EFFECTS OF CAFFEINE ON FISH LEARNING

THE EFFECTS OF CAFFEINE ON FATHEAD MINNOW BEHAVIOUR AND PHYSIOLOGY

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LAY ABSTRACT

Aquatic pollution has adverse effects on human and wildlife health, biodiversity and ecosystem function. One way aquatic pollution occurs is when the substances we consume, like caffeine, are not fully removed by wastewater treatment and enter water bodies. Concentrations of caffeine in the environment are high and yet caffeine's effects on exposed organisms are seldom studied. To redress this, we investigated how environmentally relevant caffeine concentrations affected fathead minnow (*Pimephales promelas*), a common North American baitfish. Caffeine did not affect the growth rates, length, or mass of exposed fish, but exposure to low caffeine concentrations decreased liver investment. Caffeine did not influence fish metabolism or their ability to learn to avoid a negative stimulus (a trawl), but, at low concentrations, caffeine appeared to decrease anxiety. Our results show further research is needed to better understand caffeine's effects on aquatic organisms.

THESIS ABSTRACT

Pollution is an increasing threat to health and biodiversity, especially chemical pollution in the air, land, and water. One such example is caffeine, which is a main active ingredient in coffee and is ingested by humans worldwide for its stimulant effects and cultural significance. This widespread caffeine ingestion coupled with incomplete removal during wastewater treatment results in high concentrations of caffeine in the environment. Aquatic organisms living in waterways receiving wastewater effluent are often exposed to caffeine continuously. Given this long-term and widespread exposure, caffeine is an emerging contaminant of concern. However, most research investigating the effects of caffeine on aquatic organisms use caffeine doses that are much higher and caffeine exposure durations that are much shorter than those found in the environment. Also, most caffeine exposure studies also rely on relatively simple behavioural endpoints and make use of neotropical species. In contrast, I exposed fathead minnow (*Pimephales promelas*), a common freshwater fish in North America, to environmentally relevant concentrations of caffeine (0 ng/L; 1,000 ng/L; 10,000 ng/l) for 35 days. Caffeine exposure did not affect morphology (e.g., length, mass, growth) or metabolism (maximum metabolic rate, resting metabolic rate, and aerobic scope), but decreased their hepatosomatic index (liver investment). While caffeine did not affect the number of trials taken to associative or reversal learn, or the latency of fish to avoid an aversive trawl, three weeks of exposure to low caffeine concentrations may have decreased anxiety. Taken together our results suggest that future studies perhaps with different endpoints are needed clarify our understanding of how caffeine influences metabolism, anxiety, and

learning. Overall, our results provide evidence that complex behavioural endpoints such as aversive learning can be used in ecotoxicological studies.

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THESIS ORGANISATION AND FORMAT

This thesis is organised into 2 chapters with two appendixes. **Chapter 1** provides background information on environmental pollution more generally and on caffeine contamination more specifically. I review previous caffeine research, and describe the behaviours and physiology employed in my experiment, as well as introduce the study species. **Chapter 2** provides details on an experiment I conducted to study the effects of caffeine on fathead minnow behaviour and physiology. I will be submitting an edited version of Chapter 2 for publication. **Appendix 1** describes in detail the exclusion criteria for trials in the aversive learning assay. **Appendix 2** contains a description of a research project undertaken with Megan Cyr, a thesis student in the lab, in which we examined how learning differs between wild and captive fathead minnow.

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DECLARATION OF ACADEMIC ACHIEVEMENT

CHAPTER 1: Pollution, Caffeine, and Fish Behaviour

Author: Jacqueline Bikker

CHAPTER 2: Impacts of Caffeine on Fathead Minnow Behaviour and Physiology

Authors: Jacqueline Bikker, Helen MacDougall-Shackleton, Bob Wong, Sigal Balshine

Publication: We plan to submit this manuscript to *Animal Behaviour*.

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APPENDIX 1: Trial Exclusion Criteria for the Aversive Learning Assay

Author: Jacqueline Bikker

APPENDIX 2: Learning in Captive and Wild Fathead Minnow

Authors: Megan Cyr, Jacqueline Bikker, Sigal Balshine

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CHAPTER 1. Pollution, Caffeine, and Fish Behaviour

1.1 Environmental pollution

Anthropogenic contaminants in the environment are a problem of growing concern because of the range of adverse effects these contaminants have on the organisms found in the polluted environments. Common environmental contaminants include pesticides (Meftaul et al., 2020), plastics (MacLeod et al., 2021), pharmaceuticals (Ortúzar et al., 2022), and personal care products (Hopkins & Blaney, 2016). While some of these contaminants are released directly into the environment as run-off (Tran et al., 2019) or sewer overflows (Buerge et al., 2006), many contaminants enter the environment through a less obvious source – wastewater effluent (Metcalfe et al., 2003). Wastewater effluent is the treated product released into the environment following treatment in a Wastewater Treatment Plant (WWTP) and contains a large number of contaminants including phthalates, plasticizers, disinfectants, and as mentioned above, pharmaceuticals (Deblonde et al., 2011). One of the most common substances found in treated wastewater is caffeine (Li et al., 2020).

1.2 Caffeine

Caffeine is a naturally occurring stimulant substance in the methylxanthine family (Rigueto et al., 2020). It has a low K_{OW} (octanol/water partition coefficient), meaning that it is highly water soluble, and generally stable (Rigueto et al., 2020). It is not usually affected by environmental parameters such as temperature or pH (Kurissery et al., 2012), but instead, is primarily broken down by biological and photochemical degradation (Rigueto et al., 2020).

Most of the caffeine found in aquatic ecosystems enters via treated wastewater that originates from widely consumed caffeine-containing beverages, such as coffee (Ayman & Işık, 2015; Edwards et al., 2015; Metcalfe et al., 2003; Reyes & Cornelis, 2018). Canadians, for example, consume on average 224 mg of caffeine daily (*Caffeine*, 2011), while the average Australian consumes 143 mg of caffeine daily (*Australian Health Survey*, 2014; Watson et al., 2016). Humans ingest caffeine for a number of reasons including its stimulant effects (Fisone et al., 2004) and its ancient and current significance in certain cultures (Weinberg & Bealer, 2002). While most modern WWTPs are highly effective at removing caffeine, removing 95% or more of caffeine (Li et al., 2020), because of its ubiquitous use, caffeine continues to be replenished and is almost always found in many aquatic environments, sometimes at extremely high concentrations such as 174,000 ng/L (Li *et al.*, 2020; Archana et al., 2017).

Caffeine contamination is not limited to any geographical region, or waterbody and is instead a worldwide problem (Li et al., 2020). Caffeine is often found in coastal marine areas, especially near population centres (Vieira et al., 2022). In seawater, concentrations as high as 11,000 ng/L have been detected near sewage outfalls (French et al., 2015). Caffeine has also been found in a multitude of different freshwater environments, including rivers, lakes, groundwater, rainwater, and even drinking water (Li et al., 2020). In rivers, reported maximum concentrations of caffeine ranged from 37 ng/L in the Gombak River, Malaysia (Praveena et al., 2018) to 28,440 ng/L in the Rio Grande do Sul, Brazil (Peteffi et al., 2018). In lakes, average maximum concentrations ranged from 24 ng/L in Lake Qaraoun, Lebanon (Mokh et al., 2017) to 174,000 ng/L in

Ambazari Lake, India (Archana et al., 2017). More locally, in Canada, up to ~60 ng/L of caffeine was detected in the Pictou watershed, New Brunswick (Comeau et al., 2008). In Hamilton, Ontario, the caffeine concentrations in streams and creeks dominated by wastewater effluent ranged from 16 ng/L to 1202 ng/L in the summer, and from 212 to 494 ng/L in the winter (Du et al., 2019; Nikel et al., 2021, Mehdi et al., 2020). In the nearby watershed of Lake Simcoe, Ontario, Canada, concentrations of up to 77 ng/L of caffeine were measured in one of the most anthropogenically disturbed sites studied in that relatively undeveloped lake (Kurissery et al., 2012).

Caffeine is not just found in water; it can also found in the sediment of aquatic habitats (Beretta et al., 2014; Matongo et al., 2015) and sometimes reaches higher concentrations in sediment than in the surrounding water. Caffeine appears to be more stable in sediment than in water, and sediment prevents degradation (Kurissery et al., 2012). In general, caffeine's half life varies from 3.5 days to more than 100 days, depending heavily on the microbial communities in the areas where the caffeine is being released (Benotti & Brownawell, 2009; Bradley et al., 2007).

Since caffeine is readily found in the environment, it is no surprise that caffeine has also been found in the tissues of many taxa. Coral sampled in the Maldives contained up to 37 ng/L of caffeine (Rizzi et al., 2020) while algae from the Saudi Red Sea contained up to 41 ng/g of caffeine (Ali et al., 2018). Bivalves from Singapore also contained caffeine, with Asian green mussel (*Perna viridis*) and lokan clam (*Polymesoda expansa*) tissues containing up to 11 ng/g wet weight of caffeine (Bayen et al., 2016) and up to 140 ng/g dry weight of caffeine in mussels (*Mytilus spp.*) from California USA

(Maruya et al., 2014). Concentrations up to 50 ng/g of caffeine were found in fillets of Striped bonito (*Sarda orientalis*), with even higher concentrations up to 65 ng/g found in the liver of this fish (Ojemaye & Petrik, 2019). In contrast, concentrations of only up to 2 ng/g have been found in the intestines and gill of Panga seabream (*Pteryogymnus laniarius*; Ojemaye & Petrik, 2019), indicating that caffeine enters and accumulates to a greater or lesser extent in different body tissues. Depending on the species and tissues sampled, concentrations vary with up to 74 ng/g of caffeine reported in fishes (Ali et al., 2018; Wang & Gardinali, 2012).

Caffeine metabolism

Once caffeine enters the body, up to 99% of caffeine is metabolised by the liver in humans, and it passes easily into different parts of the body including plasma, blood, the brain, placenta, and even saliva (Fredholm et al., 1999). In the liver, caffeine is broken down into metabolites. In humans, the primary metabolites are paraxanthine (1,7-dimethylxanthine), theobromine (3,7-dimethylxanthine), and theophylline (1,3-dimethylxanthine; Fredholm *et al.*, 1999), with the bulk (70-80%) broken down into paraxanthine by the liver enzyme, CYP1A2 (Thorn et al., 2012). A further 7-8% is broken down into theophylline, and another 7-8% to theobromine (Thorn et al., 2012). In addition to unmetabolised caffeine, caffeine's metabolites, specifically paraxanthine, can also affect behaviour and physiology (Fredholm et al., 1999). In humans, caffeine reaches maximum concentrations within the blood 45 – 120 minutes after consumption, and it's half life can extend to 4.5 hours in the body (Fredholm et al., 1999). However, in infants and fetuses, this half-life is much higher, and given that caffeine can move through the

placenta and has been found in neonate plasma, there is ongoing concern about the effects of caffeine on fetus and newborn development (Fredholm et al., 1999; Thorn et al., 2012). Ingesting more than 100 mg of caffeine a day during pregnancy may cause fetal growth restriction (i.e., birth weight lower than 10th centile, with maternal factors such as height and number of previous births taken into account), and ingesting more than 200 mg of caffeine a day during pregnancy can decrease birth weight (CARE Study Group, 2008). While vertebrates generally share many of the same neural pathways, the main metabolites of caffeine can differ from those in humans depending on the species in question, and the effects of those metabolites may also differ to those observed in humans (Fredholm et al., 1999). Additionally, the half life of caffeine can differ widely depending on the species, affecting how quickly effects of the chemical are observed as well as how long those effects last. For example, caffeine in mosquito fish (*Gambusia holbrooki*) had a half life of ~140 hours (Wang & Gardinali, 2013), while the half life of caffeine in Nile tilapia (*Oreochromis niloticus*) was ~ 5 hours (Gómez-Martínez, 2011).

Caffeine in the brain

Caffeine's effects in the brain are complex and not fully understood. Most of the physiologic and behavioural effects of caffeine are due to its role as an adenosine receptor antagonist (Ribeiro & Sebastião, 2010). Adenosine is a naturally occurring neuromodulator – a chemical that alters how signals between neurons act (Fredholm et al., 2005). There are multiple types of adenosine receptors, some of which act in opposing ways. For example, the A1 adenosine receptor stops adenylate cyclase, an enzyme that converts ATP (energy) to cAMP (signal molecule), from working (Daly, 2007). However,

the A2 adenosine receptor stimulates adenylate cyclase, thus encouraging the conversion from ATP to cAMP (Daly, 2007). The many different effects of each of these affected receptors highlight how difficult it is to understand caffeine's mechanism of operation.

Caffeine is thought to impact calcium signalling, which is a crucial part of cell function (Bootman et al., 2001). The A3 adenosine receptor stimulates phospholipase C which turns PIP3 into IP3, a messenger molecule that stimulates calcium release (Daly, 2007). However, while caffeine, acting as an adenosine receptor antagonist to A3, inhibits the creation of IP3, it has also been shown to help calcium move through cells (Daly, 2007), so these pathways may 'cancel' each other out, resulting in little change in the brain due to caffeine. GABA (Gamma-Aminobutyric Acid) is another important neurotransmitter in the brain that also is thought to be affected by caffeine exposure (Herden & Weissert, 2018). GABA, like adenosine, decreases wakefulness and increases relaxation (Herden & Weissert, 2018), and caffeine decreases GABA concentrations, leading to increased wakefulness and activity (Herden & Weissert, 2018). The neural pathways and mechanisms highlighted above are of great interest in therapeutic drug development, as many of these pathways are involved in chronic, potentially debilitating conditions such as asthma, diabetes, Alzheimer's, Parkinson's, and epilepsy (Daly, 2007).

Caffeine and cognition

The A2-adenosine receptors, which are inhibited by caffeine, are located in places within the brain where there are a lot of dopamine receptors (Fredholm et al., 1999), which may result in an increase in dopamine activity. Dopamine is a neurotransmitter that

helps to control learning in the brain and is used to signal the importance of information in the brain (Berke, 2018; Iversen & Iversen, 2007). However, while antagonism of the A2-adenosine receptor increases dopamine receptor activity, antagonism of the A1-adenosine receptor may diminish any effects (Fredholm et al., 1999), again highlighting the complexity of caffeine's effects in the brain and on cognitive processes.

Caffeine's effects on behaviour, cognition and physiology may differ depending on acute or chronic exposure. For example, chronic exposure to caffeine may upregulate adenosine receptor formation, meaning more caffeine needs to be ingested or consumed to see the initial stimulatory effect seen with an acute dose (Jacobson et al., 1996). However, other pathways also affected by caffeine may be so adversely impacted by the time chronic exposure is reached that any stimulatory effect may be masked by those harmful effects (Jacobson et al., 1996). While some antagonism of adenosine receptors (specifically A1) may aid in cognition, the complexity of the neural pathways affected by caffeine make it difficult to parse out its effects, highlighting the ongoing need for research in this area (Jacobson et al., 1996).

In humans, caffeine can impact a multitude of behavioural and cognitive endpoints, although these results are often not consistent across the literature. Caffeine may aid in vigilance for simple tasks, especially when there is an underlying deficit such as little sleep, by decreasing fatigue and increasing wakefulness (Smith, 2002). Despite these benefits, caffeine may increase subjective feelings of tenseness and anxiety in individuals (Loke et al., 1985). Furthermore, while caffeine is often thought to disrupt sleep, this may be more true for individuals who only consume caffeine periodically

rather than those who habitually use it (Smith, 2002). In addition to behavioural endpoints, caffeine may affect physiological endpoints as well, as evidenced by interest in caffeine as an ergogenic, a substance that could improve athletic performance. A meta analysis found that caffeine ingestion improved timed rowing trials and increased power slightly (Grgic et al., 2020). A meta analysis found that acute caffeine ingestion might affect performance on a timed trial for cycling or running, but that endpoints such as muscle power and maximum running distance were not impacted (Gonçalves Ribeiro et al., 2017). Despite its potential benefits, caffeine can also have adverse effects on physiology and performance, such as inducing hand tremors (Loke et al., 1985).

Given the conserved neural networks between species, learning outcomes may also be impacted in non-human animals. In one study, zebrafish exposed to 10 mg/L caffeine quickly found the target pattern in a discrimination/reward study, while those exposed to 50 mg/L had more difficulty finding the target pattern (Ruiz-Oliveira et al., 2019). However in another study, acute caffeine exposure at high concentrations (50 mg/L, 100 mg/L) didn't appear to impact discrimination of zebrafish in a memory assay (Santos et al., 2016). While identifying a clear pattern in how caffeine affects learning in fishes may be difficult, in invertebrates, the results are clearer. At low concentrations, caffeine aids cognition in invertebrates, especially in complex tasks and assays, while at high concentrations, caffeine adversely impacts cognition (Mustard, 2014).

Caffeine and physiology

The physiological effects of environmental contaminants are important not only for understanding which body systems are being most affected by the chemical of interest, but for also providing a broader mechanistic explanation for why behavioural endpoints may or may not be affected by a contaminant (Scott & Sloman, 2004). Caffeine is commonly used as a stimulant in pre-term human babies to increase their oxygen consumption and energy expenditure (Bauer et al., 2001). In one study on women, caffeine consumption increased metabolic rate during and 1 hour after exercise (Chad & Quigley, 1989). However, in mice, while caffeine consumption increased wheel running, it did not affect VO2 (maximum oxygen consumption), indicating that the increase in wheel running may be due to its adenosine antagonist role, rather than a change in oxygen consumption or metabolism (Claghorn et al., 2017).

Caffeine in fish

Aquatic organisms, including fish, are uniquely positioned to be exposed to caffeine given that caffeine enters aquatic ecosystems via wastewater effluent. Caffeine may have a variety of effects on fish behaviour and physiology, and many of these are dependent on dose and exposure period. For example, caffeine increased erratic movements during an acute exposure at high concentrations of caffeine (i.e., 100 mg/L; Egan et al., 2009; Wong et al., 2010), but caffeine did not alter erratic movements after 1 week of exposure under a lower acute exposure concentration of 50 mg/L (Cachat et al., 2010). Additionally, acute caffeine exposure impacted larval zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) photomotor-related endpoints and swimming

activity, even at relatively low concentrations (i.e., 39,000 ng/L caffeine; Steele et al., 2018).

In fishes, caffeine has been shown to affect activity and anxiety endpoints, with exposure to high concentrations of caffeine decreasing activity (de Farias et al., 2021; Santos et al., 2016; Tran et al., 2017; Wong et al., 2010). Acute caffeine exposure at high concentrations (50 mg/L, 70 mg/L, 100 mg/kg) increased time spent unmoving, frozen in place in zebrafish (Maximino et al., 2014; Neri et al., 2019; Santos et al., 2016); however, fish exposed to slightly lower concentrations (10 mg/L) of caffeine for a longer period (1 week) spent less time frozen (Ruiz-Oliveira et al., 2019) and chronic exposure (1 week) to the high concentrations (50 mg/L) also had no impact on freezing time (Cachat et al., 2010). These results highlight how exposure duration and concentration are both important factors dictating how caffeine affects fish behaviour. While swim distance and time were increased in zebrafish exposed to a lower concentration of caffeine (0.0006 mg/L caffeine), activity decreased with increased exposure time (de Farias et al., 2021). And while caffeine exposure at high concentrations (25, 50, and 70 mg/L) decreased swim speed in zebrafish (Ladu et al., 2015; Neri et al., 2019; Santos et al., 2016), exposure at a slightly lower concentration (10 mg/L) increased swim speed in zebrafish (Ruiz-Oliveira et al., 2019). Again, these results highlight how caffeine exposure period and concentration interact to impact behavioural endpoints.

Caffeine has been shown to impact fish anxiety using assays similar to the one I employed (see Chapter 2). For example, zebrafish exposed to acute high concentrations of caffeine (100 mg/L and 50 mg/L, respectively) displayed 1) less movement between the

top and bottom half of tank (Egan et al., 2009; Ladu et al., 2015), 2) a longer latency to enter the upper half of the tank (Egan et al., 2009), and spent more time at the edge of the behavioural assay container (10-100 mg/L; Richendrfer et al., 2012), all indicators of increased anxiety. However, with a longer exposure period (i.e., 1 week), these anxiety indicators disappeared (Cachat et al., 2010). After exposure to high concentrations of caffeine, zebrafish spent less time in the 'risky' white compartment of their tank (100 mg/kg exposure) and spent more time in thigmotaxis (hugging the walls) in a behavioural assay tank (1-100 mg/kg; Maximino et al., 2014). Thigmotaxis, or edge-seeking behaviour, is thought to be an indicator of anxiety in zebrafish (Richendrfer et al., 2012). The fish also spent more time assessing risk (i.e., making quick forays into white compartment, then back to black) when exposed to 10 and 100 mg/kg of caffeine (Maximino et al., 2014). In these examples too, caffeine concentration and exposure period modulated how caffeine affected anxiety.

Caffeine may also impact social interactions. For example, adult jewel fish (*Hemichromis bimaculatus*) exposed to 14 mg/L caffeine for 50 days had increased variability in their spacing around other conspecifics when in groups of 10 fish (Burgess, 1982). However, to date, few studies have explored the impact of caffeine on group dynamics.

1.3 Associative and reversal learning

How caffeine influences learning is an area of general interest because learning can have a central role in animal lives, influencing their fitness (Brown & Laland, 2001; Suboski & Templeton, 1989). In fishes, learning can play an important role (Kieffer &

Colgan, 1992) and appears to be especially critical during migration and foraging as well as having a central role in developing capacity for conspecific recognition and predator avoidance. Fishes' ability to learn is also of interest in the context of fisheries management. Hatchery fish often suffer deficits when released into the wild due to a lack of crucial stimuli and the necessary interactions for learning how to avoid predators (Brown & Laland, 2001). There has been an increasing push to 'train' hatchery fish on some of these stimuli before they are releasing them into the wild to improve survival rates (Edwards et al., 2021).

Associative learning is the learning of an association between a stimulus and reward through repetition (Pearce & Bouton, 2001). Reversal learning is the learning of an opposite or alternate task from the initial task by switching the outcome (Izquierdo et al., 2017). While often thought to be a measure of inhibitory control, reversal learning is now more commonly thought to be a measure of cognitive flexibility, or the ability of individuals to adapt to a change in their environment (Izquierdo et al., 2017). For example, reversal learning paradigms were used to measure cognitive flexibility in male and female guppies (*Poecilia reticulata*), with females being more cognitively flexible (able to reversal learn faster) than males, potentially due to the high social connectivity they have when compared to males (Lucon-Xiccato & Bisazza, 2014). Another study used a reversal learning assay to test if the telencephalon (the fish forebrain) was involved in cognitive flexibility, with goldfish (*Carassius auratus*) performing more poorly in reversal learning if their telencephalon was ablated, but performing normally in the initial associative learning task, indicating that the telencephalon in fish does play a part in

cognitive flexibility (López et al., 2000). Associative and reversal learning can uncover how different factors, such as sex, or exposure to contaminants may affect long-term survival and fitness.

1.4 Anxiety

Anxiety is an important behavioural endpoint because it may have significant ecological implications. For example, larval three-spined stickleback (*Gasterosteus aculeatus*) without male parental care displayed more anxiety (i.e., nosing at the sides of the assay tank) than larvae with male parental care, which made them more likely to be predated upon than individuals with male parental care (McGhee & Bell, 2014). While increased anxiety could decrease foraging and mating success, affecting fitness, decreased anxiety could render individuals more conspicuous to predators and result in higher mortality (Reyhanian et al., 2011). In recent years, anxiety has emerged as a common endpoint utilised in ecotoxicology for assessing how environmental contaminants affect fish behaviour (Hong & Zha, 2019; Huerta et al., 2016; Meijide et al., 2018; Reyhanian et al., 2011).

1.5 Aims of the thesis

The aim of this thesis was to explore how environmentally relevant concentrations of chronic caffeine exposure affected the (1) complex behaviour, (2) physiology, and (3) morphology in fathead minnow. Most previous research examining the effects of caffeine have exposed fish to caffeine concentrations that are much higher than those in found in the environment, thus limiting their potential to explain the effects of caffeine *in-situ*. Previous studies also often use short exposure periods (e.g., 15 minutes), while fish in the

environment are exposed to caffeine for longer time periods, even potentially their entire lives. While caffeine has been shown to have complex effects on the behaviour of humans, rodents, and even some fish, most studies utilise simple behavioural endpoints, such as c-start response. Finally, much of the research uses the tropical zebrafish (*Danio rerio*), the results of which may or may not be applicable to temperate species. To address these gaps, we exposed fathead minnow (*Pimephales promelas*), a common freshwater fish found in much of North America, to environmentally relevant concentrations of caffeine for 35 days. We used an aversive trawl assay and an anxiety assay to study complex behavioural responses to exposure. The motivation for using these tools and model species for my MSc research is described in detail below.

1.6 Study species: Fathead minnow

Fathead minnow are a common ecotoxicological model used by government and industry to assure compliance of environmental regulations as well as to study the toxicological effects of pollutants (Ankley & Villeneuve, 2006). Many previous studies have used fathead minnow to study the effects of different environmental contaminants, including ethynyl estradiol (Palace et al., 2002), mercury (Grippo & Heath, 2003), insecticides (Beggel et al., 2010), and microplastics (Bucci et al., 2022). Fathead minnow are a widely distributed freshwater species found across North America (Ankley & Villeneuve, 2006; Held & Peterka, 1974). Adults are small, generally 2-5g in mass (Ankley & Villeneuve, 2006) and sexually dimorphic and dichromatic, with males growing larger and developing dark vertical banding on their trunk, nuptial tubercules on their snout, and a dorsal fat pad along the dorsum (Smith, 1974; Smith & Murphy, 1974).

Females develop an ovipositor behind their anal fin to deposit eggs (Flickinger, 1969). Fathead minnow in the wild typically live in shallow, muddy environments with vegetation (McMillan & Smith, 1974), and are omnivores, generally consuming macroinvertebrates, plankton, and plants (Scott, 1954). Their wide distribution (Ankley & Villeneuve, 2006), small body size (Ankley & Villeneuve, 2006), sexual dimorphism (Smith, 1974; Smith & Murphy, 1974), tolerance to handling (Ankley & Villeneuve, 2006), short life-spans (Jensen et al., 2001), and ease of observation in the wild (Ankley & Villeneuve, 2006) make them a particularly useful model species and a fairly easy species to collect, house and rear in the laboratory.

To this author's knowledge, fathead minnow have only been made use of in two previous caffeine studies. The first study revealed that the LC50 caffeine concentration (the lethal concentration that kills 50% of individuals) for larval fathead minnow was on average, 100 mg/L after 48 hours of exposure, and 55 mg/L after 7 days of exposure (Moore et al., 2008). Another study that used fathead minnow larvae, exposed them to varying environmentally relevant concentrations of caffeine for 96 hours, and then tested their activity during repeating photoperiods (Steele et al., 2018). Larval fathead minnow exposed to 39,000 ng/L of caffeine froze (i.e., stayed unmoving) more during dark photoperiods than other exposed and control larvae (Steele et al., 2018). However, how adult fathead minnow are affected by environmentally relevant caffeine concentrations, and more specifically how chronic caffeine exposure affects more complex behavioural endpoints, such as risk avoidance and learning, has not been examined before.

1.7 Assays employed

In my MSc research I made use of an aversive assay to quantify learning, a change in behaviour that occurs as a result of past experience (Dill, 1983). The assay entailed moving a trawl (a big net) across the tank. Fish tried to avoid this trawl and to get away from it. I used this assay to ask how long it takes fish to learn to swim though a hole, reach a refuge, and avoid the trawl. This assay provided a relatively easy way to study how fish learn and could be simply altered to include a reversal learning paradigm in order to measure cognitive flexibility. The set up I used was like the one described in Lindeyer and Reader (2010). The fish encountered a moving trawl and had to escape from it and reach a refuge zone by swimming through a "correct" exit in a transparent barrier. The transparent barrier separating the dangerous trawl zone and the refuge zone had two identical sized exit holes. The "correct" hole was open and the "wrong" hole was closed (with a transparent barrier). The holes were outlined in black, and a rock illustration was placed near one of the two holes while a plant illustration was placed near the other hole to provide additional learning cues. Half the fish learned that the correct hole was initially the rock hole while the other half the fish learned that the correct hole was the plant hole. After reaching the learning threshold, fish began a reversal learning phase of the experiment.

I used a scototaxis assay, to quantify anxiety, an assay derived from light-dark box test, originally used in rodent studies (Maximino et al., 2010; Stewart et al., 2012). In the light-dark box test, the assay tank is split in half, with one side in black and the other side in white (Maximino et al., 2010). The fish's affinity for the black side of the tank can be

used as a proxy for anxious behaviour, and their affinity for the white side is used as a proxy for boldness (Maximino et al., 2010). We used a black square placed in the corner of the assay tank, while the rest of the tank was white (Polverino et al., 2021). The amount of time fish spent on the black square was used as a proxy for anxiety, and the amount of time spent on the white background was used as a proxy for boldness, or anti-anxiety behaviour (Polverino et al., 2021).

1.8 Structure of the thesis

The central question of my MSc work was how environmentally relevant caffeine exposure affects a local and common fish, the fathead minnow, a species that is likely to encounter caffeine in the wild. To address this overarching question, I had a number of smaller questions aimed at uncovering how exposure to environmentally relevant concentrations of caffeine affect fathead minnow (1) morphology, (2) physiology, and (3) complex behaviour (learning and anxiety). In Chapter 2 of my thesis, I present the major research output of my MSc where I exposed fathead minnow to environmentally relevant concentrations of caffeine for 35 days and assessed their morphology, physiology (i.e., metabolic rate), and complex behaviour (i.e., aversive learning and anxiety). In Appendix I, I include a manuscript in preparation that describes a study about how learning differs between captive and wild caught fathead minnow. These two studies shed light on how a common aquatic contaminant, caffeine, and captivity affects a well established ecotoxicological model species, the fathead minnow.

1.9 References

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CHAPTER 2. Impacts of Caffeine on Fathead Minnow Behaviour and Physiology

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2.1 Abstract

Caffeine, a commonly ingested stimulant, can be found at high concentrations in many water bodies worldwide. Caffeine's prevalence is a result of incomplete removal during wastewater treatment, and so traces of caffeine typically remain in the wastewater effluent. Aquatic organisms that live downstream of wastewater effluent, can be exposed to caffeine throughout their lives. To date, little research has been conducted on the morphology, behaviour, and physiology of aquatic organisms exposed to ecologically relevant levels of caffeine. To address this gap in our knowledge, we exposed fathead minnow (*Pimephales promelas*) to three treatments: a low environmentally relevant dose (nominal: 1,000 ng/L), a high but still environmentally relevant dose (nominal: 10,000 ng/L), and a control (nominal: 0 ng/L). We then tested the learning abilities of exposed fish, and in particular how they avoid an aversive stimulus (a trawl). We also tested the anxiety of exposed fish using a scototaxis assay and the morphological and physiological traits of exposed fish. We found that caffeine increased the anxiety of fish at low concentrations but did not alter their anxiety at high concentrations. Caffeine exposure did not affect the ability of fish to learn, nor did it affect their metabolism. While caffeine did not affect fish growth, body length, or mass, it did decrease their liver investment (Hepatosomatic Index), but this response was only observed at low concentrations of caffeine exposure. Overall, our results suggest that even relatively low concentrations of caffeine may impact the behaviour and liver size, but further research is now needed to assess how caffeine, an extremely widespread contaminant, impacts fish behaviour, reproduction, and survival.

2.2 Introduction

The average human adult consumes 135 mg of caffeine a day, mostly by drinking coffee (Drewnowski & Rehm, 2016), and caffeine consumption is only expected to increase as the world's population grows (Quadra et al., 2020). In humans, caffeine can have positive desired effects, such as increasing wakefulness and improving performance in simple tasks (Smith, 2002); however, at high doses, caffeine can make it difficult to fall asleep and can increase anxiety (Smith, 2002). Many of the physiological and behavioural effects of caffeine are a result of it being an adenosine receptor agonist; adenosine is a biomolecule that builds up while awake and promotes sleepiness (Bjorness & Greene, 2009; Smith, 2002). Once caffeine is consumed, it is broken down by the body and is excreted in urine along with its three metabolites (paraxanthine, theobromine, theophylline; Heckman, Weil, and Mejia 2010). Caffeine and its metabolites then make their way to wastewater treatment plants (WWTPs) or can directly enter into the environment wherever wastewater management infrastructure does not exist. Although wastewater treatment is common worldwide and is extremely efficient (removing 95% of the caffeine; Li et al. 2020), because such large quantities of caffeine are consumed daily, the remaining 5% still amounts to a lot of caffeine entering aquatic environments on a global scale, creating a constant exposure to caffeine for many aquatic organisms.

Many studies have examined how caffeine exposure can influence behaviours in animals; however, most of these studies have explored simple behaviours, such as movement, and only a handful of studies have investigated how caffeine can affect learning. Learning is a change in behaviour that occurs as a result of past experience

(Dill, 1983). The ability to learn is often viewed as an adaptive trait, especially in changing or challenging environments, and can be especially critical for migration, predator avoidance, mate choice, and foraging (Kieffer & Colgan, 1992). As such, any factor that influences learning may have crucial downstream effects. Because (at low doses) caffeine increases alertness it may help individuals focus and facilitate learning; however, because (at higher doses) caffeine can cause anxiety, it may also impair learning (Angelucci et al., 1999; Nehlig, 2010). In this study, we explore if and how chronic caffeine exposure affects memory, learning, and anxiety in fishes.

Most studies investigating the effects of caffeine exposure on various behaviours in fish have used extremely high concentrations of caffeine (ranging from 5 – 100 mg/L; Egan *et al.*, 2009; Wong *et al.*, 2010). However, the highest concentration of caffeine ever detected in freshwater was 19.3 µg/L (Li et al. 2020). Additionally, most caffeine studies employ short-term exposures, ranging from 15 minutes (Egan et al., 2009; Neri et al., 2019) to 96 hours (Steele et al., 2018). It is possible that such short exposures will not yield environmentally relevant responses since fish can acclimate to caffeine concentrations over time (Santos et al. 2016). Finally, most caffeine exposure studies have been conducted on zebrafish, (*Danio rerio*; Santos et al. 2016; Ladu et al. 2015; Neri et al. 2019; Egan et al. 2009; Wong et al. 2010; Maximino et al. 2014; Tran et al. 2017). Although zebra fish are easy to maintain in the lab and are therefore a popular model organism, they are tropical, and we know less about how caffeine influences organisms that live in temperate climates. To address all these shortcomings we determined how *long* exposures (35 days) to *environmentally relevant concentrations* of caffeine affects

learning, anxiety, and physiology in the fathead minnow (*Pimphales promelas*), a temperate species with a wide freshwater distribution throughout North America (Scott, 1954) that is also a common ecotoxicological model (Ankley & Villeneuve, 2006).

To test how caffeine exposure affects learning, we trained fathead minnow with an aversive learning assay. Based on a design used in previous studies (Attaran et al., 2020; Lindeyer & Reader, 2010), we used a trawl as an aversive stimulus, and first trained fish to avoid getting caught in the trawl by exiting the trawl area through one of two exit holes. Once the fish learned to do this, we then tested their ability to forget this first task and learn to exit a different, and previously blocked, exit hole (reversal learning). Reversal learning is considered to be a more cognitively complex task than associative learning, and is often used as a measure of behavioural flexibility (Buechel et al., 2018; Izquierdo et al., 2017). We considered our learning assay to be a measure of complex behaviour, as multiple decisions and reactions are made in quick succession during the assay (e.g., assessing the threat of the trawl, looking for an exit; American Psychological Association, 2022). In contrast, innate behaviours are simpler and do not require prior experience or learning (American Psychology Association, 2022).

To test how caffeine influences anxiety, we used a scototaxis assay modified from Polverino et al. (2021) where fish are placed on a white-bottomed tank containing a single small black square. Because individuals are often more conspicuous to predators on light backgrounds, many species prefer to spend time on dark-coloured, “safer” backgrounds (Maximino et al., 2010). Therefore, in our experiment we used the amount of time fish

spent on the white background as a proxy of boldness, and conversely the amount of time the fish spent on the black square as a measure of anxiety.

Finally, because caffeine consumption can increase resting and active metabolic rates (Poehlman et al. 1985; Chad and Quigley 1989; Yoshioka et al. 1990, Donelly and McNaughton 1992), we used a respirometer to measure how chronic caffeine exposure influences oxygen consumption, which is used as a proxy for metabolic rate.

We predicted that caffeine exposure at the environmentally relevant concentrations would (1) increase anxiety so that fish exposed to caffeine would spend more time in the black square and also move less between the black and white spaces and (2) the increased anxiety would make it more difficult for fish to associative and reversal learn, as they would be less likely to explore the trawl assay and try to find an exit. However, if caffeine increases metabolism in fishes, as it does in humans (Bauer et al., 2001; Chad & Quigley, 1989), we could also propose opposing predictions. If caffeine exposure increases metabolism and activity, then more active fish are likely to associative and reversal learn faster and better because active fish would explore the assay tank more and be more likely to find the open exit. If caffeine exposure indeed increases metabolism and activity, then we also predicted reduced growth in exposed fish as all fish received a similar amount of food.

2.3 Materials and Methods

Study animals and housing

In November 2020 we purchased and transported one-month-old fathead minnow from AquaTox Inc. Puslinch, Ontario to McMaster University, Hamilton, Ontario. In the lab, the fish were initially housed in two 150 L tanks for six months and then were placed into one of nine 70 L exposure tanks. These exposure tanks contained two static renewal sponge filters (AquaClear, Marina) and water temperature was kept at 17-24°C and 13 h:11 h light: dark (0600 – 1900, local time). We fed fish daily with a mixture of frozen brine shrimp, frozen bloodworm, and juvenile trout pellets ad libitum. Seven to eight days prior to the start of caffeine exposures, we tagged fish using acrylic paint (Capelle et al., 2015; Wolfe & Marsden, 1998) so we could follow individuals in the behavioural assays and measure individual growth. All animal protocols were developed in accordance with guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board (AUP # 17-12-45).

Caffeine exposures

We conducted the exposure study between June – August 2021. We exposed fish to either a low environmentally relevant dose (1,000 ng/L caffeine), a high environmentally relevant dose (10,000 ng/L caffeine), or to no added caffeine (controls) for 35 days. The low environmentally relevant dose was based on the concentration of caffeine found at sites sampled immediately downstream of a wastewater treatment plant (McCallum et al., 2017). The high environmentally-relevant dose (10,000 ng/L caffeine)

was based on concentrations of caffeine found in an urban river (Mokh et al., 2017). Each caffeine treatment contained three replicate tanks, with 33 fish in each replicate tank (99 fish per treatment, *N=297*). We ran the experiment in a staggered fashion, with one replicate tank of each treatment beginning every three weeks, and each replicate beginning on a different day of the week. We dosed the caffeine tanks to the appropriate treatment level with a concentrated stock solution (1g/L caffeine) made using Millipore Sigma ReagentPlus caffeine powder (C0750) and dechlorinated Hamilton municipal tap water. We refreshed this stock solution daily to ensure that the dose remained consistent. We performed 20% water changes every 72 – 96 hours, refreshing the tank with dechlorinated Hamilton water spiked to the appropriate dose. We removed activated carbon and Bio Ball filter media from the static renewal filters prior to caffeine exposures to prevent the uptake of caffeine by the filter media. To reduce aerosol contamination of caffeine, we put tight-fitting lids on all of the exposure tanks and used treatment-specific nets and water change equipment.

We collected paired samples of the exposure water throughout the experiment, with the first sample one-hour post-dose of the water change, and the second sample 72 or 96 hours later (immediately preceding the next water change/dose). We collected samples in 100mL amber glass bottles, then preserved them with 200g/L sodium azide and 20 g/L ascorbic acid. We stored samples at 4°C for 0 - 3 days (mean: 1 day) until the sample could be extracted and analysed at the University of Waterloo (Waterloo, Ontario). We sampled each tank twice throughout the experiment (one-hour post-dose and 72-96 hours post-dose). Caffeine in each sample was concentrated using solid phase extraction, and

then each sample was analysed via an Agilent 1260 HPLC with 6460 triple quad mass spectrometer (LC-MS/MS) with Agilent Jet Stream (AJS) electrospray ionization in both positive and negative modes (Mehdi et al., 2022).

Anxiety assay

To test how caffeine exposure affected anxiety, we ran fish through a scototaxis anxiety assay, modified from Polverino et al. (2021; Figure 2.1). Fish were placed individually in 19 L tanks (41 cm x 21 cm x 25 cm) that were filled with 10 L of aerated Hamilton dechlorinated water and dosed with caffeine, to match the exposure regimen. The bottom and sides of the anxiety tanks were lined with white, except for a single 17 cm x 17 cm black square, that was placed in one of two possible bottom corners furthest from the observer. To reduce caffeine contamination, treatment-specific tanks and equipment were used, and plastic sheeting was used in between tanks to prevent aerosol contamination. Individual focal fish were placed in a black PVC refuge tube placed vertically in the middle of the anxiety tank. After a 2-minute acclimation period, we removed the PVC refuge tube as well as the airstone in the tank and videotaped the fish for 15 minutes.

We ran about 15-20 fish per replicate tank, with three replicate tanks for each of the three treatments ($n=135$ fish total) through this assay three times: once 6 – 7 days pre-exposure, once 7 days after exposure began, and once 21 days after exposure began. Note that the control group had slightly more fish during pre-exposure testing ($n=20$) than the other groups ($n=15$) because we originally had intended to run all our fish through the

anxiety assay and opted to cut back and reduce the sample size to 45 fish per treatment due to time limitations. Twenty-one trials were removed due to missing or incomplete videos. Of these removed videos, 10 were from the control group, 6 were from the low group, and 5 were from the high group. The majority were from the pre-exposure group (10 videos), with only 6 videos from the Day 7 trial, and only 5 videos from the Day 21 trial. Videos were scored by trained observers (MD, MV, CD, JB) who were unaware of the treatment. Observers used BORIS (Friard & Gamba, 2016) and recorded (1) the amount of time the fish spent on the black square vs. the rest of the tank and (2) how many times the fish moved on and off the black square (number of transitions).

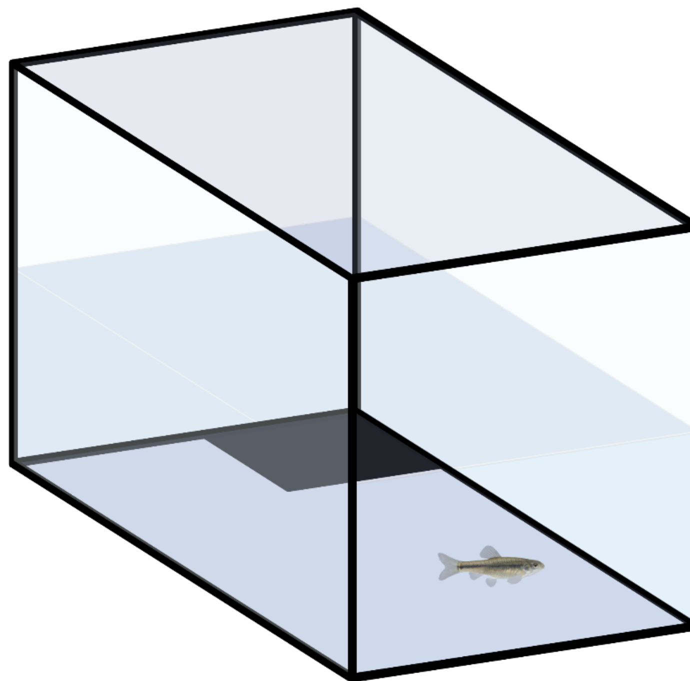

Figure 2.1 Scototaxis assay utilising a tank with a black square in the corner, and the rest of the bottom of the tank papered in white. The tank was filled with 10 litres of water.

Aversive learning assay

To investigate how caffeine exposure affected learning, we used a trawl assay modified from a design used by Attaran et al. (2020) and Lindeyer & Reader (2010). The learning trials were run in 38 L tanks (51 cm x 25.5 cm x 29 cm) divided in half by a transparent acrylic divider with two round horizontally adjacent exit holes at the bottom of the divider (1 cm from bottom). The exit holes were outlined in black to make them more visible, and we drew a plant next to one exit and a rock next to the other exit to aid with discrimination. On one side of the divider there was a black mesh trawl attached to a frame with gliders on the top which allowed us to pull the trawl back and forth through this 'trawl zone'. On the other side of the divider there was one sponge filter that was used as shelter by the fish and also maintained water quality (Figure 2.2). We filled each learning assay tank with 24 L of water dosed with caffeine, if required, to match the treatment that the fish had been exposed to. To reduce caffeine contamination, treatment-specific tanks and capture equipment were used, and plastic sheeting was placed between treatment tanks to prevent aerosol contamination. Based on pilot studies, we decided to run the fish through the learning trials in groups of three individuals as having companions significantly reduced stress and maximized movement and exploration behaviours. We performed 20% water changes of the learning tanks every 72 – 96 hours.

Associative Learning Phase: At least one hour prior to the first training session, we moved three focal fish from their chronic exposure tanks to the learning tanks, placing them in the refuge zone with both exits covered. We tried to choose a large fish, a medium-sized fish, and a small fish so that we could visually discriminate between the

fish later, when scoring behaviour using the video recordings. To begin the training session, we uncovered one exit; this same exit would be uncovered for all training sessions in this associative learning phase. We randomly chose the exit we uncovered for the associative learning phase. Fish were placed into the trawl zone and given five minutes to acclimate to allow them to recover from being moved between the refuge and trawl zone. We then moved the trawl every 30 seconds back and forth in the trawl zone four times within a two-minute period. Any fish remaining in the trawl zone after the four passes was guided with a hand net into the refuge zone through the uncovered exit. We allowed fish to rest in the refuge zone for two minutes, then moved them back to the trawl zone and let them rest there for two minutes. We repeated this trial protocol three more times, for a total of four trials per training session. We ran two training sessions per day, with at least one hour between each session. Fish were considered to have 'learned' if all three fish exited the trawl zone through the uncovered exit within the two-minute trial and did this in four consecutive trials in a row. If the fish "learned" before the end of the seventh day in the assay, they moved on to reversal learning. If the fish had not learned after seven days, they did not move on to the reversal learning phase.

Reversal learning phase: After fish associative learned, as defined above, we switched to reversal learning and did so by uncovering the previously covered exit and covering the exit used in the associative learning phase. We ran the above protocol until the fish 'reversal learned,' or until seven days in total had passed. We defined reversal learning in the same manner as in the associative learning phase; all three fish had to exit

the trawl zone through the uncovered exit within a two-minute period and do so four consecutive times.

Video analysis: To analyse these learning videos, researchers (JQ, FA, HS, JB), who were unaware of the exposure treatment, scored the videos using BORIS (Friard & Gamba, 2016) and recorded (1) the *number of trials* it took the fish to associative learn and reversal learn, and (2) the *latency to exit* the trawl zone during each trial.

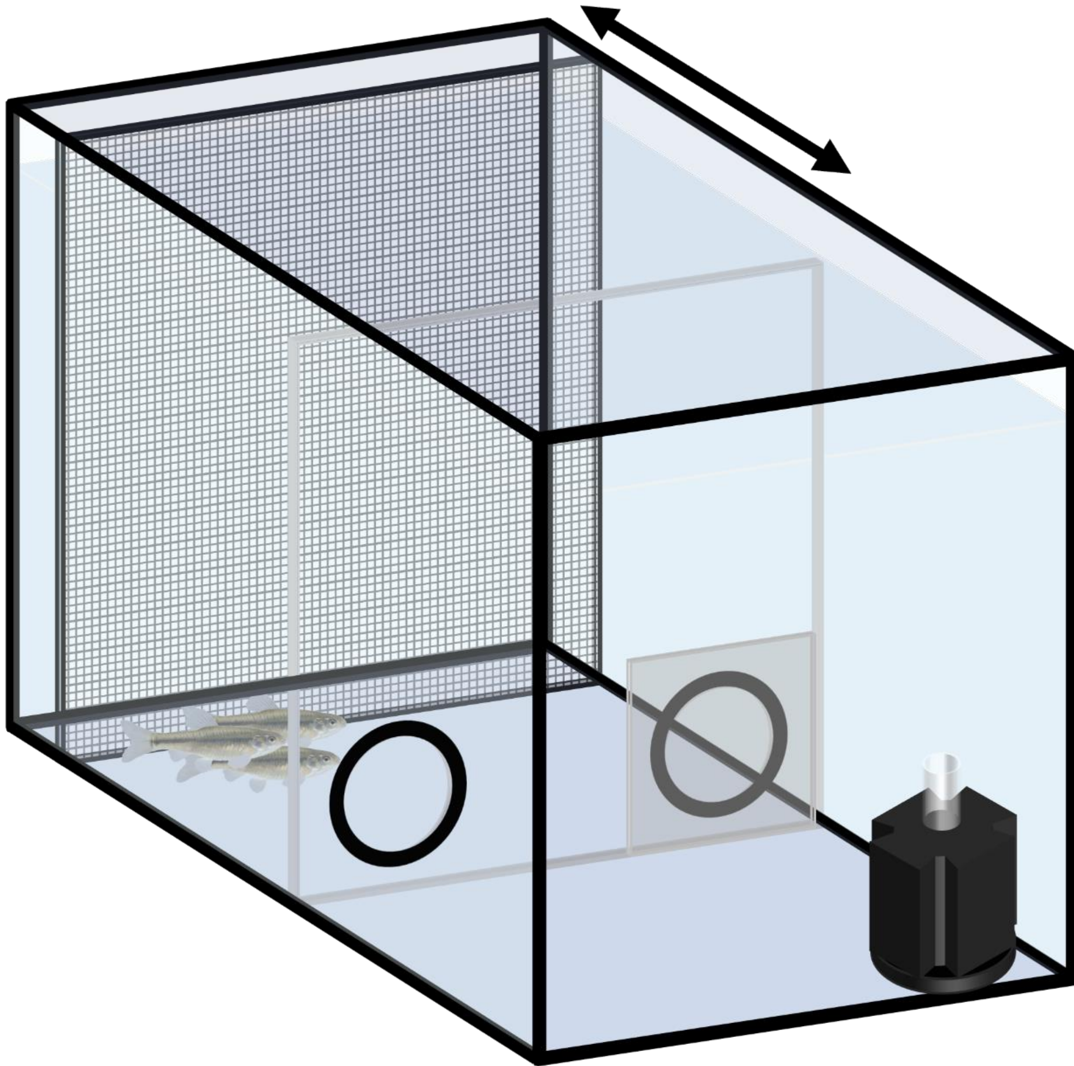

Figure 2.2 Diagram of the trawl assay. The trawl zone contains a moveable trawl, and the refuge zone contains a sponge filter. The tank is divided in half by a transparent barrier with two exit holes in it, one of which is covered by a transparent plastic barrier. Both holes were outlined in black, and while not displayed here, one exit had a plant drawing next to it, while the other had a rock drawing next to it. Once the fish associative learned (see learning criteria above), the exit that was open and the exit that was closed were switched.

Physiological measures

We measured metabolic rate using an intermittent flow respirometry system by running 4 fish on Day 34 of exposure, and another 4 fish on Day 35, for a total of 8 fish per treatment. We placed four fish individually into one of four ~75 ml respirometry chambers all placed in a dark tank at room temperature (~20°C) using dechlorinated Hamilton municipal tap water. We did not maintain caffeine exposure treatments during the respirometry testing. We placed fish in the respirometry chamber with alternating 5-minute flush periods and 5-minute measurement periods. During the 5-minute flush periods, water from the open tank would enter the chambers to refresh the oxygen concentrations and prevent hypoxic conditions from forming. During the 5-minute measurement period, the chamber was cut off from all external water and oxygen, and the exact oxygen consumption of the fish could be measured. We measured oxygen consumption during the 5-minute measurement periods using a FireStingO_2 (Pyro Science, Germany). We assessed oxygen consumption overnight to measure standard metabolic rate and calculated standard metabolic rate using the lowest 10 measurements from this time period.

To assess maximum metabolic rate, we removed fish from the respirometry chamber, placed them in a round assay tank (diameter = 46 cm), then chased them in a standardized manner with a hand net for three minutes followed by a 30 second air exposure to simulate the stress and activity of a predator chase (Mehdi et al., 2022). We then placed the recently chased fish back in the respirometry chamber and measured maximum metabolic rate for 10 minutes. We calculated maximum metabolic rate using

the highest 10 measurements from this time period. We calculated aerobic scope by finding the difference between the maximum metabolic rate and the standard metabolic rate for each individual. Fish were fasted for at least 24 hours prior to any metabolic rate measurement, and these measurements were corrected for the temperature inside the respirometry chambers.

Morphological measures

We humanely killed fish on Day 35 of exposure by placing them in a benzocaine bath, then quickly severed their spinal cords. We then measured and weighed the fish to gather the body mass, total length (TL), and standard length (SL). For all fish ($N=297$), we removed the liver and flash-froze the tissue in liquid nitrogen, then stored them at -80°C. We weighed the frozen liver to assess liver investment (hepatosomatic index).

Statistical analyses

We used R version 4.1.2 for all analyses (R Core Team, 2021). We log-transformed or square-root transformed data as necessary to meet the assumptions of normality and homogeneity of variance. We used the *emmeans* package (Lenth et al., 2022) to perform Tukey's HSD post-hoc tests when necessary to perform pairwise comparisons between treatments, sexes, and/or exposure times. We used $\alpha=0.05$ to determine statistical significance.

Anxiety: We assessed the effects of caffeine on the *amount of time* fish spent on the black square using a linear model, with amount of time on the black square as the response variable, and treatment and trial time (i.e., pre-exposure, Day 7, Day 21) as well

as their interaction as the explanatory variables. We also assessed the effects of caffeine on the *number* of times fish visited the black square using a linear model, with the number of visits to the black square as the response variable, and treatment and trial time (i.e., pre-exposure, Day 7, Day 21) as well as their interaction as the explanatory variables.

To account for baseline differences in anxiety unrelated to caffeine treatment, we calculated the difference score, or the difference between amount of time spent on the black square during exposure trials (either Day 7 or Day 21) and during pre-exposure testing. We did the same for the number of visits to the black square, then assessed how caffeine exposure affected the difference score. To do this, we used linear models with the average difference in amount of time spent on the black square between Day 7 and pre-exposure testing (or Day 21 and pre-exposure testing) as the response variable, and caffeine treatment as the explanatory variable. We used the same linear models to assess how caffeine exposure affected the average difference in the number of visits to the black square. For these analyses, we excluded fish where we could not reliably match their tags pre-exposure with any post-exposure (i.e., Day 7, Day 21) testing, resulting in a sample size of $n=122$ fish (out of the initial 135 fish).

Aversive Learning: Using the *survival* package (Therneau et al., 2022), we employed survival analysis to determine how caffeine treatment affected the number of trials it took the groups of three fish to associative learn. We also used survival analysis to determine how caffeine treatment affected the number of trials it took groups of fish to reversal learn. For the reversal learning analysis, we included the number of trials it took

to reach the associative learning criteria as a covariate because we expected that the speed it took fish to reversal learn would be positively correlated with the speed it took them to associative learn.

We assessed the effects of caffeine on the latency of fish to exit the trawl zone using linear models, with the average latency of the third fish in each group to exit the trawl zone as the response variable and caffeine treatment as the explanatory variable. We assessed groups separately during the associative and reversal learning phase, and only included groups that successfully associative learned in the associative learning analyses and included only groups that successfully reversal learned in the reversal learning analyses. Fish that did not exit the trawl zone on their own (i.e., that needed to be guided through the exit with a hand net) were given a latency of 120 seconds. Fish that had latencies greater than 120 seconds because we accidentally didn't stop the trial in time were also given the maximum latency of 120 seconds.

We assessed the effects of caffeine on how cohesive the three fish behaved by analyzing the following duration – the time between the first versus second fish in each group to exit the trawl zone, and also the time between when the second and third fish in each group exited the trawl zone. To do this, we used linear models, with the average following duration between the first and second fish to exit in each group (or the second and third fish) as the response variable, and caffeine treatment as the explanatory variable. We included both associative and reversal learning phases in these analyses, and only included groups that successfully managed to complete both associative and reversal learning. Fish that did not exit the trawl zone on their own (i.e., needed to be guided

through the exit with a hand net) were given a latency of 120 seconds. For these analyses, if the latency of the first or second fish was >120 seconds, and the second or third fish was guided through the exit with a hand net and thus given a latency of 120 seconds, we excluded the trial from the following duration analyses to avoid the following duration being a negative number.

Note that the learning trials took a long time and we had only 12 learning tanks, so we ran fish through this assay in a staggered fashion, either after 21 days or after 28 days of exposure. We aimed to run 4 groups of three fish per treatment replicate at 21 days of exposure, and another 4 groups of three fish per treatment replicate at 28 days of exposure. This would have given us a total of 8 groups of three fish per treatment replicate, or 24 groups per treatment. However, due to some technical issues, we ended up testing fewer groups in the first replicate, so overall our total was 22 groups per treatment. See Table 2.1 for exclusion criteria and number of trials excluded due to experimentation errors.

Morphological measures and physiology: We used linear models to assess how body mass (BM), total length (TL), standard length (SL), Fulton's body condition (condition factor = [body mass/length^3] x 100), average daily growth, and hepatosomatic index (HSI=[liver mass/[body mass-liver mass] x 100]) differed across treatments, with sex of the fish included as a fixed effect (Nikel et al., 2021). We calculated daily growth by finding the difference between the individual's mass during tagging and the individual's mass at dissection and divided by the number of days between tagging and dissection. Note that because tagging occurred 7–8 days prior to exposure beginning to

allow the fish to recover after tagging, the growth measurement included about 7–8 days where the fish were not exposed to caffeine. We tagged fish in advance of the exposures so that we could use tagged individuals for the pre-exposure testing, and we did not re-measure fish on Day 0 because we didn't want to cause excessive stress to the fish. Because only about half of the fish were given unique tags, and some of the tags faded over the course of the experiment, the sample size for daily growth was lower than the other morphometrics ($n=213$). We removed one fish from all morphological analyses because it could not be sexed at dissection, and we included sex as a random effect in our models. We used linear models to assess how caffeine treatment affected standard metabolic rate, maximum metabolic rate, and aerobic scope, with body mass included as a fixed effect. We attempted to randomly assign fish to treatment tanks and expected to have similar sex ratios across treatments. To check this was the case, we used a linear model to assess sex ratio differences across treatments. In the linear model, we included sex ratio as the response variable and caffeine treatment as the fixed effect.

Table 2.1. Exclusion criteria for removing trials or groups from analyses due to errors and mistakes during experimentation. In total we ran 2311 trials and excluded 204 trials completely (*n=2107* trials included or 91%). We excluded the entire latency data for 17 trials (*n= 2090* trials) and excluded only the following duration data for 18 trials (*n= 2072* trials). See Appendix 1 for more detailed information on the errors. Some errors occurred during the associative or reversal learning criteria (i.e., 4 trials where all 3 fish went through exit on their own during the 2-minute trawl period), and thus the entire group needed to be removed from analyses because they were mistakenly assumed to have associative or reversal learned when this was not the case. For some errors (e.g., where the lights that turn on and off automatically got too dark to score the video), we could still include these trials towards the number of trials it took to associative or reversal learn because we knew how many fish went through the hole into the refuge zone based on the number of fish that needed to be guided from the trawl zone to the refuge zone at the end of the trial, but we did not know the latencies at which they exited the trawl zone (as assessed on video). For these, we removed the trials only from latency and following duration analyses.

Error type	Number of times error occurred	Number of trials removed because of error	Exclusion specifics
Fish not netted into trawl zone	12	124	Trials removed completely
Fish swam underneath divider	2	60	Trials removed completely
Fish swam through blocked exit	1	1	Trial removed completely
Wrong exit open	1	2	Trials removed completely
Unnecessary trial run	17	17	Trials removed completely
Video too dark	3	4	Latencies removed completely
Missing/corrupted video	6	13	Latencies removed completely
Video lagged	10	10	Following duration removed
Latency to exit >120 seconds	8	8	Following duration removed
Total trials removed completely		204	
Total trials where only latencies removed		24	
Total trials where only following duration removed		18	

2.4 Results

Caffeine concentrations

We analysed 16 water samples from our exposure tanks. Our sampling regime was paired, with one sample collected one-hour post-dose of caffeine, and the other sample collected immediately before the next dose (72/96 hours after the initial dose). We detected caffeine in all our exposure tanks (Table 2.2), and even our control treatments contained low caffeine concentrations ranging from 4.6 – 65.7 ng/L. These levels in the control tanks may be because the water from the taps contains some low, but detectable levels of caffeine or it could be due to airborne contamination of caffeine as all exposure tanks were in close proximity to each other. The average concentrations in the control tanks were at least 16x lower than the average low caffeine concentration. We expected caffeine to decline over time and it did in our high concentration treatment, but our low caffeine tanks were fairly stable, and caffeine appeared to concentrate with more detected three days after dosing.

Table 2.2. Mean (± SE) concentrations in [ng/L] of caffeine in exposure tanks during experimentation. Samples were taken 1-hour post-dose, and 72/96 hours later, right before the next dosing event. Intended doses were 0 ng/L (control), 1,000 ng/L (low caffeine concentration), 10,00 ng/L (high caffeine concentration).

	Treatment		
Sampling time (post-dose)	Control	Low	High
1 hour	13.4 ± 8.85, *n=2*	682 ± 219, *n=2*	4094 ± 1547, *n=3*
72/96 hours	41.4 ± 12.3, *n=3*	749 ± 171, *n=3*	1707 ± 1574, *n=3*

Anxiety

Caffeine exposure affected the *amount of time* fish spent on the black square (LM, $F_{(8,385)}$=2.5, p=0.01). On Day 21, fish from the low caffeine treatment spent much less time on the black square (118 ± 19.3 s) compared to fish from the control group (279 ± 33.1 s; $t_{(ControlDay21 – LowDay21}$=3.8, p=0.005). However, when we compared the difference in the amount of time fish spent on the black square after 7 and 21 days of exposure compared to pre-exposure testing, there were no significant differences between treatments ($LM_{(Day 7 – Pre-exposure)}$: $F_{(2,119)}$=0.3, p=0.72; $LM_{(Day21-Pre-exposure)}$: $F_{(2,116)}$=2.2, p=0.11; Figure 2.3).

Caffeine exposure also affected the *number of visits* fish made to the black square (LM, $F_{(8,385)}$=3.9, p=0.0002). Fish from the high caffeine treatment group visited the black square *more often* (40 ± 3.6 visits) than fish from the low caffeine group on Day 21 (25 ± 3.1 visits; $t_{(LowDay21 – HighDay21)}$=-3.2, p=0.04). For some unknown reason, the control group fish visited the black square more on Day 21 than they did during pre-exposure testing (a mean of 38 ± 0.39 vs. 22 ± 0.63 visits, respectively; $t_{(Controlpre-exposure– ControlDay21)}$=3.7, p=0.006). When we compared the difference between the number of visits during pre-exposure testing vs. Day 7 testing, we found no differences between treatments ($LM_{(Day 7 – Pre-exposure)}$: $F_{(2,119)}$=0.2, p=0.80; Figure 2.3).However, the difference in the number of visits between pre-exposure and Day 21 testing was significantly different ($LM_{(Day21-Pre-exposure)}$: $F_{(2,116)}$=5.9, p=0.004), with control fish significantly increasing their number of visits (+16 ± 4.2 visits; t=3.4, p=0.002) compared to fish from the low caffeine group who didn't change (-2 ± 3.9 visits).

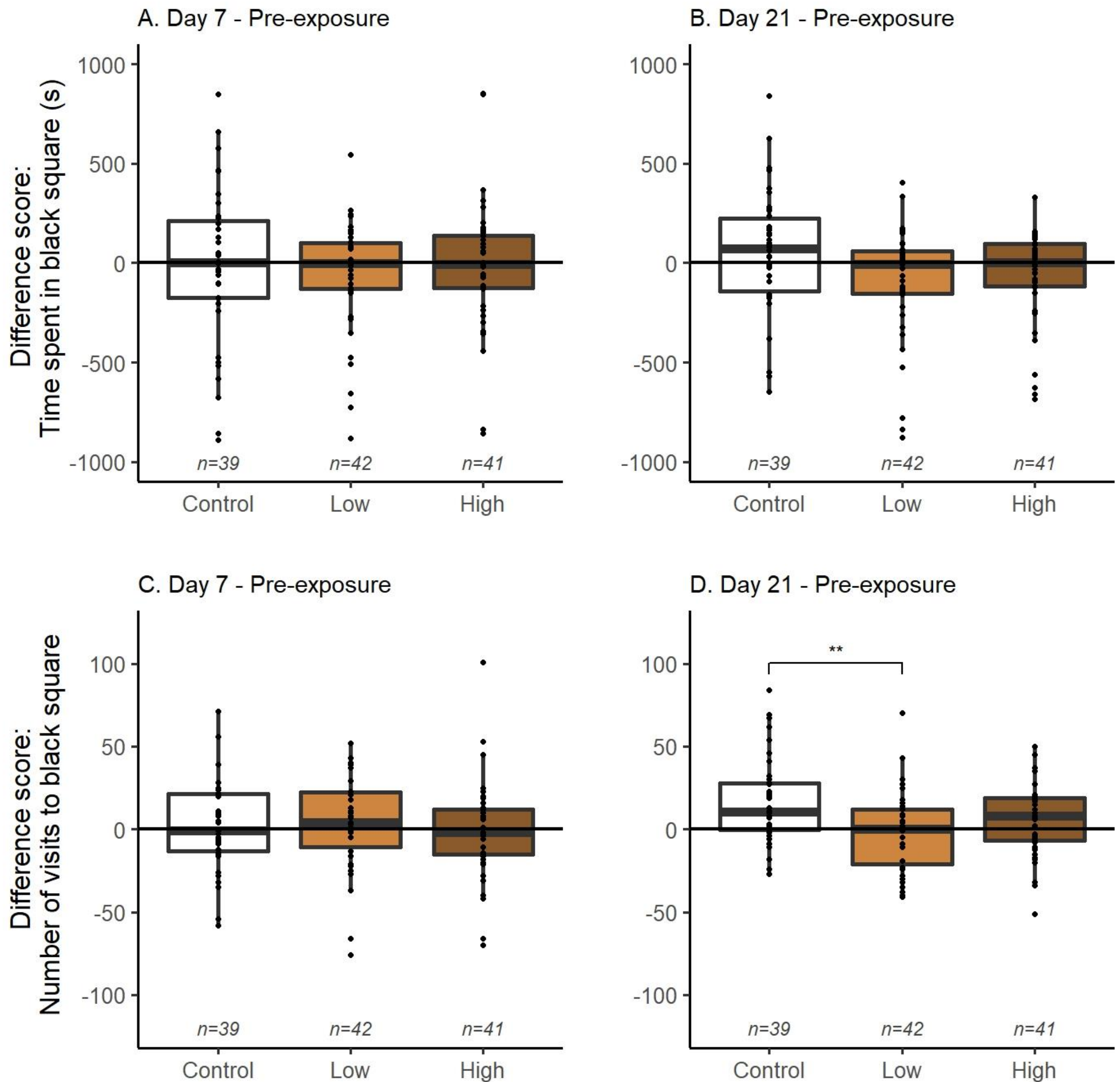

Figure 2.3 Difference score for time spent on the black square between (A) Day 7 of exposure and pre-exposure testing and (B) Day 21 of exposure and pre-exposure testing. Difference score for number of visits to the black square between (C) Day 7 and pre-exposure testing and (D) Day 21 of exposure and pre-exposure testing. The white boxplot represents control fish, the light brown boxplot represents fish exposed to low caffeine, and the dark brown box represents fish exposed to high caffeine. A significant effect of caffeine treatment on the difference in number of visits to the black square between Day 21 and pre-exposure testing is indicated by (*$**p<0.01$*).

Aversive learning

Caffeine exposure did not appear to affect how quickly fish associative learned or how fast they reversal learned. During the associative learning stage, fish from the low caffeine group took the longest to learn, some 20 ± 3.4 trials on average, compared to the control fish, who took an average of 16 ± 2.9 trials to learn and the fastest learners from the high caffeine treatment with an average of 13 ± 2.3 trials to learn (Figure 2.4A). However, this increase was not significant and so we cannot claim that caffeine influenced the number of trials required to reach the associative learning criteria ($z_{(High)}$=0.3, p=0.74, $z_{(Low)}$=0.6, p=0.52). During the reversal learning stage, control fish needed the most trials to reversal learn (13 ± 1.4 trials on average), while low caffeine and high caffeine fish took only 11 trials on average to reversal learn (11 ± 1.9 trials and 11 ± 1.8 trials, respectively; Figure 2.4B). Again, caffeine exposure did not significantly increase or decrease the number of trials required to achieve the reversal learning criteria ($z_{(High)}$=1.3, p=0.21, $z_{(Low)}$=0.7, p=0.49, $z_{(log(Number\ of\ trials\ to\ learn)}$=-0.5, p=0.59; Figure 2.4B). Overall, it took the fish 16 ± 1.7 trials on average to learn to associate one exit with escape and 12 ± 1.0 trials to stop acting on previously learned behaviour and use the other exit.

Caffeine exposure also did not significantly affect how quickly fish exited the trawl zone after the trawl began moving (Figure 2.5). During the associative learning stage, the average latency of the last fish to exit the trawl zone was comparable across all treatment groups (average: 68 ± 3.9 seconds; LM, $F_{(2,51)}$=0.2, p=0.84). Similarly, during

the reversal learning stage, the average latency of the last fish to exit the trawl zone was similar for all groups (average: 57 ± 3.6 seconds, LM, $F_{(2,36)}$=0.07, *p*=0.94; Figure 2.5b).

Caffeine exposure significantly affected the following duration (how closely fish followed each other through the exit into the refuge zone), but this was true only for the second and third fish. The following duration between the first and second fish in each group was longest in the high caffeine group taking 84 ± 9.7 s on average and was about one minute for the control fish (61± 7.5 s) and for the fish exposed to low caffeine (60 ± 6.9 s). This difference did not reach statistical significance (LM, $F_{(2,37)}$=2.7, *p*=0.08; Figure 2.6A). The following duration between the second and third fish was also longest in the high caffeine group (63 ± 7.6 s), while the following duration for the control fish was 50 ± 4.8 s, and it was 36 ± 5.6 s for the low caffeine group (LM, $F_{(2,37)}$=5.1, *p*=0.01). The post-hoc analysis showed that the following duration between the second and third fish was significantly shorter in the low caffeine treatment compared to the high caffeine treatment ($t_{(Low-High)}$=-3.2, p=0.008, Figure 2.6B).

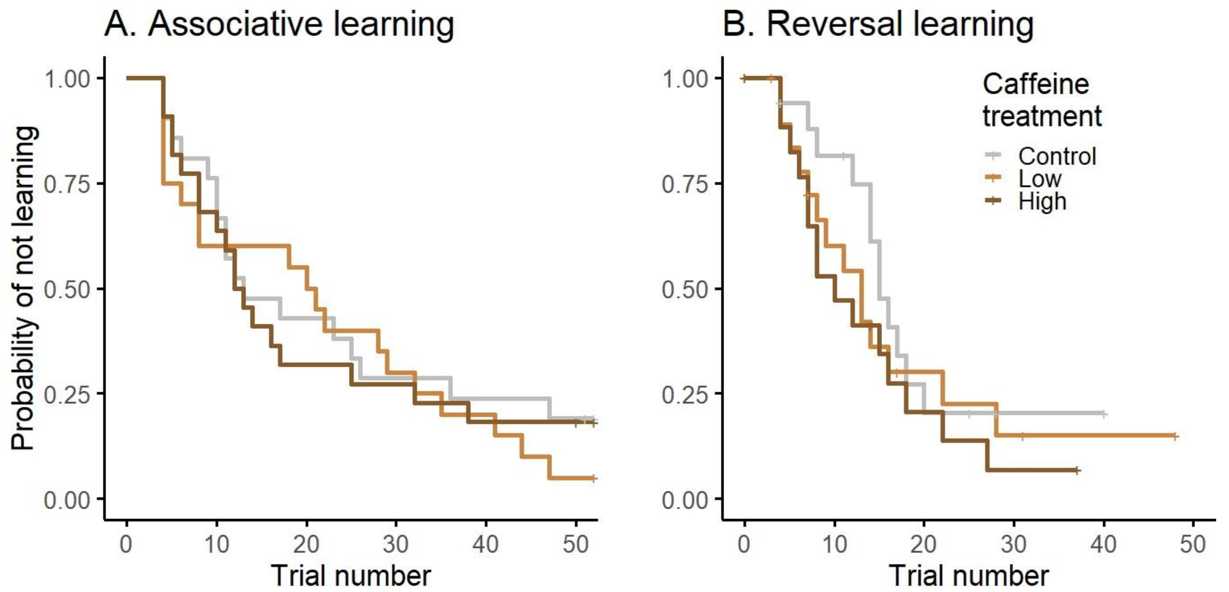

Figure 2.4. Number of trials until (A) associative learning criteria were achieved and (B) reversal learning criteria were achieved (plotted as Kaplan-Meier survival probabilities). Vertical dash marks represent censored data. Grey lines indicate control groups (*n=21*), light brown lines indicate groups exposed to low caffeine (*n=20*), and dark brown lines indicate groups exposed to high caffeine (*n=22* during associative learning, *n=21* during reversal learning; see Appendix 1 for exclusion criteria). There was no difference in the rate of associative or reversal learning depending on caffeine treatment.

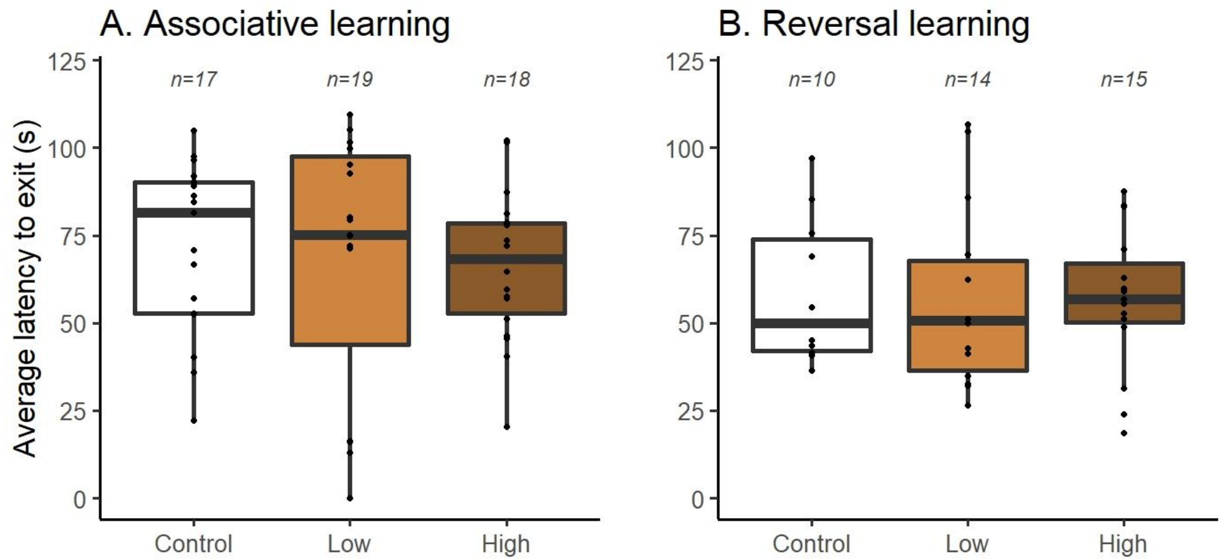

Figure 2.5. Average latency to exit the trawl zone in seconds for (A) groups that associative learned and (B) groups that reversal learned. Latencies for fish that did not exit were re-assigned as 120 seconds (maximum latency). The white boxplot represents control groups, the light brown boxplot represents groups exposed to low caffeine, and the dark brown box represents groups exposed to high caffeine. There was no significant difference in latency to exit during associative or reversal learning.

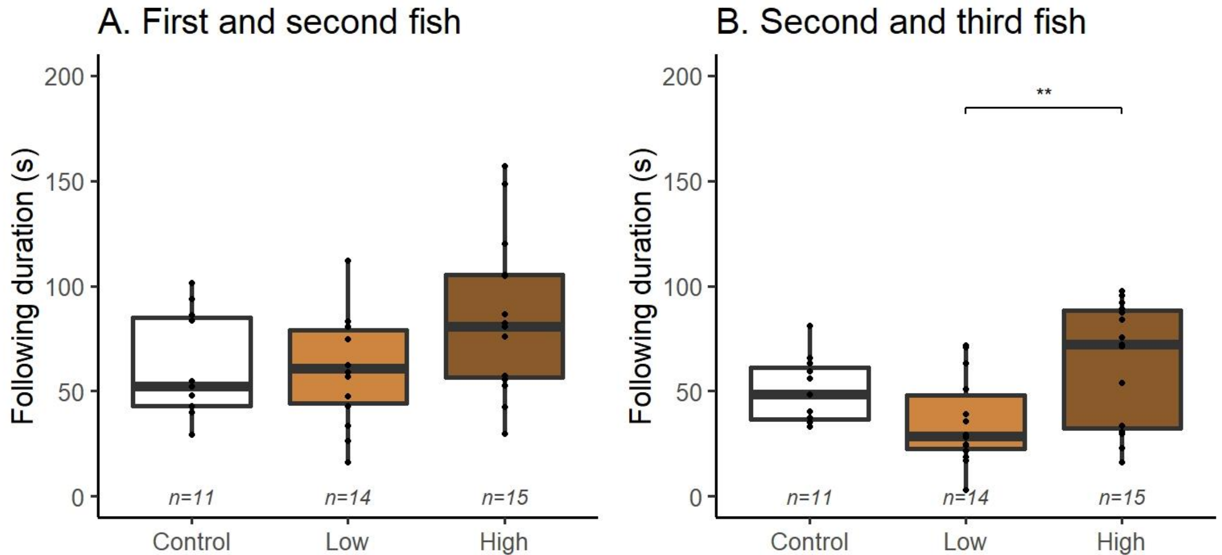

Figure 2.6. Latency duration to exit the trawl zone (A) between the first and second fish to exit and (B) between the second and third fish. Trials where fish did not exit were given a maximum latency of 120 seconds. Each dot represents the average latency difference for each group of fish. Only groups that associative and reversal learned are included. The white boxplot represents control groups, the light brown boxplot represents groups exposed to low caffeine, and the dark brown box represents groups exposed to high caffeine. A significant effect of caffeine treatment on latency difference between fish is indicated by (*$**p<0.01$*). There was no significant effect of caffeine exposure on difference in latency to exit between the first and second fish.

Physiological measures

The average standard metabolic rate (SMR) of fish exposed to low caffeine was 14 ± 0.8 mmol and 12 ± 1.7 mmol for the fish exposed to high caffeine and these rates were not significantly different from the average SMR of 14 ± 0.5 mmol for the control group (LM, $F_{(3,20)} = 2.31$, p = 0.11). Similarly, the maximum metabolic rate (MMR) of fish exposed to low caffeine (58 ± 3.5 mmol) and high caffeine (61 ± 10.3 mmol) did not significantly differ from the MMR of control fish (58 ± 3.2 mmol; LM, $F_{(3,20)} = 1.13$,

p=0.36). Unsurprisingly, the aerobic scope of fish exposed to low caffeine (43 ± 3.3 mmol) and high caffeine (49 ± 9.3 mmol) was not significantly different from the control group (44 ± 3.1 mmol; LM, $F_{(3,20)} = 0.94$, p=0.44). Note that the average mass of fish used for respirometry was 2.0 ± 0.09 g (*n=24*).

Morphological differences associated with exposure

The average body mass, total length (TL), standard length (SL), body condition (Figure 2.3), and growth per day appeared to be higher in fish exposed to high caffeine (Table 2.2); however, this pattern was driven by the fact that, by chance, our high caffeine treatment had more males than did the other two treatments ($F_{(2,6)}=9.6$, $p=0.013$). Male fathead minnow are known to be larger than females and to grow faster (Held & Peterka, 1974) and these sex differences were also true of our experimental fish (body mass: $t_{(F - M)}=-18$, $p<0.0001$; TL: $t_{(F - M)}=-18$, $p<0.0001$; SL: $t_{(F - M)}=-19$, $p<0.0001$; body condition: $t_{(F - M)}=-5$, $p<0.0001$; growth: ($t_{(F - M)}=-11$, $p<0.0001$). In contrast, the investment in the liver (hepatosomatic index, HSI) was modulated by caffeine exposure, but not by sex, with surprisingly the control fish having the largest livers controlling for body size (3.2%), while fish exposed to low and high levels of caffeine had HSI that were on average lower (2.2% and 2.9% of body mass, respectively; Table 2, Figure 2.7; $t_{(Control - Low)}=6$, $p<0.0001$, $t_{(Control - High)}=2$, $p=0.054$, $t_{(Low - High)}=-4$, $p=0.0002$).

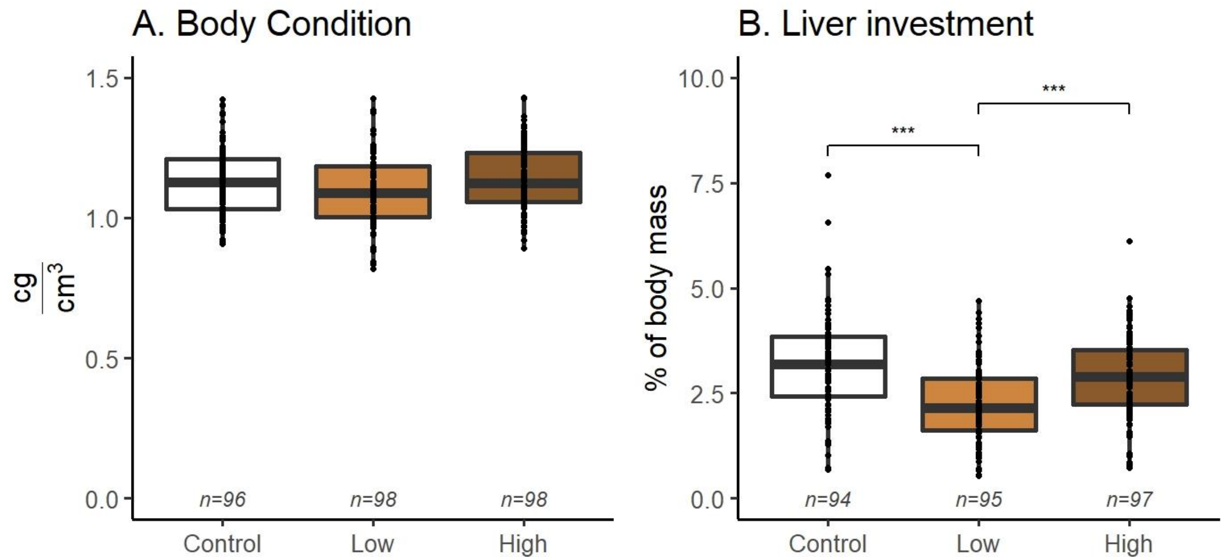

Figure 2.7. (A) Average Fulton's body condition and (B) liver investment (hepatosomatic index) of fish, on Day 35 of caffeine exposure across treatment groups. See methods section for an explanation of how body condition and liver investment was calculated. The white boxplot indicates control fish, the light brown boxplot indicates fish exposed to low caffeine, and the dark brown boxplot indicates fish exposed to high caffeine. A significant effect of caffeine treatment on liver investment is indicated by (***$p<0.001$). No significant effects were found for body condition.

Table 2.3. Mean ± standard error of each morphometric measure and their respective linear model results, with the listed morphometric measure as the response variable, caffeine exposure group as a fixed effect, and sex as a random effect. The linear model for sex ratio calculated sex ratio at the replicate tank level, and the linear model for sex ratio did not include sex as a random effect. All of these linear models were significant (as evidenced by the p-value), but post-hoc tests showed that sex, not caffeine treatment was the driving factor in differences for all morphometric measures except hepatosomatic index and sex ratio. Fish from the low caffeine treatment had a hepatosomatic index significantly lower than the control and high caffeine groups. High caffeine tanks had more males than females (as evidenced by the higher sex ratio) than the control and low caffeine tanks.

	Control	Low caffeine	High caffeine	Sample size	Df	F-statistic	*p-value*
Body mass (g)	3.20±0.14	3.01±0.11	3.47±0.13	*n=292*	3,288	112	<0.0001
Total length (cm)	6.41±0.08	6.41±0.07	6.62±0.07	*n=292*	3,288	116.4	<0.0001
Standard length (cm)	5.30±0.07	5.26±0.06	5.44±0.06	*n=292*	3,288	127	<0.0001
Body condition	1.13±0.01	1.09±0.01	1.14±0.01	*n=292*	3,288	12.7	<0.0001
Growth (g/day)	0.01±0.001	0.01±0.001	0.02±0.001	*n=213*	3,209	49.8	<0.0001
Hepatosomatic Index (%)	3.18±0.12	2.24±0.09	2.85±0.10	*n=286*	3,282	14	<0.0001
Sex ratio	1.09±0.14	0.96±0.09	1.8±0.20	*n=9*	2,6	9.6	0.013

2.5 Discussion

Aims revisited

Although caffeine is found in many aquatic habitats and ecosystems around the world (Li, Wen, et al., 2020; Vieira et al., 2022), most caffeine research to date has used non-environmentally relevant doses of caffeine (e.g., Ladu et al., 2015; Neri et al., 2019; Richendrfer et al., 2012; Ruiz-Oliveira et al., 2019; Santos et al., 2016) with short exposure periods (e.g., Ladu et al., 2015; Neri et al., 2019) and has explored its impacts only on simple measures of behaviour (e.g., de Farias et al., 2021; Richendrfer et al., 2012). Here, we investigated how environmentally relevant concentrations of caffeine affected fathead minnow (a) complex behaviour, (b) physiology, and (c) morphology.

Caffeine concentrations

We found that caffeine concentrations in the high caffeine group (10,000 ng/L) were unpredictable; however, on average, the concentrations of the tanks dosed with high concentrations (2901 ± 1122 ng/L) were higher than caffeine concentrations in the other exposure treatments (722 ± 118 ng/L in low exposure tanks and 30 ± 10 ng/L in control exposure tanks). The concentrations in the low caffeine group were close to our intended concentration of 1,000 ng/L, but the concentrations in the control group (0 ng/L) unexpectedly had some caffeine (up to 66 ng/L). Typically, caffeine concentrations would be expected to decrease over time (Lam et al., 2004), but this was not the case in our experiment, with the average caffeine concentrations increasing from 1 hour post-dose to 72/96 hours post-dose for sampled control and high caffeine exposure tanks and caffeine

concentrations remaining constant in our low concentration tanks. Other studies, too, have found caffeine to remain constant, not degrading over a 24-hour period (Cerveny et al., 2022). The increase in concentrations could have been because of aerosol contamination of caffeine (Cerveny et al., 2022), especially since all the exposure tanks were in the same room. While we took precautions to limit caffeine contamination, including putting lids on exposure tanks, using separate equipment for each exposure group, and using plastic sheeting to separate tanks, we may still have had some aerosol contamination. Additionally, we could have had contamination in our control tanks because the municipal tap water used for exposures may have contained small amounts of caffeine. Many water bodies used for drinking water (Daneshvar et al., 2012) or drinking water itself (Chen et al., 2006; Leung et al., 2013; Loos et al., 2007; Mompelat et al., 2011; Rosa Boleda et al., 2011; Stackelberg et al., 2007) contain caffeine, with concentrations up to 37 ng/L found in drinking water in Calgary, Ontario, Canada (Chen et al., 2006), which is similar to the average concentrations found in our control tanks.

Morphology

We did not find that caffeine exposure altered morphometric endpoints (i.e., TL, SL, mass) or growth in fathead minnow. While previous studies have found that caffeine alters growth, the concentrations of caffeine used in those studies were often much higher and the route of exposure (diet) differed from our study (waterborne). For example, sea bream (*Sparus aurata*) given feed with more than 1 g/kg of caffeine did not grow as much as control fish (Chatzifotis et al., 2008) and neotropical catfish (*Rhamdia quelen*) exposed to 16 mg/L of caffeine did not grow as much as control fish (dos Santos et al., 2021). In

studies utilising lower caffeine concentrations, like ours, growth was not affected. In the sea bream example above, lower concentrations of caffeine (0.1 g/kg, 1 g/kg) did not alter growth (Chatzifotis et al., 2008), while in the neotropical catfish example, fish exposed to lower concentrations of caffeine (2 – 8 mg/L) did not show a difference in growth (dos Santos et al., 2021). Furthermore, it is hypothesised that caffeine may impede growth by making it more difficult to extract nutrients from food and may increase fish metabolism (Chatzifotis et al., 2008). However, in our experiment, we did not find any difference in the metabolism (SMR, MMR, aerobic scope) of fish exposed to ecologically relevant levels of caffeine, which might explain why we did not see a difference in fish growth.

We found that caffeine concentration affected liver investment (HSI), with surprisingly the lowest liver investment in the fish exposed to low concentrations of caffeine. While previous studies have found that caffeine altered sea bream HSI, alteration was not in a predictable pattern (Chatzifotis et al., 2008). Specifically, 1 g/kg of caffeine increased HSI, but 0.1 g/kg and 5 g/kg of caffeine both decreased HSI, and 2 g/kg of caffeine didn't alter HSI (Chatzifotis et al., 2008). In the context of contaminant exposure, HSI normally either (a) increases due to higher need for decontamination (Facey et al., 2005) or (b) decreases due to starvation relating to areas with contaminant burdens (Javed & Usmani, 2017). The body condition (K) of fish at the end of the experiment was similar across our exposure groups. Hence it is unlikely that HSI was related to caffeine treatment because only the low caffeine treatment group appeared to be affected by caffeine, and the HSI in the low treatment group was lower than the control group, not higher as might be expected if the fish need to increase liver resources for

decontamination. Additionally, since starved fish usually have a lower body condition compared to those that are well-fed (Faria et al., 2011), and the body condition of fish from all three exposure groups was similar, it is very unlikely that starvation caused the decreased HSI observed in the low caffeine exposure group. Instead, it is possible that differences in the reproductive status of individuals in the treatment groups were responsible for the difference in HSI. In three-spined stickleback (*Gasterosteus aculeatus*), reproductive status, sex, and seasonality can all impact HSI, with reproductive females and males having a higher HSI than non-reproductive fish (Sanchez et al., 2008). In the Red Sea goby (*Silhouettea aegyptia*), marbled goby (*Pomatoschistus marmoratus*) and Daffodil Princess cichlid (*Neolamprologos pulcher*) the opposite pattern has been observed, with reproductive fish investing less in liver mass (Fouda et al., 1993; Sopinka et al., 2009). While we did not specifically assess reproductive status (beyond whether the fish were male or female) and sex was not a significant variable in our HSI models, it remains possible that the low caffeine group had fewer or more reproductive females and males than the other exposure groups, and thus had a lower HSI.

Physiology

Caffeine in humans and other vertebrates is known to increase resting and maximal metabolic rate (Bauer et al., 2001; Chad & Quigley, 1989); however, the studies showing these effects used concentrations that were much higher than those used in our experiment. We found that caffeine did not impact standard metabolic rate (SMR), maximal metabolic rate (MMR), or aerobic scope of fathead minnow. This lack of metabolic differences is not surprising given that the concentrations used were low

compared to those found in the literature. For example, in mice, resting metabolic rate can be increased by 60 mg/kg of caffeine, but not by 40 mg/kg (Yoshioka et al., 1990). In contrast, in our experiment, our highest concentration was only 10,000 ng/L or 0.01 mg/L. In *Daphnia magna*, resting metabolic rate did not differ at concentrations similar to those used in our experiment (i.e., 400 ng/L, 2000 ug/L, 10,000 ng/L) (Nunes et al., 2022). Hence the caffeine doses we used were likely insufficient to induce an effect and are unlikely to affect wild fish at environmental doses, given that the highest freshwater concentration was 19,3000 ng/L (Li et al., 2020), which is lower than the effect-inducing concentrations.

For respirometry testing, we tested fish in clean water rather than exposure conditions. This was done due to equipment limitations, as we were concerned that using caffeinated exposure water in the respirometers would be a major source of contamination in subsequent testing and future studies. Because we moved fish from chronic exposure to caffeine to clean water, it is possible and likely that the fish were experiencing withdrawal from caffeine. There is some evidence that withdrawal from caffeine may induce more aversive effects than caffeine exposure itself (Santos et al., 2016). In humans, however, withdrawal from caffeine does not impact maximal metabolic rate (Hetzler et al., 1994). Given the low doses used in our study and the fact that all treatment groups were tested in the same way, we assert that it is unlikely that caffeine withdrawal significantly impacted our metabolic rate and aerobic scope testing.

Aversive learning

On average, groups of fish took ~69 seconds to exit the trawl zone during associative learning, and ~57 seconds to exit the trawl zone during reversal learning. Fish in our experiment exited the trawl zone more quickly than fish in other experiments, although the experimental set-up was different because the other experiments included 'sham' demonstrators (98 – 100 seconds; Attaran et al., 2020; Lindeyer & Reader, 2010).

While we expected caffeine to either help fish learn faster or make it more difficult for them to learn, we found that caffeine did not apparently affect how quickly the fish took to learn how to avoid the aversive stimulus (i.e., the trawl) or to reversal learn the escape routes when the exits were switched. It was surprising that fish took on average less time to master the reversal task, given that this is usually observed to be the more challenging task to master, as one solution needs to be forgotten or suppressed and an alternative solution learned and employed (Buechel et al., 2018).

In humans, caffeine can either help or harm performance in complex tasks depending on the study and the paradigm or task used (Smith, 2002). Given that many neurotransmitter pathways, likely including the pathways affected by caffeine, are highly conserved across vertebrates (McArdle et al., 2020), the generalisation that the effects of caffeine differ widely depending on the experimental design and task probably also holds true in fishes, both at the species and individual level.

It is worth keeping in mind that we ran fish through the learning assay in groups of three to help prevent stress. It is possible that the support the social group provided

may have masked the effects caffeine had on learning performance. For example, in a previous study, being with conspecifics ameliorated the effects of high caffeine exposure (i.e., 70 mg/L) in zebrafish, including their ability to reach a stabilising swim speed and time spent frozen (Neri et al., 2019).

During our experiment, fish sometimes swam back through the exit, moving from the refuge zone back to the more dangerous trawl zone. It is possible that the refuge zone was not attractive enough to the fish to keep them there. We could have used plants as shelter or a group of conspecifics in an enclosure within the refuge zone end to increase the attractiveness of the refuge zone (Attaran et al., 2020) and prevent swimming back to the trawl zone, reinforcing the learning task. Recalling that we originally hypothesized that caffeine exposure would increase activity, if true, then caffeine-exposed fish may have moved from the refuge zone back to the trawl zone more often than the control fish, reinforcing their learning of which exit was open, and potentially confounding the effects of caffeine on learning with the effects of increased activity. However, active fish would also be more likely to initially move from the trawl zone to the refuge zone, and so we would expect to see them learning more quickly. Instead, in our experiment, all groups learned at similar rates, making it unlikely that fish that moved more between the refuge and trawl zone had a significant impact on our results. If time spent on the black square is used as a rough proxy for movement, then fish exposed to caffeine may have been moving more on Day 21 (see below). If fish that moved more were able to explore more then we would have expected to see a reduction in the number of trials it took them to

learn and/or reversal learn, but instead, we found no difference in the number of trials it took these caffeine exposed fish to learn and/or reversal learn.

While caffeine exposure did not affect the time between the first and second fish making it through the exit, caffeine did affect the time taken between the second and third fish exiting. In groups exposed to high caffeine concentrations, the third fish took significantly longer to exit compared to the second fish while in groups exposed to low caffeine concentrations these third fish quickly followed the second fish through the correct exit. Hence it is possible that caffeine exposure impacted the social cohesion of the group of three fish. Social group interactions have been known to be altered by various pollutants in other contexts (Mason et al., 2021), and with caffeine, the social group appears to buffer or ameliorate any negative effects (Neri et al., 2019). For example, individual zebrafish dosed with 70 mg/L of caffeine displayed decreased swim speed, but when in a group with four untreated conspecifics, their swim speed was similar to unexposed control fish (Neri et al., 2019). Given that overall latency to exit the trawl zone of the entire group was unaffected by caffeine treatment, and sociality likely buffers against negative effects of caffeine, we argue that it is highly unlikely that the difference in the following time between the second and third fish is ecologically meaningful. Instead, a more likely explanation is that more of the third fish in the groups were net to the trawl zone and given the maximum latency (120 seconds), skewing the latency higher than might otherwise be expected. For example, throughout all the trials run, there were only 89 instances where the first fish in the group was net to the trawl zone (i.e., the fish didn’t exit the trawl zone on their own), there were 249 instances where the second fish in

the group had to be net to the trawl zone and 474 instances where the third fish in the group had to be net to the trawl zone and given the maximum value of 120 seconds. This means that the latency difference between the second and third fish is likely to be higher than the latency between the first and second fish, because so many more of the third fish in the group had to be net to the trawl zone (and were given the maximum latency of 120 seconds).

Anxiety

On Day 21 of exposure fish from the low caffeine treatment groups spent less time on the black square than fish from the control groups. We had expected caffeine exposure might make the fish more anxious, and they were expected to spend more time on the black square and move between the black and white parts of the tank less often. However it is possible that caffeine was acting as an anxiolytic at low concentrations, as has been show in previous experiments with mice and rats (Hughes et al., 2014; Jain et al., 1995). However, because we specifically aimed to use environmentally relevant concentrations, both our low and high caffeine exposure concentrations were much lower than the concentrations found to be anxiolytic reported from most other studies. If caffeine were acting as an anxiolytic, we would expect both our low and high caffeine exposure groups to show decreased anxiety. Instead, only the low concentration displays decreased anxiety, despite the high concentration also being much lower than concentrations previously reported to act as an anxiolytic. It is important to note that our anxiety assay, while used by others to study anxiety, was likely not anxiety-specific, and may have also been measuring other factors, including fear and stress. While stress is considered to be a

physiological response to a stressor (American Psychological Association, 2022), anxiety is considered to be apprehension about a potential threat (American Psychological Association, 2022). Our anxiety assay could have also been measuring stress, as the fish had been moved to a new tank, by themselves, both of which are stressors to fish.

Control group fish visited the black square more times on Day 21 than they did during pre-exposure testing. This increase could be due to fish becoming more comfortable with the assay tank during subsequent trials, and being more active, crossing between the black and white more often. Previous research with zebrafish using a similar scototaxis assay showed that fish crossed between the black and white compartment fewer times during subsequent trials (Maximino et al., 2010); however, the maximum interval between the zebrafish trials in that previous study was 24 hours, while the interval between our trials was much longer (14 days).

When we controlled for pre-existing behavioural differences by quantifying the difference between pre-exposure behaviour and Day 21, we found that fish in the low caffeine group differed less in the number of visits to the black square than fish from the high caffeine group. In general, these anxiety results highlight the variability of fish within treatments and make it difficult to claim that there were any large effects of caffeine. Fathead minnow have been shown to display highly variable behaviour in other anxiety tests (i.e., novel dive test and refuge test), and it is possible that they are not the best study species for studying anxiety (Huerta et al., 2016) because the high variation in individual behaviour may mask contaminant effects.

Why is this work important?

Taken together, while our results provide little evidence that environmentally relevant concentrations of caffeine alter learning, growth, or metabolic rate in fathead minnows, we observed that caffeine at low concentrations may decrease liver investment and anxiety. However, we do not have clear explanations for why this may be occurring and or why higher, but still environmentally relevant, concentrations of caffeine exposure did not have similar effects. Despite our lack of clear results, we argue that caffeine, a chemical that is continuously added to our aquatic environments should still be studied more thoroughly, as it has been demonstrated to negatively affect multiple organisms at environmentally-relevant concentrations (Li et al., 2020). Additionally, this experiment shows that cognitively complex assays can be used in ecotoxicology research and could be effectively utilised with other contaminants that may impact learning, for example, during chronic exposures.

Future work

Our research also sets the stage for a number of future studies. First, because the refuge zone in our learning assay may not have been sufficiently attractive to the fathead minnows, and fish subsequently moved back and forth between the trawl zone and refuge, it would be worth repeating this assay with conspecifics in the refuge zone as well as artificial plants and gravel. While it is not likely that a few fish moving between the trawl and refuge zones significantly affected the results, it is still possible that this behaviour inadvertently reinforced associative learning or reversal learning. Making the refuge zone more attractive with plants, gravel, shelters, or conspecifics would ensure that the fish

didn't move between the refuge zone and trawl zone in the absence of the trawl. Making the refuge zone more attractive might also help to reduce stress in individual focal fish and allow fish to be run through the assay alone, rather than in groups of three, which made it impossible to assess precisely how sex and individual differences impacted learning outcomes. While we could not associate how individual performance during the trawl assay was associated with an individual's anxiety during the scototaxis assay because the fish from the trawl assay were run in groups of three, making the refuge zone more attractive might reduce anxiety of individuals in the assay tank, and allow them to be run individually. These changes would allow us to associate and individual's performance in the trawl assay and with relative anxiety levels from the scototaxis assay, providing additional insight into what may or may not be contributing to learning outcomes. Additionally, we cut trials off at one week (52 trials). To gain further insight and granularity into how quickly groups were learning, it would be preferable to continue the learning assay until individuals learned and reversal learned, no matter how long it took.

Finally, future work could assess metabolic rate in caffeinated water. We could not do this due to limited equipment and the risk of contamination, but we were likely measuring metabolism during withdrawal rather than exposure itself, which has been shown to have an effect on behaviour (Santos et al., 2016). Future work should measure metabolic rate in water dosed with the same caffeine concentrations as the exposures.

General Conclusion

My thesis aimed to study how caffeine, a common contaminant found in the environment, affected the behaviour and physiology of fish. While our results did not provide compelling evidence that caffeine at environmentally relevant concentrations significantly affected the morphology, behaviour, or physiology of fathead minnow, we argue that this research is important for a variety of reasons. First, most research investigating caffeine's effects on organisms used concentrations much higher than those that would be found in the environment. As such, previous research makes it difficult to direct funding, policy, and public concern to the most critical contaminants impacting biodiversity because there is not a body of evidence showing which contaminants are of the greatest concern. While my experiment did not show caffeine to have extreme effects on fish, we argue that testing these ideas and running experiments with ecologically relevant doses is important because our study utilised concentrations that would be encountered by fish on a daily basis in the wild. Second, the effects of contaminants on an aquatic organism's behaviour are often studied using simple behavioural assays. In contrast, in my thesis work, I assessed a more complex behaviour – learning – using a trawl assay. Assessing complex behaviours is a more environmentally relevant paradigm and changes in complex behaviours may have significant effects on life history and survival. While we did not find caffeine to affect learning in our study, other contaminants may alter learning. This thesis provides additional precedent for the use of environmentally relevant concentrations of contaminants in tandem with the utilisation of more complex behavioural endpoints. Even small steps towards environmental relevance

could make it easier to use research to inform policy and garner public support, as the body of literature will be more informative of what may actually be occurring in the wild compared to the many studies that use extremely high contaminant concentrations or non-informative, unnatural behavioural endpoints. While complete environmental relevance is not usually possible when studying animals in lab conditions, including as many ecologically realistic parameters and conditions as possible is key in helping to make the findings more relevant to the remediation and conservation of wild populations.

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3. APPENDICES

3.1 Appendix: Trial Exclusion Criteria for the Aversive Learning Assay

This appendix outlines why various trials and groups from the trawl assay were excluded from analyses.

Problem 1: Fish not netted to trawl zone before trial began

We excluded trials where fish were not net to the trawl zone before the trial began. Because we ran fish in groups of three and measured learning at the group level, all three fish needed to be in the trawl zone during the trawl for the trial to be used. This either occurred because a fish was forgotten during the netting of fish to the trawl zone, or because a fish was net to the refuge zone with the hand net by accident (i.e., before the trawl had started). If one of these trials was part of the four consecutive trials towards the associative learning criteria, none of the data from either the associative or reversal learning stage could be included in analyses. If one of these trials was part of the four consecutive trials towards the reversal learning criteria, none of the data from the reversal learning stage could be included, but the data from the associative learning criteria could be included.

Problem 2: Room lights went out/got too dark before trials ended

We did not include latency data for trials where the room lights got too dark to view the recorded videos of the assay. However, we included the trial in the number of trials it took to associative or reversal learn because we were able to assess this in real-time.

Problem 3: Video started recording after fish swam from trawl zone to refuge zone

We gave fish a latency of '0' if the video lag meant a fish swimming to the refuge zone immediately after being net to the trawl zone could not be viewed on the video. Since we could give the fish a value of '0' for latency, these fish were included in the latency analyses, but not in the following difference analyses.

Problem 4: Missing video/corrupted video

We excluded latency data for trials with missing or corrupted videos because we had no information about the latency at which fish exited the trawl zone. We did include these trials in the number of trials it took to associative or reversal learn because we were able to assess this in real-time.

Problem 5: Fish swimming underneath divider

We excluded trials completely if a fish swam underneath the divider (i.e., not through an exit in the divider). Because we were 'training' the fish to swim through a

specific exit, if there was a gap underneath the divider and the fish were swimming through that gap, the fish were not completing the correct task. Please note that if one of these trials were part of the 4 consecutive trials used to meet the learning (or reversal learning) criteria, none of the data from either the associative and/or reversal learning stage was used. Also included are trials where the divider wasn't flush with the bottom of the tank ("wasn't all the way down"), regardless of whether fish swam underneath or not.

Problem 6: Fish swam through wrong exit

We excluded trials completely if fish were able to swim through the wrong exit due to equipment malfunction. Because we were 'training' the fish to swim through a specific exit, if the fish went through the wrong exit because the barrier wasn't flush with the divider, we excluded these trials. Please note that if one of these trials were part of the 4 consecutive trials used to meet the learning criteria, none of the data from either the associative and/or reversal learning stage was used.

Problem 7: Fish were net thru >120 seconds after trawl started

We kept almost all of the trials where fish were net to the trawl zone >120 seconds after the trawl started. We only excluded these trials from the following difference analyses if other fish in the trial didn't go through and thus were given the maximum latency of 120 seconds. For graphing purposes, these trials were recoded as 120 seconds in R for the latency graphs.

Problem 8: Wrong exit open

We excluded any trials completely where the wrong exit was open and the fish were in the wrong phase of learning. Please note that if one of these trials were part of the 4 consecutive trials used to meet the learning criteria, none of the data from either the associative and/or reversal learning stage was used.

Problem 9: Extra trial

We excluded trials that were run unnecessarily despite the fish having already either associative or reversal learned.

3.2 Appendix: Aversive Learning in Captive and Wild Fathead Minnow

CAN YOU TEACH A CAPTIVE FISH NEW TRICKS?

AVERSIVE LEARNING IN CAPTIVE AND WILD FATHEAD MINNOW
(*PIMEPHALES PROMELAS*)

BY

MEGAN CYR

A Thesis

Submitted to the Department of Psychology, Neuroscience & Behaviour

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TITLE: Can you teach a captive fish new tricks? Aversive learning in captive and wild fathead minnow (*Pimephales promelas*)

AUTHOR: Megan Cyr

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Abstract

The vast majority of animal-based research studies are performed on captive animals in laboratory settings. As captive animals are subject to different selection pressures and experience different sensory inputs during development compared to their wild counterparts, they commonly have relatively smaller brains. Brain mass is often associated with cognitive abilities including learning, so captive animals with smaller brains may also have reduced learning ability. To test this idea, we compared the learning abilities of captive and wild fathead minnow, *Pimephales promelas.* More specifically, we investigated the ability of wild and captive populations to learn to avoid an aversive stimulus (a trawl), and also used the trawl assay to investigate reversal learning, which is considered to be a more cognitively demanding task compared to associative learning, requiring greater cognitive flexibility. We did not detect a difference in the proportion of wild versus captive fish that managed to learn to avoid the trawl by swimming to the correct escape route. We also did not detect a difference in *how long* it took for wild and captive fish to initially learn to use the correct escape route, however wild fish managed to forget a previously correct escape route and learn a new one (reversal learning) more quickly than captive fish, taking 14 fewer trials to master this more challenging task. These findings suggest that captive fathead minnows appear to have some cognitive deficits, and researchers should use caution when applying results from captive fish to wild population.

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I would like to thank my supervisors, Dr. Sigal Balshine and Jacqueline Bikker, for their guidance and encouragement throughout my thesis. I would also like to thank everyone else at the Aquatic Behavioural Ecology Lab (ABEL) for their support and feedback. I would especially like to thank ABEL members Adrienne Yau, Margaret Dobbin, Stephanie Liang, and Jessica Qiu for their endless hours spent running assays and scoring videos. Thank you to my loved ones for their emotional support. Finally, I wish to express my sincerest gratitude to the fathead minnow and to the water from which they came.

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Introduction

Humans have profoundly impacted the environment and animal behaviour throughout the past century, to the extent that many scientists have termed our current epoch the "Anthropocene" (Otto, 2018). One commonly studied aspect of the Anthropocene is how human encroachment into wild habitats may impact animals. For example, urbanization, habitat fragmentation, and other anthropogenic factors such as chemical pollutants can modify wild landscapes, resulting in altered animal behaviour (Geffroy et al., 2020; Otto, 2018; Francis et al., 2015) and contributing to the ~156% increase in the number of threatened animal species over the past two decades (Mishra, 2018). In addition to this human encroachment into wild habitats, humans have also brought a large number of animals into captivity: For example, 63% of recovery plans for the growing number of threatened species include captive breeding programs (Mathews et al., 2005). The survival rates of captive animals reintroduced to the wild are often low, indicating that captivity may impact behaviours or cognitive functions essential to survival in the wild (Mathews et al., 2005). Captive animals are also utilized in the majority of animal-based research studies (Selena et al., 2021; Viney et al., 2015; Bateson & Feenders, 2010), which are often used to guide environmental legislation intended to protect wild populations from anthropogenic factors such as chemical pollutants (Ankley & Villeneuve, 2006). The large number of animals held in captivity and their widespread use in environmental protection policies necessitates concern and careful deliberation over the wellbeing of animals in our care and how these captive animals may differ from their wild counterparts.

Animals in captivity often exhibit different morphological characteristics compared to their wild counterparts (Kruska, 1988; Burns et al., 2009). Notably, animals bred in captivity often have smaller relative brain mass than wild animals (e.g., Burns et al., 2009; Kruska, 1988; Kotrschal & Kotrschal, 2020). This reduction in captive animals' relative brain mass can be attributed to environmental factors experienced within an individual's lifetime (particularly during developmental periods), and to genetic changes across generations due to the relaxation of selective pressures. Brain morphology is plastic (Burns et al., 2009), and proper development requires appropriate environmental input (Dukas, 2004). Compared to animals raised in deprived laboratory conditions, animals raised in enriched environments (characterized by increased area, structural complexity, social stimulation, etc.) have increased brain size, more synapses per neuron, and greater dendritic branching (Dukas, 2004). Brain morphology is also heritable and subject to selection (Gonda et al., 2013). Natural selection is weakened by the benign conditions of captivity, which can allow for a wider range of phenotypes and can even select for traits which may be maladaptive in the wild (Salena et al., 2021; Tave & Hudson, 2019). For example, high rearing densities in captivity can select for increased body growth to better compete for resources (Doyle & Talbot, 1986; Salena et al., 2021), but this shift in energy allocation towards body growth may come at the expense of brain development and cognition (Kotrschal et al., 2013; Salena et al., 2021). Decreased brain mass and cognitive ability may reduce survival in the wild by reducing an individual's ability to respond to pressures such as predation (Kotrschal et al., 2015), but maladaptive

phenotypes may persist in captivity due to benign conditions and high survival rates (Tave & Hudson, 2019).

Brain size is generally correlated with cognitive ability (Kotrschal & Kotrschal, 2020; Kotrschal et al., 2013, Buechal et al., 2018), so it is plausible that the reduction in brain mass observed in captive animals may be associated with impaired cognition. Brain regions that show the greatest size reduction in captivity include the hippocampus and telencephalon, which are both brain areas implicated in learning (Kruska, 1988; Lopez et al., 2000). Learning is a cognitive ability which can be defined as the "acquisition of neuronal representations of new information" (Dukas, 2004). Learning (and other cognitive abilities) shape behaviours which impact fitness (Dukas, 2004). For example, animals can increase their chances of survival with more efficient antipredator behaviour (earlier flight times, increased vigilance in high-predation areas, etc.) by learning associations between environmental cues and the presence of a predator (Griffin et al., 2001). Since learning about predators can increase fitness (Griffin et al., 2001), predation risk is hypothesized to be a selective pressure that favors the evolution of learning (Morand-Ferron, 2017). Predation risk is often relaxed in captivity (Geffroy et al., 2020; Griffin et al., 2001), so learning may not be favoured in captive animals due to this relaxed selective pressure. Another selective pressure that favours learning is fluctuation in environmental conditions (Morand-Ferron, 2017; Stephens, 1991). Environmental conditions (e.g., predation risk, food availability) may fluctuate between day and night, winter and summer, or from one generation to the next, and learning can increase an animal's ability to quickly respond to these fluctuations with appropriate behaviour

(Bosiger et al., 2012; Kotrshal & Taborsky, 2010). For example, Bosiger et al. (2012) found that damselfish can learn to associate certain times of day (e.g., dawn, dusk) with higher risks of predation and respond by reducing their foraging behaviours during these times, likely reducing risk of mortality and increasing fitness. Therefore, environmental fluctuation may be a selective pressure that favours learning since learning can optimize the ability to switch between different fitness-promoting behaviours (e.g., antipredator and foraging behaviours) based on current environmental conditions (Bosiger et al., 2012; Kotrshal & Taborsky, 2010). Captive settings generally have low fluctuation in environmental conditions (Bhat et al., 2015), so learning may not be favoured in captive animals due to this relaxed selection pressure. An individual's learning ability is also influenced by environmental conditions experienced within their lifetime (especially during developmental periods) via phenotypic plasticity (Dukas, 2004). For example, Strand et al. (2010) found that Atlantic cod reared in tanks enriched with substrate, foliage, and weekly fluctuation in spatial features had greater learning abilities than cod raised in barren tanks. Complex environments may improve learning by evoking more neural stimulation and enhancing the brain development underlying cognition (Kotrschal & Taborsky, 2010). Captive fish are often reared in tanks with low spatial complexity (Burns et al., 2009), so they may not receive adequate neural stimulation to develop strong learning abilities. Another environmental factor that may influence learning ability is nutrition. Poor nutrition in early life can decrease neural development and hence decrease learning ability (Fisher et al., 2006). In humans, malnutrition during early childhood causes lower IQ and decreased learning ability that persists into adulthood

(Scrimshaw, 1998). Wild animals may be more likely to experience periods of malnourishment than captive animals (Burns et al., 2009), and therefore could develop decreased learning ability. Wild animals may also have increased anxiety (Wong et al., 2012) which could also negatively impact learning ability (Mandler & Sarason, 1952).

Although captivity is associated with reduced brain mass (Burns et al., 2009; Kruska, 1988), and reduced brain mass is associated with poorer cognition (Buechal et al., 2018; Kotrschal et al., 2013; Kotrschal & Kotrschal, 2020), few studies have directly compared the cognitive abilities of wild and captive animals. Therefore, in this study, we compared aversive learning capacity in wild and captive fathead minnows (Pimephales promelas) to investigate the effects of captivity on cognition. Aversive learning may be particularly relevant to antipredator behaviour, where a behavioural response results in the avoidance of predators (Griffin et al., 2001). Using an assay modified from Lindeyer and Reader (2010) and Attaran et al. (2020), we studied two types of aversive learning: associative and reversal. In the associative stage, fish learned to escape an aversive stimulus (a trawl net) using one of two escape routes. In the reversal stage, the previous correct association was reversed so that individuals had to "forget" and forge a new association. In our case, it was which of the two escape routes was available. Reversal learning is a common paradigm used to assess cognitive flexibility and general cognitive function (Izquierdo et al., 2017). Reversal learning is more cognitively demanding than associative learning, since it requires both inhibition of the previously learned response and the formation of a new response (Buechel et al., 2018). Buechel et al. (2018) found that brain size had no impact on associative learning, but larger-brained fish outperformed

smaller-brained fish at reversal learning. Similarly, Lopez et al. (2000) found that telencephalon ablation had no effect on associative learning, but telencephalon-ablated fish performed significantly worse at reversal learning than fish with intact telencephalons. Telencephalon size in fish can reduce dramatically (19%) within one generation of captivity (Burns et al., 2009), so it is plausible that captive fish may exhibit reduced learning ability given that decreased brain mass is associated with decreased cognition (Buechal et al., 2018; Lopez et al., 2000). We tested these ideas using fathead minnow (*Pimephales promelas*) as a study species. Fathead minnows make a useful model species for studies on the impact of captivity on cognition as their biology and behaviour are relatively well studied in both captive laboratory settings and in field settings (Ankley & Villeneuve, 2006). They are an easy species to rear in the laboratory (Ankley & Villeneuve, 2006), abundant in the wild, and readily located throughout southern Canada, the United States, and northern Mexico (McMillan & Smith, 1974).

Based on patterns observed in fathead minnow (Burns et al., 2009) and other species (Kruska, 1988; Kotrschal & Kotrschal, 2020), we expected that captive fathead minnow would have smaller relative brain masses compared to wild fathead minnow. If this difference in brain mass is reflected was learning ability, then we would observe differences in learning ability between captive and wild fathead minnows. In particular, given that in many species wild fish have larger brain mass (Burns et al., 2009) and brain mass is associated with cognitive ability (Kotrschal & Kotrschal, 2020), we predicted that wild fathead minnows would be better learners than captive fathead minnows. Larger brain mass and better learning ability in wild fish could be attributed to higher predation

pressure, environmental enrichment, and variability found in the wild. An alternative hypothesis is that nutrition and anxiety are key drivers of the learning differences between wild and captive fish. Given that wild fish are known to have higher anxiety (Wong et al., 2012) and are more likely to have experienced malnutrition (Burns et al., 2009), and these factors have been shown to negatively impact learning (Mandler & Sarason, 1952; Scrimshaw, 1998), we also predicted that wild fathead minnows could be worse learners compared to captive fathead minnows. In addition, we compared associative learning to reversal learning and predicted that both captive and wild fish would be better at associative learning compared to reversal learning, since reversal learning is more cognitively demanding (Buechel et al., 2018). Given that previous studies have shown that reduced brain mass and ablated telencephalon negatively impact reversal learning (Buechel et al., 2018; Lopez et al., 2000), and that these brain areas are reduced in captive fish (Burns et al., 2009), we predicted that the difference between associative learning and reversal learning would be less pronounced in wild fish.

Methods

Fish collection and housing

Captive Fish

We obtained one-month old fathead minnows (*Pimephales promelas)* from AquaTox Testing and Consulting Inc. in Puslinch, Ontario. Fish had been bred for several generations in captivity. We transported the fish to McMaster University in Hamilton, Ontario on 4 December 2020. Fish were 11 months old when we began experiments on 10 October 2021.

Wild Fish

We caught adult wild fathead minnows using a fyke net deployed for 24 hours in Windemere Basin, Hamilton, Ontario on 17 August 2021. We transported the fish back to McMaster University in aerated, chilled marine coolers.

Housing

Captive fish and wild fish were housed separately in 75 L tanks with two static renewal filters (AquaClear, Marina) and two sponge filters. We housed fish in groups of approximately 15, with 4 tanks of wild fish (n=42) and 4 tanks of captive fish (n=48). Tanks did not have substrate, but two PVC pipes were placed in each tank for shelter. We kept housing tanks at room temperature (~20°C) with a photoperiod of 13h:11h light:dark. We fed the fish 6 days a week to satiation with commercial pellets (Corey Optimum feed, 0.7 gr, New Brunswick, Canada). All animal protocols were developed in

accordance with guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board (AUP 17-45-12).

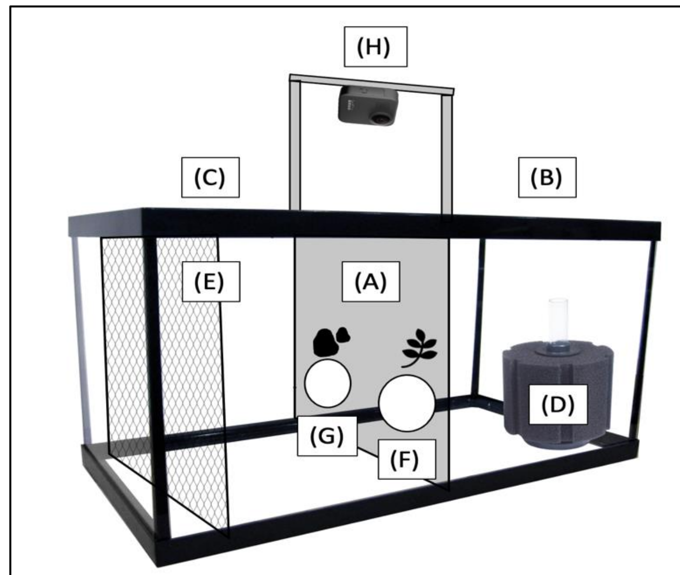
Trawl assays

Trawl tank description

We conducted trawl assays in twelve 38 L tanks (51cm x 25.5cm x 29cm) based on a design used by Lindeyer and Reader (2010) for guppies which we modified for fathead minnows. A transparent acrylic barrier (A) divided each tank lengthwise into two equal compartments called the “Refuge Zone” (B) and the “Trawl Zone” (C). Each Refuge zone had a filter (D) connected to air tubing. The trawl net (E) was moved from the back of the Trawl Zone to the divider. The divider contained two 3.2 cm holes, each 1.3 cm from the edge of the divider and 1 cm from the bottom of the divider. To make the holes visually distinctive, we outlined each hole in black marker, then drew a plant beside the left-hand hole (“Plant Exit”) (F) and a rock beside the right-hand hole (“Rock Exit”) (G). We attached a camera stand (I) to each tank and video recorded all trials. Each assay tank was filled with 24 L of charcoal-filtered City of Hamilton tap water and maintained at room temperature (~20°C) with a photoperiod of 13h:11h light:dark between 0600-1900 local time. We completed 100% water changes between groups of fish.

Figure 1

Illustration of the Trawl Tank Used in Associative Learning Trials, Reversal Learning Trials, and the Testing Phase.



Note: **(A)** Transparent acrylic divider. **(B)** Refuge Zone. **(C)** Trawl Zone. **(D)** Sponge filter. **(E)** Trawl net. **(F)** Plant exit. **(G)** Rock exit. **(H)** Camera stand.

Trawl assay set-up

As we had 12 assay tanks, we could perform assays on 6 groups of captive fish and 6 groups of wild fish simultaneously. In total, we used 16 groups of captive fish (n=48) and 14 groups of wild fish (n=42). We assigned groups to random tanks to account for possible room position effects. We ran experiments from 10 October - 6 December 2021.

Our pilot studies revealed that individual fish took much longer than groups of fish to complete the trawl assays, and the presence of conspecifics has been shown to reduce anxiety (Culbert et al., 2019). Therefore, we performed the assays on groups of 3 fish to reduce anxiety and adhere to time constraints. Groups of 3 fish were transported from their housing tanks into the Refuge Zone of the assay tanks and given a one-hour acclimation period. We randomly assigned each group to begin associative learning trials with either the Rock Exit or Plant Exit as the desired open exit. We covered the two exits with transparent plastic.

Associative learning trawl assay

To begin a training session, we started video recording and opened the desired exit. We net the fish over the divider into the Trawl Zone, where we let them acclimate for 5 minutes. We then began a 2-minute Trawl Period where we moved the trawl back and forth 4 times. Each trawl movement lasted 3 seconds and occurred every 30 seconds throughout the Trawl Period. We remained seated throughout the Trawl Period to avoid looming over the tanks. After the Trawl Period, we noted how many fish remained in the Trawl Zone and net them through the desired exit into the Refuge Zone. We waited 2 minutes before repeating the procedure 3 more times, for a total of 4 trials within one session. We completed 2 training sessions each day, separated by a minimum of 1 hour. We covered both exits between sessions. We designated a group to have associative learned when all three fish escaped through the desired exit within the 2-minute Trawl Period 4 trials in a row.

Reversal learning trawl assay

Once all three fish escaped through the desired exit four trials in a row, we switched from associative learning to reversal learning. In this assay, we swapped the desired exit, then followed the same trawl procedure as in the associative learning phase. We designated groups as having reversal learned when all 3 fish swam through the new desired exit within the 2-mintue Trawl Period 4 trials in a row. The experimental period lasted 14 days, beginning at the first day of associative learning trials. Trials were stopped after 14 days, regardless of whether the group had associative learned and reversal learned.

Testing Phase trawl assay

We began the Testing Phase after the fish completed reversal learning. We opened both exits. We net the fish over the barrier into the Trawl Zone and let them acclimate for 2 minutes before beginning the Trawl Period. If any fish remained in the Trawl Zone after the Trawl Period, we encouraged the fish to swim to the Refuge Zone by placing a net at the back of the Trawl Zone. The net was placed in the centre of the tank so as not to bias the fish towards one exit over the other.

Video scoring

We analyze five endpoints from our video recordings of the trawl assays. First, we measured the number of trials each group required to associative learn. Second, we measured the number of trials each group required to reversal learn. Third, we measured the proportion of fish within each group that managed to escape through the desired exit

in each trial. Fourth, we measured which exit each fish escaped through during the test phase when both exits were open. Fifth, we measured the latency for each fish to escape through the exits during the test phase.

Statistical analysis

Data was analyzed in RStudio version 1.4.1106 (RStudio Team, 2021). Data was inspected for normality using QQ plots and Shapiro-Wilk tests, and when not normally distributed was transformed. Data for the number of trials required to associative learn was square root transformed, but we graphed the original data.

Results

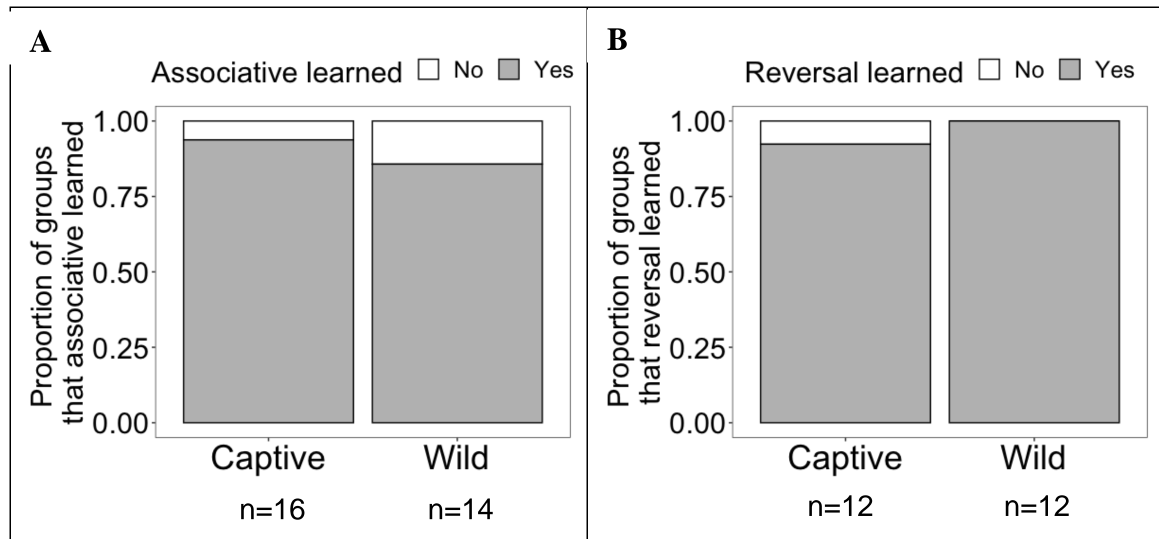
Associative learning and reversal learning

Proportion of captive and wild groups that learned

Overall, we did not detect a difference in the proportion of captive and wild groups that learned. In the associative learning assay, 94% of captive groups (n=16) and 86% of wild groups (n=14) achieved the learning criteria of escaping the trawl during four consecutive trials. We did not detect a difference in the proportion of captive and wild groups that associative learned (Fisher's exact test, odds ratio=2.4, p=0.59, Figure 2A). Of the groups that associative learned, 92% of captive groups (n=12) and 100% of wild groups (n=12) also achieved the learning criteria for the reversal learning assay. We did not detect a difference in the proportion of captive groups and wild groups that reversal learned (Odds ratio=0.0, p=1.00, Figure 2B).

Figure 2

The Proportion of Captive Groups and Wild Groups of Fathead Minnow (Pimephales promelas) That Learned



Note: Grey bars represent groups that learned and white bars represent groups that did not learn during the **(A)** Associative learning assay and **(B)** Reversal learning assay.

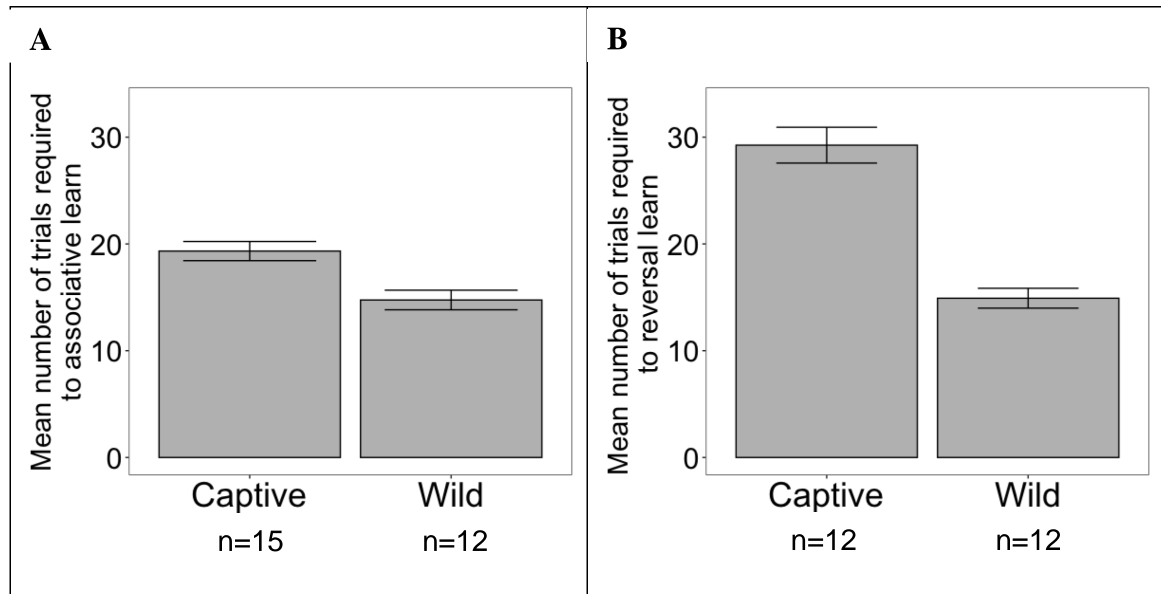
Number of trials required for captive and wild groups to learn

In the associative learning assay, captive groups (n=15) required an average of ~19 trials to reach the learning criteria and wild groups (n=12) required ~15 trials. We did not detect a difference in the number of trials required to associative learn for captive groups versus wild groups (T test, $t_{(2,25)}=1.0$, p=0.31, Figure 3A). We also did not detect a difference in the probability of associative learning over time for captive groups versus wild groups (Cox proportional hazards model, z=0.9, p=0.35, Figure 4). However, in the more challenging reversal learning assay, captive groups (n=12) required ~29 trials to

learn which was about 14 more trials than the wild groups, and here we did detect a difference (n=12; ($t_{(2,22)}$=2.2, p=0.04, Figure 3B). Wild groups required ~15 trials to reversal learn, which was similar to the number of trials they required to associative learn.

Figure 3

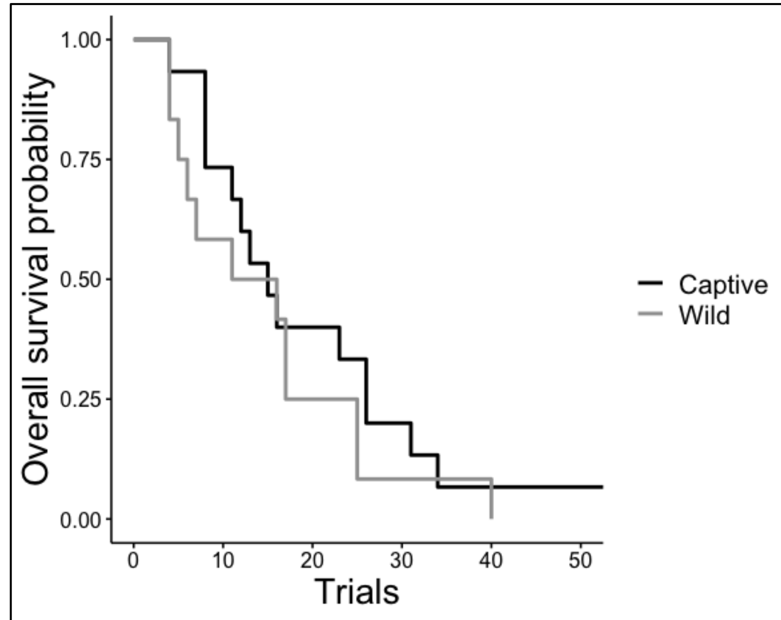
The Mean Number of Trials Required for Captive Groups and Wild Groups of Fathead Minnow (Pimephales promelas) to Learn



Note: Error bars are standard error. **(A)** Associative learning. **(B)** Reversal learning.

Figure 4

The Number of Trials Until the Associative Learning Criteria was Reached in Captive and Wild Groups of Fathead Minnows (Pimephales promelas)



Note: Plot of Kaplan-Meier survival probabilities

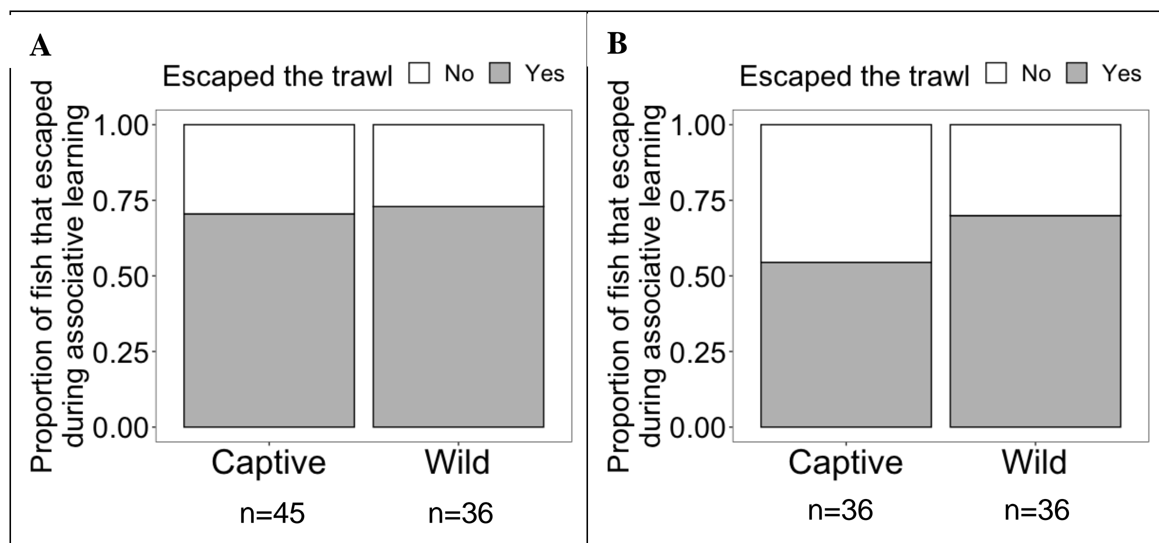
Proportion of captive and wild fish that escaped the trawl

Overall, we did not detect a difference in the average proportion of captive fish and wild fish that managed to escape the trawl. In the associative learning assay, an average of 70.5% of captive fish (n=45) and 72.9% of wild fish (n=36) managed to escape the trawl. We did not detect a difference in the average proportion of captive and wild fish that managed to escape during associative learning trials (Chi-squared test, χ^2= 0.0, p=1.00, Figure 5A). In the reversal learning assay, an average of 54.5% of captive fish (n=36) and 69.9% of wild fish (n=36) escaped the trawl each trial. We did not detect

a difference in the average proportion of captive and wild fish that managed to escape during reversal learning (Chi-squared test, χ^2= 1.2, p=0.27, Figure 5B).

Figure 5

The Average Proportion of Captive and Wild Fathead Minnow (Pimephales promelas) That Escaped the Trawl



Note: Grey bars represent the average proportion of fish that escaped the trawl and white bars represent the average proportion of fish that were required to be net through the exit during the **(A)** Associative learning assay and **(B)** Reversal learning assay.

Test Phase

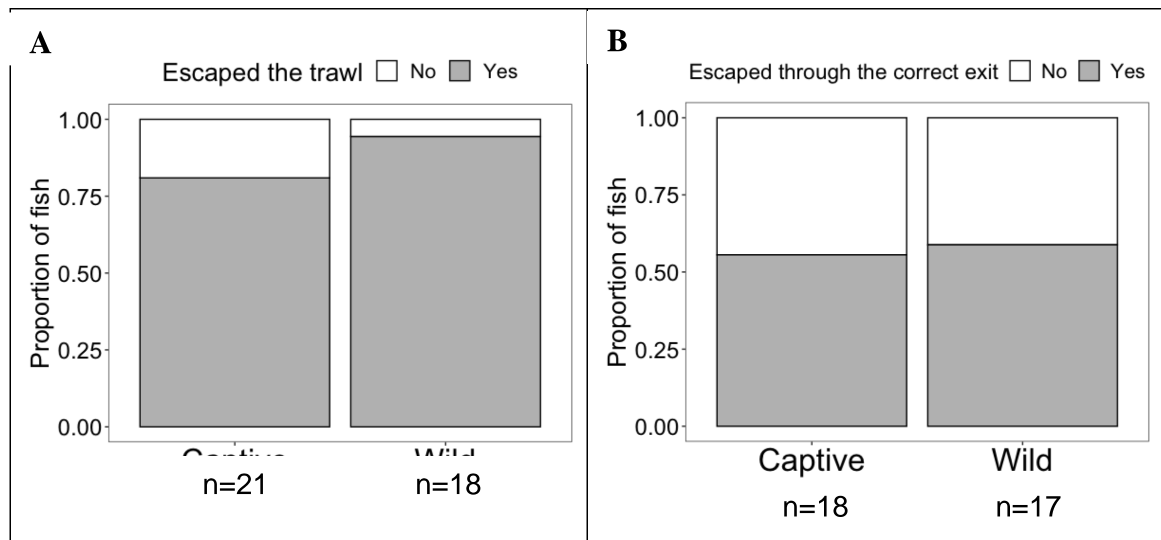
Proportion of captive and wild fish that escaped the trawl

When both exits were uncovered during the test phase, 81.0% of captive fish (n=21) and 94.4% of wild fish (n=18) managed to escape the trawl (Figure 6A). Of these

fish that escaped, 55.6% of captive fish (n=18) and 58.8% of wild fish (n=17) escaped through the correct exit that they were trained to use during the reversal learning task (Figure 6B).

Figure 6

The Proportion of all Captive and Wild Fathead Minnow (Pimephales promelas) That Escaped the Trawl During the Test Phase



Note: **(A)** The proportion of fish that managed to escape the trawl. Grey bars represent fish that escaped and white bars represent fish that did not escape. **(B)** The proportion of fish that escaped the trawl through the correct exit. Grey bars represent fish that escaped through the correct exit and white bars represent fish that escaped through the incorrect exit.

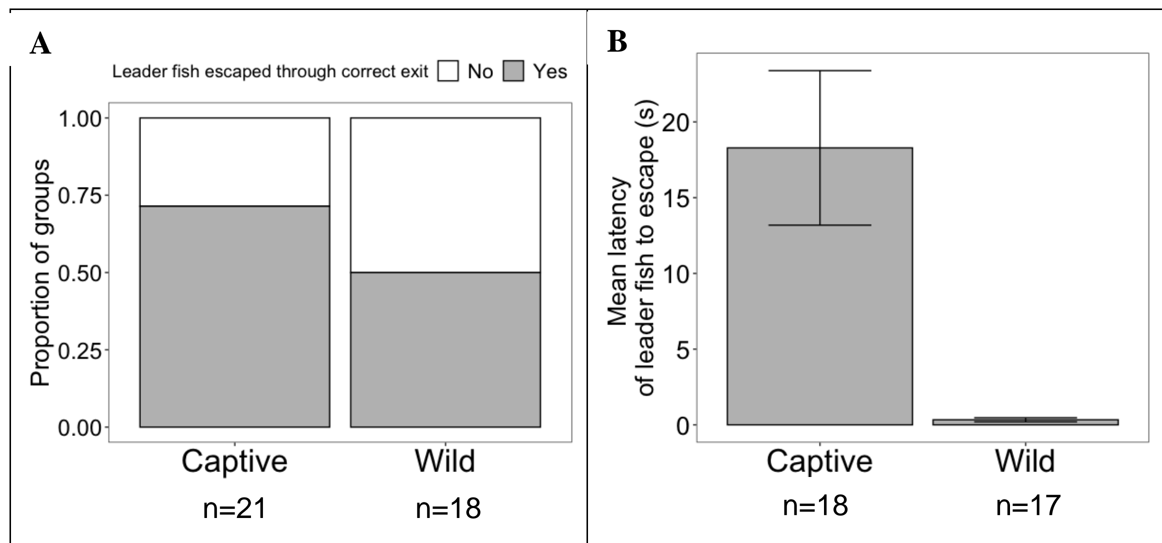
Leadership and group dynamics during the test phase

When we considered only the first fish to escape the trawl within each group, these leader fish escaped through the correct exit in 71.4% of captive groups (n=7) and in 50.0% of

wild groups (n=6, Figure 7A). The mean latency of the leader fish to escape was ~18 seconds longer in captive groups compared to in wild groups (Figure 7B).

Figure 7

The Proportion and Latency of Leader (First) Fathead Minnows (Pimephales Promelas) to Escape the Trawl During the Test Phase



Note: **(A)** The proportion of leader (first) fish to escape the trawl through the correct exit among the captive and wild groups of fathead minnows. Grey bars represent groups where the leader fish escaped through the correct exit and white bars represent groups where the leader fish escaped through the incorrect exit. **(B)** The mean latency of the leader (first) fish to escape through either exit in captive and wild groups. Error bars represent standard error.

Discussion

In this study, we tested the ability of captive and wild fathead minnow to learn to escape an aversive stimulus. We investigated associative learning as well as reversal learning, which is more complex and requires more cognitive flexibility (Buechal et al., 2018). We did not detect a difference in the proportion of captive and wild fish that associative learned or reversal learned; however, captive fish required nearly twice as many trials to reversal learn than wild fish. The reduced reversal learning speed of captive fish suggests that captive animals may have some cognitive deficits, especially in more cognitively complex scenarios.

Impact of captivity on cognition

The decrease in cognitive ability of the captive fish may be explained by previously observed morphological differences in the brains of captive and wild fish (Burns et al., 2009; Kotrschal & Kotrschal, 2020). Burns et al. (2009) found a 19.2% decrease in guppies' telencephalon size and a 17.8% decrease in optic tectum size after only one generation in captivity. Brain mass can be associated with cognitive abilities such as learning (Buechal et al., 2018; Kotrschal et al., 2013); therefore, a decrease in brain mass may underlie the decrease we observed in captive fish's learning ability. We plan to investigate this idea further by measuring the brain mass of the captive and wild fish, which we dissected and preserved after the learning assay.

We specifically found a decrease in captive fish's ability to reversal learn but not to associative learn. This finding further supports the idea that reduced brain size is responsible for the differences we observed between captive and wild fish, since previous studies have shown that reduced brain size and telencephalon ablation specifically impair reversal learning but not associative learning (Buechal et al., 2020; Lopez et al., 2000). Compared to associative learning, reversal learning is considered more complex, requiring more cognitive functions including cognitive flexibility (Buechal et al., 2020). Cognitive flexibility is the ability to adapt behaviour to shifting conditions, and therefore it can be beneficial in variable environments (Buechal et al., 2020). Fathead minnow are a generalist species capable of adapting to various environments and exist across a wide geographic range (Ankley & Villeneuve, 2006), so cognitive flexibility is likely relevant to their ecology in the wild. For example, Dunham et al (2000) found that fathead minnow readily emigrated from areas of limited food supply to unfamiliar areas, where survival can depend on rapidly learning to adjust to novel food sources and predators (Szabo et al., 2020). Therefore, cognitive flexibility may be selected for in wild fathead minnow, as fish likely gain fitness from rapidly adjusting to variable environmental conditions. However, captive environments which tend to have low variability (Bhat et al., 2015) might not favour supporting the metabolically expensive brain tissue underlying cognitive flexibility (Buechel et al., 2020; Kotrschal et al., 2013), as such selection pressures favoring cognitive flexibility may be relaxed in captivity. Previous studies suggest that stable captive environments can lead to plastic changes in phenotype (e.g., dampening behavioural flexibility, increased growth rate) within an individual's

lifetime (Hirawkawa & Salinas, 2020; Bhat et al., 2015), and prolonged exposure to stable environments over generations can select for specialists with low flexibility rather than generalists (Hirawkawa & Salinas, 2020). Therefore, our observation that captive fathead minnow have reduced reversal learning ability may be explained by a reduced brain size caused by different selection pressures and decreased phenotypic plasticity in response to low variation in captive environments.

Alternative interpretations and limitations

An alternative explanation for the reduced learning ability we observed in captive fish compared to wild fish is that it is possible that our wild fish were older and thus learned more quickly. Brain growth occurs throughout the entire lifespan of teleost fish (Burns et al., 2009), so older fish may have larger brains and increased learning ability. The ages of our wild fish were unknown, so since we could not control for age, we used similar body sizes of captive and wild fish as age and length are often correlated (Boehlert, 1985). However, captive fish often have faster growth rates than wild fish, so our wild fish may have been older than their similar-sized captive counterparts and therefore had larger brains (Burns et al., 2009) and better learning abilities (Buechal et al., 2018; Kotrschal et al., 2013). Although brain mass increases with age, Burns et al (2009) found that this increase was minimal and did not detect a difference in the relative brain mass of younger guppies compared to older guppies. Environmental conditions early in life likely have a larger effect on brain size than age and environmental conditions later in

life (Burns et al., 2009). Therefore, if our future analyses find that wild fish had larger relative brain mass, then their increased learning ability may have been minimally influenced by age but more likely caused primarily by different selection pressures and decreased phenotypic plasticity.

One limitation of our study is that when considering why captive fish might have deficient learning abilities, we were unable to differentiate the role of selection pressures from the role of phenotypic plasticity. Learning ability is affected by selection pressures (e.g., predation) experienced over generations as well as by environmental conditions experienced within one's lifetime, especially during development (Dukas, 2004). If learning ability was primarily affected by phenotypic plasticity during development, then perhaps the deficient reversal learning abilities of captive fish could be ameliorated via environmental enrichment (e.g., environmental variation). Future studies could begin to differentiate the roles of selection pressures and phenotypic plasticity by studying juvenile fish rather than adult fish, raising fish in a variety of enriched and deprived conditions, and tracking learning ability over generations.

We were also unable to fully differentiate individual learning from social learning since fish completed the assay in groups of three. In pilot studies we found that individual fish were too stressed to complete the assay. We also observed fish swimming from the refuge zone into the trawl zone, indicating that the refuge zone may not have been sufficiently less stressful than the trawl zone. Additional enrichment of the refuge zone with extra substrate and shelters could have motivated fish to swim to the refuge zone and

stay there and reduce the overall stress of the assay, potentially permitting the study of individual fish.

Although we compared captive and wild fish, our experiment only took place in a captive laboratory setting. Learning performance could conceivably vary between captive and wild settings, since individuals with high learning abilities might not demonstrate (perform) these abilities in certain settings due to factors such as anxiety (McGinnis & Milling, 2005) and lack of motivation (Duhon et al., 2019). Therefore, learning performance and learning ability are distinct concepts (Duhon et al., 2019) and we were unable to differentiate between them in our study. Few fish cognition studies (31%) utilize wild fish, and even fewer (9%) take place in wild settings (Salena et al., 2021). Future research could compare the learning performance of captive and wild fish in captive and wild settings to gain a more accurate understanding of true learning ability as well as the applicability of laboratory research to wild populations.

Implications

The reversal learning deficit we observed in captive fish has several implications for animal welfare and the validity of animal-based research. First, many animal conservation programs breed animals in captivity before releasing them into the wild (Burns et al., 2009; Sahashi & Morita, 2022). Fish with poor learning abilities (e.g., those raised in captivity) may be less able to escape predators, forage, or choose quality mates, all of which are important to fitness in the wild (Dukas, 2004). Therefore, our

finding that captive fish have dampened, or decreased learning abilities may partly explain the reduced survival and fitness observed in captive stocks re-released to the wild during conservation programs (Sahashi & Morita, 2022).

Second, the reversal learning deficit we observed in captive fish demonstrates that there may be differences between captive and wild populations and therefore research conducted on captive populations may not be applicable to wild populations. Fathead minnow are an ecotoxicological model species and studies on captive fathead minnow or other captive species often guide legislation on tolerable water concentrations of pollutants in the wild (Ankley & Villeneuve, 2006). Traditional ecotoxicological research uses measures of lethality (e.g., LC50) to inform environmental legislation of pollutants; however, sublethal endpoints (e.g., altered behaviour, cognition) are becoming more popular since they can indicate loss of fitness (ecological death) at much lower concentrations than lethality (physiological death; Leonard et al., 2014). Cognitive endpoints in ecotoxicological research using captive fathead minnow may fail to accurately predict the cognitive effects of pollutants on wild fathead minnow since we demonstrated that these populations differ in their cognitive abilities. Therefore, environmental protection policies based on ecotoxicological research using captive populations may not be the most effective strategy to protect wild populations since these populations may have different needs according to their different cognitive abilities.

Finally, fishes are increasingly used as model organisms in the study of human development and senescence, processes which involve many cognitive changes (Burns et al., 2009; Adams & Kafaligonul, 2018). Many of these studies make use of captively bred

fish for research such as zebrafish. Researchers should be aware of any existing cognitive deficits in captive fish before using them as models for human cognition since captive fish may not be representative of normal cognitive function. When unaccounted for in research studies, cognitive deficits of captive fish may limit our ability to understand basic mechanisms of cognition (Burns et al., 2009), how these mechanisms impact survival, and how they are likely to be impacted by anthropogenic factors such as chemical pollutants in the future.

Conclusion

To our knowledge, this was the first study to compare aversive learning in wild fathead minnows to aversive learning in fathead minnows raised in generations of captivity. Our finding that captive fish required more trials to reversal learn suggests that captive environments may lack certain selection pressures or developmental inputs essential for cognitive function (e.g., cognitive flexibility). Future research could investigate the causes of the reversal learning deficit we observed in captive fish, and this will provide future directions for animal conservation and the creation of valid research models. We believe that our findings will strengthen our understanding of how captivity impacts learning, a sublethal endpoint with high relevance to fitness in the wild. Our findings will also provide insight into the differences between captive and wild animals, which are rarely utilized in animal-based research studies.

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