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No evidence that the widespread environmental contaminant caffeine alters energy balance or stress responses in fish

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Abstract

Anthropogenic sources of environmental pollution are ever-increasing as urban areas expand and more chemical compounds are used in daily life. The stimulant caffeine is one of the most consumed chemical compounds worldwide, and as a result, has been detected as an environmental contaminant in all types of major water sources on all continents. Exposure of wildlife to environmental pollutants can disrupt the energy balance of these organisms, as restoration of homeostasis is prioritised. In turn, energy allocated to other key biological processes such as growth or reproduction may be affected, consequently reducing the overall fitness of an individual. Therefore, we aimed to investigate if long-term exposure to environmentally relevant concentrations of caffeine had any energetic consequences on wildlife. Specifically, we exposed wild eastern mosquitofish (*Gambusia holbrooki*) to one of three nominal concentrations of caffeine (0, 100 and 10,000 ng/L) and assayed individuals for metabolic rate, general activity, antipredator and foraging behaviour and body size as measures of energy expenditure or energy intake. We found no differences in any measured traits between any of the given exposure treatments, indicating that exposure to caffeine at current environmental levels may not adversely affect the energy balance and fitness of vulnerable freshwater fish.

KEYWORDS
activity, antipredator response, foraging behaviour, *Gambusia holbrooki*, metabolic rate, pharmaceutical pollution

1 | INTRODUCTION

Rapid urbanisation and exponential population growth are two leading drivers of global anthropogenic change (Steffen et al., 2015). This increased urbanisation and growth of urban populations has led to a massive increase in the use of household and industrial chemical compounds in the past three decades (Bernhardt et al., 2017; OECD, 2021). The number of chemicals registered for production and use is greatly underestimated, as monitoring efforts and risk assessments struggle to keep up with the rapid increase in the production of novel chemicals (Persson et al., 2022; Wang et al., 2020). In turn, this has resulted in the pollution of the

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environment with a wide range of chemical compounds, such as caffeine (Li, He et al., 2020; Wilkinson et al., 2022).

The central nervous system stimulant caffeine is among the most consumed chemical compounds worldwide, with coffee consumption approximately doubling between 1990 and 2016, matching the trend in global population growth (Quadra et al., 2020). Caffeine is acknowledged as being an extremely ubiquitous environmental contaminant (Dafouz et al., 2018) due to its widespread consumption and the many pathways by which it enters the environment (Hillebrand et al., 2012; Li, He et al., 2020). As evidence of its ubiquity, caffeine has been detected as an environmental pollutant on all continents, including Antarctica (Wilkinson et al., 2022), and in all major water sources around the world, including in raw and treated wastewater, as well as rivers, lakes, reservoirs, estuaries, groundwater, seawater, rainwater and even in drinking water (Dafouz et al., 2018; Li, Wen et al., 2020). Additionally, caffeine has been found to be pseudo-persistent in aquatic environments due to constant input, negligible sorption and sedimentation, and relatively low rates of biodegradation and chemical degradation (Buerge et al., 2003; Cormier et al., 2015; Korekar et al., 2020). Caffeine pollution is also often worse in more urbanised and densely populated areas due to high anthropogenic input, with concentrations in surface waters typically ranging up to the tens of thousands of nanograms per litre (Li, Wen et al., 2020). Whilst these levels of caffeine pollution in the environment may not necessarily be a threat to human health (with daily average caffeine consumption estimated at 70 mg in adults; Nehlig, 2018), there is still a potential for wildlife to be impacted in an increasingly caffeinated world.

Importantly, the major and minor adenosine receptors targeted by caffeine and its metabolites are evolutionarily conserved (Fredholm et al., 2001, 2011), suggesting that caffeine can affect non-target animals. Indeed, this is the case across various taxa (e.g. Al-Amin et al., 2016; Garrett & Holtzman, 1995; Maguire et al., 2017; Min et al., 2015; Santos-Silva et al., 2018). However, many of these animal studies focus on the neurotoxic or developmental effects of exposure to high doses (e.g. tens to hundreds of mg/kg body mass) of caffeine (Li, He et al., 2020). This is true even though caffeine typically persists at a much lower concentration in the environment than is generally employed within those studies. As a point of comparison, the maximum concentration of caffeine found to bioaccumulate in the tissue of wild freshwater and marine fish was approximately 0.074 mg/kg (Ali et al., 2018; Scott et al., 2018). Therefore, rather than severe neurotoxic effects, the kinds of impacts that caffeine might be expected to exert as an environmental contaminant are likely to be more subtle, including potential changes to ecologically important physiological and behavioural processes.

Energy metabolism, which encompasses energy assimilation, conversion and utilisation, is a key physiological trait that is linked to animal behaviour (Sokolova, 2021), growth and reproduction (White et al., 2022). By disrupting the internal homeostasis of an organism, environmental stressors, like chemical contaminants, can alter an organism's rate of energy metabolism (metabolic rate) and consequently their energy balance, as restoring homeostasis requires energy (Sokolova, 2021). Exposure to environmental contaminants can, therefore, change how organisms prioritise energy allocation among the competing processes of self-maintenance, locomotion, growth and reproduction, which can affect how they prioritise specific behaviours and ultimately reduce their fitness (Killen et al., 2013; Sokolova, 2021). Caffeine has the potential to alter the energy balance of organisms via effects on both organismal metabolic rates and behaviour. For instance, in human and rodent models, caffeine increases metabolic rates, decreases energy intake, and increases spontaneous activity levels, which may result in a negative energy balance that leads to a loss in body mass (Harpaz et al., 2017; Nehlig et al., 1992). Whether environmental concentrations of caffeine exert similar effects on aquatic wildlife is less well known, but studies in fish, amphibians, bivalves and crustaceans suggest that caffeine may result in increased metabolic rates and reduced energy reserves, whereas activity levels may either increase or decrease (Aliko et al., 2019; Cruz et al., 2016; Fraker & Smith, 2004; Steele et al., 2018). Other observed behavioural responses to caffeine suggest that caffeine may also cause animals to be more anxious, with fish and tadpoles displaying increased erratic and freezing behaviours, reduced exploration and increased startle responses when disturbed (Aliko et al., 2019; Fraker & Smith, 2004). Behavioural changes associated with caffeine exposure may, therefore, act to conserve energy if animals reduce their activity or exploratory behaviour, or they may further contribute to a negative energy balance if, instead, animals become more active, or if anxious animals are less inclined to search for food. Given the varied effects of caffeine on animal behaviour, it is important that studies conduct metabolic rate measurements in conjunction with multiple behavioural measurements to better evaluate the potential energetic and fitness consequences of caffeine exposure for aquatic wildlife.

Here, we investigate the effects of environmentally relevant concentrations of caffeine exposure on metabolic rate, and several behavioural and morphological traits of wild-caught mosquitofish (*Gambusia holbrooki*) to determine if caffeine has the potential to induce adverse effects on wildlife as an environmental contaminant. We exposed fish to one of three nominal caffeine concentrations (control: 0 ng/L, low-caffeine: 100 ng/L, high-caffeine: 10,000 ng/L) for 21–42 days and measured their routine metabolic rates, activity (total distance moved), foraging behaviour (number of food items consumed and latency to feed) and body size (body mass and length) to determine how the energy balance of individuals might be altered. We also measured the activity of fish following a simulated predator strike as an indicator of anxiety (i.e. fearfulness; Sih et al., 2023). We expected that if caffeine made fish more anxious then they might respond differently to a predatory threat and exhibit behaviours described previously, such as increased erratic movements or immobility (Aliko et al., 2019; Fraker & Smith, 2004).

2 | MATERIALS AND METHODS

2.1 | Animal collection and housing

Wild juvenile and adult male and female mosquitofish were collected from the Science Centre Lake (37°54′28″ S, 145°08′16″ E), Monash University, Victoria, Australia in July 2020. Following collection, fish were transported back to Monash University to be acclimated to and housed in a controlled-temperature room for 3 weeks in mixed-sex glass housing tanks ($n = 24$ tanks; 54 L; 60 × 30 × 30 cm; 18.5–19.4°C; 12:12 h light: dark photoperiod; ~16 fish per tank) prior to caffeine exposure and experimentation. Fish were distributed equally across all housing tanks by sex to maintain equal sex ratios across tanks. Housing tanks were filled with aged reverse osmosis water (20 cm water depth), were aerated by an air stone, contained a 2 cm gravel substrate (6 mm grain size), and were covered with plastic film as a tank lid. One-third of the volume of water in each tank was changed once per week, and fish were fed ad libitum once daily with a mix of commercial pellets (Aquasonic Nutra Xtreme C1 pellets; 0.8 mm) and chironomid larvae during the acclimation, exposure and experimentation periods. Animal collection and experiments complied with Australian law and were approved by the Monash University Animal Ethics Committee (Project ID 23461).

2.2 | Exposure regime

Following acclimation in freshwater, each of the 24 housing tanks were randomly assigned to one of three caffeine exposure treatments: a freshwater control (nominal concentration of 0 ng/L; $n = 8$ tanks), a low-caffeine treatment (nominal concentration of 100 ng/L; $n = 8$ tanks) and a high-caffeine treatment (nominal concentration of 10,000 ng/L; $n = 8$ tanks). These nominal caffeine levels were chosen to represent the average upper and lower range of concentrations typically detected in polluted surface waters. Due to the extremely pervasive nature of caffeine, a background level of caffeine was also detected in controls (see Results, verification of caffeine concentrations), which represents an ever-present baseline level of caffeine in the environment. Fish were exposed to their respective caffeine exposure treatment for a minimum of 21 days prior to the start of experiments. The housing room was monitored daily for air temperature (mean ± SD = 19.13 ± 0.16°C, $n = 21$) and all housing tanks were monitored twice a week for pH (mean = 7.32, range = 6.84–7.71, $n = 144$) during the exposure period.

2.3 | Exposure dosing, monitoring and analytical verification

The desired caffeine concentrations in each exposure tank were maintained via static renewal. This involved first dissolving 36 mg of caffeine powder (1,3,7-Trimethylxanthine; ≥99.0% purity; CAS: 58-08-2; Sigma-Aldrich) in 100 mL of reverse osmosis water to produce a stock solution for the high-caffeine treatment. A 1 mL aliquot of the high-caffeine stock solution was further diluted in 99 mL of reverse osmosis water to produce a stock solution for the low-caffeine treatment. Once per week, for all caffeine exposure tanks, a 1 mL aliquot of stock solution (low or high) was used to dose each exposure tank. Freshwater control tanks were dosed with 1 mL of reverse osmosis water once per week.

Water samples (50 mL) from each of the low- and high-caffeine exposure tanks, and from half of the unexposed control tanks were taken weekly, and a subset of samples from the initial 21-day exposure were haphazardly chosen to represent all exposure tanks at multiple weekly exposure time points ($n = 60$) and tested for analytical verification of caffeine concentrations. Briefly, analysis was performed using liquid chromatography-tandem mass spectrometry based on previously reported methods (Anumol et al., 2013). For a detailed description of the analytical protocol, see Data S1, *'Analytical verification of caffeine treatment concentrations'*.

2.4 | Metabolic rate, behavioural and morphological measurements

The effects of caffeine on the physiology, behaviour and morphology of mosquitofish were assayed across three separate experimental trials: a metabolic rate experiment, a general activity and antipredator response behavioural experiment and a foraging behaviour experiment. Following the initial 21-day exposure period, fish were subjected to either a metabolic rate experiment or a foraging behaviour experiment, with individuals only completing one of the two experiments. Following these two experiments, mosquitofish were returned to their respective exposure treatments for another 21 days, after which they were then assayed for activity and antipredator behaviour. Immediately following each experiment, all fish were weighed and photographed to extract wet body mass and body length measurements. As fish were group-housed, individual identities were not tracked between the two experimental trial periods. Prior to commencing experimental trials, all mosquitofish were not fed for 24 h to standardise hunger levels for the foraging behaviour experiment and also to ensure that fish were in a post-absorptive state for the metabolic rate experiment (Niimi & Beamish, 1974).

2.4.1 | Metabolic rate

To test for the impacts of caffeine on metabolic rate, the rate of oxygen consumption was measured in 96 fish (freshwater control: $n = 32$; low-caffeine: $n = 32$; high-caffeine: $n = 32$) as a proxy for their routine metabolic rate, using 4-channel closed-system respirometry (adapted from Alton et al., 2007; Martin et al., 2022). Experiments were conducted in a dimly lit controlled-temperature

room kept at 19°C, consistent with the room temperature focal individuals were housed in. At the start of the experimental trial, a focal individual was placed into a sealed glass respirometry chamber (100 mL; 56 mm diameter Schott bottle) filled with aerated aged reverse osmosis water. The chamber was submerged in a tank (25 × 15 × 15 cm) filled with aged reverse osmosis water that contained an air stone and a temperature probe (PT100 sensor; PreSens Precision Sensing GmbH). The respirometry chambers had three holes in the lid to accommodate inflow and outflow tubing (3 mm diameter) and an oxygen probe (Oxygen Dipping Probe DP-PSt7; PreSens Precision Sensing GmbH). The inflow and outflow tubing were connected to a peristaltic pump (Watson Marlow 323 U/MC) to ensure constant gentle circulation of water (flow rate of 2.5 mL/min) and constant mixing of water within the respirometry chamber during the trial. Pilot trials showed that the flow rate used to circulate the water during the trial was not strong enough to displace the fish within the chamber. The oxygen probe was connected to an oxygen meter (OXY-4 trace; PreSens Precision Sensing GmbH) and PreSens Measurement Studio 2 (v3.0.3.1653; PreSens Precision Sensing GmbH) was used to record the dissolved oxygen levels (recorded as % air saturation) within the chamber. Experimental trials lasted 2 h whereby dissolved oxygen levels within the respirometry chamber were recorded every second. Fish were not given an acclimation period prior to initiation of oxygen measurements. Instead, any increased metabolic oxygen consumption potentially caused by handling stress was accounted for during post-trial calculations of oxygen consumption rates (see Table S1). During the trial, respirometry chambers were video-recorded so that activity levels could be quantified to take into account any sources of variation in the oxygen uptake measurements. Specifically, the external wall of each respirometry chamber was lined with 1 × 1 cm grid squares, and activity levels were quantified by counting the number of grid squares crossed by the fish during a 5 min period in the middle of each hour of the trial. Water within respirometry chambers was emptied following each round of respirometry trials and chambers were refilled with clean aerated reverse osmosis water, to remove any waste products left by the focal individual. To correct for background bacterial respiration, oxygen levels of each replicate respirometry chamber without fish were recorded for 45 min at the end of each day of trials. Aquatic rates of oxygen uptake ($\dot{V}_{O2}$, μg h^{-1}) were calculated from the slope of oxygen saturation against time for chambers with fish (m_f, % h^{-1}), the equivalent slope for chambers without fish for background correction matched to replicate chamber and experimental day (m_c), the oxygen solubility of air-saturated water (β_{O2}, 9.28 mg/L at 19°C) and water volume (V, L; i.e. water volume of respirometry chamber and circuit tubing minus the body mass of focal fish assuming a density of 1 g mL^{-1}):

$$\dot{V}o_2 = -\frac{(m_f - m_c)}{100} \times V\beta o_2$$

For a detailed list of methodological information regarding the respirometry trial following guidelines for reporting methods of aquatic respirometry in Killen et al. (2021), see Table S1.

2.4.2 | Activity and antipredator response

To test for the effects of caffeine on both activity and antipredator behaviour, 147 fish (freshwater control: $n = 69$; low-caffeine: $n = 40$; high-caffeine: $n = 38$) were assayed for general activity and response to a predatory stimulus (adapted from Martin et al., 2017). Experiments were conducted in an experimental tank (25 × 15 × 15 cm; 3 cm water depth) filled with aged reverse osmosis water, and tank walls were covered with an opaque film to prevent external disturbances from affecting mosquitofish behaviour. At the start of the trial, fish were released into the experimental tank and left to freely acclimate for 10 min. Following acclimation, the activity of fish was recorded for 10 min as a baseline. Following the baseline measurement, fish were presented with a simulated predatory strike, which involved striking the water with a probe approximately 3 cm away from the fish. The trial was video-recorded so data could be extracted post-trial. Experimental tank water was changed after every trial to remove any chemical cues left by mosquitofish. For this experiment, we quantified the distance travelled by fish prior to any predatory stimulus to compare any differences in general baseline activity levels between the caffeine exposure treatments. We then quantified the distance travelled by fish following the predatory stimulus, to determine if there was an antipredator response and if this response differed between exposure treatments. Distances travelled (cm) were extracted from videos using the commercial tracking software, Ethovision XT v. 14.0.1326 (Noldus Information Technology bv).

2.4.3 | Foraging behaviour

To test for the impacts of caffeine on foraging behaviour and food intake, 172 fish (freshwater control: $n = 52$; low-caffeine: $n = 58$; high-caffeine: $n = 62$) were subjected to a foraging trial (adapted from Bertram et al., 2018; Martin et al., 2019). Experiments were conducted in an experimental tank (60 × 30 × 30 cm; 15 cm water depth) filled with aged reverse osmosis water and containing a white sand substrate of 2 cm depth. At one end of the experimental tank, a foraging zone was designated (12 × 24 cm). This foraging zone consisted of 64 shallow cylindrical wells (well diameter: 17 mm, depth: 5 mm), into which five food items (chironomid larvae) were randomly placed. At the start of the experimental trial, a focal individual was introduced into a cylindrical acclimation chamber at the end of the tank opposite to the foraging zone and allowed to acclimate for 15 min. Following this acclimation period, the acclimation chamber was removed, and the fish was released into the experimental tank to forage for 20 min. The trial was

video-recorded so that data could be collected post-trial. Behaviours quantified during this experiment included the number of food items consumed and the time taken to first consume a food item.

2.5 | Statistical analysis

Statistical analyses were conducted in R v. 4.0.2 (R Core Team, 2020). Where necessary, data were transformed to approximate a Gaussian distribution. For metabolic rate, a linear mixed effects model (LME; *lme4* package; Bates et al., 2015) was used to test for the effects of caffeine exposure on rate of oxygen consumption. Sex, body mass, round of trial (two rounds of metabolic rate trials were conducted each day) and activity levels were included as covariates, and experimental tank identity (to account for the specific respirometry probe used to measure oxygen uptake) was included as a random intercept in the metabolic rate model. For general activity and antipredator behaviour, an LME (*lme4* package; Bates et al., 2015) was used to test for the effects of caffeine exposure and predator stimulus on distance travelled. Sex and body mass were included as covariates, and fish identity and exposure tank identity were included as a random intercept in general activity and antipredator behaviour models. For foraging behaviour, a zero-inflated Poisson regression model (*pscl* package; Zeileis et al., 2008) and a Cox proportional hazards regression model (*survival* package; Therneau, 2022) were fitted to test for the effects of caffeine exposure on the number of food items eaten and the time to first consume a food item, respectively. Sex and body mass were included as covariates in the final foraging behaviour models. For morphology, wet body mass and total lengths were compared between exposure treatments and time since start of exposure (21 or 42 days) using Type II analysis of variance (ANOVA) linear models, with no significant interaction term found between exposure treatment and time since start of exposure.

3 | RESULTS

3.1 | Verification of caffeine concentrations

Individual values for all analysed water samples are presented in Table S2. The caffeine exposure concentrations for the low- and high-caffeine treatments were 417.9 ± 132.3 ng/L (mean ± SD, $n = 24$) and 8095.8 ± 2218.0 ng/L (mean ± SD, $n = 24$), respectively. Unfortunately, as with at least one other published study (Cerveny et al., 2022), caffeine was also found in all measured control treatment water samples (at a mean ± SD concentration of 349.2 ± 79.5 ng/L; $n = 12$) despite water being treated by reverse osmosis. The reasons for this contamination are likely to be the same as those previously discussed in Cerveny et al. (2022), including the presence of caffeine in the water source used to supply freshwater to exposure tanks (including control treatment tanks), and/or transfer of caffeine between exposure tanks through aeration and airborne particulates since control and caffeine treatment tanks were housed in the same room to standardise experimental conditions. In urban settings, caffeine has been previously detected in airborne particulates, typically in the hundreds of nanograms per square meter (Cecinato et al., 2017). Therefore, despite the presence of caffeine in our control treatment, it nevertheless provides an accurate representation of the ever-present background level of caffeine in urban ecosystems. Moreover, it is important to highlight that the high-caffeine treatment still represented a concentration of caffeine more than an order of magnitude greater than concentrations detected in the controls and is still representative of aquatic environments subjected to high levels of caffeine pollution. With that said, we will hereafter refer to our nominal control treatment as a 'background level' treatment to reflect the fact that there were background concentrations of caffeine in these tanks.

3.2 | Metabolic rate

Rate of oxygen uptake was assayed as a proxy for routine metabolic rate and as a measure of energy expenditure. We found no effect of exposure treatment on the rate of oxygen uptake of mosquitofish ($F_{2,81.7} = 0.18$, $p = 0.833$; Figure 1), nor was there an effect of sex ($F_{2,81.3} = 0.32$, $p = 0.724$). We also found no significant relationship between rate of oxygen uptake and activity levels ($F_{1,81.9} = 2.37$, $p = 0.128$). There was, however, an effect of body mass on the rate of oxygen uptake ($F_{1,80.4} = 27.61$, $p < 0.001$), with heavier fish having a higher rate of oxygen uptake. Full model output can be found in Table S3.

3.3 | General activity and antipredator behaviour

Activity levels were assayed as a measure of energy expenditure, and antipredator behaviour was assayed as an indicator of anxiety-related stress responses. Here, we found no effect of exposure treatment on the general baseline activity of mosquitofish ($F_{2,14.9} = 0.31$, $p = 0.738$; Figure 2). Furthermore, whilst there was a clear antipredator response, with mosquitofish exhibiting higher activity levels following the stimulus predatory strike ($F_{1,144} = 29.85$, $p < 0.001$; Figure 2), exposure treatment did not alter antipredator response ($F_{2,144} = 0.17$, $p = 0.843$; Figure 2). There were also no effects of body mass ($F_{1,138.6} = 0.23$, $p = 0.631$), nor sex ($F_{1,137.8} = 1.94$, $p = 0.148$), on the antipredator responses of mosquitofish. Full model output can be found in Table S4.

3.4 | Foraging behaviour

Foraging behaviours were assayed as a measure of energy intake. Here, we found that the time taken for mosquitofish to first

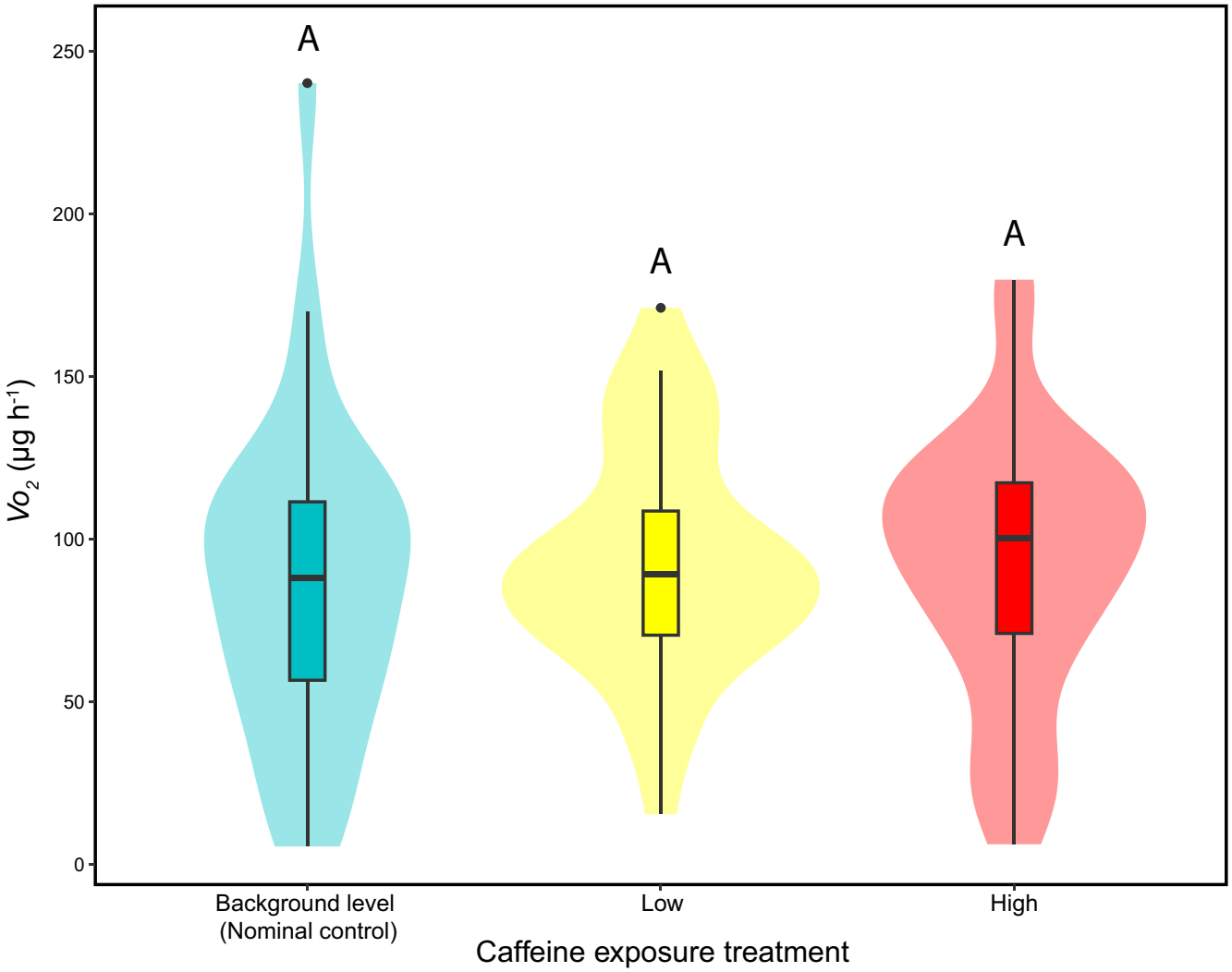

FIGURE 1 Aquatic rates of oxygen uptake ($\dot{V}_{O2}$, µg h^{-1}) in mosquitofish plotted by exposure treatment and normalised to mean body mass of all individuals. Box plots show the median (centre line), lower and upper quartiles (bottom and top of each box, respectively), variability outside the quartiles (whiskers) and outliers (circles). Box plots sharing the same letter are not significantly different.

consume a food item was not significantly affected by exposure treatment ($\chi^2 = 1.71$, df = 2, $p = 0.425$; Figure 3a), nor body mass ($\chi^2 = 0.31$, df = 1, $p = 0.579$), nor sex ($\chi^2 = 0.69$, df = 2, $p = 0.707$). Similarly, the number of food items consumed was not significantly affected by exposure treatment ($\chi^2 = 3.76$, df = 2, $p = 0.153$; Figure 3b), nor sex ($\chi^2 = 4.22$, df = 2, $p = 0.121$). There was, however, a significant effect of body mass on the number of food items consumed ($\chi^2 = 6.92$, df = 1, $p = 0.009$), with heavier fish consuming more food items.

3.5 | Morphology

Exposure treatment did not significantly affect total lengths ($F = 1.01$, df = 2, $p = 0.365$; Figure 4a) or body mass ($F = 1.22$, df = 2, $p = 0.297$; Figure 4b) of mosquitofish, regardless of exposure period. There was, however, a difference in both total lengths ($F = 5.89$, df = 1, $p = 0.016$) and body mass ($F = 7.76$, df = 1, $p = 0.006$) between the two exposure periods, with fish being, on average, longer and heavier at 42 days than 21 days.

4 | DISCUSSION

We investigated whether a long-term chronic exposure to environmentally realistic levels of the widespread contaminant, caffeine, affected routine metabolic rates, general activity, antipredator and foraging behaviour, and body mass and lengths of wild-caught mosquitofish. Contrary to our hypotheses, we found that exposure to all of the given concentrations of caffeine did not affect any of these traits. Similar to our findings on the routine metabolic rates of mosquitofish, individuals of a freshwater crustacean (*Daphnia magna*) exposed to a range of environmentally relevant concentrations of caffeine showed no changes in oxygen uptake and glycogen content, indicating that caffeine did not induce any metabolic change (Nunes et al., 2022). Additionally, individuals

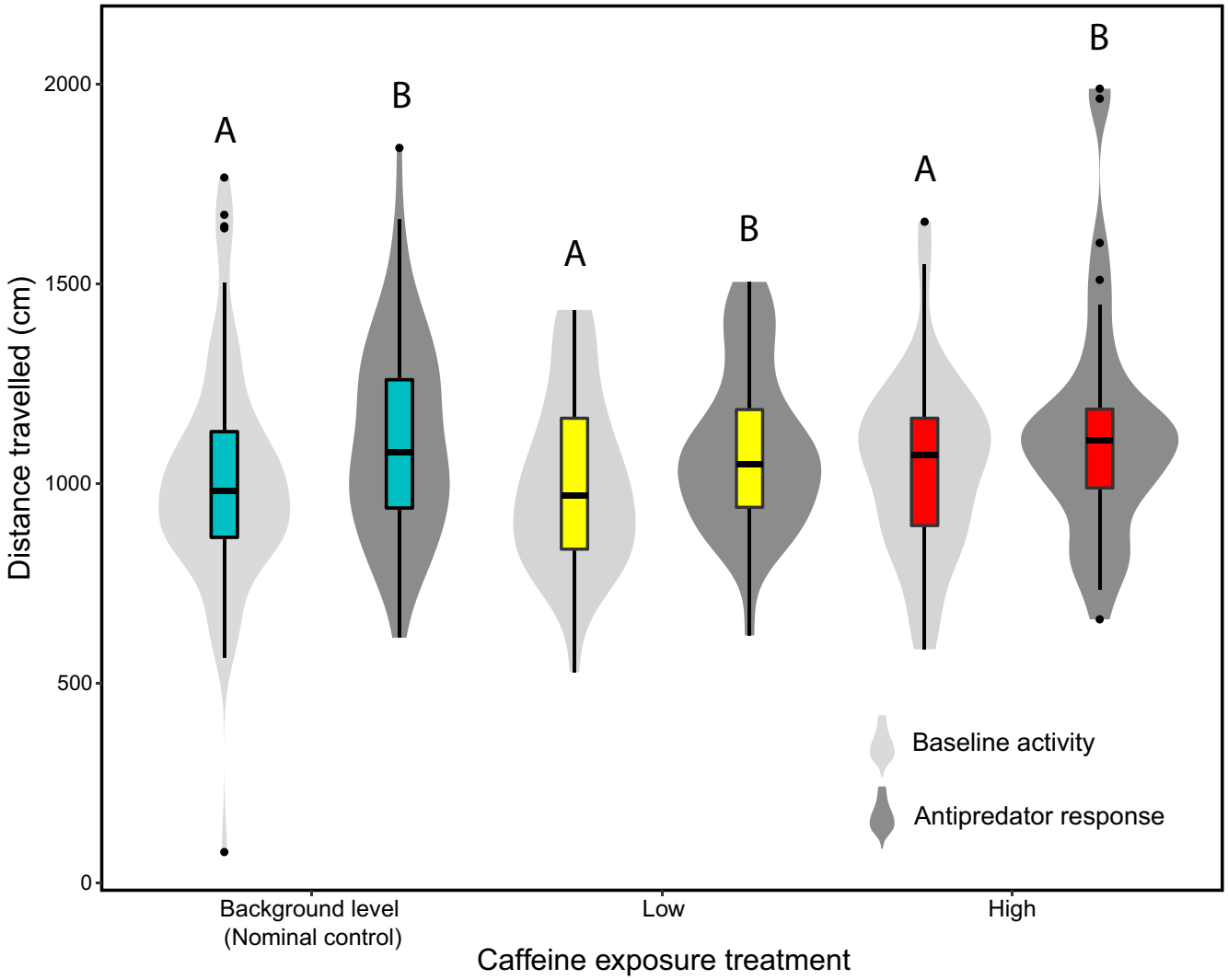

FIGURE 2 Distance travelled (cm) by mosquitofish as a measure of baseline activity (light grey) and an antipredator response following a stimulus predatory strike (dark grey) plotted by exposure treatment. Box plots show the median (centre line), lower and upper quartiles (bottom and top of each box, respectively), variability outside the quartiles (whiskers) and outliers (circles). Box plots sharing the same letter are not significantly different.

of a freshwater fish species (*Prochilodus lineatus*) exposed to caffeine at 3 and 30 μg/L exhibited biochemical changes in the brain and liver, but this was not sufficient to induce a state of oxidative stress indicating no major physiological change (Santos-Silva et al., 2018). However, past studies have observed caffeine exposure to induce oxidative stress and, therefore, increasing metabolic activity in polychaetes and bivalves even at lower environmentally relevant levels (Aguirre-Martínez et al., 2016; Cruz et al., 2016; Pires et al., 2016a, 2016b). Therefore, there may be taxon-specific differences in physiological changes observed in caffeine-exposed non-target organisms. That said, unlike in human and rodent models exposed to high pharmacological doses of caffeine that showed increased metabolic rates and, therefore, increased energy expenditure (Harpaz et al., 2017; Nehlig et al., 1992), there does not appear to be any profound alterations to energy utilisation or a clear adverse physiological effect in wildlife exposed to lower environmentally relevant levels of caffeine.

We also found no differences in the general activity of mosquitofish in any of the given concentration treatments of caffeine. Consistent with our results on general activity, exposure of wild perch (*Perca fluviatilis*) to 10,000 ng/L of caffeine (i.e. a nominal concentration identical to the high-caffeine treatment in this study) did not affect their general activity during the day nor circadian activity (Cerveny et al., 2022). Similarly, exposure of larval zebrafish (*Danio rerio*) to 1 μg/L caffeine did not impact swimming speed (Zhou et al., 2019), and exposure between a range of 1 and 4070 μg/L did not affect total distance travelled compared to controls (Steele et al., 2018). However, exposure to much higher concentrations of caffeine at 48.46 and 193.82 mg/L did inhibit activity and reduced total distance travelled in larval zebrafish (Steele et al., 2018). Additionally, at higher doses of caffeine, adult zebrafish exposed to 70 mg/L (Neri et al., 2019) and goldfish (*Carassius auratus*) exposed to 50 mg/L caffeine (Aliko et al., 2019) also exhibited a reduction in general activity. In humans, caffeine is known to cause dose-dependent effects

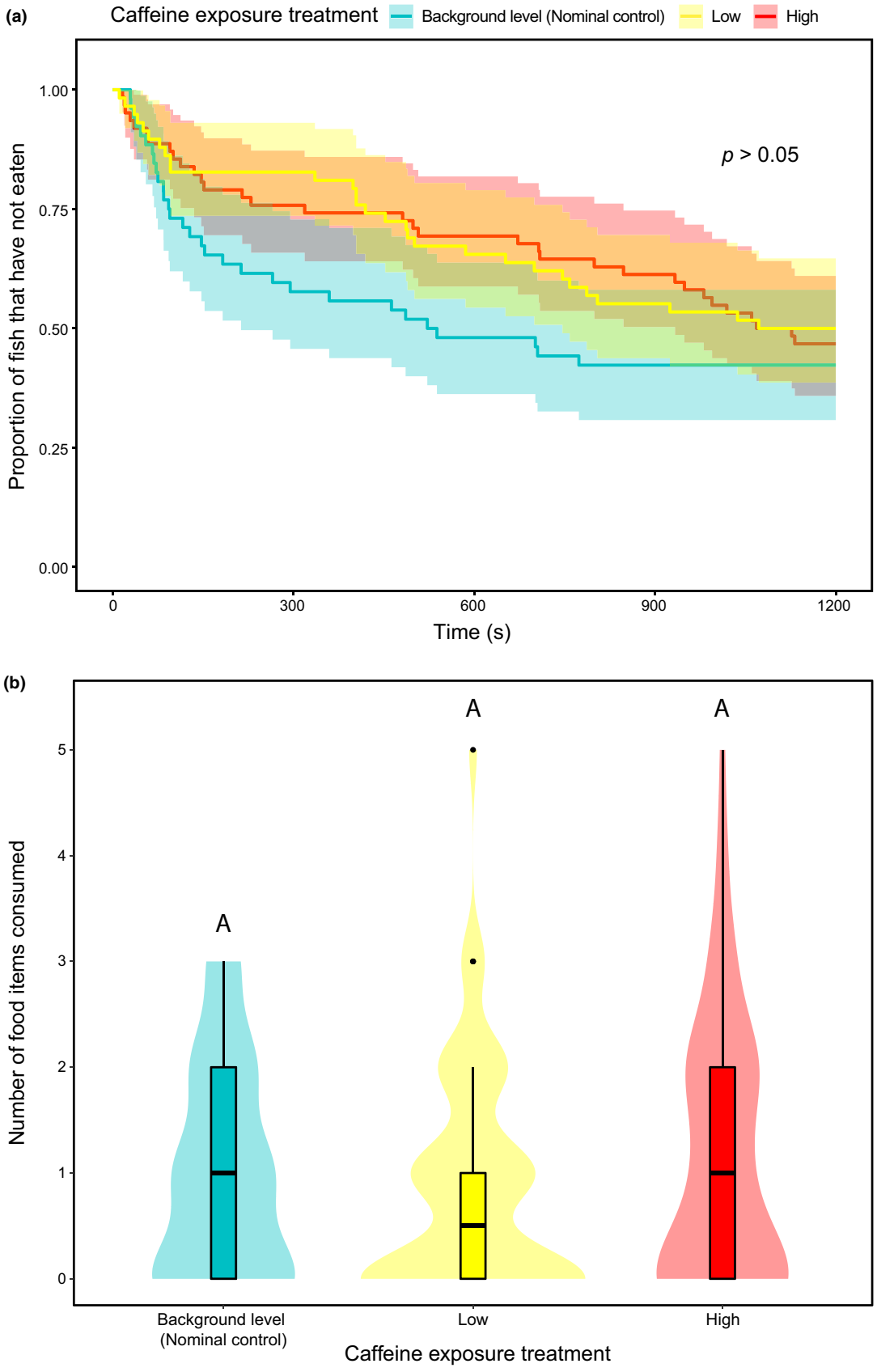

FIGURE 3 Plots showing (a) the proportion of mosquitofish individuals (with 95% shaded confidence intervals) that have not yet consumed a food item over the trial period (time, s) split by exposure treatment and (b) the number of food items consumed split by exposure treatment. For (b), box plots show the median (centre line), lower and upper quartiles (bottom and top of each box, respectively), variability outside the quartiles (whiskers) and outliers (circles). Box plots sharing the same letter are not significantly different.

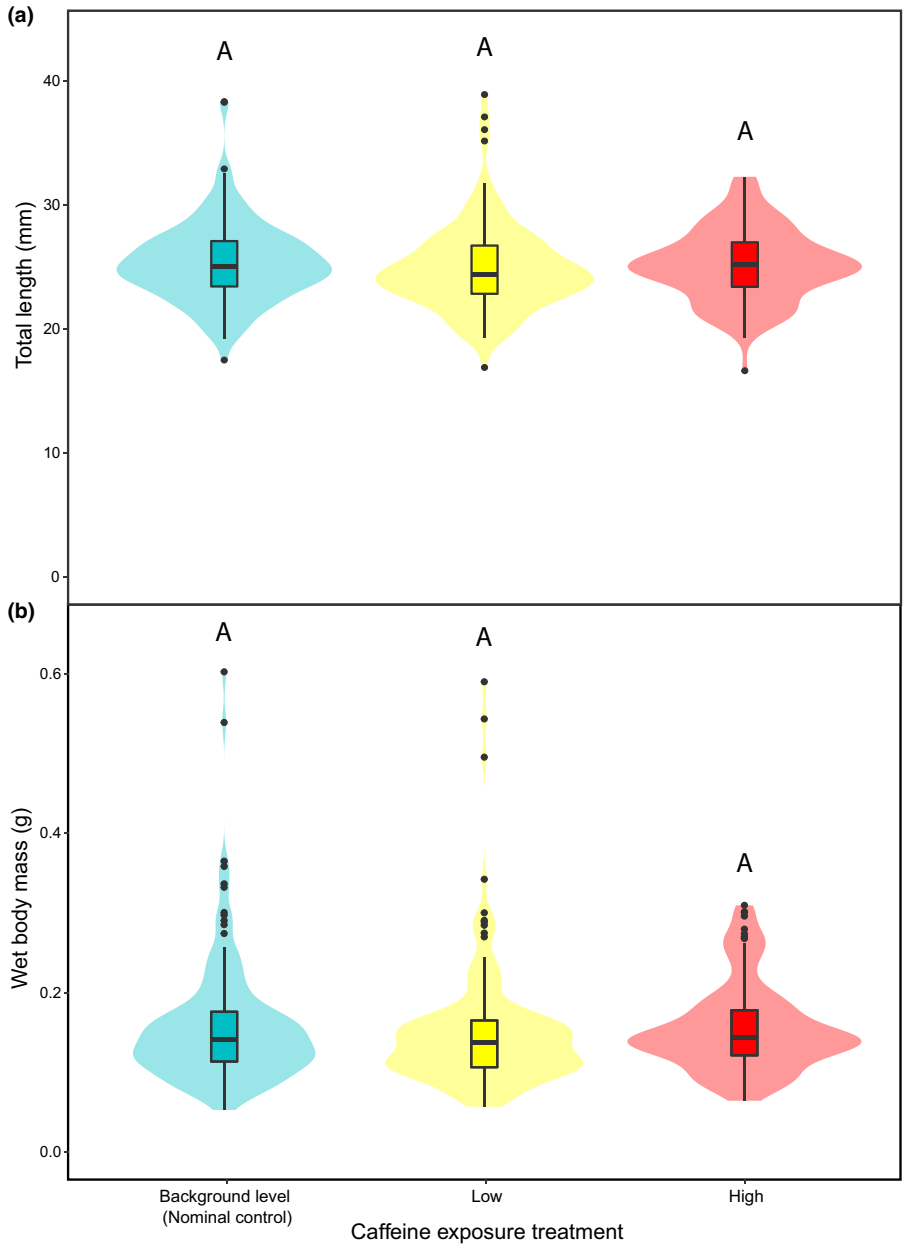

FIGURE 4 Plots showing (a) the total lengths (mm) of mosquitofish split by exposure treatment and (b) the wet body mass (g) of mosquitofish split by exposure treatment. Box plots show the median (centre line), lower and upper quartiles (bottom and top of each box, respectively), variability outside the quartiles (whiskers) and outliers (circles). Box plots sharing the same letter are not significantly different.

on the central nervous system, inducing anxiety-related effects at higher doses (Fredholm et al., 2017). Freezing behaviour (i.e. periods of immobility) is a common indicator for increased anxiety (i.e. fearfulness) in fish (Blaser et al., 2010; Maximino et al., 2010). Thus, the reduction in activity seen in previous studies may be a result of exposure to higher doses of caffeine causing a stress response and exerting anxiogenic effects in fish. At these higher exposure doses, an increased fear response and freezing behaviour associated with exposure to caffeine may indicate a behaviourally driven change, or potential increase in energy expenditure, as energy has to be allocated to both common 'fight or flight' physiological responses such as increased respiration and blood circulation and to compensatory or protective mechanisms such as antioxidant defence. Yet, as these exposure doses are typically at levels much higher than what has been detected in the environment, it is unlikely that current environmental exposure to caffeine would induce anxiogenic effects or compensatory behavioural alterations in activity that would consequently alter the energy balance of wildlife.

Mosquitofish exposed to the given concentrations of caffeine in this study also exhibited no changes in their antipredator and foraging behaviour. However, exposure to caffeine has been shown to increase anxiety-related antipredator responses and induce appetite alterations in previous studies, contrary to what was observed in this study. For example, caffeine-exposed goldfish increased

freezing behaviour (Aliko et al., 2019), and a larger proportion of caffeine-exposed tadpoles of the northern leopard frog (*Rana pipiens*) exhibited a startle response when disturbed compared to controls (Fraker & Smith, 2004). *Rana pipiens* tadpoles exposed to higher concentrations of caffeine also tended to be smaller in size; however, it was not clear whether this reduction in mass was a result of a decrease in food consumption (Fraker & Smith, 2004). There is some evidence that caffeine causes appetite suppression in humans (Schubert et al., 2017) and non-human animals such as rats (Pettenuzzo et al., 2008), and food avoidance behaviour in a nematode (*Caenorhabditis elegans*; Min et al., 2017). Alterations in the foraging and antipredator behaviour of individuals can have direct and indirect effects on energy intake leading to an altered energy balance. An increase in food avoidance behaviour or reduction in foraging behaviour will directly result in decreased energy intake, whilst increased antipredator behaviour can indirectly lead to a reduction in energy intake due to costs to foraging opportunities (Killen et al., 2013; Sih et al., 2023). Together, these reductions in energy intake can then lead to a negative energy balance and losses to body mass. However, as with effects exerted on activity, many of these anxiety-inducing effects of caffeine are typically seen at high experimental doses not typically reflective of environmental concentrations. Interestingly, adult zebrafish exposed to 50–100 mg/L of caffeine exhibited increased anxiety in a novel tank test, whereas zebrafish exposed to 0.5–25 mg/L of caffeine showed decreased anxiety (Clayman & Connaughton, 2022), highlighting the dose-dependent differences in the effects of caffeine. At the least, between the exposure range employed in this study, we found no evidence for any dose-dependent differences. As there is currently a lack of studies that have explored the energetic and anxiety-inducing effects of caffeine under an environmentally relevant setting, it remains inconclusive whether caffeine exposure at current environmental levels may be affecting energy intake and stress responses in exposed wildlife, highlighting the need for further research.

Ultimately, we also found no evidence for any differences in mosquitofish body mass and body lengths across any of the given caffeine concentrations employed in this study. Previous studies that have found evidence for increased metabolic rates (Aguirre-Martínez et al., 2016; Cruz et al., 2016; Harpaz et al., 2017; Nehlig et al., 1992; Pires et al., 2016a, 2016b) and stress responses (Aliko et al., 2019; Fraker & Smith, 2004; Neri et al., 2019) indicated that exposure to caffeine would result in a reduction in energy intake and alteration in energy expenditure. Overall, these changes to energy intake and expenditure would then contribute to a negative energy balance and reductions in body mass and growth, as these traits are tightly linked (White et al., 2022). As body size determines reproductive output and is, therefore, a key component in determining individual fitness (Barneche et al., 2018), any reductions in body size caused by exposure to caffeine can be expected to have adverse fitness effects. However, we found that there were no differences in metabolic rates, foraging behaviour, general activity and antipredator behaviours across any of the caffeine treatment groups within this study, indicating that there were no changes in energy intake and expenditure, and hence, no alterations in body size of individuals. As with the background level of caffeine detected in our controls, it is possible that mosquitofish used in this study were already exposed to a background concentration of caffeine in the wild. Therefore, perhaps the constant exposure at background levels has led to a long-term adaptation to caffeine in these individuals. However, when taking into consideration findings from previous studies that have also found no adverse effects of caffeine exposure at low environmental levels on various fish species, it appears that current environmental levels of caffeine pollution does not adversely affect the energy balance nor overall fitness of exposed individuals, based on the traits measured within this study. Whilst we found no effects of caffeine on any measured traits, one future avenue of research would be to investigate the effects of caffeine on the circadian rhythms of wildlife as there may be longer-term diurnal or nocturnal effects that were not captured within this study. For example, caffeine has been shown to disrupt sleep, inducing signs of insomnia in rodent models (Paterson et al., 2009) and reducing sleep duration in *Drosophila* flies (Wu et al., 2009). A chronic reduction in periods of sleep and, therefore, higher overall activity levels across the day and night could have long-term deleterious effects on the energy balance of caffeine-exposed organisms.

In conclusion, we found that exposure to caffeine at environmentally relevant levels did not induce changes in key physiological, behavioural and morphological traits in wild fish, with no adverse effects on their energy balance or stress responses. Although we cannot exclude the possibility that the traits exhibited by our control fish were affected by the background caffeine content in the control treatments, nevertheless, there was at least one order of magnitude difference in the measured caffeine concentrations between our high-caffeine treatment and controls. Furthermore, past studies have also shown that caffeine exposure at current environmental concentrations are not likely to induce any strong adverse effects in non-target animals, albeit with some differences between taxa. Whilst chemical pollution as a result of rapid urbanisation and increased anthropogenic activity has been shown to have severe deleterious effects on wild populations and natural ecosystems in general, it seems likely that caffeine exposure at current environmental levels, does not exert strong adverse ecological impacts on vulnerable fish species.

AUTHOR CONTRIBUTIONS

Hung Tan: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; visualization; writing – original draft; writing – review and editing. **Jack A. Brand:** Investigation; writing – review and editing. **Bradley O. Clarke:** Validation; writing – review and editing. **Jack L. Manera:** Data curation; writing – review and editing. **Jake M. Martin:** Conceptualization; formal analysis; methodology; supervision; writing – original draft; writing – review and editing. **Bob B. M. Wong:** Conceptualization; funding acquisition; methodology; project administration; resources; supervision; writing – review and editing; writing – original draft. **Lesley A. Alton:** Conceptualization; funding

acquisition; methodology; resources; supervision; writing – review and editing; writing – original draft.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data sets generated during the current study are available at the Dryad data repository, (DOI: 10.5061/dryad.6djh9w16c).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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