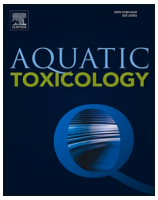
Aquatic Toxicology 273 (2024) 106982

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



Impacts of caffeine on fathead minnow behaviour and physiology

Jacqueline Bikker [a,*], Helen MacDougall-Shackleton [a], Leslie M. Bragg [b], Mark R. Servos [b], Bob B.M. Wong [c], Sigal Balshine [a]

[a] Department of Psychology, Neuroscience & Behaviour, McMaster University, 1280 Main Street West, Hamilton ON L8S 4K1, Canada
[b] Department of Biology, University of Waterloo, 200 University Avenue West, Waterloo ON N2L 3G1, Canada
[c] School of Biological Sciences, Monash University, 25 Rainforest Walk, Melbourne, Victoria 3800 Australia

ARTICLE INFO

Keywords:
Contaminant
Learning
Fathead minnow
Ecotoxicology
Metabolic rate
Anxiety

ABSTRACT

Pollution from regularly used substances such as pharmaceuticals, cleaning agents, and even food and beverages is an increasing problem in the environment. Caffeine, a commonly ingested stimulant, is one such contaminant that has been detected in aquatic environments worldwide. Yet, little is known about how ecologically relevant concentrations of caffeine influence the morphology, behaviour, and physiology of exposed organisms. To address this knowledge gap, we exposed fathead minnow (*Pimephales promelas*) to three caffeine treatments: a freshwater control (nominal: 0 ng/L), a low (nominal: 1,000 ng/L) and high environmentally relevant dose (nominal: 10,000 ng/L), for 35 days. We tested the learning abilities, anxiety, metabolic rates, and morphological features of exposed vs. control fish. Caffeine exposure did not affect the ability of fish to learn but did influence anxiety levels. Over the course of repeated anxiety testing, unexposed control fish visited a black square more often while fish exposed to low levels of caffeine did not, potentially indicating that these fish remained in a more anxious state. While caffeine did not impact metabolism, fish growth, or body size, it was associated with lower liver investment—although this response was only observed in our low caffeine treatment. Overall, our results suggest that even relatively low concentrations of caffeine may impact the liver size and anxiety of exposed fish, but further research is needed to assess how extended exposure to caffeine impacts fitness. Given the increase in anthropogenic contaminants in aquatic environments, it is important that we continue to investigate their effects on the organisms exposed to them.

1. Introduction

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

While a number of studies have examined how caffeine exposure can influence behaviours in animals, most of these have explored simple behaviours, such as movement (Connaughton and Clayman, 2022). By contrast, only a handful of studies have investigated how caffeine can affect cognitively complex behaviours, such as learning. Learning is a change in behaviour that occurs as a result of past experience (Shettleworth, 2010). The ability to learn is often viewed as an adaptive trait, especially in changing or challenging environments, and can be especially critical for foraging, predator avoidance, mate choice, and

* Correspondence author at: Department of Psychology, Neuroscience & Behaviour, McMaster University, 1280 Main Street West, Hamilton ON L8S 4K1, Canada.
E-mail address: jacqueline.bikker@mail.utoronto.ca (J. Bikker).

https://doi.org/10.1016/j.aquatox.2024.106982
Received 11 February 2024; Received in revised form 24 May 2024; Accepted 30 May 2024
Available online 1 June 2024

migration (Kieffer & Colgan, 1992). Thus, any factor that influences learning may have crucial downstream fitness effects. At low doses, caffeine increases alertness, thus raising the possibility that it may help individuals focus and facilitate learning. However, at higher doses, caffeine is also known to cause anxiety, which may impair learning (Nehlig, 2010).

To address this question, we determined how long-term exposure (35 days) to caffeine affected learning, anxiety, and physiology in the fathead minnow (*Pimephales promelas*) — a common ecotoxicology model and species with a wide freshwater distribution throughout North America (Ankley and Villeneuve, 2006). We used environmentally relevant concentrations of caffeine treatments in our study, in contrast to many previous studies investigating the effects of caffeine exposure on fish behaviours that often used unnaturally high doses (Egan et al., 2009; Li et al., 2020b; Wong et al., 2010). Additionally, most caffeine studies employ short-term exposures, ranging from 5 min to 96 h (Steele et al., 2018; Egan et al., 2009). It is possible that such short exposures will not yield environmentally relevant responses since fish can acclimate to caffeine over time (Santos et al., 2016), so in our study we employed a chronic exposure. Finally, we used fathead minnows as a study species, because most caffeine exposure studies to date have been conducted on zebrafish (*Danio rerio*; Santos et al., 2016; Neri et al., 2019; Egan et al., 2009; Wong et al., 2010), with much less known about the impacts of caffeine on other species. The toxicity of contaminants can differ depending on whether a species is tropical or temperate; this is due to differences in the solubility of contaminants that depend on water temperature, and on a fish's metabolism and ability to detoxify which are usually faster at higher temperatures (Kwok et al., 2007). As temperate fish are more likely to experience more colder temperatures compared to tropical fish, this may mean they will have an increased response to a contaminant like caffeine. Indeed, fathead minnow exposed to wastewater containing high levels of caffeine paired with winter temperatures (4 °C) were more metabolically taxed, while fish exposed to wastewater in summer temperatures (20 °C) did not experience any measured changes in metabolism, perhaps suggesting that temperate fish might be more metabolically influenced by caffeine than tropical fish since they experience contaminants often at lower water temperatures, for a considerable period of the year (Mehdi et al., 2022).

To test how caffeine exposure specifically affects learning, we trained fathead minnow to avoid getting caught in a trawl by escaping via one of two exits and then reversed which of the two exits represented safe passage. Such reversal learning is considered to be more cognitively complex and is often used as a measure of behavioural flexibility (Buechel et al., 2018; Izquierdo et al., 2017). Because caffeine consumption can increase anxiety and metabolic rates (Chad and Quigley, 1989; Donelly and McNaughton, 1992), we allowed fish to move between a white and black area of a tank and used the number of visits and the amount of time spent on the black as a measure of anxiety. Given that individuals are often more conspicuous to predators on light backgrounds, there is commonly a preference in fishes to spend time in dark coloured, "safer" backgrounds (Maximino et al., 2010b). We predicted that caffeine exposure at environmentally relevant concentrations would reduce learning ability and increase anxiety so that exposed fish would 1) be less likely to explore and find an exit and 2) spend more time on the black square and visit the black square less often. Finally, we used a respirometer to measure how chronic caffeine exposure influences oxygen consumption, a proxy for metabolic rate, and also measured body size and growth rates.

2. Methods and materials

2.1. Study animals and housing

Commercially purchased six-month-old fathead minnow (AquaTox Inc) were housed in 70 L exposure tanks at McMaster University (Hamilton, Ontario, Canada). Tanks contained two filters (AquaClear and Marina brands) and were maintained at 17–24 °C with a 13 h:11 h light: dark (0600 – 1900, local time). Activated carbon and ceramic bead media were removed from the filters prior to caffeine exposures to prevent any uptake of caffeine by the filter media. We fed fish daily with a mixture of frozen brine shrimp, bloodworms, and juvenile trout pellets ad libitum. Seven to eight days prior to the start of caffeine exposures, we tagged fish using non-toxic acrylic paint (Wolfe and Marsden, 1998) so that we could follow individuals in the behavioural assays and measure individual growth. All animal protocols were carried out in accordance with guidelines established by the Canadian Council on Animal Care and approved by the McMaster University Animal Research Ethics Board (AUP # 17–12–45).

2.2. Caffeine exposures

We conducted this study between June – August 2021, exposing fish to either no added caffeine (freshwater control), a low (1000 ng/L) or high (10,000 ng/L) environmentally relevant dose of caffeine, for 35 days. The low dose was based on the concentration of caffeine found at sites sampled immediately downstream of a wastewater treatment plant in Hamilton Ontario, Canada (McCallum et al., 2017), while the high dose was based on concentrations of caffeine found in an urban river (Beirut River, Lebanon; Mokh et al., 2017). Each treatment contained three replicate tanks, with 33 fish in each replicate tank (99 fish per treatment, $N = 297$). To be able to logistically collect behavioural and physiological data on so many animals, 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 (1 g/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 concentrations remained consistent. We performed 20 % water changes every 72 or 96 h, refreshing the exposure tank with dechlorinated Hamilton water spiked to the appropriate dose. To reduce caffeine contamination during exposures and testing, we used tight-fitting lids on all tanks and used treatment-specific testing tanks, nets and water change equipment. In addition, we had treatment specific capture equipment and hung plastic sheeting from the ceiling between treatment tanks to prevent aerosol contamination. Experimenters also refrained from consuming caffeine during the months of experimentation.

We collected paired samples of the exposure water throughout the experiment, with the first sample occurring one-hour post- water change/caffeine dosing, and the second sample occurring immediately before the next dose (72/96 h after the initial dose/water change). To accommodate the scattered behavioural and physiological testing schedule, dosing and water changes alternated between either every 72 or 96 h, but this schedule was consistent between all tanks and treatments. Our sampling regime for the exposure tanks spanned the exposure period from 0 – 28 days, and extended throughout the experimentation period (June – late July 2021; see Supplementary 6 for sampling dates). We also sampled municipal tap water and the carbon filtered reservoir water that was used for making our caffeine stock solution and filling our tanks. In total, we analysed 16 water samples from our exposure tanks and 6 samples from the tap water. Five of these water samples were from control exposure tanks (2 immediately pre-dose, 3 1-h post-dose), five samples were from the low caffeine exposure tanks (2 immediately pre-dose, 3 1-h post-dose), and six samples were from the high caffeine exposure tanks (3 immediately pre-dose, 3 1-h post-dose). We collected these water samples in 125-mL amber glass bottles, then preserved them with 200 g/L sodium azide and 20 g/L ascorbic acid, before transporting them on ice to the University of Waterloo (Waterloo, Ontario) where samples were extracted and analysed. Caffeine in each sample was concentrated using solid phase extraction and analysed via an Agilent 1260 HPLC with 6460 triple quad mass spectrometer (LC-MS/MS) with Agilent Jet Stream (AJS) electrospray ionization in positive

mode (Mehdi et al., 2022).

2.3. Aversive learning assay

To investigate how caffeine exposure affected learning, we used a trawl assay modified from a design used by Lindeyer & Reader (2010; Fig. 1A). The learning trials were all 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 exit holes (diameter = 3.2 cm) at the bottom of the divider (1 cm from bottom). The exit holes were outlined in black to make them more visibly obvious, and we drew a plant illustration next to one exit and a rock next to the other exit to further aid with discrimination. At the back of the tank there was a black mesh trawl attached to gliders which allowed us to pull the trawl back and forth through this 'trawl zone'. On the other side of the clear divider with the two exit holes there was a large sponge filter that served as a shelter creating a 'refuge zone' (Fig. 1A). Each learning assay tank contained 24 L of water dosed with the treatment-appropriate amount of caffeine. Water changes for the learning tanks followed the procedures used for the exposure tanks.

Associative Learning Phase: Based on pilot studies, we ran fish through the learning trials in groups of three as having companions maximized movement and exploration behaviours. For each trio of fish tested, we deliberately mismatched the size of the three individuals so that we could visually discriminate between the fish when scoring behaviour from the video recordings. 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. To begin the training session, we uncovered one exit (rock or plant); this same exit would be uncovered for all training sessions in this associative learning phase until the group learned the task. In each tank, using a random assignment calculator, we determined which of the two exits (rock vs. plant) to uncover first for the associative learning phase. After the one-hour habituation period, we moved the trawl back and forth in the trawl zone every 30 s for a total of four times within a 2-minute period. Any fish remaining in the trawl zone after the four passes was gently guided with a hand net into the refuge zone through the uncovered exit. We allowed the three fish to then rest in the refuge zone for two minutes before moving them back to the trawl zone and gave them five minutes to acclimate to the trawl zone and to recover from transport and handling. We would then repeat the training 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 training session.

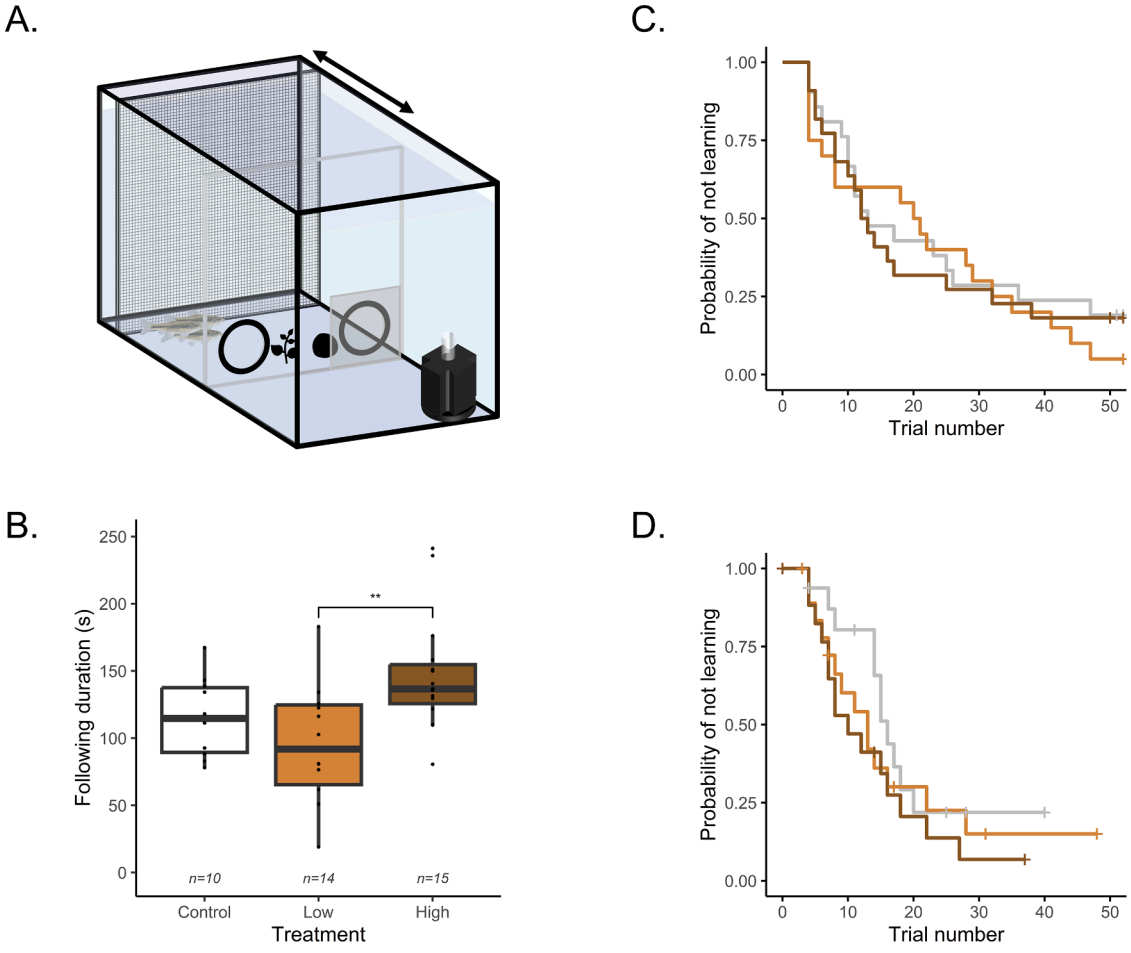


Fig. 1. (A) Aversive learning trawl assay with one side containing a moveable trawl, and the refuge zone containing a sponge filter. Once the fish reached the threshold for associative learning, the exit that was open and the exit that was closed previously were switched. (B) Following duration to exit the trawl zone between the first and third fish. Trials where fish did not exit were given a maximum latency of 120 s. 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 following duration between fish is indicated by ($^{**}p < 0.01$). (C) Number of trials until associative learning criteria were achieved (plotted as Kaplan-Meier survival probabilities). (D) Number of trials until reversal learning criteria were achieved (plotted as Kaplan-Meier survival probabilities). Vertical dash marks represent censored data (fish that didn't learn by the end of testing). Grey lines indicate control groups ($n = 21$ during associative learning, $n = 20$ during reversal learning), 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 Supplementary 1 for exclusion criteria).

Fish were considered to have 'learned' if all three fish exited the trawl zone through the uncovered exit within the 2-minute trial and did this four times in a row. If the fish 'learned' within seven days, they moved on to the reversal learning phase of the experiment. If the group of three fish had not learned after seven days, they did not move on to the reversal learning phase and testing ceased.

Reversal learning phase: After fish trios had been successfully trained in the associative learning task, we initiated the reversal learning phase of the experiment. To achieve this, we changed the exit through which the fish could escape from the trawl (i.e., the previously uncovered exit (rock or plant) was blocked, and vice versa). We then ran the fish through this protocol until they had either 'reversal learned' the task, or until seven days had elapsed since beginning the associative learning phase of the experiment. 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 2-minute period and do so for four consecutive trials. To analyse the learning videos, researchers who were unaware of the exposure treatment 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 for each fish during each trial.

2.4. Anxiety assay

To test how caffeine exposure affected anxiety, we ran a subset of the exposed fish (140 fish) through a scototaxis anxiety assay, modified from Polverino et al. (2021; Fig. 2A). Adult fathead minnow have previously been shown to have a preference for dark-coloured areas (Vignet and Parrott, 2017), and in our study, the fish across all treatments and assay dates, on average, spent 215.5 ± 13.7 s of the trial time on the black square, with the black square representing 1/7 of the aquarium bottom. This suggests that the black square was preferred. Fish were placed individually in 20 L tanks (41 cm x 21 cm x 25 cm) filled with 10 L of aerated, dechlorinated water and either dosed with low ($n = 45$ fish) and high ($n = 45$) caffeine or left undosed (control, $n = 50$), to match the exposure treatments. The bottom and sides of the anxiety tanks were lined with white adhesive paper, except for a single 11 cm x 11 cm black square, that was placed randomly in one of two possible bottom corners furthest from the front of the tank. Initially individual focal fish were placed in the middle of the tank within a vertically positioned black PVC refuge tube. After a 2-minute acclimation period, we removed the refuge tube and videotaped (using GoPro or Sony HDR cameras) the fish's activity and location for 15 min. Fish were tested repeatedly at seven days pre-exposure and then again at 21 days post-exposure. We used BORIS (Friard and Gamba, 2016) to score: 1) the amount of time the fish spent on the black square and 2) the number of times the fish moved off the black square. Behavioural data were extracted by trained observers who scored the videos unaware of the treatment to prevent the possibility of any observer bias. To account for underlying differences in anxiety unrelated to caffeine treatment, we calculated a behavioural difference

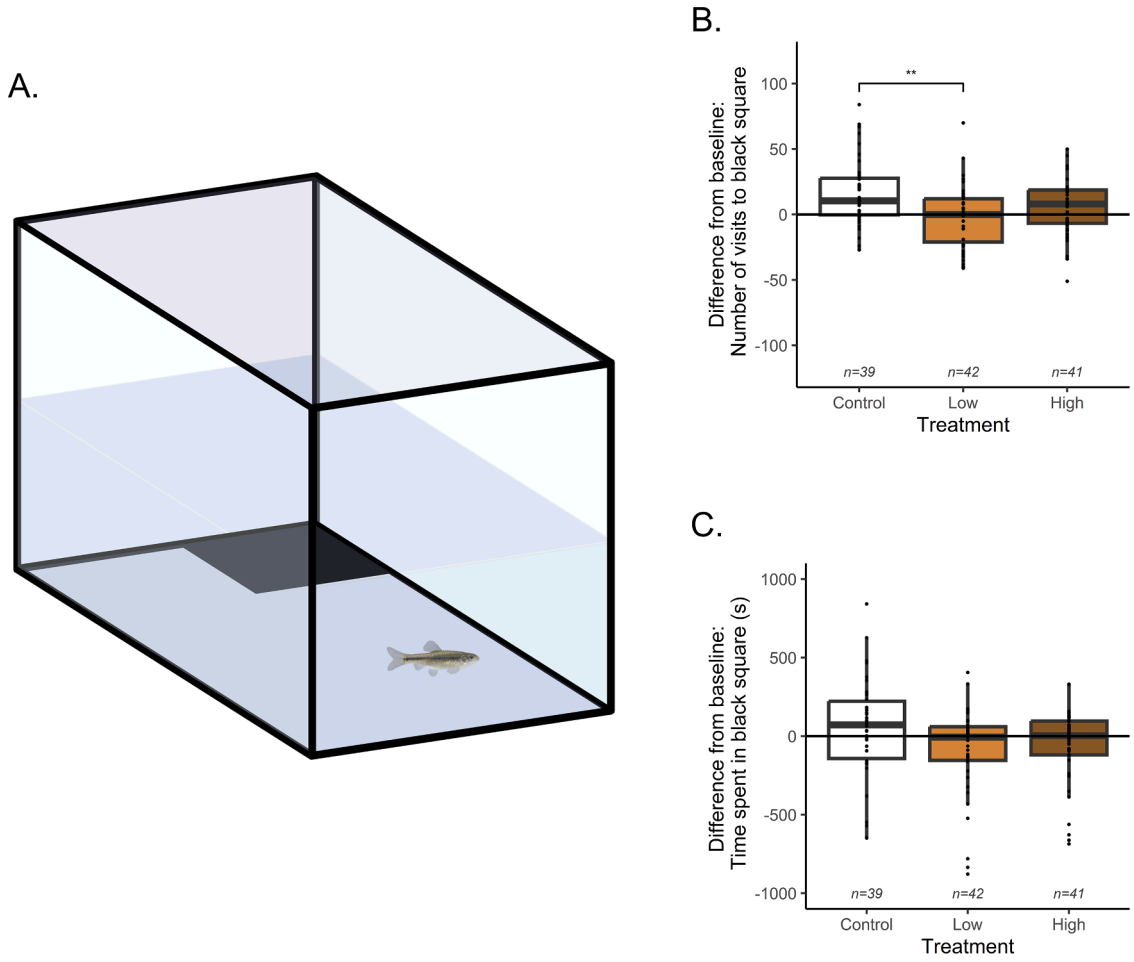


Fig. 2. (A) Anxiety assay where there is a black 11×11 cm square in one corner, while the rest of the bottom and sides of the tank are white. (B) Difference score for number of visits to the black square between Day 21 of exposure and pre-exposure (baseline) testing. The white boxplot represents control 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 is indicated by (*$p < 0.01$). (C) Difference score for time spent on the black square between Day 21 of exposure and pre-exposure (baseline) testing.

score, taking the difference between amount of time spent on the black square or the number of visits to the black square during pre-exposure testing (baseline) versus at the end of exposure (Day 21).

2.5. Physiological measures

We measured metabolic rates using an intermittent flow respirometry system by running four fish on Day 34 of exposure, and another four fish on Day 35, for a total of eight fish per treatment. We placed fish individually into one of four ~75 mL respirometry chambers all placed in a dark tank at room temperature (~20 °C) using dechlorinated municipal tap water. During the respirometry trials all fish were tested in freshwater and were placed in the respirometry chamber with alternating 5-minute flush periods and 5-minute measurement periods. During the 5-minute flush periods, fresh water from the open tank would enter the chambers to refresh the oxygen concentrations and prevent hypoxic conditions from forming (Mehdi et al., 2022). 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 was measured using a FireStingO_2 (Pyro Science, Germany).

Fish were in the respirometry chambers for ~22 – 24 h, and standard metabolic rate was measured continuously during this time. To measure standard metabolic rate, we assessed oxygen consumption overnight and used the lowest 10 measurements from this period. To measure maximum metabolic rate, we removed fish from the respirometry chamber, placed them in a round assay tank (diameter = 46 cm), and then chased them in a standardized manner with a hand net for three minutes followed by a 30 second air exposure; together these procedures simulated 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 continuously immediately post-chase (for about 5 min). We utilised the highest measurement from the post-chase period to calculate maximum metabolic rate. There was no flush period during this time, and fish were only chased once. We calculated aerobic scope by calculating the difference between the maximum metabolic rate and the standard metabolic rate for each individual. Throughout all of the respirometry trials, the average temperature was 21.6 °C and temperatures ranged from 19.8 °C to 22.9 °C. Fish were fasted for at least 24 h prior to any metabolic rate measurement, and all measurements were corrected for the temperature inside the respirometry chambers and for body mass (Clark et al., 2013). Note that the average mass of fish used for testing metabolic rates in the respirometer was 2.0 ± 0.09 g (n = 24).

2.6. Morphological measures

On Day −6/−7 and Day 35 of exposure, all experimental fish (N = 292; $n_{Control}$=96, n_{Low}=98, n_{High}=98) were measured (body mass in grams, total length (TL in mm), and standard length (SL in mm)) to measure post-exposure body condition and growth rate. We were able to determine the growth rate of most but not all fish because tag loss meant we could not reliably match up some tags at the end of the experiment (N = 213; $n_{Control}$=64, n_{Low}=73, n_{High}=76). Prior to the last measurement on Day 35, fish were humanely sacrificed using a benzocaine bath followed by spinal cord severance. We then removed and weighed the liver mass (N = 286; $n_{Control}$=94, n_{Low}=95, n_{High}=97), to assess relative liver investment (hepatosomatic index, or HSI=[liver mass/[body mass-liver mass] x 100]. We calculated body condition using Fulton's body condition factor = [body mass/length^3] x 100.

2.7. Statistical analyses

We used R version 4.1.2 for all analyses (R Core Team, 2021). We tested for normality and homogeneity of variance (Shapiro and Levene's tests), and whenever possible, we transformed the data (using log or square-root functions). When we could not achieve normality by transforming the data, we used non-parametric tests. We used a Kruskal-Wallis Test to assess the effects of treatment on all morphological endpoints. We used a linear model to determine if sex ratio within exposure tanks differed between treatments. For all other analyses determining treatment effects, we used linear models (unless the assumption of normality and homogeneity of variance were not met, in which case we used a Kruskal-Wallis test). We used linear mixed models for all our analyses determining how the interaction between treatment and sex affected endpoints, with sex/sex ratio and caffeine treatment as fixed effects. See Supplementary Materials for further details and all exclusion criteria. 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 and sexes. We used α=0.05 to indicate statistical significance.

3. Results

3.1. Caffeine concentrations

We detected caffeine in all our water samples (Table 1), even in our tap water and carbon-filtered water controls (2.2 ± 0.8 ng/L, n = 6). However, the average caffeine concentrations in the control tanks were at least 23x lower than the average concentration found in the low caffeine tanks and at least 96x lower than the average concentration found in the high caffeine tanks. This meant that the average caffeine concentrations in the control samples were one order of magnitude lower than in the low caffeine samples, and almost two orders of magnitude lower than in the high caffeine samples. We also expected caffeine to decline over time, which it did in our high caffeine treatment, but in our control and low caffeine tanks, the levels were fairly stable, and even appeared to concentrate.

3.2. Aversive learning

Caffeine exposure did not appear to affect if and how quickly fish learned or how fast they managed to reverse what they had previously learned. For the associative task, 88 % of the exposed fish and 81 % of the control groups learned. During the associative learning stage, fish from the low caffeine group took the longest to learn, needing 20 ± 3.4 trials on average to reach the learning criteria, compared to the control fish, who took an average of 16 ± 2.9 trials to learn. The fastest learners were from the high caffeine treatment with an average of 13 ± 2.3 trials to learn. However, difference in number of trials taken to learn across treatments was not significant and so we cannot claim that caffeine influenced the time required to reach the associative learning criteria

Table 1
Mean (± SE) concentrations in [ng/L] of caffeine in exposure tanks during experimentation and of tap water and the carbon-filtered reservoir water used during the experiment. In the exposure tanks, samples were taken 1 hour post-dose, and 72/96 h later, right before the next dosing event. Target nominal concentrations of exposure tanks were 0 ng/L (control), 1000 ng/L (low caffeine concentration), and 10,000 ng/L (high caffeine concentration). Due to analysis errors with the machine, the control and low exposure tanks are missing one sample each (see Supplementary 6). Samples of tap water and carbon-filtered water were taken at one sampling time and are included for comparison.

	Exposure tanks			Source water	
Sampling time (post-dose)	Control	Low	High	Tap water	Carbon-filtered water
1 h	15.2 ± 10.6, n = 2	682 ± 219, n = 2	4094 ± 1547, n = 3	3.63 ± 0.91, n = 3	0.68 ± 0.39, n = 3
72/96 h	40.2 ± 13.1, n = 3	749 ± 171, n = 3	1707 ± 1574, n = 3		

(z_{High}=0.3, p = 0.74; z_{Low}=0.6, p = 0.52; Fig. 1B). During the reversal learning stage, control fish needed the most trials to reversal learn (14 ± 1.5 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). Again, caffeine exposure did not affect the number of trials required to achieve the reversal learning criteria (z_{High}=1.4, p = 0.18; z_{Low}=0.8, p = 0.43; $z_{log(Number\ of\ trials\ to\ learn)}$= −0.7, p = 0.51; Fig. 1C), but while 71 % of the fish groups exposed to caffeine learned, only 55 % of the control groups learned the reversal task. Across all treatments, we found that it took fish 16 ± 1.7 trials to learn to associate one exit with escape and it took fewer trials, only 12 ± 1.0 trials, for them stop acting on previously learned behaviour and use the other exit.

Caffeine exposure did not significantly alter how quickly fish escaped (i.e., how fast the fish exited the trawl zone after the trawl began moving). During the associative learning stage, the average latency until the last fish exited the trawl zone was similar across all treatment groups (average: 69 ± 3.9 s; Kruskal-Wallis Test: H(2)=1.1, p = 0.58). Similarly, during the reversal learning phase, the average latency until the last fish exited the trawl zone was similar across treatments (average: 57 ± 3.7 s, LM: $F_{(2,36)}$=0.008, p = 0.99).

Of those fish that successfully associative and reversal learned, caffeine exposure influenced how closely fish followed each other through the exit and into the refuge zone (i.e., the following duration). The duration between the first and third fish in each group escaping into the refuge zone (across the associative and reversal learning phases; see Supplementary 5 for more information) was 115 ± 9.5 s in the control group, 96 ± 11.6 s in the low caffeine group, and 147 ± 11.2 s in the high caffeine group (LM: $F_{(2,36)}$ = 6.0, p = 0.006; Fig. 1B). Post-hoc analysis showed that following duration was significantly shorter in the low caffeine treatment compared to the high caffeine treatment ($t_{Low\text{-}High}$=−3.4, p = 0.004, Fig. 1B).

3.3. Anxiety

Caffeine exposure significantly altered the number of visits to the black square made by fish (LM: $F_{(2116)}$=5.9, p = 0.004), with control fish significantly increasing the number of visits by 16 ± 4.2 visits (t = 3.4, p = 0.002), from 23.2 ± 2.9 visits to 38.7 ± 4.2 visits, compared to fish in the low caffeine group that reduced the number of visits over subsequent testing, from 28.0 ± 3.67 visits to 25.4 ± 3.56 visits (2 ± 3.9 fewer visits; Fig. 1B). However, after accounting for individual behavioural differences (by examining the behavioural change from pre-exposure), we found that caffeine exposure did not clearly affect the amount of time fish spent on the black square (Kruskal-Wallis Test: H(2)=4.6, p = 0.10; Fig. 2C).

3.4. Physiological measures

The average standard metabolic rates (SMR) of fish exposed to low and high caffeine were 14 ± 0.8 mmol and 13 ± 1.7 mmol, respectively. These rates were not significantly different from the average SMR of 14 ± 0.5 mmol for the control fish (LM, $F_{(2,21)}$ = 1.36, p = 0.28). 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, $F_{(2,\ 21)}$ = 0.002, p = 1.00). Furthermore, the aerobic scopes of fish exposed to low caffeine (44 ± 3.3 mmol) and high caffeine (49 ± 9.3 mmol) were also not significantly different from the control group (44 ± 3.1 mmol; Kruskal-Wallis Test: H(2)=0.38, p = 0.83).

3.5. Morphological measures

The average body mass, total length (TL), standard length (SL), body condition, and growth per day appeared to be highest in fish exposed to high caffeine (Table 2 and Supplementary Table 3). However, this pattern was likely driven by the fact that, just by chance, our high caffeine treatment contained more males than the other two treatments ($F_{(2,6)}$=9.6, p = 0.01) and male fatheads grow larger and faster than females (Held and Peterka, 1974). In contrast, relative investment in the liver (hepatosomatic index, HSI) was modulated by caffeine exposure, but not by sex (Supplementary Table 4), with control fish having the largest livers (3.2 % of body mass), while fish exposed to low and fish exposed to high levels of caffeine had smaller HSIs on average (2.2 % and 2.9 % of body mass; Fig. 3; $t_{(Control\ -\ Low)}$=7, p < 0.0001, $t_{(Control\ -\ High)}$=2, p = 0.07, $t_{(Low\ -\ High)}$=−4, p = 0.0001).

4. Discussion

4.1. Caffeine concentrations

In this study we investigated how environmentally relevant concentrations of caffeine affected fathead minnow cognition, physiology, and morphology. Although overall, the high caffeine concentrations (2901 ± 1122 ng/L) remained higher than in the low exposure treatments (722 ± 118 ng/L) and the controls (30 ± 10 ng/L), we expected to have no caffeine in the controls and for concentrations to decrease over time (Lam et al., 2004), but this did not happen. Constant caffeine levels have been reported in other studies (Cerveny et al., 2022). The very low levels of caffeine in our municipal and carbon-filtered water (2.2 ± 0.8 ng/L) suggest that tap water was not our caffeine source. We surmise that, despite our best efforts to minimize contamination (i.e., using lids, exposure-specific equipment, and separating tanks with plastic sheets), the low levels of caffeine in our controls were from aerosol contamination between exposure tanks, as reported in other caffeine exposure

Table 2
Mean ± standard error of each morphometric measure and their Kruskal-Wallis Test results, with the listed morphometric measure as the response variable, and caffeine treatment as the independent variable. Sex ratio was calculated at the replicate tank level (n = 9). We used a linear model to assess sex ratio due to a small sample size, and instead of an H-value, an F-statistic is listed. For all of the significant Kruskal-Wallis Tests (as evidenced by the p-value), post-hoc tests showed that sex, not caffeine treatment was the driving factor in differences for all morphometric measures except hepatosomatic index. 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. Note that although we started with 297 fish, 5 fish died during the 35 days of exposure (death occurring in different treatments), so most of these morphometrics apply only to the 292 remaining fish. An additional 6 fish were not included in the liver analyses because they did not have measurable livers (excluded fish: $n_{Control}$=2, n_{Low}=3, n_{High}=1).

	Control	Low caffeine	High caffeine	Sample size	Df	H	*p-value*
Body mass (g)	3.15±0.14	3.01±0.11	3.47±0.13	*n = 292*	2	6.9	0.03
Total length (cm)	6.41±0.08	6.41±0.07	6.62±0.07	*n = 292*	2	6.3	0.04
Standard length (cm)	5.25±0.07	5.26±0.06	5.44±0.06	*n = 292*	2	6.0	0.05
Body condition	1.13±0.01	1.09±0.01	1.14±0.01	*n = 292*	2	6.7	0.04
Growth (g/day)	0.01±0.001	0.01±0.001	0.02±0.001	*n = 213*	2	12.2	0.002
Hepatosomatic Index (%)	3.18±0.12	2.24±0.09	2.85±0.10	*n = 286*	2	39.1	<0.0001
Sex ratio	1.11±0.14	0.97±0.09	1.8 ± 0.20	*n = 9*	2,6	9.6	0.01

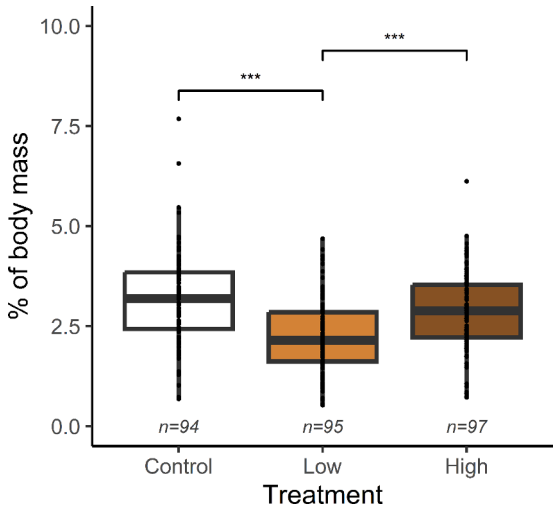


Fig. 3. Liver investment (hepatosomatic index) of fish on Day 35 of caffeine exposure across treatment groups. See Methods Section 2.6 for an explanation of how liver investment was calculated. The white boxplot indicates control fish, the light brown boxplot indicates fish exposed to low caffeine, and the dark brown boxplot indicates fish exposed to high caffeine. A significant effect of caffeine treatment on liver investment is indicated by (***$p < 0.001$).

studies (Cerveny et al., 2022; Tan et al., 2023). Although we had a small amount of caffeine contamination in the control treatment, because the average concentrations were an order of magnitude lower than the values measured in the low caffeine treatment, and almost two orders of magnitude lower than in the high caffeine treatment, we argue that the comparisons made between treatments are still valid. Additionally, average concentrations in the low caffeine treatment were 4x lower than in the high caffeine treatment, so overall the caffeine exposure treatments are sufficiently different to compare behaviour, morphology, and physiology in relation to caffeine concentrations.

Despite the variability in caffeine concentrations, we are confident that fish were exposed to caffeine during the experiments and that uptake happened quickly. Mosquitofish (*Gambusia holbrooki*) exposed to ~9000 ng/L caffeine achieved almost a maximum uptake concentration in their tissue after only 5 h of exposure, and with a daily renewal, concentrations remained constant for about 7 days (Wang and Gardinali, 2013). In the mosquitofish, the half-life of caffeine in clean water following exposure was 141 h (Wang and Gardinali, 2013). The half-life of caffeine in Nile tilapia (*Oreochromis niloticus*) was much shorter at 4.95 h, but the fish were injected with caffeine, rather than exposed to it via their tank water (Gomez-Martinez, 2011). Given that our fish were exposed to caffeine via tank water, we expect that the half-life of caffeine in the tissues of our fish would also be longer than those of the Nile tilapia, and that even if the concentrations of caffeine in the exposure tanks were variable between doses, the fish would still maintain a somewhat higher concentration of caffeine in their tissues because the half-life was so long.

While we did not measure the caffeine concentrations of the brain or liver tissue, an important next step would be to investigate and determine how much caffeine is located in these tissues (i.e., the two tissues associated with our observed behavioural and morphological effects). This would address whether these observed effects are due to caffeine buildup in the tissues, or if they are just a byproduct of caffeine circulating throughout the bloodstream.

4.2. Aversive learning

On average the fish in our study took ~69 s to exit the trawl zone during associative learning, and ~57 s to exit the trawl zone during reversal learning. While we expected caffeine to either help fish learn faster or make them more anxious and therefore make it more difficult for them to learn, caffeine exposure did not clearly influence how quickly the fish learned to avoid an aversive stimulus (i.e., the trawl) or reverse this learning when escape routes were switched. It was surprising that fish took less time on average to master the reversal task (see the steeper survival slope on Fig. 1D), given that this is considered to be the more challenging task as one solution needs to be forgotten or suppressed and an alternative solution learned and employed (Buechel et al., 2018).

Caffeine exposure influenced the following duration between fish (as measured by the exit latency between the first and third fish). Groups exposed to high caffeine concentrations had a longer following duration between the first and third fish to exit than in groups exposed to low caffeine concentrations, suggesting that caffeine exposure and the concentration of caffeine could impact the social dynamics of the fish. Social dynamics and group interactions are known to be altered by other psychoactive pollutants (Mason et al., 2021). Furthermore, the effects of caffeine may be buffered or ameliorated by shoal interactions (Neri et al., 2019). For example, while isolated zebrafish dosed with 70 mg/L of caffeine decreased swim speed, when fish were exposed in groups, they swam as quickly as unexposed control fish (Neri et al., 2019), suggesting that social support received by group living may mask the impacts of caffeine on learning performance.

Many of the fish used in the learning assay had undergone anxiety testing earlier in the experiment (i.e., 7 days before exposure began and after 21 days of exposure). While it is possible that the experience of being tested in the anxiety assay may have affected the fish's ability to learn, this is highly unlikely. The fish spent a week in the learning assay in total, and thus were well habituated to the assay. Additionally, the fish underwent many trials as part of the learning assay (i.e., up to 52 trials). The anxiety assay was only 15 min long, so it is unlikely that such a short assay would affect the learning endpoint, especially given the length of the learning assay. We also employed the same methodology across all treatments, so reusing the fish in both assays should not have affected the results of the learning assay.

4.3. Anxiety

Control group fish visited the black square more frequently on Day 21 compared to during pre-exposure testing (baseline), suggesting that fish were more comfortable and more active in the anxiety assay as time progressed. Previous research with zebrafish using a similar anxiety scototaxis assay showed that fish crossed between the black and white compartment fewer times during subsequent trials (Maximino et al., 2010a). In our study, this increase in movement did not occur to the same extent in the low and high caffeine treatments and is suggestive that caffeine at low concentrations may keep fish more anxious, as they weren't moving around as much. However, our other measure of anxiety, time spent on the black square, provided a more muted difference between treatments when we calculated the difference between amount of time spent on the black square on Day 21 compared to during pre-exposure testing (baseline). Given that only one of our anxiety results reached the threshold of statistical significance, we cannot claim that there were any large anxiety effects of caffeine on exposed fish.

4.4. Physiological measures

While caffeine is known to increase resting and maximal metabolic rate in humans (Donelly and McNaughton 1992; Chad and Quigley, 1989), caffeine exposure did not impact standard or maximum metabolic rate or the aerobic scope of fathead minnows in this study. Similarly, the resting metabolic rate of water fleas (*Daphnia magna*) also did not increase when exposed to concentrations similar to the ones we used in our experiment (i.e., 400 ng/L, 2000 ug/L, 10,000 ng/L; Nunes et al., 2022). The previous studies that managed to show metabolic effects all used caffeine concentrations that were much higher (60 mg/kg) than

those used in our experiment (0.01 mg/L). Hence the environmentally relevant caffeine doses we used were likely insufficient to induce a metabolic effect.

4.5. Morphological measures

Caffeine exposure did not influence body condition or growth of the fathead minnow in our study. Altered growth has been observed in sea bream (*Sparus aurata*; Chatzifotis et al., 2008) and neotropical catfish (*Rhamdia quelen*; dos Santos et al., 2021) exposed to caffeine, although again these studies used much higher caffeine doses (1 g/kg and 16 mg/L) and a different exposure route (diet). Caffeine exposure did, however, affect liver investment patterns in our study, with the lowest liver investment surprisingly observed in fish exposed to low caffeine concentrations. We predicted altered liver investment with caffeine exposure because of the greater need to detoxify the caffeine (Facey et al., 2005). Caffeine has been shown to decrease lipid accumulation in the livers of zebrafish (Zheng et al., 2015), and in mice, it encourages the breakdown of lipids in hepatic cells (Sinha et al., 2014), so it is possible that this decrease in HSI that we observed is due to decreased lipid accumulation. However, this does not explain why we saw this effect only at low caffeine concentrations. Other studies too have found that caffeine altered HSI in unpredictable ways with 1 g/kg of caffeine increasing HSI of sea bream but 0.1 g/kg and 5 g/kg of caffeine decreasing HSI, and 2 g/kg of caffeine not altering HSI (Chatzifotis et al., 2008). Further research is needed to determine the precise mechanistic reasons underpinning HSI responses to caffeine exposure.

We studied the effects of caffeine on the fathead minnow, a temperate species, while most other research has focused on tropical species, such as the zebrafish (*Danio rerio*; Santos et al., 2016; Neri et al., 2019; Egan et al., 2009; Wong et al., 2010). It is difficult to accurately compare the response of our study organism to caffeine with that of a tropical species, given that most studies use much higher caffeine concentrations (Egan et al., 2009; Li et al., 2020b; Wong et al., 2010). In our study, we found there to be only a few modest effects of caffeine on the behavioural and morphological endpoints we used. While we expected that our fish would be more affected by caffeine compared to tropical species because of the colder water temperatures experienced by temperate species, we completed this study in the summer, and the effects of caffeine in the winter may differ, as they do with other contaminants, such as wastewater (Mehdi et al., 2022). Our results highlight both the need to investigate other temperate species, as well as the need to investigate the impact of caffeine in colder temperatures, when aquatic ectotherms are more likely to experience more extreme effects of contaminants.

We found that exposure to our low concentration of caffeine (1000 ng/L) decreased both the number of times fish visited the black square when compared to baseline testing as well as the following duration between fish in the aversive learning assay. However, we did not find these endpoints to be altered at our high concentration of caffeine (10,000 ng/L). Caffeine has been shown to have a biphasic effect on fish and mice; normally researchers have observed that lower caffeine concentrations decrease anxiety, while higher concentrations increase anxiety (Santos et al., 2017; El Yacoubi, 2000). Instead, we found that low caffeine concentrations reduced one measure of anxiety, while fish exposed to high caffeine concentrations did not show altered measures of anxiety. Caffeine blocks both the A_1 and A_2 adenosine receptors (Fredholm, 1999), and in zebrafish when A_1 receptors are blocked it increases anxiety, while blocking A_2 receptors increases locomotion (Maximino et al., 2011; Santos et al., 2023). Hence it is possible that in our study, the low caffeine exposure blocked A_1 receptors, causing an increase in anxiety, while the high caffeine concentration instead blocked A_2 receptors. Further research investigating the role of the A_1 and A_2 adenosine receptors in these endpoints and at these caffeine concentrations would be needed to confirm whether this is indeed the case.

5. Conclusion

Taken together, our results show that environmentally relevant but low concentrations of caffeine decrease liver investment and may alter one aspect of anxiety (specifically, the number of visits to the black square). Caffeine levels also altered the following duration between fish but we found little evidence that caffeine alters learning, growth, or metabolic rates in fathead minnow. Our other measure of anxiety — time on the black square — was not as sensitive to our concentrations of caffeine. We currently do not have a clear explanation for why liver investment and anxiety decreased under low caffeine or why the high-dose exposure did not have similar effects. Despite not finding consistent results as to how caffeine effects fish behaviour and physiology, we argue that because caffeine is continuously added to our aquatic environments, it needs to be studied more thoroughly. Caffeine has the potential to negatively affect multiple organisms even at environmentally relevant concentrations (Li et al., 2020a). 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. Given a world with an increasing number and concentration of pollutants, continued focus on studying anthropogenic contaminants and their effects on aquatic environments is crucial.

Funding

This work was supported by funding from NSERC Discovery (#RGPIN2017–03816 to MS and #RGPIN2022–05353 to SB), Canada Research Chairs (#2017–00230 to MS), the Australian Research Council (FT190100014 and DP220100245 to BBMW), and Ontario Graduate Scholarship to JB.

CRediT authorship contribution statement

Jacqueline Bikker: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Helen MacDougall-Shackleton:** Conceptualization, Methodology, Writing – review & editing. **Leslie M. Bragg:** Conceptualization, Methodology, Writing – review & editing. **Mark R. Servos:** Conceptualization, Methodology, Writing – review & editing. **Bob B.M. Wong:** Conceptualization, Writing – review & editing, Supervision. **Sigal Balshine:** Conceptualization, Writing – review & editing, Funding acquisition, Resources, Project administration, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data and code will be submitted to Mendeley Data Repository.

Acknowledgements

We thank Hossein Mehdi for running the respirometry trials, and Andy Turko for helping with analysis. We thank Margaret Dobbin, Stephanie Liang, Adrienne Yau, Charlotte Day, Fatima Al-Mosawi, Hannah Scholtens, and Megha Vinod for scoring the behaviour videos. Thank you to Amy Kalbfleisch and Victoria Winslow for assisting with dissections. Thank you to Hung Tan for assistance with caffeine methodology and thank you to Dave Thompson for building the learning trawl apparatus. We thank Max Kirby for extracting the samples in the lab.

Thank you to Joanne Parrott for your advice for juvenile fish husbandry. Thank you to Upama Aich for proofreading the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.106982.

References

Ankley, G.T., Villeneuve, D.L., 2006. The fathead minnow in aquatic toxicology: past, present and future. Aquatic Toxicol. 78 (1), 91–102. https://doi.org/10.1016/j.aquatox.2006.01.018.

Buechel, S.D., Boussard, A., Kotrschal, A., van der Bijl, W., Kolm, N., 2018. Brain size affects performance in a reversal-learning test. Biolog.l Sci. 285 (1871), 20172031 https://doi.org/10.1098/rspb.2017.2031.

Cerveny, D., Cisar, P., Brodin, T., Mccallum, E.S., Fick, J., 2022. Environmentally relevant concentration of caffeine—Effect on activity and circadian rhythm in wild perch. Environm. Sci. Pollut. Res. 29 (36), 54264–54272. https://doi.org/10.1007/s11356-022-19583-3.

Chad, K., Quigley, B., 1989. The effects of substrate utilization, manipulated by caffeine, on post-exercise oxygen consumption in untrained female subjects. Eur. J. Appl. Physiol. Occup. Physiol. 59 (1–2), 48–54. https://doi.org/10.1007/bf02396579.

Chatzifotis, S., Kokou, F., Ampatzis, K., Papadakis, I.E., Divanach, P., Dermon, C.R., 2008. Effects of dietary caffeine on growth, body composition, somatic indexes, and cerebral distribution of acetyl-cholinesterase and nitric oxide synthase in gilthead sea bream (*Sparus aurata*), reared in winter temperature. Aquac. Nutr. 14 (5), 405–415. https://doi.org/10.1111/j.1365-2095.2007.00541.x.

Clark, T.D., Sandblom, E., Jutfelt, F., 2013. Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. J. Experim. Biol. 216 (15), 2771–2782. https://doi.org/10.1242/jeb.084251.

Connaughton, V.P., Clayman, C.L., 2022. Neurochemical and behavioral consequences of ethanol and/or caffeine exposure: effects in zebrafish and rodents. Curr. Neuropharmacol. 20 (3), 560–578. https://doi.org/10.2174/1570159X19666211111142027.

Donelly, K., McNaughton, L., 1992. The effects of two levels of caffeine ingestion on excess postexercise oxygen consumption in untrained women. Eur. J. Appl. Physiol. Occup. Physiol. 65 (5), 459–463. https://doi.org/10.1007/bf00243514.

dos Santos, J.A., Quadra, G.R., Almeida, R.M., Soranço, L., Lobo, H., Rocha, V.N., Bialetzki, A., Reis, J.L., Roland, F., Barros, N., 2021. Sublethal effects of environmental concentrations of caffeine on a neotropical freshwater fish. Ecotoxicology. 31 (1), 161–167. https://doi.org/10.1007/s10646-021-02498-z.

Drewnowski, A., Rehm, C., 2016. Sources of caffeine in diets of US children and adults: trends by beverage type and purchase location. Nutrients. 8 (3), 154. https://doi.org/10.3390/nu8030154.

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav. Brain Res. 205 (1), 38–44. https://doi.org/10.1016/j.bbr.2009.06.022.

El Yacoubi, M., Ledent, C., Ménard, J., Parmentier, M., Costentin, J., Vaugeois, J., 2000. The stimulant effects of caffeine on locomotor behaviour in mice are mediated through its blockade of adenosine A2A receptors. Br. J. Pharmacol. 129 (7), 1465–1473. https://doi.org/10.1038/sj.bjp.0703170.

Facey, D.E., Blazer, V.S., Gasper, M.M., Turcotte, C.L., 2005. Using fish biomarkers to monitor improvements in environmental quality. J. Aquat. Anim. Health 17 (3), 263–266. https://doi.org/10.1577/h04-055.1.

Fredholm, B.B., Bättig, K., Holmén, J., Nehlig, A., Zvartau, E.E., 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol. Rev. 51 (1), 83–133.

Friard, O., Gamba, M., 2016. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol. Evol. 7 (11), 1325–1330. https://doi.org/10.1111/2041-210x.12584.

Gomez-Martinez, L.E., 2011. Disposition kinetics of caffeine and paraxanthine in Nile tilapia (*Oreochromis niloticus*): characterization of the main metabolites. Arch. Environ. Contam. Toxicol. 60, 654–664.

Heckman, M.A., Weil, J., de Mejia, E.G., 2010. Caffeine (1, 3, 7-trimethylxanthine) in foods: a comprehensive review on consumption, functionality, safety, and regulatory matters. J. Food Sci. 75 (3), R77–R87. https://doi.org/10.1111/j.1750-3841.2010.01561.x.

Held, J.W., Peterka, J.J., 1974. Age, growth, and food habits of the fathead minnow, Pimephales promelas, in North dakota saline lakes. Trans. Am. Fish. Soc. 103 (4), 743–756. https://doi.org/10.1577/1548-8659(1974)103<743:AGAFHO>2.0.CO;2.

Izquierdo, A., Brigman, J.L., Radke, A.K., Rudebeck, P.H., Holmes, A., 2017. The neural basis of reversal learning: an updated perspective. Neuroscience 345, 12–26. https://doi.org/10.1016/j.neuroscience.2016.03.021.

Kieffer, J.D., Colgan, P.W., 1992. The role of learning in fish behaviour. Rev. Fish Biol. Fish. 2 (2), 125–143. https://doi.org/10.1007/bf00042881.

Kwok, K.W., Leung, K.M., Lui, G.S., Chu, V.K., Lam, P.K., Morritt, D., Maltby, L., Brock, T. C., Van den Brink, P.J., Warne, M.S.J., Crane, M., 2007. Comparison of tropical and temperate freshwater animal species' acute sensitivities to chemicals: implications for deriving safe extrapolation factors. Integr. Environ. Assess. Manage 3 (1), 49–67. https://doi.org/10.1002/ieam.5630030105.

Lam, M.W., Young, C.J., Brain, R.A., Johnson, D.J., Hanson, M.A., Wilson, C.J., Richards, S.M., Solomon, K.R., Mabury, S.A., 2004. Aquatic persistence of eight pharmaceuticals in a microcosm study. Environ. Toxicol. Chem. 23 (6), 1431. https://doi.org/10.1897/03-421.

Lenth, R.V., Bolker, B., Buerkner, P., Giné-Vázquez, I., Herve, M., Jung, M., Love, J., Miguez, F., Riebl, H., & Singmann, H. (2022). *emmeans: estimated marginal means, aka least-squares means* (Version 1.7.3) [Computer software]. https://CRAN.R-project.org/package=emmeans.

Li, S., He, B., Wang, J., Liu, J., Hu, X., 2020a. Risks of caffeine residues in the environment: necessity for a targeted ecopharmacovigilance program. Chemosphere 243, 125343. https://doi.org/10.1016/j.chemosphere.2019.125343.

Li, S., Wen, J., He, B., Wang, J., Hu, X., Liu, J., 2020b. Occurrence of caffeine in the freshwater environment: implications for ecopharmacovigilance. Environm. Pollut. 263, 114371 https://doi.org/10.1016/j.envpol.2020.114371.

Lindeyer, C.M., Reader, S.M., 2010. Social learning of escape routes in zebrafish and the stability of behavioural traditions. Anim. Behav. 79 (4), 827–834. https://doi.org/10.1016/j.anbehav.2009.12.024.

Mason, R.T., Martin, J.M., Tan, H., Brand, J.A., Bertram, M.G., Tingley, R., Todd-Weckmann, A., Wong, B.B.M., 2021. Context is key: social environment mediates the impacts of a psychoactive pollutant on shoaling behavior in fish. Environ. Sci. Technol. https://doi.org/10.1021/acs.est.1c04084.

Maximino, C., Lima, M.G., Olivera, K.R.M., Picanço-Diniz, D.L.W., Herculano, A.M., 2011. Adenosine A1, but not A2 receptor blockade increases anxiety and arousal in zebrafish. Basic Clin. Pharmacol. Toxicol. 109 (3), 203–207. https://doi.org/10.1111/j.1742-7843.2011.00710.x.

Maximino, C., Marques de Brito, T., Colmanetti, R., Pontes, A.A.A., de Castro, H.M., de Lacerda, R.I.T., Morato, S., Gouveia, A., 2010a. Parametric analyses of anxiety in zebrafish scototaxis. Behav. Brain Res. 210 (1), 1–7. https://doi.org/10.1016/j.bbr.2010.01.031.

Maximino, C., Marques de Brito, T., Dias, C.A.G., de, M., Gouveia, A., Morato, S., 2010b. Scototaxis as anxiety-like behavior in fish. Nat. Protoc. 5 (2), 209–216. https://doi.org/10.1038/nprot.2009.225.

McCallum, E.S., Du, S.N.N., Vaseghi-Shanjani, M., Choi, J.A., Warriner, T.R., Sultana, T., Scott, G.R., Balshine, S., 2017. In situ exposure to wastewater effluent reduces survival but has little effect on the behaviour or physiology of an invasive great lakes fish. Aquatic Toxicol. 184, 37–48. https://doi.org/10.1016/j.aquatox.2016.12.017.

Mehdi, H., Morphet, M.E., Lau, S.C., Bragg, L.M., Servos, M.R., Parrott, J.L., Scott, G.R., Balshine, S., 2022. Temperature modulates the impacts of wastewater exposure on the physiology and behaviour of fathead minnow. Chemosphere 294, 133738. https://doi.org/10.1016/j.chemosphere.2022.133738.

Mokh, S., El Khatib, M., Koubar, M., Daher, Z., Al Iskandarani, M., 2017. Innovative SPE-LC-MS/MS technique for the assessment of 63 pharmaceuticals and the detection of antibiotic-resistant-bacteria: a case study natural water sources in Lebanon. Sci. Total Environ. 609, 830–841. https://doi.org/10.1016/j.scitotenv.2017.07.230.

Nehlig, A., 2010. Is caffeine a cognitive enhancer? J. Alzheimer's Dis. 20 (s1), S85–S94. https://doi.org/10.3233/jad-2010-091315.

Neri, D., Ruberto, T., Mwaffo, V., Bartolini, T., Porfiri, M., 2019. Social environment modulates anxiogenic effects of caffeine in zebrafish. Behav. Pharmacol. 30 (1), 45–58. https://doi.org/10.1097/fbp.0000000000000415.

Nunes, B., Santos, J., Dionísio, R., de Alkimin, G.D., 2022. Investigation of potential behavioral and physiological effects of caffeine on D. magna. Environmental Sci. Pollut. Res. 29 (28), 43237–43250. https://doi.org/10.1007/s11356-022-18695-0.

Polverino, G., Martin, J.M., Bertram, M.G., Soman, V.R., Tan, H., Brand, J.A., Mason, R. T., Wong, B.B.M., 2021. Psychoactive pollution suppresses individual differences in fish behaviour. Biolog. Sci. 288 (1944), 20202294 https://doi.org/10.1098/rspb.2020.2294.

Quadra, G.R., Paranaíba, J.R., Vilas-Boas, J., Roland, F., Amado, A.M., Barros, N., Dias, R.J.P., Cardoso, S.J., 2020. A global trend of caffeine consumption over time and related-environmental impacts. Environmental Pollution 256, 113343. https://doi.org/10.1016/j.envpol.2019.113343.

R Core Team, 2021. R: A language and environment for statistical computing. [Computer software]. R Foundation for Statistical Computing. https://www.R-project.org/.

Santos, L.C., Ruiz-Oliveira, J., Oliveira, J.J., Silva, P.F., Luchiari, A.C., 2016. Irish coffee: effects of alcohol and caffeine on object discrimination in zebrafish. Pharmacol. Biochem. Behav. 143, 34–43. https://doi.org/10.1016/j.pbb.2016.01.013.

Santos, L.C., Ruiz-Oliveira, J., Silva, P.F., Luchiari, A.C., 2017. Caffeine dose-response relationship and behavioral screening in zebrafish. InTech. https://doi.org/10.5772/intechopen.68341.

Santos, N., Picolo, V., Domingues, I., Perillo, V., Villacis, R.A., Grisolia, C.K., Oliveira, M., 2023. Effects of environmental concentrations of caffeine on adult zebrafish behaviour: a short-term exposure scenario. Environm. Sci. Pollut. Res. 30 (23), 63776–63787. https://doi.org/10.1007/s11356-023-26799-4.

Shettleworth, S., 2010. Cognition, evolution, and Behavior. Oxford university press.

Sinha, R.A., Farah, B.L., Singh, B.K., Siddique, M.M., Li, Y., Wu, Y., Ilkayeva, O.R., Gooding, J., Chong, J., Zhou, J., Martinez, L., Xie, S., Bay, B.H., Summers, S.A., Newgard, C.B., Yen, P.M., 2014. Caffeine stimulates hepatic lipid metabolism by the autophagy-lysosomal pathway in mice. Hepatology 59 (4), 1366–1380.

Steele, W.B., Mole, R.A., Brooks, B.W., 2018. Experimental protocol for examining behavioral response profiles in larval fish: application to the neuro-stimulant caffeine. J. Visualiz. Experim. (137) https://doi.org/10.3791/57938.

Tan, H., Brand, J.A., Clarke, B.O., Manera, J.L., Martin, J.M., Wong, B.B.M., Alton, L.A., 2023. No evidence that the widespread environmental contaminant caffeine alters energy balance or stress responses in fish. Ethology 129 (12), 666–678. https://doi.org/10.1111/eth.13403.

Vignet, C., & Parrott, J. (2017). Maturation of behaviour in the fathead minnow. *Behavioural Processes*, 138, 15–21. doi:10.1016/j.beproc.2017.02.004.

Wang, J., Gardinali, P.R., 2013. Uptake and depuration of pharmaceuticals in reclaimed water by mosquito fish (*Gambusia holbrooki*): a worst-case, multiple-exposure scenario. Environ. Toxicol. Chem. 32 (8), 1752–1758. https://doi.org/10.1002/etc.2238.

Wolfe, K.R., Marsden, E.J., 1998. Tagging methods for the round goby (*Neogobius melanostomus*). J. Great Lakes Res. 24 (3), 731–735. https://doi.org/10.1016/s0380-1330(98)70857-3.

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., Goodspeed, J., Suciu, C., Tan, J., Grimes, C., Chung, A., Rosenberg, M., Gaikwad, S., Denmark, A., Jackson, A., Kadri, F., Chung, K.M., Stewart, A., Gilder, T., Beeson, E., Zapolsky, I., Wu, N., Cachat, J., Kalueff, A.V, 2010. Analyzing habituation responses to novelty in zebrafish (*Danio rerio*). Behav. Brain Res. 208 (2), 450–457. https://doi.org/10.1016/j.bbr.2009.12.023.

Zheng, X., Dai, W., Chen, X., Wang, K., Zhang, W., Liu, L., Hou, J., 2015. Caffeine reduces hepatic lipid accumulation through regulation of lipogenesis and ER stress in zebrafish larvae. J. Biomed. Sci. 22 (105) https://doi.org/10.1186/s12929-015-0206.