.3 cm ± 0.5) and four eight-month-old brook char (fork length: 23.6 cm ± 0.6) were used to collect conspecific (CAC) and heterospecific (HAC) alarm cues, respectively. The fish used to procure cues were approximately four times the length of the fish subjected to behavioural testing in the first part of Experiment 1 and in Experiment 2 and about 30% longer than the five to six-month-old trout tested in the second half of Experiment 1, but they still had visual the markings characteristic of juveniles (oval spots along their sides). Trout were euthanized with a single, lethal blow to the head following approved institutional ethics protocols (20070083, as per guidelines from the Canadian Council on Animal Care) and a thin layer of skin was removed from each side of their body and measured. Skin fillets were placed in chilled water and homogenized using a Polytron homogenizer (Brinkmann Instruments, Mississauga, ON). The solution was filtered through cotton batting to remove the remaining tissue. A concentrated solution of 0.1 cm^2 of skin / mL water was stored frozen at −20ºC in 30-mL aliquots. Prior to use, the alarm cue aliquots were thawed overnight in the dark at temperature (approximately 14ºC).

Predator odour (PO) was collected from three northern pike (19.1 cm ± 0.2). The pike were held in 60-L glass tanks (Hagen, Montreal, QC) apart from other fish for a full week prior to odour collection and were not fed during this time to avoid any chemical cues that might result from the consumption of related prey species (Mirza & Chivers, 2001a). Each pike was then placed individually in a tank containing 60 L of clean, dechlorinated water, with an air stone but no filtration system. They remained in these tanks for 24 h with no food, after which they were returned to their original holding tanks. The water in which they were kept for 24 h was filtered through cotton batting and then frozen at −20ºC in 125-mL aliquots until needed, at which point it was allowed to thaw in the dark overnight at ambient temperature (approximately 14ºC).

4.2.3 Experiment 1: Embryonic learning and memory of predator information

4.2.3.1 Experimental outline

Trout embryos were taught (or not) to recognize the odour of a novel predator, a northern pike, as a threat, using conspecific or heterospecific alarm cues, and their antipredator response to pike odour was recorded after two–three months. To further investigate the potential memory window of the fish, only trout taught with conspecific alarm cues were tested for their response to pike odour after five – six months.

4.2.3.2 Conditioning phase

Trout embryos were each conditioned once a day on 3, 4, 5, and 6 May (four conditioning events each) and began to hatch on 7 May 2013 (approximately 31 days post-fertilization at 10°C). Prior to conditioning, the trout embryos were placed in groups of approximately 60 into 450-mL plastic cups filled with 300 mL of dechlorinated water. After an hour of acclimation, one of five different conditioning treatments was applied to each cup: 1) a water control (DW + DW) consisting of 11 mL distilled water to control for the disturbance associated with cue injection; 2) a predator odour control (DW + PO) consisting of 1 mL of distilled water and 10 mL predator odour to control for potential sensitization or habituation arising from multiple exposures to predator odour; 3) an alarm cue control (CAC + DW) consisting of 1 mL conspecific alarm cue control and 10 mL of distilled water to control for behavioural biases arising from embryos being exposed to risk stimuli during early ontogeny; 4) the conspecific alarm cue conditioning (CAC + PO) consisting of 1 mL conspecific alarm cue and 10 mL predator odour; and finally, 5) the heterospecific alarm cue conditioning (HAC + PO) consisting of 1 mL heterospecific alarm cue and 10 mL predator odour. There were 36 replicates (cups) of the DW + DW control and 27 replicates of each of the other treatments. The cues were injected gently into each cup to minimize disturbance. The final concentration of skin was 1 cm^2 / 3 L, a concentration known to elicit an overt antipredator response in this species, even early life stages (Mirza & Chivers, 2003; Horn & Chivers, 2017, Chapter 2). After one h, each egg cup underwent two complete water changes, whereby the conditioning treatment and water were drained and the cup was refilled with fresh water (twice), and then the eggs were returned to their respective hatching tanks.

4.2.3.3 Testing phase

Fish from all five treatments were tested for their responses to water or predator odour alone 33 – 67 days (two to three months) after receiving their last conditioning treatment (fork length: 45 mm ± 0.7). Based on the results we obtained, we decided to further investigate the duration of the memory window by testing fish from the CAC + PO group between five and six months after their last conditioning treatment (14.1 cm ± 0.17).

Behavioural assays followed established methodology (Chivers & Smith, 1998), consisting of observing the behaviour of the fish for five min prior to and five min after the injection of stimuli into the test arena. The smaller juveniles were tested in round plastic containers (745-mL, no name brand, Loblaws, Brampton, ON) filled with 600 mL of dechlorinated water and visually divided into quarters by external perpendicular lines. Larger juveniles were tested in 37-L glass tanks (Hagen) partly filled with 25 L of dechlorinated water and divided by a three-by-four grid (8.9 × 12.4 cm quadrats) on the front of the tank. Each large tank was also equipped with a semicircular shelter cut from PVC piping and an airstone to provide aeration. Unfortunately, airstones created too much vibration for the small test bowls and were, thus, not used; time in the test bowls was, therefore, limited to a maximum of 2 h. The outer sides of each arena were darkened by heavy black plastic to minimize visual disturbance. All test arenas were equipped with airline tubing attached to the side of the arena, which facilitated the injection of stimuli into the tanks while minimizing disturbance of the fish. Observations were recorded from above the small arenas and from the side of the larger ones.

Typical antipredator behaviours in fish include decreases in movement and foraging behaviour and increases in shelter use. We recorded movement as number of lines crossed using the grids in the testing arenas. We considered a line to be crossed when 3/4 of a fish's body crossed a dividing line. Foraging was quantified by counting the number of feeding bites or strikes an individual attempted during the test period. Foraging behaviour and time spent using shelter (in seconds) were only evaluated with the larger individuals due to differences in behaviour between the two ontogenetic groups.

Trout were placed in their individual testing tanks and left undisturbed for at least an hour prior to testing. Before each trial, either 30 or 60 mL of water (depending on arena size, see

below) was drawn from each tank and set aside to be used at a later time to flush the stimuli into the testing arenas. Thirty seconds prior to the prestimulus observations, a small quantity of food (1 mL of live Artemia for the small and fifteen pellets of food for the large: 1.5 mm FinFish slow sinking pellets (Zeigler, Gardners, PA)) was added. Behaviours were recorded for five min. A second allotment of the same quantity of food was added immediately prior to the injection of the test stimulus. The test stimulus (distilled water or predator odour) was injected slowly into the tanks via blue airline tubing, which was then completely flushed into the arena with the previously reserved tank water. For small arenas, we injected 20 mL of stimulus followed by a 30-mL flush, whereas 30 mL of stimulus with a 60-mL flush was used for the larger tanks. Given the time elapsed between conditioning and testing of the older juveniles, we needed a positive control to ensure that the fish were capable of showing significant antipredator response, in case they failed to respond to the other cues. As a positive control, we exposed some of them to a third test stimulus: a CAC solution (7.5 mL of CAC mixed with 22.5 mL of distilled water). Behaviours were recorded for another five min poststimulus injection.

4.2.4 Experiment 2: The role of ontogeny in the learning and retention of predator-related information

4.2.4.1 Experimental outline

Trout were conditioned to recognize a novel predatory threat at one of three ontogenetic stages: as embryos, newly hatched alevins, or free-swimming larvae and were tested at a later date for their response to predator odour.

4.2.4.2 Conditioning phase

Trout embryos were each conditioned once a day on 11, 12, 13, and 14 January 2014 approximately three weeks postfertilization, and began to hatch on 24 January. The newly hatched alevins were conditioned approximately one week after hatching on 1, 2, 3, and 4 February, while still in the low-mobility stage. Free-swimming larvae were treated on 22, 23, 24, and 25 February after their yolk sacs were completely resorbed and they were fully mobile (approximately three weeks posthatch). Conditioning protocol followed that used in Experiment 1 with the difference that the fish received only three types of conditioning treatments: DW + DW, CAC + PO, and HAC + PO.

4.2.4.3 Testing phase

Trout were tested 28 – 155 days (1 – 5 months) after their last conditioning treatment (fork length: 46 mm ± 1.1). Fish were tested in the small arenas using the same protocol outlined above for Experiment 1, but testing was limited to PO to decrease the complexity of the analysis as the capacity for learning was established in Experiment 1.

4.2.5 Statistical analyses

Prestimulus and poststimulus behavioural data (numbers of line crosses, feeding strikes, and time spent unsheltered, as applicable) were computed into proportional change in behaviour from the prestimulus baseline ((poststimulus – prestimulus) / prestimulus). Proportional change in behaviour was used as a response variable in subsequent analyses. Prestimulus activity, foraging, and shelter, along with fish length (fork length in mm), were also analyzed to ensure no baseline biases between experimental groups. Any fish that crossed fewer than 30 or more than 200 lines remained completely stationary for longer than two min, or failed to forage at all during the prestimulus test, and any fish that increased their movement by more than 100% poststimulus was eliminated from analysis to avoid the use of fish with particularly aberrant behaviour that might signify an external disturbance. Removing particularly inactive or active fish may bias the results by excluding the extremes inherent to any population of individuals; however, including fish who are already frightened would create a greater bias by obfuscating any potential reactions with an altered baseline. The remaining fish provided groups of 14 – 80 for each sample subset, depending on the experiment.

4.2.5.1 Experiment 1

For the small juveniles, a two-way general linear model (GLM) was used to test the effect of conditioning treatments (DW + DW, DW + PO, CAC + DW, CAC + PO, and HAC + PO) and testing cues (DW vs PO) on the line crosses of the fish, both for prestimulus data and proportional change data (n = 14 – 15 per subset). For the large juveniles, one-way multivariate GLM was performed to evaluate the effect of testing cues (DW, PO, or CAC) on prestimulus and proportional change data for line crosses, foraging, and time spent unsheltered (n = 16). To use the most conservative measure, we reported p-values for Pillai's Trace (Olson, 1976). Shapiro–Wilk's test was used to assess normality and Levene's test was performed to assess for heteroscedasticity. Where significant interactions were observed, data was split to further

investigate the nature of the interaction. Where necessary, non-normal data were rank transformed before analysis was performed. Although rank transformation removes the magnitude information from the data, it has the benefit of still allowing for a complex analysis.

4.2.5.2 Experiment 2

Length, prestimulus, and proportional change in activity levels were each assessed using a univariate GLM testing the effects of ontogenetic stage at conditioning (embryos, newly hatched, and free-swimming larvae), conditioning (DW + DW, CAC + PO, and HAC + PO) and testing latency (number of days since conditioning, included as a continuous variable) tested with PO. Outliers were assessed using a Cooks Distance (4 / n). From an initial 671 individuals, 27 outliers were identified, leaving a total of 644 individuals for assessment (n = 30 – 80 per subset). Normality and homoscedasticity were visually assessed using Q-plots and residual plots. Where significant interactions were assessed by the GLM, subsequent analyses were performed by splitting the data to further investigate the nature of the interaction. All analyses were performed using SPSS Statistical Software [Version 17.0] (IBM).

4.3 Results

4.3.1 Experiment 1

Fish tested at one–two months of age did not differ in length or baseline activity among conditioning treatments or testing cues (length: treatment: $F_{4,148} = 0.587$, $p = 0.672$, cue: $F_{1,148} = 0.827$, $p = 0.365$, treatment × cue: $F_{4,148} = 0.035$, $p > 0.99$; prestimulus: treatment: $F_{4,148} = 1.48$, $p = 0.211$, cue: $F_{1,148} = 0.129$, $p = 0.720$, treatment × cue: $F_{4,148} = 1.22$, $p = 0.307$). When exposed to the testing cues, their change in activity depended on both the conditioning treatment they received and the testing cue to which they were exposed (treatment × cue: $F_{4,148} = 6.19$, $p < 0.001$). Fish in the three control groups did not respond differently to testing with water versus testing with predator odour (cue: DW + DW: $F_{1,29} = 1.38$, $p = 0.25$; DW + PO: $F_{1,29} = 0.29$, $p = 0.60$; CAC + DW: $F_{1,29} = 0.17$, $p = 0.68$), indicating that they failed to learn the pike as a threat. However, both alarm cue learning groups showed a significant antipredator response to the predator odour as compared with water (cue: CAC + PO: $F_{1,28} = 39.81$, $p < 0.001$; HAC + PO: $F_{1,29} = 25.55$, $p < 0.001$, Figure 4.1).

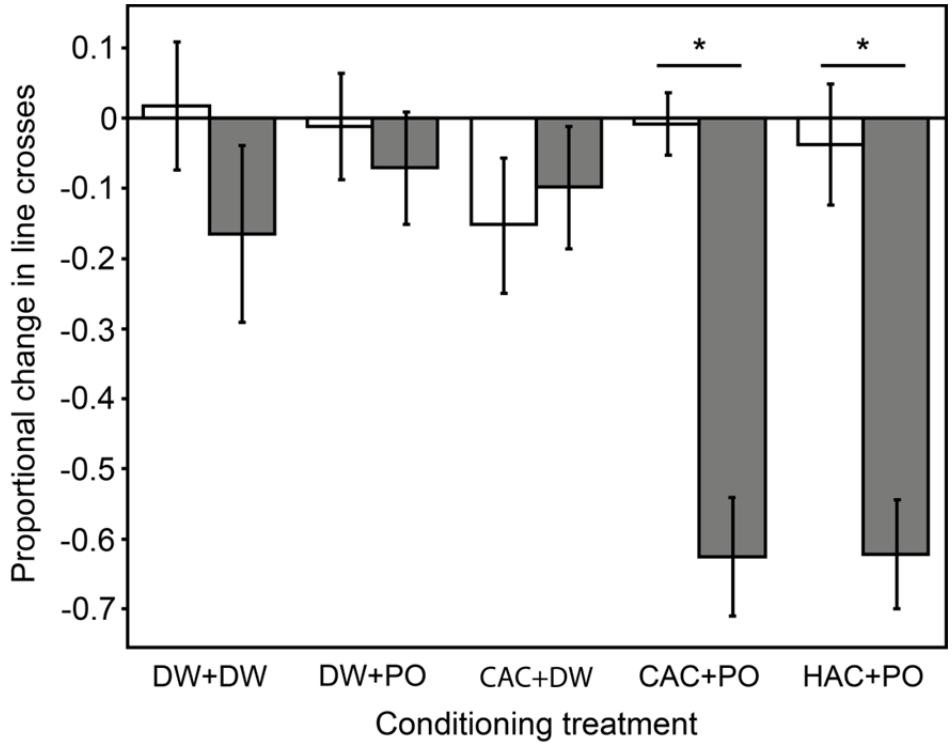

Figure 4.1. Mean (±SE) proportional change in movement (line crosses) for one to two-month-old trout exposed to water (open bars) or predator odour (grey bars). The fish received one of five conditioning treatments as embryos: water only (DW + DW), predator odour only (DW + PO), conspecific alarm cues only (CAC + DW), conspecific alarm cues paired with predator odour (CAC + PO), or heterospecific alarm cues paired with predator odour (HAC + PO).

Embryos conditioned with alarm cue and predator odour (CAC + PO) and tested after five to six months did not differ in length ($F_{2,25} = 0.707$, $p = 0.503$) or baseline activity level (MANOVA: Pillai's Trace: $F_{6,88} = 0.823$, $p = 0.555$), indicating no pre-existing bias among the groups. Fish altered their behaviour depending on the testing cue (DW, PO, or AC) they received (MANOVA: Pillai's Trace: $F_{6,88} = 5.943$, $p < 0.001$). All types of activity were affected by testing cue (line crosses: $F_{2,47} = 13.72$, $p < 0.001$; bites: $F_{2,47} = 10.07$, $p < 0.001$; time unsheltered: $F_{2,47} = 3.337$, $p = 0.045$; Figure 4.2). Juveniles displayed an antipredator response to alarm cues compared with water control and predator odour based on their movement and foraging activity (line crosses, bites: all $p \leq 0.001$), with no difference between predator odour and water (line crosses: $p = 0.465$; bites: $p = 0.669$, time unsheltered: $p = 0.167$), indicating that the fish failed to respond to pike odour as a threat. In terms of time unsheltered, the fish responded more to alarm cue than predator odour ($p = 0.013$) but not to water ($p = 0.246$).

4.3.2 Experiment 2

Neither length nor prestimulus activity was affected by interactions between testing latency (day), ontogenetic stage, and conditioning treatment (length: $F_{4,537} = 2.21$, $p = 0.067$; prestimulus activity: $F_{4,643} = 0.72$, $p = 0.58$), but both were affected by two-way interactions involving day (length: stage × day: $F_{2,537} = 3.42$, $p = 0.034$, conditioning × day: $F_{2,537} = 2.21$, $p = 0.067$, stage × conditioning: $F_{2,537} = 2.21$, $p = 0.067$; prestimulus activity: stage × day: $F_{2,643} = 4.48$, $p = 0.012$, conditioning × day: $F_{2,643} = 3.74$, $p = 0.024$, stage × conditioning: $F_{2,643} = 1.00$, $p = 0.406$). The effects of day on length and prestimulus activity are expected; as the fish grow over time, their size and baseline activity levels increase. Our use of proportional change in behaviour for analysis should minimize bias from this unavoidable consequence of our experimental design.

The proportional change in activity of the fish was affected by an interaction between conditioning treatment and ontogenetic stage at conditioning ($F_{4,643} = 2.47$, $p = 0.044$) and conditioning treatment and testing latency ($F_{2,643} = 9.48$, $p < 0.001$) and a near significant three-way interaction among the three factors ($F_{4,643} = 2.36$, $p = 0.052$). Individuals conditioned with DW + DW were not affected by ontogeny or testing latency ($F_{2,224} = 2.38$, $p = 0.095$). Conditioning with CAC + PO resulted in an interaction between ontogeny and day ($F_{2,233} = 3.98$, $p < 0.001$; Figure 4.3), indicating that individuals conditioned at different stages retained the

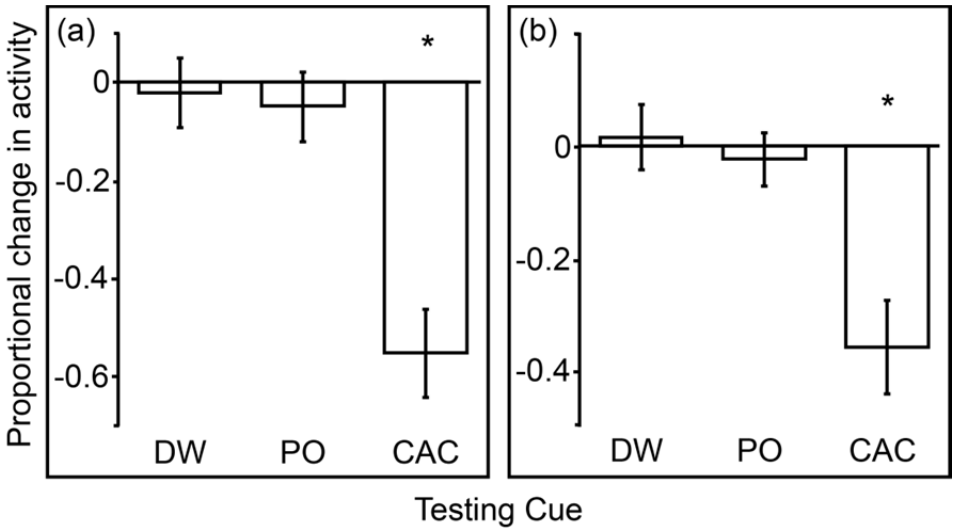

Figure 4.2. Mean (±SE) proportional change in (a) line crosses and (b) foraging bites in response to testing with a water control (DW), predator odour (PO), or alarm cue (CAC) five months after predator recognition conditioning (CAC + PO) of embryos.

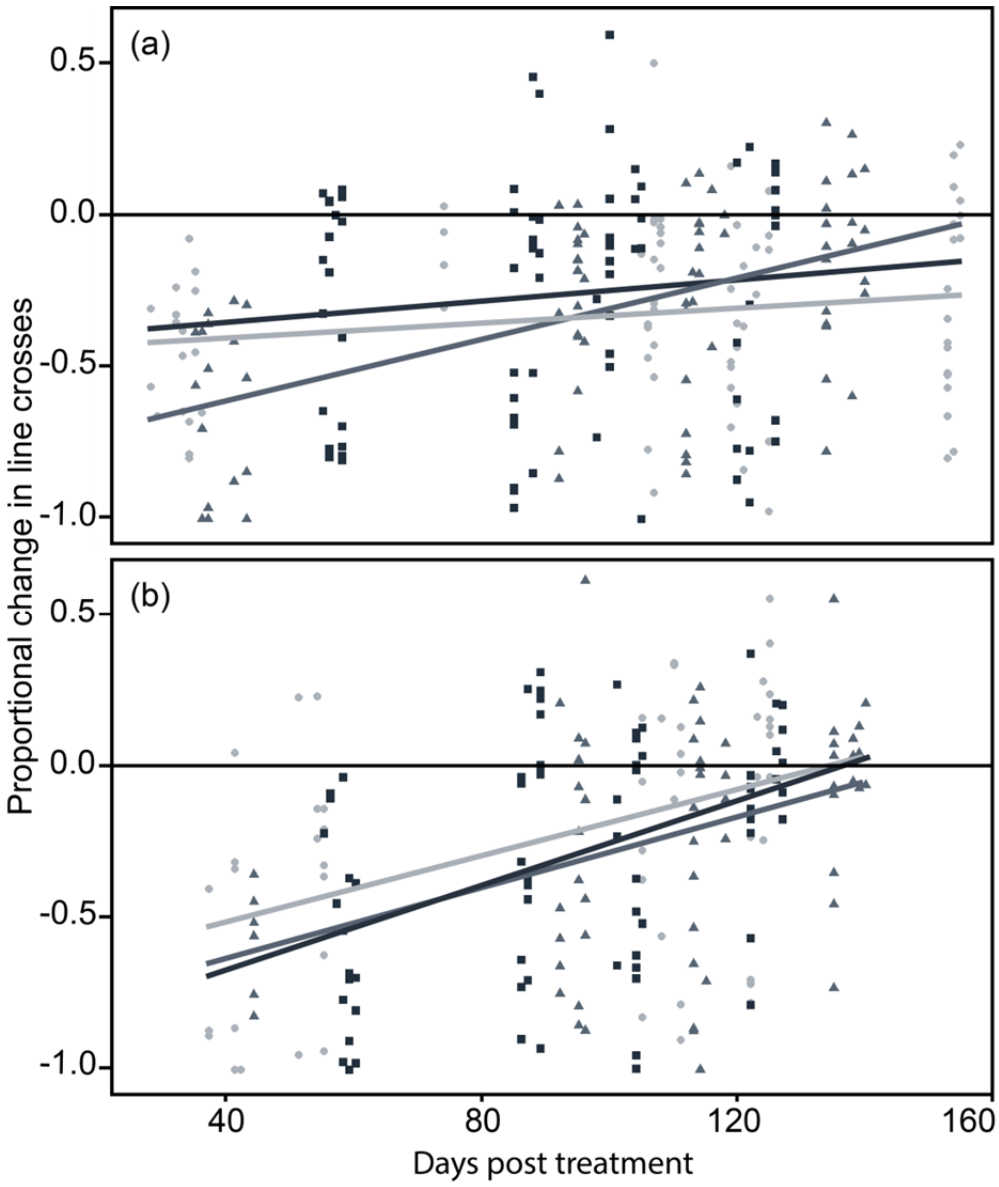

Figure 4.3. Proportional change in movement (line crosses) in response to testing with predator odour over time after conditioning treatment with either (a) conspecific alarm cue and predator odour (CAC + PO) or (b) heterospecific alarm cue and predator odour (HAC + PO) at one of three life stages: embryo (circle), newly hatched (triangle), or free swimming (square).

information for different amounts of time. Specifically, embryos and free swimmers did not differ in their rate of forgetting (stage x day: $F_{1,156} = 0.063$, $p = 0.80$). However, newly hatched individuals displayed a steeper decline of response over time as compared with embryos (stage x day: $F_{1,156} = 9.71$, $p = 0.002$) but not significantly when compared with free swimmers (stage x day: $F_{1,153} = 2.81$, $p = 0.096$). Individuals conditioned with heterospecific cues were not affected by the interaction between ontogeny and testing latency ($F_{2,184} = 0.17$, $p = 0.84$) but were affected by testing latency ($F_{1,184} = 37.86$, $p < 0.001$), indicating that all individuals, regardless of ontogeny, show a similar rate of forgetting (Figure 4.3). For clarity, only p-values of interest have been reported in this section; the remainder can be found in Table 4.1.

4.4 Discussion

Embryonic rainbow trout exposed to a combination of either conspecific or heterospecific alarm cues and predator odour showed a significant post-hatch antipredator response to pike odour, indicating that they successfully learned to recognize a novel predator odour as a threat while in the egg. To the best of our knowledge, this study is the first to demonstrate embryonic learning of predator recognition in salmonids and to show that such learning can occur via heterospecific alarm cues. This result concurs with previous studies in amphibians demonstrating highly sophisticated learning abilities in embryos, including the ability to learn the time of day at which a predator poses the greatest danger or the ability to match intensity of response to the risk level posed by the predator (Ferrari & Chivers, 2010).

Unlike the only two previous experiments looking at embryonic learning of predator recognition in fish (Nelson *et al.*, 2013; Atherton & McCormick, 2015), we used alarm cues from significantly older juveniles (six- to eight-month-old) rather than larval cues. This is of interest because learned responses may be stronger when conditioned with cues from conspecifics that are from a more similar ontogenetic group (Mirza & Chivers, 2002; Lönnstedt & McCormick, 2011; Mitchell & McCormick, 2013). The capacity of embryonic fish to learn predators using alarm cues from fish from significantly different ontogenetic groups (and thus representing a very different prey guild) is also novel. The adaptive value of this depends on the closeness of the fish to which it responds. In this case, the rainbow trout responded to fish that were larger but in the same life stage and, thus, probably from a similar prey guild. They also responded to brook char, a visually similar fish of comparable size, with similar habitats, so the

Table 4.1. Statistical table of the GLM performed on the proportional change in activity over time, by ontogenetic stage and conditioning cue. Significant values in bold.

Factors	F	df	p-value
Overall GLM			
conditioning * ontogeny * date	2.36	4, 643	0.052
conditioning * ontogeny	2.47	4, 643	**0.044**
conditioning * date	9.48	2, 643	**<0.001**
ontogeny * date	1.44	2, 643	0.24
Conditioning	11.39	2, 643	**<0.001**
Ontogeny	1.25	2, 643	0.29
Date	39.03	1, 643	**<0.001**
Split by Conditioning: DW			
ontogeny * date	2.38	2, 224	0.095
Ontogeny	2.21	2, 224	0.12
Date	0.30	1, 224	0.59
Split by Conditioning: CAC+PO			
ontogeny * date	3.98	2, 233	**0.020**
Ontogeny	3.64	2, 233	**0.028**
Date	15.50	1, 233	**<0.001**
Split by Conditioning: HAC+PO			
ontogeny * date	0.17	2, 184	0.84
Ontogeny	0.39	2, 184	0.68
Date	37.86	1, 184	**<0.001**
Conditioning by CAC+PO: Paired stages			
Embryo vs new hatch	9.71	1, 156	**0.002**
Embryo vs free swimming	0.063	1, 156	0.80
New hatch vs free swimming	2.81	1, 153	0.096

response is adaptive. However, if this response also occurs with less similar species, it may prove detrimental by causing a fish to miss out on important foraging opportunities when a different prey is at risk.

In terms of information retention, our study indicates that embryos that learned to recognize pike using conspecific alarm cues displayed an overt antipredator response to pike odour two to three months after training, but the antipredator response is absent after five months. The significant response to alarm cues by this age class confirmed the ability of fish to display an antipredator response in a perceived risky situation. Adaptive forgetting is essential to allow prey to stop responding to former predators that are no longer a threat, as individuals grow and move through different prey guilds. Prey that maintain responses to predators that they have outgrown may miss out on important foraging and mating opportunities.

Our second experiment provided valuable insights into the effects of ontogeny on the retention of learned predator recognition. Regardless of their ontogenetic stage at conditioning, all fish taught to recognize the predator with either conspecific or heterospecific alarm cues displayed strong antipredator responses to pike odour when tested five weeks post-conditioning. The intensity of this response decreased over time, a trajectory leading to extinction of the learned response after four to six months. For conspecific alarm cue learning, the rate of forgetting was slower when the information was learned as embryos as compared with the newly hatched alevins, with free swimmers displaying an intermediate rate, leading to a fluctuation in the predicted retention times depending on age at learning. In contrast, the antipredator response intensity decreased evenly in all age groups that learned from heterospecific alarm cues.

Fish may have a period of cognitive sensitivity early in their development, as they are taking in vast quantities of crucial information about their habitat, their kin, their food, and their predators—a sort of predetermined period of sensitivity built into their development. Ferrari, Horn and Chivers (2019) found that tadpoles that learned predator recognition as embryos retained it longer than conspecifics that learned as larvae (see Chapter 6). The article further proposes that, although young organisms generally have greater cognitive plasticity than older ones, embryonic learners may suffer from “cognitive resonance,” a phenomenon by which information learned during certain stages of development have a relatively augmented impact on

later life as compared with similar information learned later on. Specifically, information learned during these periods of cognitive resonance is better conserved, for better or for worse, than similar information gathered at different ontogenetic stages. Similarly, in Experiment 2, we also observed a longer retention in embryonic learners as compared with the newly hatched. However, by adding a third early ontogenetic stage, we observed a complexity than might not be entirely explained by a single period of sensitivity. Intrinsic factors such as growth rate, vulnerability to predation, hormone levels, and rate of developmental change are all greatly affected by ontogeny (Sibly *et al.*, 2015) and may all have an effect on learning and memory. Although multiple periods of sensitivity are possible, we instead propose that relevant extrinsic and intrinsic factors modulate periods of sensitivity creating a complex landscape of learning and retention.

Embryos and newly hatched alevins are both limited in their mobility. Embryos cannot move at all and, although physically capable of some movement, alevins have tiny fragile bodies, encumbered by large egg sacs and scant muscles. Neither stage has much hope of dashing into a hiding spot or out of the reach of a predator, whereas free swimmers, slender and muscular, do. Low-mobility, high-vulnerability prey may put a higher premium on predator information and, thus, retain it for a longer time, as they are at greater overall risk for predation. A parallel trend is represented by growth rate. Brown *et al.* (2011b) found that faster-growing trout extinguished their learned predator recognition more rapidly, which is logical as these fish outgrow their predators more rapidly. If growth rate alone were the predictor, we would expect that embryos would retain the information longest, followed by alevins, and then free swimmers, as alevins grow faster than their egg-bound embryonic counterparts but more slowly than the free-swimming conspecifics who are able to feed rather than relying entirely on their yolk sacs for nutrition (Alami-Durante *et al.*, 2014). Because the free swimmers retained the information longer than the alevins, growth rate alone cannot explain the trend we observed. Although this trend may justify why embryos might retain predator information better than newly hatched counterparts, it does not explain the lack of significant difference between free swimmers and embryonic learners.

Other factors might bias free swimmers to retain information better than embryos. Specifically, the rate of growth of the nervous system in the early days of a larval fish is inverse

to that of their general mass, such that their brains grow fastest immediately post hatch and the development slows over time (Alami-Durante, 1990). This high rate of neural development can most likely be projected back into the embryonic stage as well. During periods of rapid brain growth and development, there is often a high level of plasticity, such that new information may more easily replace older information (Hattori & Wasterlain, 1990). Additionally, a more fully developed brain may have a greater physical capacity to retain information than a less developed brain, as the structures involved are more complete and less neural restructuring is occurring (Akers *et al.*, 2014).

Perhaps the pattern we observed is a consequence of a nexus of these opposing trends. Although the embryos have a lower growth rate and higher vulnerability, which induce greater retention of information, and the free swimmers have a slower neural development to reduce the extinction of memory, alevins are less influenced by these factors. The net result may be that, by not retaining the information as well as the embryos and possibly by extinguishing it more rapidly than the free swimmers, the intensity of newly hatched learners' reactions decrease more quickly.

Another factor which may have played a less predictable role in the retention is the semipermeable membrane surrounding the trout eggs (Gray, 1932; Groot & Alderdice, 1985). Although our first experiment demonstrates that alarm cues and predator odour can permeate the membrane, we cannot predict from this experiment the degree to which this membrane impacted the complete profile or the relative quantities of the chemicals that diffused through. It is possible that the concentration of alarm cues permeating the embryo was significantly lower than that surrounding the alevins or free swimmers, though that would have the opposite effect of what we observed, decreasing retention rather than increasing it. Alternatively, the cues might have gotten trapped in the membrane of the embryo, causing a much longer exposure period, potentially increasing the duration of their learned response. Unfortunately, without a greater understanding of the extremely complex chemistry of alarm cues and without related testing of membranes, it is impossible to predict what role embryonic membranes might play in the relative retention of cue-learned information.

Interestingly, the differentiated retention discussed above occurred only in individuals that learned from conspecific cues. Perhaps, heterospecific alarm cues carried some uncertainty as to their relevance to the juveniles, thus giving a lower value to the learned information at the time of conditioning. Mitchell *et al.* (2012) showed fish respond most strongly to cues from the most closely phylogenetically related donors. Information from a closely related yet distinct species may be considered less relevant and may, therefore, elicit a weaker antipredator response. The lower relevance of heterospecific alarm cues may have prevented the increased retention observed in the embryonic trout that learned from conspecific cues.

For any potential prey species, the ability to recognize predators is essential to survival. If an organism is able to learn to recognize its predators while it is still an embryo, it will have an immediate advantage over others who do not have that ability. However, to retain that information indefinitely would be detrimental as it would remove opportunities for growth and reproduction. Here, we have demonstrated that embryonic trout can not only learn from conspecifics, but also heterospecifics, and that they cease to respond as the information loses value. The information retention varies depending on ontogeny at the time of learning when fish learn from conspecific alarm cues, most likely as a result of a variety growth and vulnerability factors that impact a period of sensitivity, or cognitive resonance, in the immature trout.

CHAPTER 5: Threat-sensitive learned predator recognition in embryonic minnows[4]

5.1 Introduction

Most animals spend much of their lives under threat of predation. They must constantly assess danger to evaluate optimal timing for foraging and mating or, conversely, for restricting activity in an effort to mitigate risk of death or injury (Pfeiffer, 1977; Lima & Dill, 1990; Sih *et al.*, 2000). Fish do not typically respond innately to their predators (Brown, 2003), but do demonstrate an innate fright response to chemical alarm cues released by physical damage to their conspecifics (Chivers & Smith, 1998; Wisenden *et al.*, 2004b). Such cues may present in the water at an injury event or may present later as dietary cues after passing through the digestive system of a predator (Mathis & Smith, 1993b; Ferrari *et al.*, 2007a; McCormick *et al.*, 2019). Like many other aquatic organisms, fish learn to identify potential predators when they detect alarm cues paired with sight or smell of novel predators (Suboski, 1990; Ferrari *et al.*, 2010c). After even a single pairing (Magurran, 1989; Mathis & Smith, 1993b; Chivers & Smith, 1994b), fish may identify newly-learned predator odour and be able to respond early, thus maximizing their chances of escape (Mirza & Chivers, 2000; Lönnstedt *et al.*, 2012). For example, fathead minnows do not innately respond to pike but can learn to recognize them as predators through exposure to pike concurrent with either injury-released alarm cues or dietary cues (Mathis & Smith, 1993b; Chivers & Smith, 1994b, 1995a). In one study, Brown *et al.* (1997) demonstrated that a population of 78 000 minnows in a four ha pond could learn the odour of pike within two to four days of introduction of the pike.

Learned antipredator responses are often highly sophisticated. Organisms may gather more information than simply which novel predator was present when they detect alarm cues. They may learn the time of day, the habitat characteristics present or the intensity of the alarm cue they

[4] The content of this chapter has been submitted to Animal Behaviour and is currently under review. For this publication, I designed and executed the experiment, did the analyses and wrote the first draft of the manuscript. DP Chivers contributed to the final draft. Changes have been made to avoid redundancy and for consistency among chapters.

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encountered (Chivers & Smith, 1995b; Sih *et al.*, 2000; Ferrari & Chivers, 2009c). This contextual information can allow them to make more accurate inferences about the degree of threat involved when they next encounter the same predator (Helfman, 1989; Lima & Dill, 1990; Brown *et al.*, 2011a). For instance, fathead minnows are capable of discerning the proximity, density and even size of the predators based on olfactory information (Kusch *et al.*, 2004; Ferrari *et al.*, 2006).

The intensity or concentration of alarm cues provides prey with highly valuable information, and many aquatic organisms are capable of detecting variance in the concentrations of cues (reviewed in Brown *et al.*, 2011a). A high concentration of alarm cues is typically representative of a high degree of physical damage, for example if a fish were being injured close by, or if multiple individuals were injured simultaneously. A higher risk situation typically requires a more drastic defense and fish are capable of adjusting the intensity of their antipredator responses accordingly. Such a graded response to different intensities of alarm cues is referred to as threat sensitivity (Helfman, 1989). The intensity of the threat can also be correlated to a novel predator odour in learning events, such that an individual exposed to a high concentration of alarm cues in combination with predator odour will learn to associate that predator with a higher threat than one exposed to a low concentration of alarm cues associated with predator odour. Indeed, threat-sensitive learning has been documented in fish (Ferrari *et al.*, 2005; Zhao *et al.*, 2006), amphibians (Ferrari & Chivers, 2009c) and even insects (Kesavaraju *et al.*, 2007; Ferrari *et al.*, 2008a).

Organisms may also use background information to make generalizations about the baseline level of risk in their environments. For instance, organisms which are continually exposed to risk (for example in the form of alarm cues) may learn to approach all novel stimuli with caution, a phenomenon known as neophobia. Neophobia has been observed across a wide range of taxa, including birds, mammals, fishes and amphibians (reviewed in Crane & Ferrari, 2017a; Crane *et al.*, 2020). Brown *et al.* (2013b) compared the responses of guppies from high- and low-risk environments to novel odours and found that those individuals from high-risk environments showed a dramatically different response to a novel odour than those from low-risk environments. They further showed that neophobia can be induced by repeated exposure to

conspecific alarm cues. Likewise, Ferrari, Brown and Chivers (2015) demonstrated that repeated exposure of embryos to alarm cues induced neophobia in tadpoles.

Various aquatic organisms are capable of responding to risk prior to hatching (Chivers *et al.*, 2001; Mathis *et al.*, 2008; Atherton & McCormick, 2015). Though these organisms, still captive in their membranes, have little recourse by way of antipredator behaviours, they are aware from the presence of alarm cues that there is danger, as evidenced by elevated heart rates at the time of exposure (Atherton & McCormick, 2015) and early hatching responses (Chivers *et al.*, 2001; Mirza *et al.*, 2001; Kusch & Chivers, 2004). In addition to recognizing the presence of a threat while in the embryo, some species are able to learn from these exposures. Embryonic amphibians and fishes (wood frogs, cichlids, damselfish and trout) exposed concurrently to alarm cues and predator odour have been shown to subsequently provide a fright response to the predator odour alone, demonstrating a capacity for learned predator recognition by embryos in amphibians and fish (Mathis *et al.*, 2008; Nelson *et al.*, 2013; Atherton & McCormick, 2015; Horn *et al.*, 2019, Chapter 4). In fact, some species demonstrate great complexity in the information acquired as an embryo. Wood frog embryos, for example, retain both temporal and threat-intensity information from embryonic events. Tadpoles demonstrate a stronger response to predators when they are conditioned with higher concentrations of alarm cues. They also respond more strongly to learned predators when the time of day when they were conditioned matches the time at which they are tested (Ferrari & Chivers, 2010). Some species, such as the rainbow trout, are also capable of embryonic learning from heterospecific alarm cues (Horn *et al.*, 2019, Chapter 4).

In this study, we expose embryonic fathead minnows to three concentrations of conspecific alarm cues paired with a novel predator odour to assess whether they are capable of learning to recognize predators from alarm cues and whether that learning is threat-sensitive, such that a more intense fright response is attributed to a predator learned at a higher threat-level.

5.2 Methods

5.2.1 Holding conditions

Adult fathead minnows were purchased from Osage Catfisheries Inc. of Osage, MO. One hundred and twenty-five mating pairs were allowed to breed in individual ten-L glass tanks

(Hagen, Montreal, QC), equipped with air stones and shelters (semi-circular halves of PVC pipe). Once eggs were deposited on the inside of a pipe, the mating pair was removed to minimize any parenting bias in egg exposure. Each clutch of eggs was held in the same tank with the same conditions through hatching and development up until testing. The light:dark cycle was 16:8 h and the tanks were maintained around 25ºC, with water changes of approximately ten percent each week. Small bubbler filters with charcoal and polywool batting were added once the juveniles reached a fork length of one cm. The newly-hatched minnows were fed twice daily with live brine shrimp to promote growth for the first two months and then graduated to crushed commercial fish flakes once a day.

5.2.2 Collection of cues

Four adult minnows from our breeding stock were sacrificed with a blow to the head (as per the guidelines from the Canadian Council on Animal Care) to collect alarm cues [AC]. A one cm^2 piece of skin was carefully removed from the flank of each fish (total four cm^2) and then homogenized in 40 mL chilled, distilled water. The homogenate was filtered through polywool to remove tissue remnants, and serially diluted to produce low, medium and high concentrations of skin solution, at 1 cm^2 / 240 L, 1 cm^2 / 120 L and 1 cm^2 / 40 L respectively. The skin solutions were stored in frozen aliquots of approximately 30 mL at –20ºC and thawed at room temperature in water prior to use.

Three pike (fork length: mean ± SE: 19.1 cm ± 0.2) were used to produce the predator odour [PO] for our experiment. The pike were starved for one week prior to cue collection to minimize the presence of dietary cues resulting from feeding on related prey species (Mirza & Chivers, 2001b). Fish were then placed in clean 60-L glass collection tanks (Hagen) full of fresh dechlorinated water with air stones but no filtration. After 24 h they were transferred back into their holding tanks and the water from the collection tanks was then filtered through polywool and stored in aliquots of approximately 125 mL at –20ºC until use. Frozen aliquots were brought to room temperature in water prior to use for conditioning or testing.

5.2.3 Conditioning

Each clutch of eggs was exposed to conditioning cues for one hour in the morning and one hour in the afternoon for two days (for a total exposure time of four h) starting 24 – 36 h after

deposition. Fish developed eye spots around 43 h after they were laid and hatched at an average of five days. The timing of cue application allowed for the fish to be exposed after their eye spots developed because this pigmentation occurs after some significant developmental milestones including completion of fundamental structural neural development, and commencement of circulation (US EPA, 1996). Very few fish hatched before the completion of the conditioning, but any that did were removed from the experiment to eliminate the possibility of non-embryonic learning. The purpose of conducting exposures in both morning and evening was to decrease the possibility of a temporal bias in learning and to allow for more exposures in the short period of embryonic development.

For each conditioning treatment, the PVC pipe to which the eggs were adhered was transferred into a secondary container containing 1.5 L of water and the conditioning cues. The six conditioning treatments were as follows: 25 mL distilled water control [DW]; 20 mL predator odour control [PO] + 5 mL DW; 5 mL high conspecific alarm cue control [AC] + 20 mL DW; 5 mL low concentration AC with 20 mL PO [LO]; 5 mL medium concentration AC with 20 mL PO [MED]; 5 mL high concentration AC with 20 mL PO [HI]. The final concentrations of alarm cue in the LO, MED and HI treatment buckets were 1 cm^2 / 73 200 L, 1 cm^2 / 36 600 L, and 1 cm^2 / 12 200 L, respectively. The positive control AC treatment was prepared to match the high concentration in the learning treatment (1 cm^2 / 12 200 L). Lawrence and Smith (1989) found that one cm^2 of skin was enough to elicit alarm responses in an active space of at least 58 000 L. We created two stimuli that exceeded this threshold concentration and one below it to elicit a range of responses that might demonstrate threat sensitivity. After conditioning, the egg-covered pipes were transferred to a clean water bath for two min before they were transferred back into a number-coded holding tank. Using number-coded tanks ensured that the observer was blind to the treatments, effectively eliminating observer bias.

5.2.4 Testing conditions

Juvenile fish were tested in groups of three, 102 – 319 days after they were conditioned (fork length: mean ± SE: 26.9 mm ± 0.26). This timeframe is appropriate given that adult wild fathead minnows retain their fright response to pike for a minimum of one year when held in captivity without there being any reduction in the intensity of the response (Chivers & Smith, 1994a). In the current experiment, fish were tested in the order in which the clutches were laid. Testing

tanks were ten-L glass tanks, each filled with fresh water and containing an air stone and a PVC shelter, with gravel covering the bottom. A cue injection line was fastened to the airline to allow the cues to be injected into the bubbles to minimize physical disturbance and speed up cue dispersal throughout the tank. The tanks were marked on one long wall with a three by four grid, creating rectangular 7.4 by 5.3 cm quadrats. The three other walls of the tank were covered with dark plastic and a light was positioned directly above each tank to minimize visual disturbance by the observers. Behavioural trials were video recorded through the grid-marked, uncovered side of the tank for subsequent analysis.

Groups of three fish were transferred to testing tanks 24 h prior to testing to allow the fish to acclimate to their new environment. Each clutch provided one test group for distilled water and one test group for predator odour; clutches were not reused. Fish were fed approximately 30 min prior to testing to prevent a hunger bias. One fish was selected at random to observe movement; all three fish were assessed at thirty second intervals for shelter use. Behaviours were observed for a five-min period prior to the injection of cues and then again for a five-min period starting approximately twenty s after the injection of cues. This procedure allowed us to calculate change in behaviour following stimulus injection. Twenty mL of test stimulus (predator odour or distilled water) was used for each injection, followed by a 60-mL flush of tank water (removed prior to the experiment) to clear the line.

We used two metrics to assess antipredator behaviour: decrease in movement and decrease in time spent out of the shelter. We quantified movement as “line crosses” occurring when 3/4 of the focal fish’s body passed over a grid line on the testing tank. Line crosses were measured over the entire observation period. Shelter use was initially quantified by recording the number of fish using the shelter at each time interval. However, there was some aggression among shoal members which precluded all of the fish from entering the shelter. As a result of this shelter avoidance, we inverted this number to represent the number of fish outside of the shelter at each interval. This allowed us to calculate the proportional change in shelter avoidance. We recorded this variable at each of ten 30-s time intervals, using (1) to indicate that one fish was outside of the shelter, (2) to indicate that two fish were outside the shelter and (3) to indicate that all three fish were outside the shelter.

5.2.5 Statistical Analysis

We calculated a proportional change in behaviour ((poststimulus – prestimulus) / prestimulus) for the two behaviours observed (line crosses and shelter avoidance). We used proportional change as the variable in a two-way multivariate general linear model (GLM) that tested the effects of the conditioning treatments (DW, PO, AC, LO, MED, HI) and testing cues (distilled water vs predator odour) on the observed behaviours (line crosses, shelter avoidance). We assessed outliers using Cook's Distance (4 / n) and removed two outliers from our pool of 231 test groups, leaving 229 for analysis (n = 18 – 20 individuals). We ran the same two-way multivariate GLM on the prestimulus data to ensure that there were no prestimulus biases. We reported Pillai's trace for these multivariate GLMs to use the most conservative measure (Olson, 1976). We also analysed fish length (fork length in mm) and day of testing (number of days after conditioning) to ensure there were no biases between groups. We used a Shapiro-Wilks' test to assess normality, Box's test to check the covariance in our multivariate GLM, and Levene's to test for heteroscedasticity. Because proportional changes in line crosses and shelter avoidance were both non-heteroscedastic, these two measures were rank-transformed to allow us to proceed with a complex analysis, as rank transformation corrected for unequal covariance identified by the Box's test. Where the multivariate GLM showed significant interactions, we split the data to allow further investigation of the interactions. Specifically, we split by the testing cues (water vs predator odour) to allow us to examine the relative dynamics and any possible threat-sensitivity. Finally, we used one-way univariate GLMs with age (days after treatment) as a covariate to examine the responses over time across conditioning cues as tested with predator odour; there was no interaction between age and treatment (MANOVA: Pillai's Trace: $F_{10,202} = 0.701$, $p = 0.723$). All analyses were performed using SPSS Statistical Software [Version 17.0] (IBM).

5.3 Results

Age on the day of testing (treatment x cue: $F_{5,217} = 0.042$, $p = 0.999$, cue: $F_{1,217} < 0.001$, $p = 0.990$, treatment: $F_{5,217} = 1.42$, $p = 0.218$), fish length (treatment x cue: $F_{5,217} = 0.623$, $p = 0.683$, cue: $F_{1,217} = 3.83$, $p = 0.052$, treatment: $F_{5,217} = 0.671$, $p = 0.646$) and baseline activity level (MANOVA: Pillai's Trace: treatment x cue: $F_{10,434} = 1.043$, $p = 0.406$, cue: $F_{2,216} = 0.746$, $p = 0.475$, treatment: $F_{10,434} = 0.769$, $p = 0.659$) did not differ based on conditioning treatment,

testing cue or their interaction. These non-significant results indicate that there is no apparent underlying bias between groups.

Proportional change in behaviour differed significantly between groups (MANOVA: Pillai's Trace: treatment x cue: $F_{10,434} = 2.25$, $p = 0.014$, cue: $F_{2,216} = 16.1$, $p < 0.001$, treatment: $F_{10,434} = 2.23$, $p = 0.016$). After we split by test cue, we found no significant difference across treatment groups for those tested with the distilled water cue (MANOVA: Pillai's Trace: $F_{10,220} = 0.638$, $p = 0.780$). However, we did find significant differences for those tested with the predator odour cue (MANOVA: Pillai's Trace: $F_{10,214} = 3.17$, $p = 0.001$; Figure 5.1). Both line crosses and shelter avoidance were affected by the conditioning treatment. Pairwise comparisons between conditioning treatments show a significant difference in the number of line crosses for the control treatments DW and PO as compared to all three paired learning treatments (DW vs LO: $p = 0.025$, DW vs MED: $p = 0.001$, DW vs HI: $p = 0.001$; PO vs LO: $p = 0.023$, PO vs MED: $p = 0.001$, PO vs HI: $p = 0.001$) and a significant difference between the AC control and the MED and HI learning treatments (AC vs MED: $p = 0.006$, AC vs HI: $p = 0.006$) but no difference between DW, PO and AC, between AC and LO, or between LO, MED and HI (see Table 5.1).

For the shelter avoidance, pairwise comparisons show a significant difference between the control treatments and LO learning treatment as compared to the HI learning treatment (DW vs HI: $p = 0.025$, PO vs HI: $p = 0.025$, AC vs HI: $p = 0.001$, LO vs HI: $p = 0.047$), but no differences between the control treatment and the LO, or between the MED and any other treatment (see Table 5.1). There was no effect of age on proportional change in behaviour when tested with predator odour (MANOVA: Pillai's Trace: $F_{2,105} = 2.10$, $p = 0.128$).

5.4 Discussion

Embryonic exposure of fathead minnows conditioned with a combination of alarm cue and predator odour resulted in post-hatch antipredator responses to predator odour, indicating that fathead minnows are capable of learning to recognize predators prior to hatching. The significant difference between the proportional change in line crosses of the three paired learning conditioning treatments as compared to the distilled water and predator odour control groups demonstrates that the minnows are learning to recognize their predators at all three of the concentrations we tested. The response in those taught at the lowest concentration suggests that

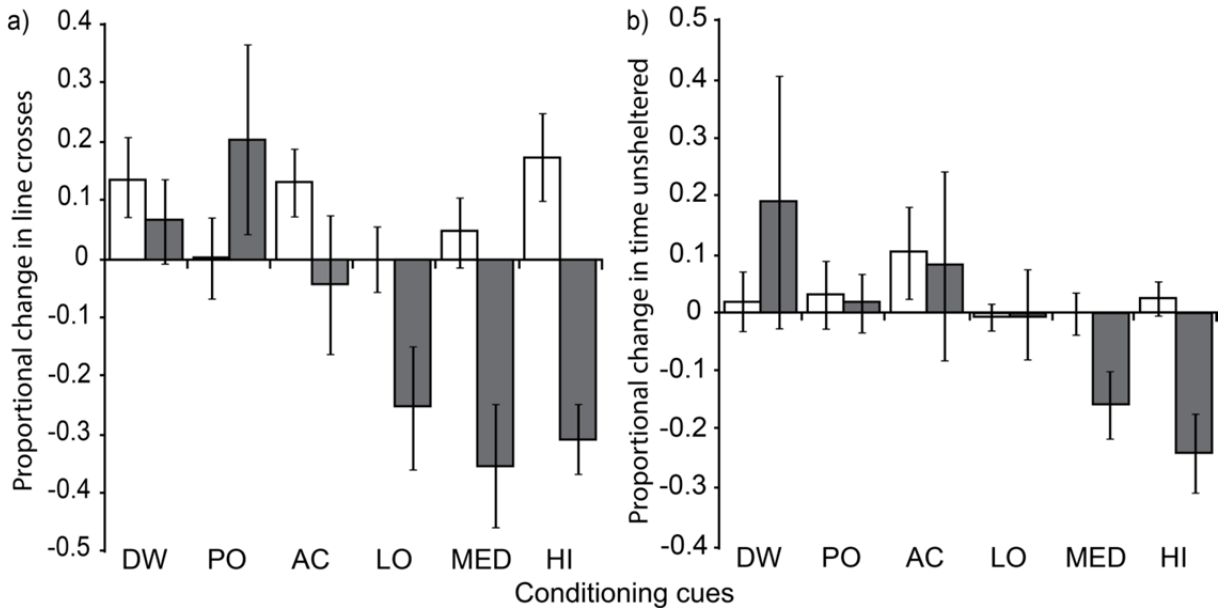

Figure 5.1. Mean (±SE) proportional change in (a) line crosses (movement) and (b) shelter avoidance amongst juvenile fish tested with a distilled water cue (open bars) or a predator odour cue (grey bars) after embryonic exposure to different conditioning cues (DW = water, PO = predator odour, AC = alarm cue, LO = PO + low concentration AC, MED = PO + medium concentration AC, HI = PO + high concentration AC).

Table 5.1. P-values of pairwise comparisons of proportional change in line crosses and proportional change in shelter avoidance for fish tested with predator odour. Significant values in bold.

		Proportional Change in Line Crosses					
		DW	**PO**	**AC**	**LO**	**MED**	**HI**
Proportional Change in Shelter Avoidance	**DW**		0.974	0.620	**0.025**	**0.001**	**0.001**
	PO	0.940		0.598	**0.023**	**0.001**	**0.001**
	AC	0.646	0.700		0.084	**0.006**	**0.006**
	LO	0.375	0.417	0.678		0.272	0.944
	MED	0.062	0.073	0.162	0.315		0.290
	HI	**0.004**	**0.005**	**0.018**	**0.047**	0.340	

the previous estimate of a 58 000 L active space from a single cm^2 of minnow skin (Lawrence & Smith, 1989) may be conservative; our results suggest an active space of at least 73 200 L. Interestingly, we see a significant change in shelter use only in learning pairing with the highest concentration of alarm cue. Because we know that the minnow embryos learned from all three concentrations, we can interpret this as a threat-sensitive response, rather than an inability to detect the lower cue concentrations.

These results provide a textbook example of threat-sensitive behavioural modification. The change in line crosses (movement) represents the baseline antipredator response; any level of threat can incite this modification. However, we only see modification of shelter use in individuals responding to a higher-level threat. This escalation of behavioural responses concomitant with risk, matches the findings of Magurran and Pitcher (1987) which showed that seeking shelter was a last resort for European minnows exposed to fish predators. By decreasing movement, preys reduce the risk of being observed and although their foraging is somewhat limited, it is not completely terminated. Conversely, while effectively eliminating predation threat, shelter use also completely precludes foraging opportunities.

Another interesting result is the absence of a difference between the alarm cue control and the low learning group in terms of line crosses in response to a predator odour cue. While we cannot draw definitive conclusions as the alarm cue control did not differ significantly from the distilled water or predator odour controls, its similarity to the lowest learning group suggests the possibility that embryonic exposure to alarm cue is resulting in fear-induced neophobia in fish. Although embryonic induction of neophobia has been demonstrated in wood frogs (Ferrari *et al.*, 2015), it has not been well-studied in fish. Further testing with a higher concentration of alarm cue and a longer cumulative exposure time might allow for a definitive demonstration of predator-induced neophobia in embryonic fish.

Previous work investigating the capacity of embryonic fish to learn to recognize predators has focused on cues from conspecifics of more similar ages than were used in this experiment. Both Nelson *et al.* (2013) and Atherton and McCormick (2015) used larval alarm cues to induce learning in embryos, and Horn, Ferrari and Chivers (2019) used alarm cues from juveniles (Chapter 4). Here we used adult fish to produce our alarm cues, as we believe this to be an

ecologically relevant choice for our species. Embryonic minnows receive paternal care, and as such are in the immediate vicinity of an adult that might fall victim to a predator. In some species and age groups, such as brook char (Mirza & Chivers, 2002), spiny chromis (Mitchell & McCormick, 2013) and ambon damselfish (Lönnstedt & McCormick, 2011), the fish respond more strongly to alarm cues from conspecifics of the same ontogenetic group. However, rainbow trout do not appear to differentiate by ontogeny (Horn & Chivers, 2017, Chapter 2). This experiment does not compare reactions across ontogenetic stages, but demonstrates strong learning by embryos to adult cues, the most ontogenetically disparate source available.

Consistent with Chivers and Smith (1994a) work on minnows, we did not observe any change in strength of response to predator odour over time. Previous work on trout showed that the strength of response diminished greatly over time (Horn *et al.*, 2019, Chapter 4). The trout experiment was specifically designed to address the question of changes in response over time, and therefore had a greater array of data than this experiment, where the question of change in response over time was a consequence of the execution of the experiment rather than an inherent part of the experimental design. The time span over which the trout were observed was shorter than the span over which the minnows were observed (one to six months as opposed to three to ten months) and yet the trout almost entirely extinguished their responses over this time period, while the minnows showed no change in response. Though it would be prudent to further investigate the time component with an experiment designed expressly for that purpose, the results here can serve as preliminary findings to suggest an interesting disparity between retention for the two species.

A great many factors may contribute to differences in predator retention between fathead minnows and rainbow trout, including alarm cue ontogeny, life histories, shifts in habitat and prey guilds, and differential growth rates. As mentioned earlier, the alarm cues used in this experiment were from adult donors, whereas the alarm cues used for the trout experiment were juveniles. It is possible that the fish are capable of discerning from which ontogenetic group the alarm cues originate and retain the information until it is ontogenetically relevant. However, it is perhaps more plausible that the difference in size disparity between life stages is responsible for the different retention trajectories of these two species. Although juvenile minnows may occupy the same prey guild as their adult counterparts, the same cannot be said for trout, which increase

almost exponential in size until adulthood, and typically occupy several prey guilds over that time. Fathead minnows have a much shorter lifespan than do rainbow trout, but also have a considerably slower growth rate and a much smaller disparity in size between hatching and adulthood. Brown *et al.* (2011b) demonstrated that trout on a faster growth trajectory retain predator information for a shorter time. It is conceivable that this is also true across species: that species which typically grow faster simply dispense with learned predator information more rapidly than species that grow more slowly. In the case of either growth rate or relative size at different life stages, the prediction is the same: a fish that maintains the same prey guild for longer may benefit from retaining its predator information for longer periods of time, as its predators are less likely to shift than for an individual who moves from one prey guild to another. Furthermore, trout have much more diverse life histories than minnows. Where rainbow trout occupy a variety of habitats over the course of their lifespans, minnows may spend their entire lives in the same microhabitat and may therefore benefit more from retaining predator information as they are less likely to move into a habitat where their learned predator information is obsolete. Simply, the minnows' less dynamic life histories may require less cognitive plasticity. Unfortunately, because of the complexities of varying sensitivities from one species to the next, as well as the obvious differences in life histories, it is hard to design an experiment to truly test differences between these species.

Another interesting point to consider is the role of phenotypic plasticity and subsequent sensitivity in early learning. For learning to occur, embryos are exposed (often repeatedly) to olfactory cues. Research has shown that early exposure to olfactory cues can induce upregulation of the specific receptors that correspond to those cues (Harden *et al.*, 2006; Murray *et al.*, 2011; Broad & Keverne, 2012). Harden *et al.* (2006) showed that young zebrafish exposed continuously to an artificial odorant upregulate the receptors specific to that odorant (physically creating more receptors) such that they display greater sensitivity to it as adults. In this experiment, we found that the minnows did not diminish their fright response to predator odour over the entire length of the study (ten months). However, as mentioned earlier, research on trout demonstrated that they do not retain the information indefinitely (Horn *et al.*, 2019, Chapter 4). While is possible, it is highly unlikely that these two examples of embryonic learning in fish are occurring via completely independent mechanisms. We therefore contend that although phenotypic plasticity may play a role and potentially increase retention, it is most probable that

learning is still the primary mechanism by which fish are gaining this predator recognition. It would nevertheless be interesting to investigate physiological aspects to determine if upregulation is indeed occurring in this situation. If it is, this might even suggest the potential for epigenetic effects; multigenerational studies would be required to verify this possibility.

This experiment provides a first example of threat-sensitive learning in embryonic fish, in which embryos exposed to predator odour in combination with one of three concentrations of alarm cue learned to recognize a novel predator. Learners from all three concentrations reduced their movement in response to post hatch exposure to predator odour, and those that learned at the highest concentration also increased their shelter use, demonstrating a threat-dependent variation in antipredator behaviour.

CHAPTER 6: Ontogeny of early learned predator recognition in wood frogs[5]

6.1 Introduction

Predator-prey interactions are strong drivers of evolution among animals (Lima & Dill, 1990). Responding to predators in a timely manner reduces the intensity of physical encounters and thus the risk of injury or death (Pfeiffer, 1977; Lima & Dill, 1990; Sih *et al.*, 2000). Diverse organisms can tell when a conspecific has been injured based on chemical alarm cues released following physical damage to the conspecific's skin, and many learn to recognize their predators by detecting a novel predator odour concurrent to the release of these alarm cues (Chivers & Smith, 1998). This type of learning is an important mechanism by which prey can come to recognize predators without themselves being injured (Suboski, 1990).

Animals that avoid too many things, or are overly cautious, may miss opportunities to forage and mate, which may reduce their overall fitness (Lima & Dill, 1990). To prevent over-reactions to minor threats, and thus missed opportunities, prey may retain contextual information relating to predation events, such as the time of day or the intensity of the alarm cue, and use this information to temper their responses to later threats (Chivers & Smith, 1995b; Sih *et al.*, 2000; Ferrari & Chivers, 2009b). Prey may also stop responding to a learned predator after a period of time. An organism that ceases to respond to a predator may retain the knowledge that it was once a threat, but may no longer provide an overt response to its presence. For example, wood frog tadpoles ceased to respond to a learned predator after a period of time, but increased their subsequent responses to a predator they had encountered in the past (Chivers & Ferrari, 2013). This terminated response is referred to as "adaptive forgetting," because an organism is gaining a fitness advantage by no longer responding to threats that it is has outgrown (Bouton, 1994;

[5] The data from this chapter was published as part of the following publication. For this chapter, I designed the experiment with DP Chivers and MCO Ferrari, DP Chivers and MCO Ferrari executed the experiment, and MCO Ferrari did the analyses. I have completely rewritten the content of the original publication for this thesis as the manuscript includes an additional experiment. My figures are modified replicates of the originals created by MCO Ferrari.

Ferrari MCO, Horn ME, Chivers DP. (2019) *Cognitive resonance: When information carry-over constrains cognitive plasticity.* ***Functional Ecology, 33(4): 703-711.***

Kraemer & Golding, 1997; Ferrari *et al.*, 2010a). Various contextual factors may impact the rate of adaptive forgetting, including both environmental factors (such as encounter rate or habitat shifts) and intrinsic factors (such as growth rate or ontogeny) (reviewed in Ferrari *et al.*, 2010a).

In this study, I chose to focus on the ontogeny of the learner because age at the time of learning has been well-established as having an impact on learning capacity (Campbell & Spear, 1972; Wyss *et al.*, 2000; Madsen & Kim, 2016). I investigate the possibility that certain ontogenetic stages provide greater sensitivity in terms of learning. Sensitive periods, or periods in which an individual is particularly influenced by experiences or its environment, have been observed in the development of a wide range of species (Westeberhard, 1989; Travis, 1994; Panchanathan & Frankenhuis, 2016; Arnett & Kinnison, 2017). A classic example is newly-hatched salmonids, that imprint on their natal streams during an early sensitive period so that they may one day return to spawn (Cooper *et al.*, 1976; Dittman *et al.*, 1996), but do not imprint on habitats experienced at subsequent life stages. In Chapter Four, I investigated periods of sensitivity in predator learning in early life stages of rainbow trout, and found that rainbow trout that learned as embryos retained predator information for longer than those that learned as newly-hatched alevins (Horn *et al.*, 2019).

Predator learning has been extensively studied in wood frogs, and they are known to have sophisticated learning beginning in the embryonic stages, making them an excellent study organism with which to investigate sensitive periods of learning. Here, I compare retention of learned predator information between individuals conditioned as embryos and as larvae to assess the impacts of ontogeny on learning in tadpoles to look for trends in sensitivity of learning and retention.

6.2 Methods

6.2.1 Holding conditions

Eight freshly-laid clutches of wood frog eggs were collected in early May and subdivided in four similar-sized groups to make 32 sub-clutches, each of which was maintained in two L of water in a seven-L food-grade plastic pail. After they hatched out, the tadpoles were fed alfalfa pellets, and received a partial water change every four days.

6.2.2 Collection of cues

Alarm cues (AC) were procured from tadpoles hatched out of six freshly-lain clutches collected at the end April. The tadpoles hatched in large pools approximately five days after collection and were then fed alfalfa pellets and algae until they were approximately 10 mm long. Sixty-four individuals were euthanized by a blow to the head. After we homogenized them with a mortar and pestle, we added 320 mL of aged well water and filtered the resulting solution through polywool. The resulting alarm cue solution was frozen in aliquots at –20 °C.

Our predator cues (PO) were collected from two tiger salamanders (snout-vent length 13 – 14 cm) that had been maintained in the lab for five years on a diet of earthworms. Each salamander was soaked individually in two L of water for 24 h. We combined the water from both predators before freezing the predator odour at –20°C in aliquots.

6.2.3 Conditioning

We conditioned the wood frogs at one of two stages (embryonic or larval), with one of two treatments (true or false conditioning), and one subgroup from each clutch was allocated to one of these four treatments (Figure 6.1).

Embryonic exposure was performed in early May, approximately 36 h before the tadpoles hatched. Each pail received two treatments (1100h and 1600h). Those receiving embryonic conditioning treatments received an injection of either a true conditioning treatment of 10 mL of AC paired with 20 mL PO, or a false conditioning treatment of 10 mL of water paired with 20 mL PO. The embryos slated for larval conditioning treatments each received 30 mL of water as a sham to control for the disturbance of the treatment experience. An 80% water change was performed one hour after the administration of each conditioning treatment.

Larval exposure was performed when the tadpoles reached Gosner stage 25 (approximately eleven days after embryonic exposure) and were therefore adequately developed for testing. At this time, those receiving larval conditioning treatments received either a true conditioning treatment of 10 mL of AC paired with 20 mL PO, or a false conditioning treatment of 10 mL of water paired with 20 mL PO. The tadpoles that received embryonic conditioning treatments each

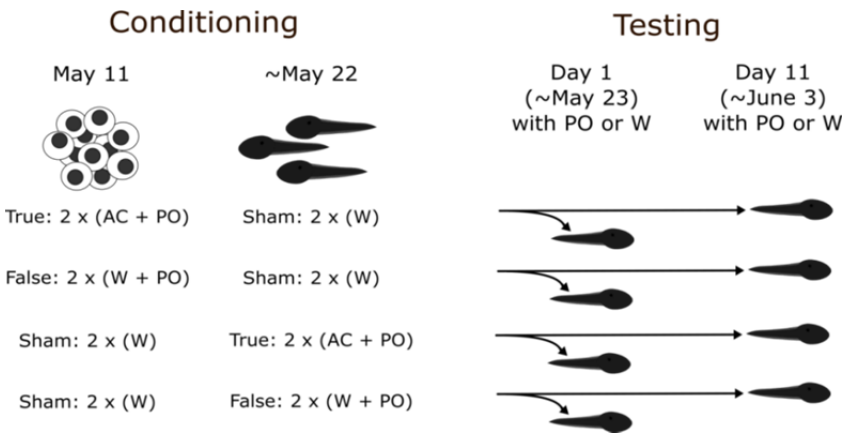

Figure 6.1. Conditioning, exposure and testing design. Wood frogs were conditioned either with a true conditioning pairing of alarm cues and predator (salamander) odour (AC + PO), or a false conditioning pairing of water with predator odour (DW + PO). Individuals not being conditioned received a sham water (DW) exposure to mimic the disturbance. Each tadpole was tested once, either one day, or eleven days after larval conditioning ended with predator odour (PO) or DW.

received 30 mL of water to control for the disturbance of the treatment experience. An 80% water change was performed one h after the administration of each conditioning treatment.

6.2.4 Testing

Tadpoles were tested with water or predator odour for antipredator responses either one day after their larval exposure or eleven days later. A randomly selected tadpole was chosen from each pail and placed in a 0.5-L cup of water outdoors. After a two-hr acclimation period, the tadpoles were tested for responses to predator odour. Each tadpole was observed for four min prestimulus and four min poststimulus, separated by a 45-s injection period in which five mL of either water or PO was slowly injected into the cup from a ten-mL syringe. During the pre- and poststimulus observation periods, the number of times a tadpole's entire body crossed a line across the middle of the cup was recorded to assess its level of movement. Observers were blind to treatments during data collection.

6.2.5 Statistical analysis

Proportional change in activity was calculated for each tadpole from the pre- and poststimulus data, and used this as the response variable for analysis. Prestimulus activity was also analysed to ensure no baseline biases between experimental groups. Any tadpole that did not complete at least six line crosses during the prestimulus period was excluded as per (Ferrari & Chivers, 2009a). A total of 391 individuals remained (n = 21 – 30 per treatment group).

The tadpoles were raised in pails with conspecifics and can therefore not be considered as independent. To accommodate this, we used a mixed-model nested design (Type I SS) with the pail as a random factor, which allows the pail rather than the individual to be used as the level of replication, avoiding the pseudo-replication that would have resulted from the ontogenetic stage at conditioning and conditioning type being the same within each pail.

A five-way mixed-model ANOVA (four fixed, one random) was used to compare the activity level between ontogenetic stage at conditioning (embryonic vs larval), conditioning type (true vs false), testing day (day one vs day eleven), testing cue (water vs predator odour), and pail. This analysis was performed on both the prestimulus data and the proportional change data. The data was then split by testing day and a four-way nested ANOVA was used to explore biases and

further interactions. Where appropriate, the data was then split by conditioning groups to allow further exploration of patterns of response. Most of the data met parametric assumptions, but in cases where the data was heteroscedastic a Scheirer-Ray-Hare extension of the Kruskal-Wallis test (Sokal & Rohlf, 1995) was used, allowing for a more robust, albeit less powerful, non-parametric ANOVA (reported as H-values).

6.3 Results

Baseline activity was affected by testing day ($F_{1, 347} = 26.2$, $p < 0.001$), with an average increase of eight lines in tadpoles tested on day eleven as compared to those tested on day one (mean ± SE: 23.3 mm ± 1.0 vs 31.1 ± 1.0). It is expected that the tadpoles tested on day eleven should have higher baseline activity due to their larger size. There were no other significant interactions (two-, three- or four -way) (Table 6.1).

The proportional change in movement was affected by significant interactions between ontogenetic stage at conditioning, cue and testing day ($H_{1, 347} = 4.1$, $p = 0.043$, Figure 6.2), demonstrating that tadpole responses to cues varied based on the day of testing and the stage at which they were conditioned.

Among tadpoles tested on day one, responses were not affected by the ontogenetic stage at conditioning (ontogenetic stage: $H_{1,23.6} = 0.19$, $p = 0.66$; ontogenetic stage x conditioning type: $H_{1,23.6} = 0.04$, $p = 0.84$; ontogenetic stage x testing cue: $H_{1,161} = 0.31$, $p = 0.58$; ontogenetic stage x conditioning type x testing cue: $F_{1,161} = 0.27$, $p = 0.60$), but were affected by an interaction between conditioning and testing cue ($H_{1,161} = 38.7$, $p < 0.001$, Figure 6.2). Specifically, a two-way nested ANOVA indicated that individuals that were conditioned with a true conditioning responded differently (more strongly) to predator odour than to the water control ($H_{1,82} = 141.0$, $p < 0.001$, Figure 6.2), while those that received false conditioning did not respond differently to predator odour.

Responses of tadpoles tested on day eleven were significantly affected by interactions between ontogenetic stage at conditioning and cue ($F_{1,159} = 5.9$, $p = 0.016$), ontogenetic stage at conditioning and conditioning type ($F_{1,26.6} = 2.6$, $p = 0.011$), and conditioning type and cue ($F_{1,159} = 5.4$, $p = 0.021$, Figure 6.2). As with the testing on day one, tadpoles that had received false conditioning did not respond differently to predator odour and water (three-way nested ANOVA:

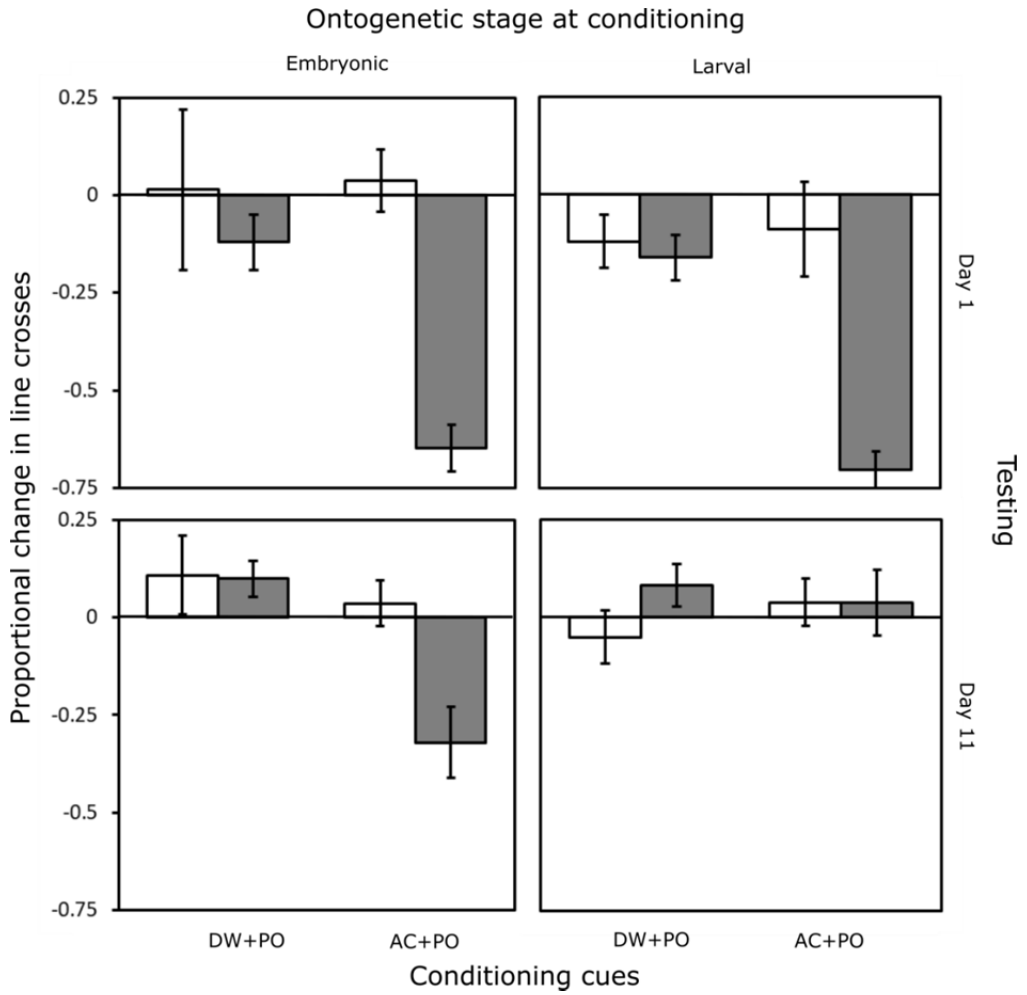

Figure 6.2. Mean (±SE) proportional change in line crosses in response to water (open bars) or predator odour (grey bars), when tested on day one (top) or day eleven (bottom) following larval exposure. Wood frogs were exposed as embryos or as larva (two distinct ontogenetic stages) to either a false conditioning control (water and predator odour, DW +PO) or a true conditioning (alarm cue and predator odour, AC + PO).

Table 6.1. Results from the five-way nested ANOVA performed on prestimulus activity. Significant values in bold.

Factors	F	df	p-value
Conditioning	0.05	1, 26.23	0.825
Ontogeny	0.157	1, 26.43	0.695
Conditioning * Ontogeny	1.37	1, 26.44	0.252
Pail	1.838	28, 347	**0.007**
Cue	0.017	1, 347	0.896
Testing	26.206	1, 347	**<0.001**
Conditioning * Cue	0.1	1, 347	0.752
Conditioning * Testing	0.077	1, 347	0.782
Ontogeny * Cue	0.132	1, 347	0.717
Cue * Testing	0.272	1, 347	0.603
Ontogeny * Testing	0.139	1, 347	0.710
Conditioning * Ontogeny * Cue	3.215	1, 347	0.074
Conditioning * Cue * Testing	0.015	1, 347	0.904
Conditioning * Ontogeny * Testing	0.118	1, 347	0.732
Ontogeny * Cue * Testing	1.65	1, 347	0.200
Conditioning * Ontogeny * Cue * Testing	0.411	1, 347	0.522

cue: $F_{1,77} = 1.4$, $p = 0.24$), nor did their responses differ based on ontogenetic stage at conditioning (stage: $F_{1,13.2} = 0.69$, $p = 0.42$, stage x cue: $F_{1,77} = 0.74$, $p = 0.39$) when tested on day eleven. In contrast, the responses of tadpoles conditioned with true conditioning were affected by an interaction between ontogenetic stage and cue (three-way nested ANOVA, $F_{1,82} = 6.8$, $p = 0.011$). Among tadpoles tested on day eleven, embryonic conditioning resulted in an antipredator response to predator odour (two-way nested ANOVA on cue: $F_{1,38} = 14.7$, $p < 0.001$), while larval conditioning did not (cue: $F_{1,44} = 0.02$, $p = 0.96$, Figure 6.2).

6.4 Discussion

Ontogeny at the time of learning has a strong impact on the retention of learned predator recognition in tadpoles. Wood frogs that learned as embryos could not be tested until they had hatched and developed adequately to not be damaged by handling, resulting in testing almost two full weeks after their conditioning. Their response at this time was of the same intensity as their peers who had received larval conditioned only a day earlier. Furthermore, ten days later, the embryonic learners continued to demonstrate a significant antipredator response whereas the larval learners showed no response. This great difference in the retention between embryonic and larval learners is fascinating.

As previously mentioned, adaptive forgetting is an advantageous way for individuals to discontinue use of information that no longer has relevance if, for example, an animal has outgrown its predator's gape limit (Ferrari *et al.*, 2010a), and therefore it is no surprise to confirm that the tadpoles that learned as larvae forget a predator that they may theoretically have outgrown. However, embryonic learners would certainly also have outgrown those predators too, but are still responding after two weeks, while the larval learners forget in less than eleven days. Among the many intrinsic and environmental factors known to influence information retention, the most relevant factors to this experiment are growth rate, stress levels, relevance of cues, intensity of exposure, and cognitive flexibility (e.g. sensitive periods).

The results of this experiment, showing that embryonic learners retain information far longer than their larval learner counterparts, fit well with the results from another experiment with tadpoles which looked at increased latent inhibition in embryonic learners as compared to larval learners (Ferrari *et al.*, 2019), and a third experiment on trout showing that these rates of

retention are even more nuanced when looking at additional developmental stages (Horn *et al.*, 2019, Chapter 4). The combined evidence points strongly towards a sensitive period in early development. Specifically, this early stage of development is associated with the acquisition of a large quantity of information; at this stage may animals imprint on their environment learning about their habitat, their kin, or their diet – information that they will typically need to retain for application throughout their lives (Immelmann, 1975; Cooper *et al.*, 1976; Dittman *et al.*, 1996; Crane *et al.*, 2018). The term "cognitive resonance" can be used to describe a sensitive period in which learned information is given special priority, giving it greater impact on future decision-making (Ferrari *et al.*, 2019). In this case, because this is an important stage for learning general information, the wood frog embryo may retain the information that it gathers about predators longer simply because it is gathering so much other information at that time. This may be advantageous in a stable environment, but may actually prove costly if the environmental circumstances are highly variable, as in the areas subject to human impacts (Sih, Ferrari, & Harris, 2011; Sih, 2013).

The growth rate of an individual has a significant impact on its retention of information, and in both trout and wood frogs the slowest growers retain information for the longest time – a logical trend when considering the rate at which one might outgrow a predator (Brown *et al.*, 2011b; Ferrari, Brown, & Chivers, 2012). Wood frog embryos are contained within their egg membranes and grow with a limited supply of food. They may therefore grow more slowly than their hatched out, larval conspecifics, at least in these well-fed experimental conditions. To this end, growth rate may contribute to the shorter retention of learned predator information in the larval learners. However, the growth rates and morphologies of tadpoles may also be impacted by embryonic exposure to alarm cues, resulting in a deeper bodied morphotype, while the larvally-exposed tadpoles lack morphotypic variation from the same exposure (unpublished data, MCO Ferrari). These variations in growth and development from cue exposure may increase the disparity in retention based on timing of exposure.

Another interesting possibility that merits exploration is that the ontogeny of the cue "donor" has an impact on retention. Many species of fish are capable of discerning the ontogeny of the individual from which the alarm cues are produced (Mirza & Chivers, 2002; Lönnstedt & McCormick, 2011; Mitchell & McCormick, 2013) and tadpoles may have the same capacity. In

fish, the age and size of the cue donor may affect the degree of response, such that an organism responds most strongly to individuals of similar size and ontogeny. It is conceivable that an organism might gather information about the age of the individual that produced the alarm cues and use that information to adjust its response based on relevance. For example, it might theoretically use cues from an individual of the same size or age immediately, but put aside the information from a different ontogenetic stage for later use due to the age mismatch, by making assumptions about the duration of the relevance of that information. Future studies addressing this idea will have to take care not to inadvertently presenting the different stages with different concentrations because of differences in quantity of alarm cues produced by individuals at different stages.

It is well-documented that stress levels have an impact on learning and retention of information, due to the interference of cortisol on cognitive functions (Demuth *et al.*, 2017; Merz *et al.*, 2018). Logically it follows that the stress levels of the embryos and larvae may impact their retention. One could speculate that given the vulnerability of newly hatched tadpoles (after hatching they often die simply from being handled), the cortisol levels of the larval subjects may have been higher than the membrane-protected embryos, thus negatively impacting their capacity for retention. This would be a fascinating subject for future research.

Without further research it is also difficult to clearly understand what the impact of the egg membrane may have been on embryonic learning. It is possible that the embryonic membrane may be sufficiently permeable to the alarm cues that there is no impact on exposure. However, if the egg membrane affects the intensity or duration of exposure to the olfactory cues, it could alter the effective exposure of an embryo as compared to a larva. For example, if the membrane excludes some elements of the alarm cue or predator odour, it could lead to decreased exposure during this stage. This is not the trend I observed, but hypothetically if this were the case, the retention could have been even greater had it not been for the membrane. The opposite, and perhaps more plausible explanation, would be that the membrane prevents easy removal of the alarm cues once they permeate the membrane, thus leading to what is essentially an extended exposure. Extended exposure could be expected to extend retention, or possibly even cause a neophobic response. Neophobia has been observed in embryonic tadpoles as a consequence of extended exposure to alarm cues (Ferrari *et al.*, 2015). A more complex experimental design

would be necessary to accurately assess the role of neophobia in retention in this experiment. Of course, a neophobic response could also be subject to cognitive sensitivity. Future research with greater knowledge of the precise chemistry of the alarm cues as well as extensive investigation of the permeability of the membrane to these chemicals, might be able to more accurately predict the impact the membrane might have on the relative exposure of an embryo as compared to a larva.

Indeed, all of the discussed factors may contribute to the differential retention of predator information from one stage to another. I propose that the primary mechanism of this variation in retention is the consequence of a period of cognitive sensitivity which takes place during the embryonic stage, but that this sensitive period is augmented, or possibly even caused, by a variety of other mechanisms, possibly including growth rates, cortisol levels, and exposure intensity.

CHAPTER 7: Discussion

In this thesis, I have provided the first evidence that fish are capable of threat-sensitive embryonic learning and of embryonic learning from heterospecifics. I also present the first evidence that the ontogeny at learning has significant impacts on a fish and tadpoles' retention of learned predator information, such that embryonic learners are slower to forget than their slightly older larval conspecifics, suggesting that embryonic learners may have increased cognitive plasticity as a result of sensitive period in their development. The effect of a difference in age between the alarm cue producer and receiver varies between species and in different situations.

7.1 Impacts of alarm cues from donors of different ages

Some fish are capable of differentiating between alarm cues from different ages and sizes of fish (Mirza & Chivers, 2002; Lönnstedt & McCormick, 2011; Mitchell & McCormick, 2013). This capacity is advantageous as it allows a fish to respond only to threats to individuals that typically have similar predators, such as those of a similar age group or size. In Chapter 2, I tested whether very young juvenile rainbow trout would respond differently to alarm cues from conspecifics of two age groups: newly-hatched as compared six-month-old juveniles. The six-month-old fish were nearly ten times the average length of the newly-hatched (19.3 cm as compared to 2.2 cm) – easily large enough to be assumed to be in a distinct prey guild. I found that the rainbow trout responded equally to the alarm cues from newly-hatched and six-month-old individuals and that their response did not vary significantly based on the three different concentrations of alarm cue that I used (0.001, 0.01 and 0.1 cm^2 / ml). I also found that juvenile rainbow trout are capable of responding to alarm cues as early as twelve days post hatch, in contrast to minnows or zebrafish which are reported to fail to respond until they are over a month old (Pfeiffer, 1963; Waldman, 1982; Carreau-Green *et al.*, 2008).

The contrast between the early antipredator responses I recorded in trout and the literature records for minnows and zebrafish may be a consequence of the testing systems. Many testing methods for antipredator behaviours in juveniles are based on the behaviours of adults, but often different strategies benefit smaller, less mobile juveniles, so different metrics may better represent the antipredator responses of different age groups. To address this problem in my research, I treated the young juvenile rainbow trout more as larva (based on the standard tadpole

testing method) than as mature fish. A more nuanced approach might provide even more representative results. The impacts of using wider variety of antipredator behaviour metrics and test systems for early juvenile fish merit further investigation. Due to the large inherent behavioural differences between different phyletic groups, generalizations would be difficult, but for any species that will receive regular scientific attention, a greater understanding of the ontogenetic differences in antipredator behaviour would allow for improved metrics and therefore more robust results.

The complexity of responses to alarm cues from different ontogenetic groups was further emphasized when I found that embryonic minnows did not demonstrate an early hatching response when exposed to alarm cues from adult minnows (Chapter 3). Two pieces of information from other research make this result interesting: 1) that embryonic minnows demonstrate a strong early hatching response to alarm cues from other embryonic minnows (Kusch & Chivers, 2004), and 2) that minnows are capable of embryonic learning from exposure to adult alarm cues (Chapter 5), which means that embryos can recognize that these alarm cues represent a threat. Therefore, I can conclude that although the embryonic minnows have assessed the threat, they can differentiate between a future threat and a current one. They retain this information as a learned predator response, but do not respond to it immediately – a sophisticated response that helps them to avoid the mismatch of responding by hatching out into the stage which is at higher risk. Fish are clearly capable of gathering valuable information based on the ontogeny of the cues and the extent of this capacity merits further exploration.

7.2 Fish are capable of sophisticated embryonic learning

In 2013, Nelson *et al.* (2013) published a paper demonstrating that convict cichlids are capable of embryonic learning and, in 2015, Atherton & McCormick published their work on embryonic responses and the capacity for learning in cinnamon clownfish. I chose to look at species that were from two different orders than those previously published: minnows (Order: Cypriniformes) and rainbow trout (Order: Salmoniformes) and to extend beyond the basic capacity to learn by assessing whether other information was gathered concurrently. I used rainbow trout to provide the first evidence of heterospecific learning in embryonic fish (Chapter 4), and fathead minnows for the first demonstration of the capacity for threat-sensitive learning in embryonic fish (Chapter 5). The results of the threat-sensitive learning experiment in minnows

were of particular interest because the fish actually shifted their antipredator behaviours in response to a more intense threat level. Specifically, I exposed the embryonic minnows to pairings of predator odour with one of three different concentrations of alarm cues. Minnows from all three concentrations subsequently restricted their movement in response to predator odour. However, only those that learned at the highest concentration increased their shelter use in response to predator odour, indicating a shift from a less costly antipredator behaviour to a more costly one in accordance with the degree of threat present.

Future research could assess precisely how early in their development fish become capable of embryonic learning. In my experiments, I emphasized the capacity for the learning rather than the specific timing of the learning during the embryonic stage. I designed the experiment using multiple exposures and covering a time a period that included both before and after eye spots had appeared to ensure that they were sufficiently well-developed to learn if they had the capacity to do so. However, now that I have established that embryos do have this capacity, there is the opportunity to do a more detailed investigation to ascertain how early in development this learning can take place. Taking a close look at the relationship between developmental milestones and the capacity to learn could teach us a great deal about the physical parameters of learning.

I did not test the trout for a threat-sensitive response because typically rainbow trout are considered to have non-graded threat responses (Mirza & Chivers, 2003). However, in consideration of the differences in sensitivity of embryos as compared to older individuals (Chapter 4), it may be worthwhile to test young juvenile trout for a threat-sensitive response in spite of older trout having been observed to have a dichotomous response rather than a graded one. Furthermore, artificial testing facilities, as well as the strict behavioural parameters that observed to quantify traditional antipredator responses may sometimes obfuscate shifts that may, in fact, represent more subtle antipredator behaviours. That said, without a higher resolution lens (i.e. more species- and ontogeny-specific testing setups and parameters), it might be difficult to observe a threat-sensitive response in a species like rainbow trout even if it exists. Furthermore, because of the large investment of resources required to set up specially-tailored parameters, those type of adjustments would only practical for species and ages that will receive a great deal of research attention.

I did not design my testing of minnows to evaluate the duration of retention of antipredator behaviours after learning as embryos. However, the minnows were tested over a 10-month period, and I checked for a decline in response over that time and found none, so the minnows clearly retain the information for at least ten months. Although minnows were selected in part because of their long retention of learned predator recognition (Chivers & Smith, 1994a), it would be interesting to investigate whether the intensity of a learning event impacts retention. This could be assessed with various strategies, such as looking at different numbers of learning events and different concentrations, to determine what aspects of the learning events impact the retention time.

7.3 Sensitive periods of learning for embryonic learners

Retention of learned predator information is impacted by a wide range of factors (Ferrari *et al.*, 2010a) and in this thesis I chose to focus on a key intrinsic factor which has been largely overlooked in the literature: ontogenetic stage. I aimed to see how the developmental stage of an organism might impact its rate of adaptive forgetting to explore the lifetime impacts of embryonic learning.

I exposed three different early life stages of rainbow trout (embryos, alevins and fry) to pairs of alarm cues (conspecific and heterospecific) and novel predator odour to induce learning, and then monitored their responses over time to assess how long the different learning groups maintained their antipredator responses (Chapter 4). The results were fascinating; newly-hatched alevin learners stopped responding to their learned predator far more rapidly than their embryonic learner conspecifics. However, the oldest group – the free-swimming fry learners – fell in between in their rate of adaptive forgetting, not significantly different from either other group of learners. Furthermore, these trends only applied to the fish that learned from conspecific alarm cues; those fish that learned from heterospecific alarm cues stopped responding to predator cues at an even faster rate regardless of learning age, and this rate was much faster than the extended retention of the embryonic learners, suggesting that heterospecific alarm cues are less conserved, most likely due to their slightly lower relevance as compared to conspecific cues. I propose that this differential retention of predator information is the consequence of a variety of intrinsic factors, all of which cumulatively amount to a period of cognitive sensitivity, such that the fish intrinsically place a premium on information acquired during certain developmental

periods. In the case of trout, the embryonic stage is such a sensitive period because individuals in this developmental stage are gaining a great deal of information about their habitat, their kin and their environment in general. During the newly hatched stage, physical development is the priority as they build their ability to swim, and then as trout reach the free swimming stage, they again begin to imprint on their environment, this time for the purpose of homing (Cooper *et al.*, 1976).

I also found that ontogeny also impacted retention of learned predator recognition in wood frogs, with the same trend of embryonic learners retaining the information far longer than their older tadpole conspecifics (Chapter 6). Embryonic tadpoles gather large amounts of long-term information about their surroundings – their kin, their habitat, their food sources – and they may retain learned predator information longer at this stage simply because it is gathered along with other information that is retained over a lifetime (Immelmann, 1975; Crane *et al.*, 2018).

Although trout and wood frogs are from completely different phylogenetic classes, they demonstrate parallel trends of increased retention of information gathered as embryos. Perhaps this similar trend is related to their similar life history paths, including rapidly outgrowing predators, trout through simple physical growth and tadpoles through development into a terrestrial adult life stage. Also, both trout and frogs also have a tendency to imprint on their home territory and return to it to spawn (Durham & Bennett, 1963; Cooper *et al.*, 1976), and may therefore be particularly strong candidates to have a sensitive period of learning in early development.

Further research into cognitive resonance and sensitive periods would benefit from exploring organisms that do not share these life history trends. I have proposed that the relatively long retention in minnows as compared to trout is a result of their dissimilar life histories, in that the minnow does not grow through very many prey guilds, nor does it stray far from its natal habitat over the course of its life (Chapter 5). Although retention was not the focus in the minnow learning experiment, I did not observe any decline in response over a ten-month period (Chivers & Smith, 1994a). Considerable modifications to the conditioning treatments (e.g. one low concentration treatment rather than four) might make it possible to carry on the minnow experiment long enough to observe forgetting. Experimenting with various species in each of a

variety of life histories would allow the possible establishment of life-history based trends in cognitive resonance. Ideally, these life history categories should include more species with extensive rapid growth and imprinting (like trout), some with rapid growth but no imprinting, some with lower growth and imprinting, and more with low growth and no imprinting, which would clearly demonstrate the roles of these factors in cognitive resonance.

In my thesis proposal, I proposed looking at retention of latent learning – variations based on timing of learning of fear or safety in minnows and trout, work which was ultimately beyond the scope of a single PhD thesis. However, perhaps even more-so now, I believe that this research would contribute significantly to our understanding of retention, as it develops a comparison between adaptive forgetting and genuine lack of information retention. This has since been explored using tadpoles (Ferrari *et al.*, 2019) in an experiment that demonstrated embryonic learners had a reduced capacity to update acquired information as compared to larval learners. Tadpoles are a great system for this, as the research can be done rapidly. However, they are somewhat restrictive in terms of the handling of newly hatched tadpoles, as well as the short duration of development in which conditioning and testing can take place. Exploring the phenomenon of latent learning in fish would allow more flexibility in terms of the developmental stages of conditioning, testing and retention. Additionally, these studies could provide further insights into the differences in retention between different orders of fish (as in the case of the minnows and trout studied here) by demonstrating relative levels of cognitive flexibility or varying trends in cognitive resonance between different groups.

Sensitive periods of learning and cognitive resonance, as well as their mechanisms, merit a great deal more investigation. Creating a detailed analysis of retention following different learning times with concurrent observation of growth rates of the individual, of the brain tissue, and of other environmental information gathered by the organism would allow for a far greater understanding of the mechanisms that drive sensitive periods in early development.

My experiments were unable to truly explore the impact of the embryonic membrane on embryonic learning. The embryonic membrane has the theoretical capacity to slow the penetration of odorants into the embryo, or conversely to prevent them from escaping, thus altering the relative concentrations and durations of exposure, and thus the effective “dose”.

However, the consistency in retention across age groups for the three heterospecific groups might suggest that the membrane is not having an effect after all. Additionally, my observations provide valuable information even if there is a difference in the net dose; I am still observing what would happen if the two different stages experience the same environmental learning exposure – the environmentally relevant measure – and provide a basis for future investigations into this subject.

The impact of the egg membrane on learning and retention deserve further attention. With extensive investigation, including a greater understanding of the chemistry of the alarm cues and odorants and the permeability of the membrane, as well as flow within the egg, it might be possible to predict the impact of the egg membrane on the passage of the cues, and possibly their retention. With that information, one could ostensibly calculate the concentrations and durations of exposure experienced by the embryo to try to figure out whether they are retaining the information longer solely because they are bathing in the cues for a longer period, or whether they actually have a special sensitivity to this information.

Alternatively, the impact of the membrane could be investigated using an entirely different system that would allow a greater distinction between the effects of development and the egg membrane. Acoustic cues, for example, would not be retained by the membrane. Any modulation or distortion as a result of membrane and its contents could be verified using acoustic measurements within the membrane. Birds are known to use acoustic alarm signals to learn to recognize predators (Potvin *et al.*, 2018) and are capable of learning as embryos (Sneddon, Hadden, & Hepper, 1998; Turatto, Dissegna, & Chiandetti, 2019) and could provide an excellent alternative study system, which would allow comparison of retention of predator learning by embryos versus juveniles without any differential cue retention as a result of the egg shell. This research would offer fascinating insights into the mechanism of the sensitive period of retention that I observed in fish and tadpoles.

Another idea that merits investigation is that of the impact of phenotypic plasticity and epigenetics on responses to early exposure to cues. I believe, particularly based on the trend of trout ceasing to respond over time, that the observed response to predators is a consequence of recognition learning: a response resulting from simultaneous exposure to a known trigger (alarm

cue) paired with a novel odour. However, particularly in early developmental stages, phenotypic plasticity may also impact retention. Repeated exposure to olfactory cues can cause corresponding receptors to be upregulated, causing the adults to have a greater number of receptors and thus a greater sensitivity to a particular group of odorants (Harden *et al.*, 2006; Murray *et al.*, 2011; Broad & Keverne, 2012). With organisms that continue to demonstrate a response over a long period of time, it is conceivable that the phenotype has been modified to increase this response based on embryonic exposure. I did not see a decline in antipredator responses over time in the minnows that learned as embryos, as I did with the rainbow trout. This may be the result of the organism discontinuing its response, rather than no longer having that information, which would be possible even in the case of physiological variations as a result of plasticity. Learning and adaptive forgetting have been studied at length and are certainly believed to be the primary mechanisms observed here, but the extended retention of information, particularly in embryonic learners, could be the result of an augmented sensitivity due to phenotypic plasticity. Physiological variations would need to be studied in depth to investigate this possibility, and the possibility that these effects are epigenetic could be verified by looking at the retention of so-called "learned" antipredator responses between generations or by investigating changes in DNA methylation.

It would also be interesting to explore whether embryonic fish develop neophobia. In the experiment looking at threat-sensitive learning in minnows (Chapter 5) there was no difference in the response to predator odour between the alarm cue control and the low concentration learning conditioning groups. There was also no difference between the alarm cue, water or predator odour conditioning groups, so I was unable to draw any definitive conclusions, but the results hint that neophobia may be possible in fish embryos. Long-term low-level embryonic exposure to alarm cues has a strong potential to induce neophobic behaviour, as such changes have been observed in older fish as well as in embryonic tadpoles (Brown *et al.*, 2013b; Ferrari *et al.*, 2015).

7.4 Greater implications

There is great value in understanding periods of cognitive resonance. On a basic level, understanding fluctuations in cognitive sensitivity allows us to understand the mechanisms driving learning and memory, and how they may be impacted by ontogeny. This understanding

has far reaching implications ranging from techniques to improve learning and memory, to therapies to deal with trauma and anxiety (Crane & Ferrari, 2017b). Studying these trends in fish and amphibians allows us the opportunity to explore specific aspects with a model system that offers a much greater degree of experimental control and can therefore offer insight into more complex human behaviours.

Furthermore, greater understanding of the mechanisms for learning and memory may have increased importance for conservation efforts in the face of climate change. Indeed, as climate change and human-induced rapid environmental change transform the globe, species will be forced to adapt to these changes or go extinct. Studying learning and memory allows us to better predict the ramifications of these changes on specific species. For example, species that have high cognitive flexibility may be better at adapting to rapidly-transforming habitats compared to species with less flexibility, which, conversely may perform better in stable environments (Sih, 2013). These differences in cognitive flexibility will likely have large implications in terms of which species live and die over the coming years. To understand the implications of cognitive resonance, it will also be important to explore its prevalence.

In the shorter term, learning and memory may have an impact on currently ongoing conservation efforts. The literature suggests that learned predator recognition may have a limited capacity to enhance survival of hatchery-reared fish (Brown, Ferrari, & Chivers, 2013a). However, the fact remains that hatchery-reared fish have significantly lower survival rates than wild caught fish (Araki & Schmid, 2010) and perhaps with the proper methodology predator recognition could benefit resource management efforts (Wisenden *et al.*, 2004a). It is conceivable that with a greater understanding of the nuance of learning and memory in early life stages, it may yet be possible to find a way to increase survival rates to assist with stocking and conservation efforts.

Finally, the value of information has an intrinsic impact on what can be gained by retaining it. There may be little value in continuing to use obsolete data, and it may in fact be costly to maintain it. I have presented trends in which different species, different ontogenies and different information values have impacted retention of data. There are instances in which constant, repeated exposure and intermittent exposure have different implications for retention. It is clear

that the drivers of information retention are highly complex and merit further investigation to assess the relative impacts of different factors and to determine whether there are consistent patterns based on life history.

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